



Purification and Partial Characterization of Esterase from Marine *Vibrio fischeri*

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Abstract

Lipolytic enzymes from marine microbes have been the focus of intense and growing research. The bioluminescence bacterium *Vibrio fischeri* was produced esterase enzyme when the medium contained specific substrate. The esterase was purified from the concentrated culture supernatant. The most active fractions were obtained using the technique of precipitation with 1N HCl. The precipitated fraction was purified by ion exchange chromatography (DEAE-Cellulose) and gel filtration chromatography (Sephadex G200). The purified active fraction exhibiting final specific activity of 300U/mg and characterized; the optimum pH was 7.5, the optimum temperature was 30°C. The enzyme was very stable at the temperature 30°C and at wide range of pH. The enzyme was monomeric protein having molecular mass of 37 KDa estimated by native PAGE assay.

Keywords: *Vibrio fischeri*, Lipolytic enzymes, Extracellular esterase

1. Introduction

Marine microorganisms which are salt tolerant, provide an interesting alternative for therapeutic purposes. Marine microorganisms have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with novel enzymes. Especially, halophilic microorganisms possess many hydrolytic enzymes and are capable of functioning under conditions that lead to precipitation or denaturation of most proteins. Further it is believed that sea water, which is saline in nature and chemically closer to the human blood plasma, could provide microbial products, in particular the enzymes, that could be safer having no or less toxicity or side effects when used for therapeutic applications to humans (Sabu, 2003).

The *Photobacterium (Vibrio) fischeri* group consists of rod-shaped cells with a light yellow, cell-associated pigment and a tuft of sheathed flagella (Hendrie et al., 1970). The species is restricted to the marine environment and has a specific requirement for sodium ion for growth (Reichelt and Baumann, 1973). It occurs both free living in sea water (Ruby and Nealson, 1976) and as the specific luminous symbiont of the monocentrid fish and squid.

Esterases are distinguished from lipases in that their action is generally restricted to short-chain fatty acids. Esterases catalysis of a large number of aliphatic and aromatic esters. Although the molecular and catalytic properties of this protein from mammalian sources have been well studied, only limited investigations have been made in to properties of isolated microbial esterases. Because of the potential food applications, the general economic attractiveness of extracellular enzyme is higher than the intracellular microbial industrial enzymes (Meghji et al., 1990).

Exterases have a wide range of industrial applications. The main applications are in cosmetics, paper and pulp, feed processing detergents or detergent compositions, synthesis of carbohydrate derivatives, food additives, e.g. flavor enhancer and animal feed moreover they can be used, wherever enantiopure compounds are needed and find application as research reagents in studies on plant cell wall structure. The global market for industrial enzymes is constantly growing with a rate of 5-10% per year. The numerous advantages of the new set of esterases compared to commonly used esterases present a great economical potential to a suitable industrial partner in each of the application sectors. Information of lipolytic enzymes produced by marine *Vibrio* spp is particularly limited. Therefore, the objective of this study is to focus on the esterase production of *V. fischeri* isolated from squid and on the purification and partial characterization of its esterase.

2. Experiments

2.1 Bacteria and Growth Conditions

The squids (*Sepia* sp.) were collected and cooled to about 8°C before opening the mantle cavity along the ventrum. The following two methods were used to obtain samples of symbiotic luminescent bacteria. Light organ fluid containing bacteria was obtained from pore that leads into channels with in the organ tissue. Whole light organs were removed by dissection and homogenized in 700µl of sterile seawater (Ruby and Nagai, 1992). Material obtained in either of these ways was serially diluted in seawater complete (SWC) broth and the samples were spreaded on SWC (0.38M NaCl, 0.02M MgCl₂·6H₂O, 0.025M MgSO₄·7H₂O, 8mM KCl, 0.5% peptone, 0.3% yeast extract, 2% agar and 0.3% glycerol) agar plates and identified the luminous bacteria based on the work of Reichelt and Baumann (1973) and VHA (*Vibrio* Hareyi Agar) differential media (Harris *et al.*, 1996).

2.2 Screening of Esterase Enzyme

2.2.1 ZoBell 2216E Modified Media

All strains were precultivated on the solid maintenance SWC medium. For detection of esterase activity the following basal medium (Zobell 2216E, slightly modified) was used. It contains 10g of peptone, 1g of yeast extract, 0.05g of CaCl₂, 15 g of bacto agar made to 1 L with aged seawater, pH was adjusted to 7.6. The media supplements with either of 1% tween 20 or 0.25% Triacylglycerol (tributylin) were then poured into petri dishes, after solidification of these media inoculated and incubated at 30°C for 10 days. The total diameter, minus the diameter of the colony was considered to be proportional to the esterase activity rate. After 1-10 day incubation the halos, clear (on tributyrin) or turbid (on all other substrates) were measured.

2.2.2 Spirit Blue Agar

Broth culture was streaked on the spirit blue agar plates with substrate (Tween or Triacylglycerol). Then the plates were incubated at different temperature 6°C, 17°C, 30°C for up to 15 days. The plates were observed after 6 hrs and every 12 hrs for the clearing of the blue or deep blue color around each streak. Esterase activities at different hours were compared by measuring the width (millimeter) of areas of cleaning or area of deep blue color around the colonies.

2.3 Physical and Chemical Conditions for Esterase Production

Different culture conditions were used to obtain the maximum levels of enzyme productivity by *V. fischeri*.

2.3.1 Incubation Temperature

Bioluminescence bacterium was grown on production medium and incubated at 24 hrs at different temperatures Viz: 10, 20, 30, 40, 50 and 60°C respectively.

2.3.2 Different pH Values

The different buffers were prepared at different pH values (3, 5, 7 and 9). The production medium was adjusted using a standard pH meter and incubated 30°C for 24 hrs.

2.3.3 Incubation Period

The effect of incubation period was determined by incubating the production medium at different incubation periods viz. 6, 12, 18, 24, 30, 36, 42 and 72 hrs at 30 °C.

2.3.4 Different Carbon Sources

A defined minimal salt medium was added with carbon sources included triacylglycerol (tributylin), tween 20, edible oil (olive, cotton seed, coconut oil) free fatty acids (oleic acid, lauric acid), glycerol and ethanol. All carbon sources were sterilized separately (ethanol by filtration) and added to a final concentration of 2% (w/v).

2.3.5 Time Course of Esterase Enzyme Production

Lipolytic enzyme of esterase production was observed from the initial time of inoculation to decline phase of the *V. fischeri*.

2.4 Purification of Esterase

2.4.1 Enzyme Production and Preparation of Cell Free Filtrate

150µl of culture was inoculated into 200ml of sea water medium with substrate and incubated at 30°C for 3 days. The portion was centrifuged at 20,000 xg at 4°C for 30 min. The supernatant was filtered using 0.45 µm cellulose acetate filter units. The cell free filtrate was used as the crude enzyme for the purification experiments.

2.4.2 Acid Precipitation

The crude extract was precipitated by slowly adding 1N HCl at 4°C with stirring until pH 4.3 was attained. The precipitate containing the esterase activity was collected by centrifugation 10,000 rpm for 30 min. The pellet was dissolved in 10mM phosphate buffer and brought buffer pH 7.5 by the addition of 1N NaOH.

2.4.3 Ion Exchange Chromatography (DEAE- Cellulose)

Dissolved pellet was first applied to a column of DEAE-Cellulose, equilibrated with 20 mM phosphate buffer, pH 7.5. The proteins were eluted at a flow rate of 18 ml/h with a linear NaCl gradient (0.1 to 0.6M). The fractions containing strong esterase activity were pooled (fraction size of 3ml) and concentrate.

2.4.4 Gel Filtration Chromatography (Sephadex G200)

Esterolytic fractions collected from DEAE cellulose chromatography were redissolved in a small volume of 50mM phosphate buffer, pH 7.0, and further purified by gel filtration on Sephadex G200, equilibrated with 50mM phosphate buffer, pH 7.0, containing 0.15M NaCl. Elution with the same buffer was at the flow rate of 12ml/h, 3 ml of fractions were collected. The elution containing esterase activity was pooled and used for further characterization.

2.5 Enzyme Activity Assay

Enzyme assay used for the determination of esterase activity upon emulsified substrate and Tween solution. The final concentration of Triacylglycerol (TAG) was 10-50mM, Tween 0.8-20% (v/v). The mixture of the substrate and buffer (final volume 8ml) was adjusted to pH 8.0 by 1N NaOH and the pH was maintained for 3 min by titration with 50mM NaOH solution (blank). Then 1 ml of the esterase solution in the buffer was added and the esterolytic reaction was observed for 40 min by titration as mentioned above. Enzyme activity is expressed as U/ml and one unit (U) of activity is defined as µmols of free fatty acids liberated /min/ml by the enzyme solution under assay conditions.

2.6 Protein Mass Determination (Native PAGE)

In order to study the undenatured protein profile of esterase enzyme from *V. fischeri* in cell free broth, electrophoresis performed by the method described by Lammeli *et al.*, (1970) with some modifications (Vorderwulbecke *et al.*, 1992). Gel was casted by using discontinuous buffer system having 10% resolving gel and 5% stacking gel. For measurement of molecular mass of protein, commercial broad range molecular mass standard proteins were used. Protein bands were located by coomassie blue staining.

2.7 Characterization of Lipase Enzyme

2.7.1 pH Stability

Enzyme solutions at a concentration of 70mg/ml were adjusted to various pH ranging from pH 2 to 11 with either 0.1N NaOH or 0.1 N HCl and aliquots were incubated at 30°C for 4 hrs. Then aliquot was removed and assayed for activity.

2.7.2 pH Optimum

Enzyme assays were conducted at various pH in an emulsified reaction mixture containing 0.4 ml of Tween or TAG, 4ml 50mM Tris, 0.1 ml enzyme. The pH was maintained by pH stat with 0.02 NaOH. After incubation of the reaction mixture for 40 min at 30°C, it was titrated to pH 9. The quantity of free fatty acids released was calculated from the total quantity of base used. Control reaction mixture contained heat inactivated enzyme.

2.7.3 Temperature Stability

The solution of enzyme at the concentration of 45mg/ml was adjusted to pH 7.0 with 0.02 NaOH and aliquots were incubated at temperatures ranging from 5°C to 65°C for 4 hrs. Then the aliquots were assayed for activity.

2.7.4 Temperature Optimum

Enzyme activity was determined at various temperatures ranging from 5°C to 65°C. The reaction mixtures (except enzyme) were held at the respective temperature for 5 min before the addition of the enzyme.

2.7.5 Substrate and Enzyme Kinetics

The esterase enzyme was incubated with various concentrations of substrate and the final substrate concentration ranged from 0.5 to 10% of tween 20. Different concentrations of enzyme also studied in the concentration of 5, 10, 15 and 20 microlitres.

2.7.6 Enzyme Stability on Metal Ions and Other Chemicals

Enzymes were preincubated for 1 h at 30°C (pH 7.0) and in 0.1M Tris-HCl buffer with various ions and other chemicals (one at a time). Assay was performed with the mixture, which did not contain CaCl₂ (except in test sample). The ions used were included NaCl (10mM), BaCl₂ (0.001M), MgCl₂ (0.001M), KCl (2mM), FeSO₄ (0.001M), CaCl₂ (0.001M), SrCl₂ (0.001M), NaF (2mM), MnCl₂ (2mM), CuO₂ (2mM). Other chemicals tested were ethylene diamine tetra acetic acid (0.5% EDTA), the ammoniac detergent, sodium dodecyl sulphate (0.5% SDS).

2.7.7 Enzyme Stability During Storage

Enzyme solutions from 2 days culture were stored at -20, 1, 8, and 20°C. Formaldehyde was added to a final concentration of 0.04% to prevent bacterial growth. 30 ml of a 2 days culture of esterase solution was sterilized by membrane filtration (0.22µm, Millipore corp.). The esterase activity in the stored samples was determined periodically by the pH stat technique and expressed as percent initial activity.

3. Results and Discussion

3.1 Bacteria and Growth Condition

Vibrio fischeri encountered 100% of luminous bacteria. Total viable luminous count range was varied from 4 to 18 CFU/ml. The habitats of this squid species must receive a significant input of cells of symbiotic *V. fischeri* (Lee and Ruby, 1994). The luminous *V. fischeri* isolate was motile Gram negative rods. They produced yellow colonies on SWC agar plates. They were halophiles, unable to grow in the absence of NaCl. The colony morphology of the luminous *V. fischeri* strains tested on VHA was small (2 to 5mm) dark blue green colonies. Harris et al., (1996) reported that the VHA media displays great potential as primary isolation medium and offers significant advantages over thiosulfate-citrate- bile salts- sucrose agar.

3.2 Screening of Esterase Enzyme

In ZoBell modified media, the *V. fischeri* was showed 36 mm of halos after 10 days incubation indicates that the strain *V. fischeri* showed significant esterase activity. In fact it actively splits tween 20 than tributyrin as good substrate. Bruni et al., (1982) reported that the most strains of *Pseudomonas* sp. NCMB 1082 was split all tweens, tributyrin, but not triolein, 9 strains showed good activity on water soluble tweens, 4 on tween 85. In spirit blue agar, the width of hydrolysis areas were measured to observe the esterase activity of luminous *V. fischeri* bacteria at different temperatures. At 30°C, the esterase activity of strain was appeared after 7 hrs with a large discoloration area or dark blue halos and the widest area observed after 1 day. At 17°C, the activity began after 12 hrs, the size of halos increase after 3 days but the area of hydrolysis was smaller than that at 30°C. Apparently low activity was observed at 6°C. More or less similar mm of halos as that in ZoBell modified agar was observed in spirit blue agar with the substrate (tween, tributyrin) at 30°C. The higher activity at 30°C may be attributed to maximum growth of the organism and subsequently increased esterase secretion.

3.3 Physical and Chemical Conditions for Esterase Production

3.3.1 Temperature

From this study, observed that 30°C was generally more favorable for esterase production. However, the temperature below or above the 30°C caused a sharp decrease in enzyme yield as compared to the optimal temperature. Wood et al., (1995) found that esterolytic activity at moderate temperatures (eg. 20°C - 30°C) was high with structurally diverse ester substrates including aliphatic, cyclic and sugar esters.

3.3.2 pH

This test found that the best buffer was phosphate buffer at optimum pH for the production of esterase enzyme was recorded at pH 7.0. A notable decline in the enzyme productivity occurred at both higher and lower pH values. In order to favour the secretion of extracellular lipolytic enzymes the effect of temperature and carbon source has been studied by Dominguez et al., (2007). In the dynamic field of biocatalysis, several lipolytic enzymes active and stable in extreme conditions of temperature, salinity and pH have been characterized by Encarnacion et al., (2005).

3.3.3 Carbon Source

The ability of *V. fischeri* to utilizing tween 20, tributyrin as a carbon source and energy material to produce esterase. Oils were not utilized by this strain. Interestingly *V. fischeri* exhibited their maximum ability to biosynthesize esterase enzyme with in 48 hrs. The production level of esterase enzyme of *V. fischeri* depended on the substrate utilized by the cells. The specific production level of the enzyme examined was very low when ethanol serves as the sole carbon source. In contrast, with the water immiscible glycerol and triglycerides such as tributyrin, the expression of the enzyme was markedly enhanced. More over the use of sorbitan monoesters (tween 20) increased the esterase enzyme production. Different substrates were used, but the maximum esterase productivity was attained in the tween 20. The addition of fatty acids did not repress the production of the enzyme. The similar result was observed by Shabtai and Mishne (1992).

3.3.4 Incubation Period

Highest enzyme production was observed in the incubation period of 72 hrs at 30°C.

3.3.5 Time Course for Esterase Production

Esterase from the *V. fischeri* produced maximum activity in stationary phase (Fig 1). Similarly, the maximal *Lactobacillus casei* esterase production was obtained intracellularly during the late logarithmic phase, but during the stationary phase, the esterase activity was released in the culture medium (Choi and Lee, 2001). In contrast Kakariari et al., (2000) reported that the production esterase, as assess in the intracellular extract during growth, reached maximum in the middle of the logarithmic phase and then decline rapidly.

3.4 Purification of Esterase

1N HCl precipitate of crude extract of cell free supernatant eluted in DEAE-Cellulose chromatography. The esterolytic fractions were assayed in chromogenic agar plate with the substrate of tween 20 showed yellow zone around the active fractions. The pooled material was eluted in gel filtration (Sephadox G200) chromatography. The esterase activity was represented by fractions between 28-34. Gel filtration (Sephadox G200) chromatogram shown in figure 2. This esterase had substrate specificity on tween 20, tributyrin and exhibited high activity only on the compound of sorbitan monoester. The purified esterase from gel filtration chromatography showed single peak corresponding to protein peak and exhibiting final specific activity of 300U/mg. In native PAGE electrophoresis it showed single band indicates esterase from *V. fischeri* was monomeric protein.

An esterase hydrolyzing tween 80 (polyoxyethylene sorbitan monooleate) was purified from sonicated cell lysates of *Mycobacterium smegmatis* ATCC 14468 by DEAE-Cellulose, Sephadex G150, phenyl sepharose and diethyl – (2-hydroxypropyl) aminoethyl column chromatography and by subsequent preparative polyacrylamide gel electrophoresis. The esterase had narrow substrate specificity; it exhibited a high activity only on compounds having both polyoxyethylene and fatty acyl moieties such as tweens (Tomioka, 1983). Purification of the esterase was performed in three steps of anion exchange chromatography and finally by gel filtration chromatography. Separation on Sephacryl S-300 gave a symmetrical single peak (Kakariari et al., 2000). Smacchi et al., (2000) reported that the chromatography in both DEAE-cellulose and on Sepharose 6B resolved the esterase activity into one peak corresponding to the major protein peak.

3.5 Protein Mass

The esterase from *V. fischeri* was showed molecular mass of 37 KDa. Tomioka (1983) estimated that the molecular weight of esterase was to be 36,000 by sodium dodecyl sulfate polyacrylamide. The purified extracellular *Arthrobacter nicotianae* esterase showed a single band on SDS-PAGE corresponding to a molecular mass of about 32 kg mol⁻¹, suggesting that the enzyme is a monomer (Smacchi et al., 2000). Kakariari et al., (2000) obtained only one band with the crude cell free extract in SDS-PAGE, indicating a simple enzyme system, significantly different from the complex esterase system of the *P. freudenreichii* ssp *freudenreichii* strains described by Dupuis and Boyaval (1993).

3.6 Characterization of Esterase

3.6.1 pH, Temperature Optima

Vibrio fischeri esterase had an optimum pH of 7.5 on tween 20 substrate (Fig 3). In this pH the esterase enzyme was retained 100% of the optimum activity. As indicated in figure 4, the enzyme had a temperature optimum of 30°C. At the temperatures below 25°C, activity slowly decreased. Sixty percentage of the activity available at 37°C. Smacchi et al., (2000) stated that the extracellular esterase of *A. nicotianae* 9458 had pH and temperature optima of 7.0 and 30°C, respectively. Similarly crude preparations of esterases from lactic acid bacteria (Khalid and Marth, 1990) and purified esterase from *L. plantarum* (Gobbetti et al., 1997) had pH and temperature 6.5-7.0 and 30-40°C respectively. Rhee et al., (2005) determined that the optimal pH for Est E1 esterase protein at various pH values of pH 3.0 to 9.5. The temperature range was 30 to 90°C, at lower temperature the enzyme still showed activity.

3.6.2 pH and Temperature Stability

The enzyme was stable over a wide pH range. In pH 6 retained 60% and pH 9 retained 70% activity. At pH below 4 or above 10 the loss of enzyme activity was appreciable. Gradual loss of activity with increased temperature up to 65°C. The temperature above 30°C, 30% actively retained at 50°C, 70% of the activity retained at 40 and 45°C, 90% activity at 35°C. Tomioka (1983) reported that tween-hydrolyzing esterase of *Mycobacterium smegmatis* was stable to heat treatment at 100°C and to a wide range of pH.

3.6.3 Substrate and Enzyme Kinetic

The esterase activity was reached at maximum in between the concentration of 0.5 to 8 % of tween. The concentration above 8 % reduced 50 % of the enzyme activity. Macarie et al., (1999) assayed the esterase activity in the range of substrate concentration from 0.3 to 2.7 mM using pNPC6. More than 2mM inhibit the esterase activity. Bendikiene et

al., (2005) found the tween 85 was the best substrate among all the detergents studied and 10% concentration was optimal for the hydrolysis by lipolytic enzyme from *Pseudomonas mendocina* 3121-1. A linear relationship was also evident when enzyme activity was measured as a function of protein concentration. The same direct proportionality was found when crude extract were used as the source of enzyme (Oterholm et al., 1970).

3.6.4 Effect of Metal Ions, Chelators and Other Chemicals on Esterase

Figure 5 showed the results on the effect of various inhibitors on the activity of the esterase. Except NaCl, CaCl₂, which were showed no effect on enzyme activity. All other ions tested in the present study showed inhibition with relative degree of variation. Evidently even the low concentration of NAF and CuO₂ highly retarded the esterase activity. The inhibitory action of former was stronger than that of latter. Action of these two compounds could be attributed to their effect in creating the imbalance of ions in the reaction mixture by absorption or release, respectively. In this study the SDS was highly favored the activity of esterase (133%). The esterase enzyme activity was not inhibited by the EDTA. It produced 67% of the activity in the presence of EDTA. Similarly Moskowitz *et al.*, (1977) reported that the esterase of *Mucor miehei* was relatively unaffected by the high concentration of various salts, ethylene diamine tetraacetate (EDTA) or sulfhydryl inhibitors. Kakariari *et al.*, (2000) showed that the esterase from *P. freudenreichii* ssp *freudenreichii* did not affected by EDTA, other metal ions except Cu²⁺, Hg⁺, Fe²⁺.

3.6.5 Enzyme Stability During Storage

Figure 6 showed the esterase activity as a function of storage time at different temperatures. Frozen storage did not affect the enzyme activity to any greater extent. These lipolytic enzymes stability suggests that the accelerated destruction of enzyme at higher temperatures.

Conclusion

The demand for industrial enzymes particularly of microbial origin is ever increasing owing to their applications in a wide variety of processes. Enzyme mediated relations are attractive alternatives to tedious and expensive chemical methods. In the above scenario enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates. However with the realization of the biocatalytic potential of microbial lipases and esterases in the last one and a half decades, industrial fronts have shifted towards utilizing lipase, esterase enzymes for a variety of reactions of immense importance.

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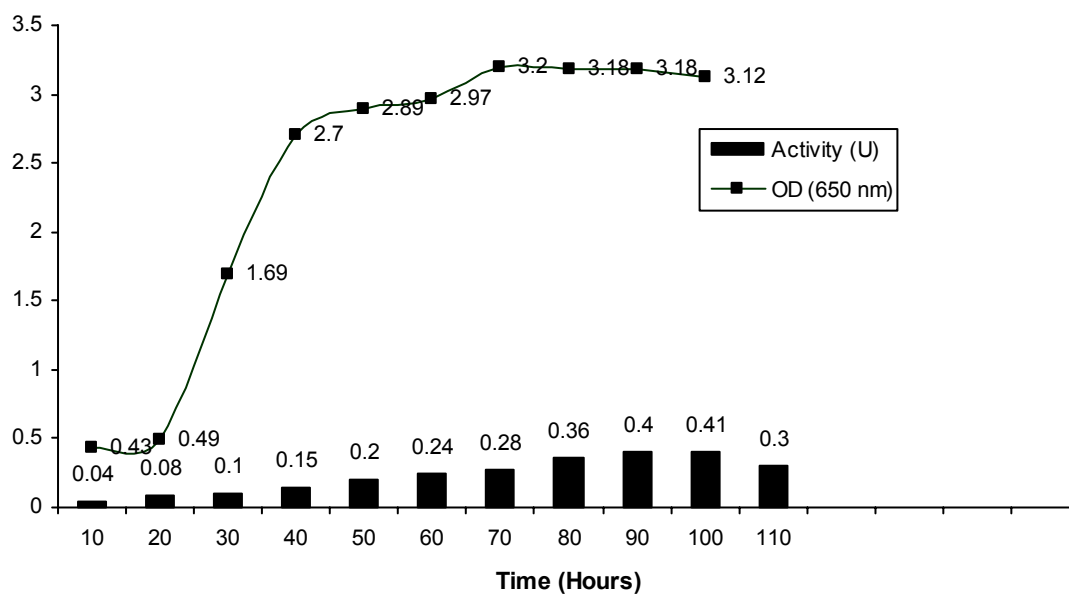


Figure 1. Esterase Production during Growth of *V. fischeri*

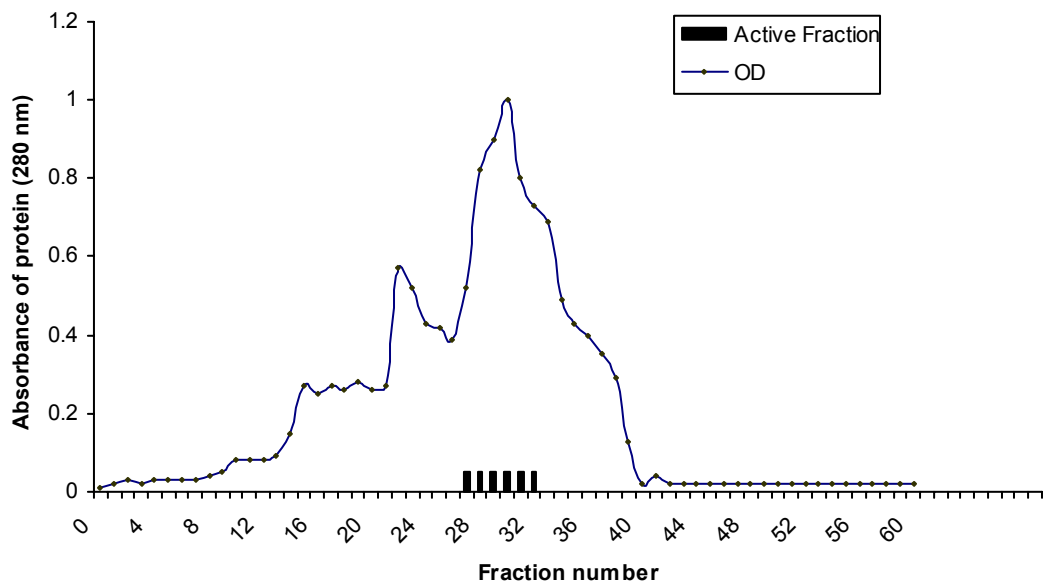


Figure 2. Elution Profile of Esterase from the Sephadex G200 Column

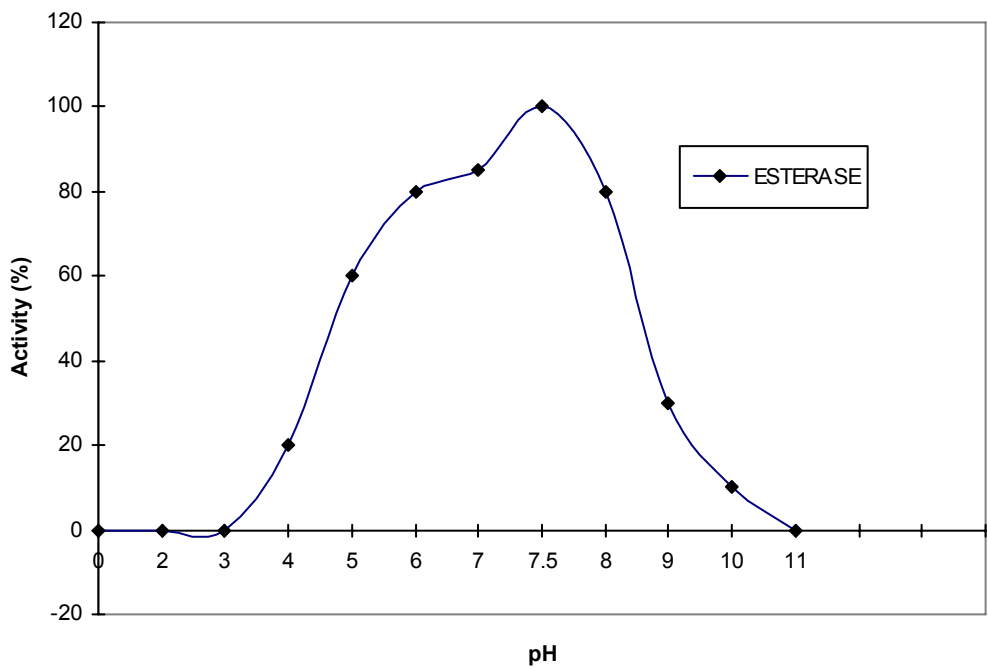


Figure 3. Effect of pH on the Esterase from *V. fischeri*

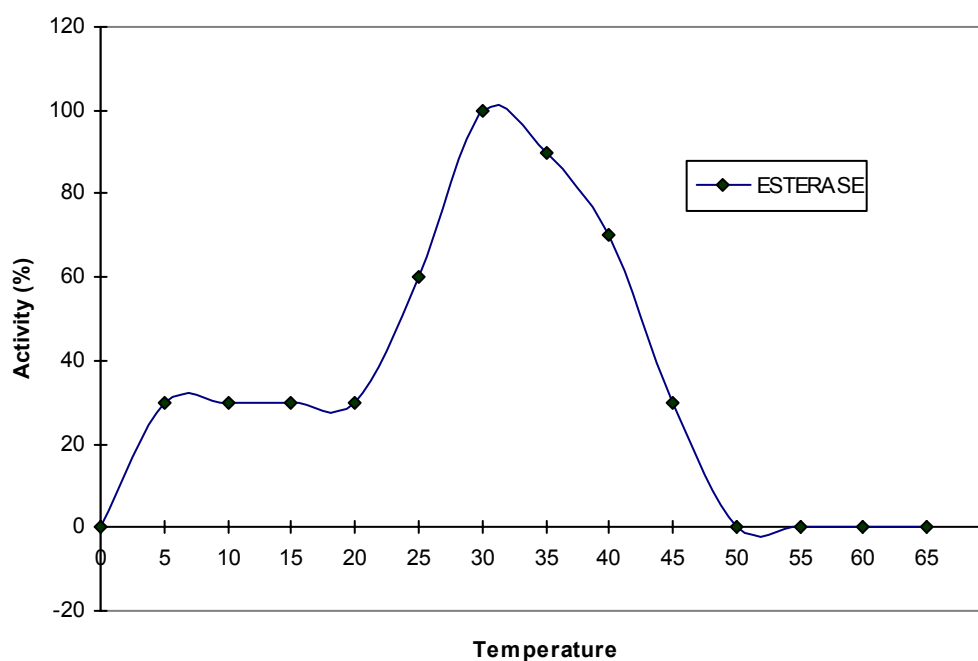


Figure 4. Effect of Temperature on the Esterase from *V. fischeri*

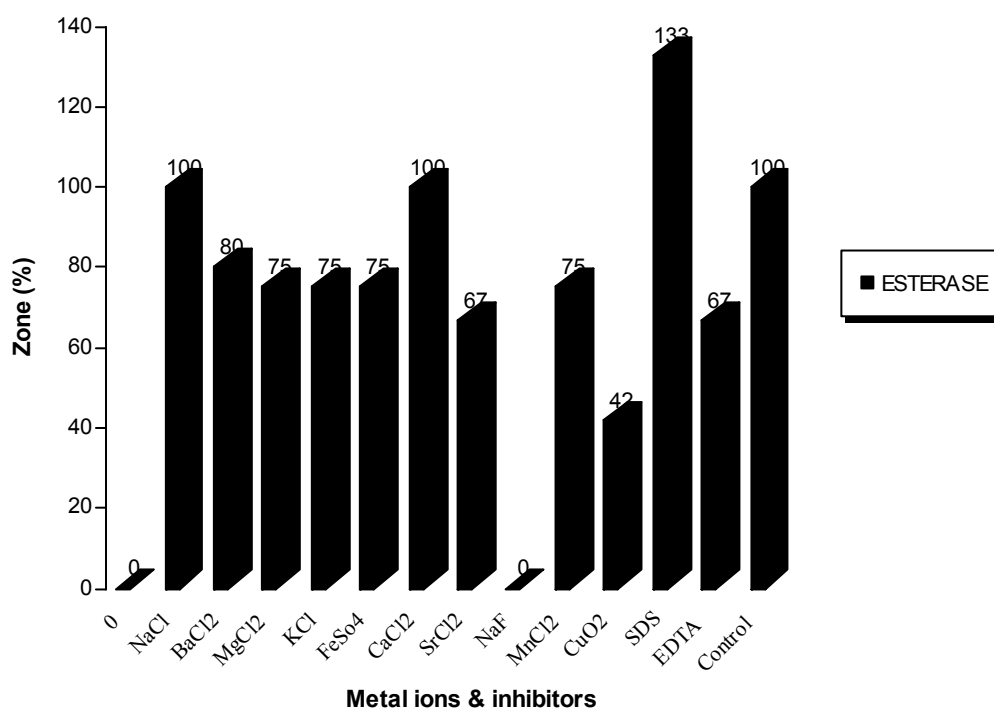


Figure 5. Effect of inhibitors on Esterase enzyme

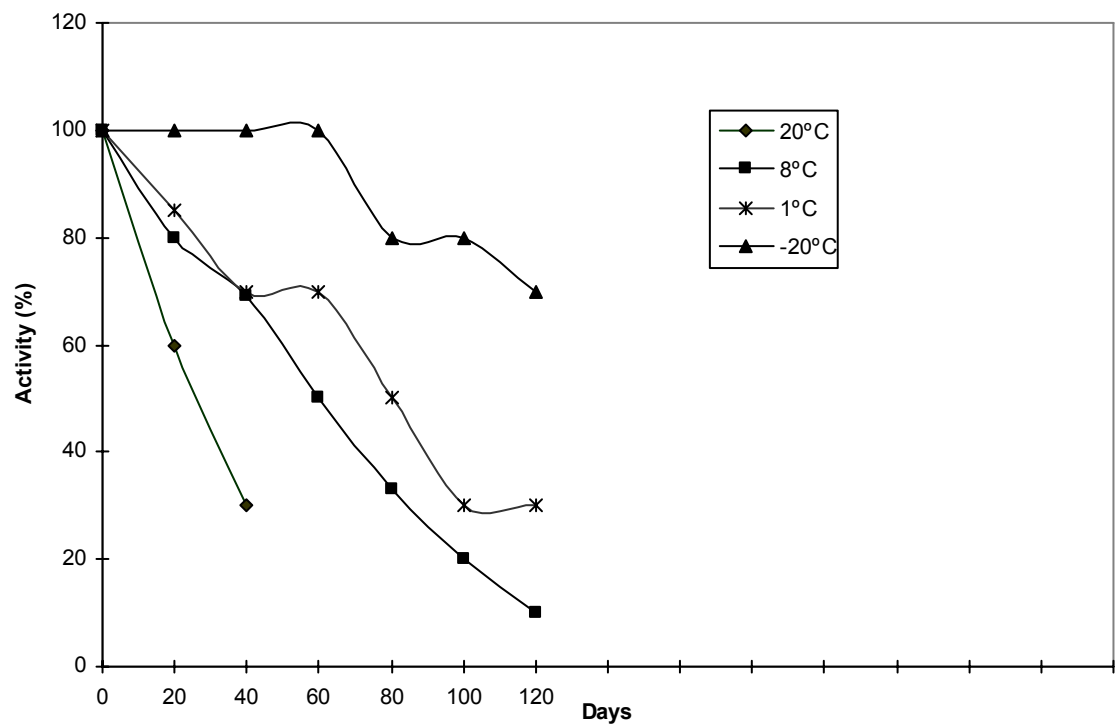


Figure 6. Storage Stability of *V. fischeri* Esterase at Different Temperature