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Isolation and Characterization of Lipase producing Bacteria from Vegetable Oil Spillage Site

Miral Patel*, Jemisha Mistry, Suraj Desai, Swetal Patel and Shreya Desai

C.G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Maliba campus, Bardoli, Surat, 394350, Gujarat, India

*Corresponding author

ABSTRACT

Study was conducted to isolate the lipase producing bacteria and characterize the lipase enzyme for its optimal activity. The samples were collected from vegetable oil spillage site. A total of 26 isolates producing lipase were isolated from three different samples processed. Lipolytic activity was confirmed by observing the zone of hydrolysis formed around the growth of colony on TBA plate. Out of 26 isolates, 4 isolates, viz A2, B4, C1 and C2 having zone diameter more than 1 mm were considered to be potential isolates and were subjected for further characterization. Lipase was quantified by determining the EU/ml by butyric acid titrimetric assay. All the potential isolates were also subjected to morphological and biochemical characterization for their partial identification. B4 and C1 isolates from 4 selected isolates were Gram positive while remaining A2 and C2 were Gram negative. Isolate B4 and C1 were identified as belongs to genus Bacillus, while other isolates required further characterization for their identification. Lipase enzyme produced by these potential isolates was also characterized for determining its optimal activity. Effect of different pH and temperature on lipase activity was determined. The optimum pH for A2, B4, C1 and C2 was found to be 7.5, 8, 8 and 7.5 respectively, while the optimum temperature for lipase activity for A2, B4, C1 & C2 was found to be 37°C respectively.

Keywords

Lipolytic Activity, zone of hydrolysis.

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Introduction

Lipases

Lipids are key elements in the chemistry of life. Most organisms use the supramolecular chemistry inherent to phospholipids to form their exterior and compartmental membranes. Many plants and animals store chemical energy in the form of triglycerides, which are sparingly soluble in water (Schmid and Verger, 1998).

To be able to carry out these functions, lipids require lipolytic enzymes during their metabolism. Lipolytic enzymes catalyze the turnover of these water-insoluble compounds (Gilham and Lehner, 2005).

Lipase is defined by the enzyme commission of International Union Of Biochemistry and Molecular Biology (IUBMB) as the enzyme that catalyzes hydrolysis of ester bonds at

the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved, under a natural conditions. Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reactions lead to esterification and formation of glycerides from fatty acids and glycerol (Jaeger *et al.*, 2002).

According to Enzyme Nomenclature given by International Union of Biochemistry and Molecular Biology (IUBMB), Lipolytic enzymes are grouped into 2 main categories, those which can hydrolyze triglycerides at the water/oil boundary are termed lipases or, more systematically, triacylglycerol hydrolases, and those which attack phospholipids are termed Phospholipases (Schmid and Verger, 1998).

Both types of enzymes have recently received considerable attention; phospholipases are involved in key metabolic events such as membrane turnover and signal transduction, lipases have diverse functions in the degradation of food and fat; they have qualified as valuable drugs against digestive disorders and diseases of the pancreas. They also find applications in biotechnology (in particular as detergent additives) and as catalysts for the manufacture of specialty (oleo) chemicals and for organic synthesis (Schmid and Verger, 1998).

Now a day, lipase arises amongst the most important biocatalysts carrying out novel reactions in aqueous and non-aqueous media. This is mainly due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents, and show chemo-regio- and enantio-selectivity. In recent times, the determination of their three dimensional structures has thrown light in to

their unique structure- function relationship (Berglund, 2001).

Lipases were first discovered in 1856 by Claude Bernard when he studied the role of the pancreas in fat digestion (Peterson and Drabløs, 1994). Since then, many different lipases have been identified in and isolated from bacteria, fungi, plants, and animals. The presence of lipases has been noticed as early as in 1901 for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which are now called *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively (Jaeger *et al.*, 1999; Hasan *et al.*, 2006).

It is very important to know three-dimensional structures of lipases in order to make them fit for specific applications. Human pancreatic lipase and the lipase from the fungus *Rhizomucor miehei* were the first ones whose 3D structures elucidated. Various other fungal and bacterial lipase structures were determined slowly. The first bacterial lipase structure, from *Pseudomonas glumae*, was clarified in 1993 (Noble *et al.*, 1993).

Sources of Lipases

Lipases are found throughout all kingdoms of life, which are prokaryotes including bacteria and archaea and eukaryotes including plants, animals and fungi (Caihong *et al.*, 2008). However, lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reaction including alcoholysis, aminolysis, hydrolysis, esterification and interesterification (Pandey *et al.*, 1990). Besides, microbes can be easily cultivated and the lipases produced are mostly extracellular. Several *Bacillus sp.* have been reported to be the main source of lipolytic enzyme (Schmidt *et al.*, 1994).

Lipase producers have been isolated mainly from soil, or spoiled food material that contains vegetable oil. Lipase production from a variety of bacteria, fungi and actinomycetes has been reported in several works (Sztajer *et al.*, 1988; Kulkarni and Gadre, 2002).

Bacterial Lipases

Bacterial enzymes are more preferred over fungal enzymes because of their higher activities and neutral or alkaline pH optima. 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).

A variety of lipases are produced from both Gram-positive and Gram-negative bacteria. Greater part of bacterial lipases comes from Gram-negative bacteria and the most important Gram-negative genus is *Pseudomonas* which contains at least seven lipase producing species that are *P. aeruginosa*, *P. alcaligenes*, *P. fragi*, *P. glumae*, *P. cepacia*, *P. fluorescens* and *P. putida* (Kojima *et al.*, 2003). Besides *Pseudomonas* species *Achromobacter*, *Alcaligenes*, *Burkholderia* and *Chromobacterium* strains are the most common lipase producing gram-negatives (Gupta and Rathi, 2004).

Gram-positive lipase producers are *Staphylococcus* (especially, *S.aureus* and *S.hyicus*), *Streptomyces* and *Bacillus* species. The most useful lipase producer genus used in industry is *Bacillus* among Gram-positive bacteria (Jaeger *et al.*, 1994).

Detection of Lipase Producing Microorganisms

There must be three factors to detect a lipase-positive bacterium by culturing it. These factors include (i) growth of the organism, (ii) production of lipase by that organism under suitable growth conditions and (iii) the presence of a sensitive method to detect lipase activity (Shelley *et al.*, 1987).

Microorganisms are often tested for lipase production on solid media. All methods containing agar can be grouped into two categories; (1) Methods based on substrate changes in appearance as a result of lipolysis and (2) methods including the usage of an indicator dye to detect lipolysis (Thomson *et al.*, 1999).

It is possible to visualize lipase-producing microbial colonies on solid media depending on the used lipase substrate. A diffusion assay can be used to identify lipase-producing microorganisms. In this method, a growth medium with tributyrin or triolein is prepared and microbial culture is put into wells cut in the agar. Lipase activity is defined by a clear zone formation around colonies after incubation (Thomson *et al.*, 1999; Shelley *et al.*, 1987) concluded that agar media can be useful for only screening of lipase production, but not to measure lipase activity.

Parameters for Optimum Lipase Production

Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration (Brune and Gotz, 1992; Jaeger *et al.*, 1994).

The major factor for the expression of lipase activity has always been carbon, since lipases are by and large inducible enzymes (Lotti *et al.*, 1998) and are thus generally produced in the presence of a lipid source such as an oil or any other inducer, such as triacylglycerols, fatty acids, hydrolyzable esters, tweens, bile salts and glycerol (Ghosh *et al.*, 1996).

Besides carbon source, the type of nitrogen source in the medium also influences the lipase titers in production broth (Ghosh *et al.*, 1996). Generally, organic nitrogen is preferred, such as peptone and yeast extract, which have been used as nitrogen source for lipase production by various *Bacillus* and *Pseudomonas* spp (Wang *et al.*, 1995). Divalent cations stimulate or inhibit enzyme production in microorganisms. Rathi *et al.*, (2001) observed stimulation in lipase production from *Burkholderia* sp. in the presence of Ca_2^+ and Mg_2^+ .

In addition to the various chemical constituents of a production medium, physiological parameters such as pH, temperature, agitation, aeration and incubation period also play an important role in influencing production by different microorganisms. The initial pH of the growth medium is important for lipase production. Largely, bacteria prefer pH around 7.0 for best growth and lipase production, such as in the case of *Bacillus* spp. (Sugihara *et al.*, 1991), *Acinetobacter* spp. (Barbaro *et al.*, 2001) and *Burkholderia* spp. (Rathi *et al.*, 2001). However, maximum activity at higher pH (>7.0) has been observed in many cases (Nashif and Nelson 1953; Wang *et al.*, 1995; Khyami-Horani 1996; Dong *et al.*, 1999). The optimum temperature for lipase production corresponds with the growth temperature of the respective microorganism. For example, the best temperature for growth and lipase

production in the case of *Bacillus* spp. was 50°C (Sharma *et al.*, 2002). It has been observed that, in general, lipases are produced in the temperature range 20–45°C. Incubation periods ranging from few hours to several days have been found to be best suited for maximum lipase production by bacteria. An incubation period of 12 h was optimum for lipase production by *A. calcoaceticus* and *Bacillus* spp. (Sharma *et al.*, 2002). While maximum lipase was produced after 72 h and 96 h of incubation, respectively, in the case of the *Pseudomonas* spp. including *P. fluorescens* (Dong *et al.*, 1999).

Analytical Determination of Lipase

Lipases are often analyzed by their hydrolytic action on triglycerides in a heterogeneous reaction medium of water and oil (Schmid and Verger, 1998).

Applications of Lipases

Microbial lipases have assumed a great deal of importance as industrial enzymes in view of their potential for use in various biotechnological purposes. Lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity and activity in organic solvents. Lipases are the most widely used enzymes in organic synthesis and more than 20% biotransformations are performed with lipases (Gitleson *et al.*, 1997). The field of industrial enzymes is now experiencing major R and D initiatives, resulting in both the development of a number of new products and in improvement in the process and performance of several existing products. Today nearly 4000 enzymes are known and out of these, about 200 are commercially used (Sharma *et al.*, 2011).

In eukaryotes, lipases are involved in various stages of lipid metabolism including

fat digestion, absorption, reconstitution and lipoprotein metabolism. Bacterial lipases are extensively used in food industry for quality improvement, dairy industry for hydrolysis of milk fat, cheese ripening, in beverages to improve aroma and in health foods for trans esterification. In detergent industry for removal of oil stains from fabrics by hydrolysis of fats, in textile industry to increase fabric absorbency. In addition the enzyme is used in chemical industry for enantioselectivity & synthesis of different products. In pharmaceuticals monoglycerides are used as binders in tablets enabling slow drug release, for the synthesis of biodegradable polymers or compounds, in pulp and paper industry to improve quality, degreasing leather in leather industry and for the production of various products in cosmetic industry (Sumanjelin *et al.*, 2013).

Industrial Applications of Microbial Lipases

Lipases found promising application in chemical processing, dairy industry, agrochemical industry, paper industry, oleochemical industry, cosmetics, pharmaceuticals, synthesis of surfactants, detergent industry, polymer synthesis and personal care products (Sharma *et al.*, 2011). The lipases can be employed in industry by cultivating lipase producing microorganisms in the medium containing suitable substrate (especially in the food industry), which is name *in situ* application or by using immobilized pure enzymes (especially in the production of fine chemicals) called as *ex situ* application (Pandey *et al.*, 1999).

Lipases in the food industry

Lipases have become an integral part of the modern food industry. It is desirable for the production of flavours in cheese and for

interesterification of fats and oils. It also accelerates the ripening of cheese and lipolysis of butter, fats and cream. Addition of lipases to such products primarily releases short-chain (C12 and C14) fatty acids leads to formation of a smooth taste (Saxena *et al.*, 1999).

Lipases are also used as emulsifiers in food, pharmaceuticals and cosmetics industries (Fleming, 1991). Lipases are used for the production of maltose-and lactose-like sugar fatty acid esters.

Lipases are also commonly employed in dairy industry for the hydrolysis of milk fat. Improvement of flavor in cheeses, the acceleration of cheese ripening, the production of cheese like products and the lipolysis of butterfat and cream is achieved by the help of lipase enzymes. Lipases have been used to produce meat like fish meat without excess amount of fat. The fat is removed during the processing of the fish meat by adding lipases and this procedure is called biolipolysis (Sharma *et al.*, 2001; Seitz, 1974).

Lipases in the detergents

The addition of lipases to detergent formulations has been investigated in the context of removal of fat stains (Schmid and R. Verger, 1998). Ever since the discovery of lipases it became part and parcel of the detergent industry along with proteases, amylases and cellulases. Lipases, protease, amylase and celluloses were used as they can split fats, proteins, starch and cotton-fluff, respectively.

Nowadays they were extensively used in household detergent, industrial cleaner and leather processing. Lipase has also been used to clean clogged drain which is due to the food and non-food material deposition.

In 1992, Novo Nordisk introduced the first commercial recombinant lipase 'Lipolase' originating from fungus *Thermomyces lanuginose* which was expressed in *Aspergillus oryzae* (Jaeger and Reetz, 1998). In addition to their application in laundry, lipases are also included in dish washing, contact lens cleaning, degradation of organic wastes on the surface of exhaust pipes and toilet bowls etc (Hasan *et al.*, 2006).

Lipases in pulp and paper industry

Wood is the main source of paper and pulp industry and the presence of the hydrophobic components (mainly triglycerides and waxes), also name pitch, in wood causes serious problems in the production of paper and pulp. Lipases are used to remove the pitch from pulp produced for paper making. The enzymatic pitch control method using lipase was put into practice in a large-scale paper-making process as a routine operation in the early 1990s and was the first case in the world in which an enzyme was successfully applied in the actual paper-making process (Bajpai, 1999). *Candida rugosa* lipases were used by Nippon Paper Industry, Japan to develop a pitch control method to hydrolyse 90 % of wood triglycerides (Reetz and Jaeger, 1998).

Lipases in cosmetics and perfumery

Lipases have potential application in cosmetics and perfumeries because it shows activities in surfactants and in aroma production (Metzger and Bornscheuer, 2006). Monoacyl glycerols and diacyl glycerols are produced by esterification of glycerols and are used as a surfactant in cosmetics and perfume industries.

Lipases in leather industry

The main process in leather industry is the removal of subcutaneous fat and dehairing.

Conventional methods including organic solvents and surfactants to remove fat from animal skins can be harmful to environment due to the production of dangerous final products like volatile organic compound (VOC) emissions (Hasan *et al.*, 2006).

Usage of lipases in association with other hydrolytic enzymes like proteases is a new approach in leather processing. Since the process is carried out at alkaline pH, alkalophilic lipases are used in combination with alkaline or neutral proteases and other necessary hydrolytic enzymes (Pandey *et al.*, 1999).

Lipases in bioremediation

Employment of lipases in bioremediation process is a new aspect in lipase biotechnology. Oil spills in refinery, shore sand and processing factories could be handled by the use of lipases from different origins (Nakamura *et al.*, 1994). It has been also used for the degradation of wastewater contaminants such as olive oil from oil mills. Another important application has been reported for the degradation of polyester waste and removal of biofilm deposits from cooling water systems (Sharma *et al.*, 2011).

Lipases as biosensors

The quantitative determination of triacylglycerol is of great importance in clinical diagnosis and in food industry. The lipid sensing device as a biosensor are rather cheaper and less time consuming as compared to the chemical methods for the determination of triacylglycerols. An analytical biosensor was developed for the determination of lipids for the clinical diagnosis (Masahiko *et al.*, 1995). Here, in quantitative determination of lipases are used to generate glycerol from

triacylglycerol in the analytical sample and to quantify the released glycerol by enzymatic or chemical methods. This principle enabled the physician to diagnose the patients with cardiovascular complaint. *C. rugosa* lipase biosensor from *Candida rugosa* has been developed as a DNA probe (Benjamin and Pandey, 2001).

Materials and Methods

Sample Collection

Samples from vegetable oil spillage site were collected from hotel and restaurant along Bardoli area in a sterile container. The samples were then brought to the laboratory without further contamination. The samples were then processed for isolation of lipolytic bacteria.

Screening for Lipase Production

Primary screening for lipase producing bacteria

1 gm of each samples were dissolved in 10 gm of sterile distilled water and was serially diluted. Screening of lipase producers was carried out using tributyrin agar plates (0.5% peptone, 0.3% yeast extract, 1% tributyrin and 2% agar, pH 7.0) according to method described by Lawrence (1967) with slight modifications. The dilutions were then spread onto tributyrin agar plates and incubated at 37°C temperature for 24-48 hr.

Following incubation, the plates were observed for the zone of clearance around colonies, indicative of lipase activity. The zone diameters of all the positive isolates for lipase production were measured. All the potent isolates were then preserved on sterile NA slants under refrigeration for further studies.

Secondary screening to determine Enzyme Units by shake flask study

All the potent isolates with higher zone diameter were then subjected to shake flask study for determination of enzyme units produced by individual isolates.

Lipase production

Each potential isolates were inoculated in the 15 ml of inoculum media (20 gm glucose, 10 gm yeast extract, 10 gm peptone, 10 gm CH₃COONa.3H₂O, 0.09 gm MgSO₄, 0.03 gm MnSO₄, 1.5 mg CuSO₄.5H₂O, 0.5 gm KCL, 5 ml Olive oil, 1000 ml Distilled water and pH 10.8). The inoculum flasks were then incubated at 37°C temperature for overnight on rotary shaker at 100 rpm.

The actively growing inoculums of individual potent isolates were then transferred in to 135 ml of production media (same as inoculums media but different in the olive oil concentration i.e. 20 ml). The production media flasks were then incubated at 37°C temperature for 24 hours on a rotary shaker at 120 rpm. After 24 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4°C and the cell free culture supernatant fluid was used as the crude extracellular enzyme for quantitative determination of enzyme units per ml.

Lipase Assay

Lipase activity was determined titrimetrically with modification of the method described by Arima *et al.*, (1972). In the standard assay conditions, the substrate was prepared by emulsifying 2.5 ml of tributyrin with 22.5 ml of polyvinyl alcohol (2 gm polyvinyl alcohol was dissolved in 50 ml of distilled water using magnetic stirrer). Then 0.25 ml of crude enzyme solution was added into the assay medium and the mixture was incubated for 30 minutes at

37⁰C. The lipase reaction was terminated with the addition of 4 ml of acetone-ethanol mixture (1:1 v/v) into the reaction mixture. The amount of free fatty acids liberated during the incubation was titrated with 0.01 N NaOH and 1 % phenolphthalein as the indicator. One unit of lipase activity is defined as the amount of enzyme required to liberate 1 μ M of fatty acid per ml per minute under the specified conditions (such as incubation temperature etc.).

Characterization of Isolates

The potential isolates were further characterized for their partial identification. Morphological and biochemical characteristics of the isolate have been studied for the identification of the isolate. Bergey's Manual of Systematic Bacteriology (2nd edition) was used as a reference for identification based on the result of various biochemical tests.

Morphological characterization

Gram Staining

Morphological characterization of the potential isolates involved use of Gram's staining. The cells were studied on the basis of their size, shape, arrangement and Gram reactivity. A smear of the selected strains were prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat-fixed smear was flooded with crystal violet and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95 % ethyl alcohol, washed with water and then counter-stained with safranin for 45 seconds. After washing with water, the smear was dried with tissue paper and examined under oil immersion (100x).

Cultural characterization

The cultural characteristics such as size, shape, margin, elevation, surface,

consistency, opacity and pigmentation were studied after growing the most potential isolates on nutrient agar plates

Biochemical Characterization

The biochemical test such as Sugar utilization test, IMViC test, Starch hydrolysis, Gelatin liquefaction, Nitrate reduction, Phenylalanine, TSI, Catalase, Lipid hydrolysis, Oxidase, Haemolysis and Indole production test were studied for their characterization.

Sugar Utilization test: A loopful culture of selected potential isolates were inoculated in to the sugar broth [10 % aqueous test sugar solution (glucose, maltose, lactose, mannitol, rhamnose, sucrose, xylose, mannose etc.) in 10 ml basal medium (nutrient broth or 1% peptone water), 1 ml 1% Andrade's indicator and ph 7.5] and incubated at 37⁰C for overnight. Acid production change the colour of medium to pink and gas production was concluded from a small bubble in inverted Durham's tube kept in the test tube.

Indole Production test: A loopful culture of selected potential isolates were inoculated in to tryptone broth (1% Tryptone Water, 0.5 gm NaCl, 100 ml distilled water and pH 7.4) and incubated at 37⁰C for overnight. After incubation, 3-4 drops of xylene were added in medium and shaken vigorously. The two layers were allowed to separate and 1ml of Kovac's reagent was added slowly. The formation of pink colour ring was considered as indicative of positive test.

Methyl Red test: A loopful culture of selected potential isolates were inoculated in to glucose phosphate broth (5 gm glucose, 5 gm K₂HPO₄, 5 gm peptone, 1000 ml distilled water and pH 7) and incubated at 37⁰C for 48-72 hours. Following incubation,

5 drops of methyl red indicator (0.1 gm methyl red in 300 ml 95 % ethanol) were in the medium. Development of red colour was considered as indicative of positive test.

Voges-Proskauer test: A loopful culture of selected potential isolates were inoculated in to glucose phosphate broth (5 gm glucose, 5 gm K_2HPO_4 , 5 gm peptone, 1000 ml distilled water and pH 7) and incubated at $37^{\circ}C$ for 48-72 hours. Following incubation, 0.6 ml of α -naphthol and 0.2 ml KOH solution were added and shaken well. Development of red colour was considered as indicative of positive test.

Citrate Utilization test: The test determines ability of bacteria to use citrate as a sole source of carbon and energy. Heavy streaking of all potential isolates were done on the surface of Simmons's citrate agar slant (0.2 gm sodium citrate, 0.02 gm $MgSO_4$, 0.5 gm NaCl, 0.1 gm ammonium dihydrogen orthophosphate, 0.005 gm bromothymol blue, 100 ml distilled water, 4 gm agar, pH, 6.9) and incubate slant at $37^{\circ}C$ for 48-72 hours. Development of deep blue colour within 24 -48 hours was considered as indicative of positive test.

Nitrate Reduction test: Organisms possessing nitrate reductase use nitrate as sole source of nitrogen will reduce nitrate to nitrite. All potential isolates were inoculated into peptone nitrate broth (0.3 gm meat extract, 0.5 gm peptone, 0.1 gm potassium nitrate, 100 ml distilled water, pH 7.4) and incubated at $37^{\circ}C$. 0.5 ml of α -naphthylamine reagent (5.7 ml glacial acetic acid, 14.3 ml distilled water and 0.1 gm α -naphthylamine) and sulphanilic acid reagent (5.7 ml glacial acetic acid, 14.3 ml distilled water and 0.16 gm sulphanilic acid).The development of red colour in 30 seconds was considered as indicative of positive test.

Gelatin Hydrolysis test: Gelatin is used in culture media for determining gelatinolysis

by bacteria A loopful culture of selected potential isolates were inoculated in to nutrient gelatin agar tube (3 gm meat extract, 10 gm peptone, 150 gm gelatin, 1000 ml distilled water, pH 7.2) and incubated at $37^{\circ}C$ for 48-72 hours. Following the incubation, the tubes are placed at $5-10^{\circ}C$ for 30-60 min. The test would be positive if the inoculated medium remains in a liquid state even after refrigeration, while control remains solidified.

Phenylalanine Deamination test: Heavy streaking of potential isolates were done on the surface of phenylalanine agar slant (0.4 gm DL-phenylalanine, 0.2 gm Na_2HPO_4 , 1 gm NaCl, 200 ml distilled water, 4 gm agar, pH 7.4) and incubated at $37^{\circ}C$ for 18-24 hours. After incubation, 4- 5 drops of 10 % aqueous ferric chloride were added on the surface of slant. Immediate appearance of an intense green colour indicates presences of phenyl pyruvic acid and was considered as indicative of positive test.

Starch Hydrolysis test: The presences of amylase in microorganism can determine from starch hydrolysis test. All potential isolates were culture on the starch agar plate (3.0 gm beef extract, 10.0 gm soluble starch, 12.0 gm agar and 1000 ml distilled water) as spot or line and incubated at $37^{\circ}C$ for 48-72 hours. Following incubation the plates were flooded with Lugol's iodine (2.5 gm iodine, 5 gm potassium iodide, and 10 ml distilled water). Appearance of clear colourless zone around growth was considered as indicative of positive test.

Catalase test: Catalase is an enzyme that spilt up hydrogen peroxide into oxygen and water. Catalase is present in high concentration in majority of aerobic organisms, but absent in most obligate anaerobes Heavy streaking of potential

isolates were done on the surface of nutrient agar slant and incubate at 37⁰C for 24 hours. 1 ml of hydrogen peroxide was then added over the growth on agar slant. Rapid appearance and sustained production of gas bubble constitute positive test.

Triple Sugar Iron (TSI) Agar test: Heavy streaking was done on the surface of TSI agar slant, and stab same culture into butt of the slant (3 gm beef extract, 3 gm yeast extract, 20 gm peptone, 10 gm lactose, 10 gm sucrose, 1 gm D-glucose monohydrate, 0.2 gm iron sulphate, 5 gm sodium chloride, 0.3 gm sodium thiosulphate, 24 mg phenol red, 13 gm agar, 1000 ml distilled water 1000 ml and pH 7.1) and incubated at 37⁰C for 24 hours. Appearance of yellow coloration of the medium was considered as a positive result for carbohydrate fermentation. If the medium in the butt of the tube turns yellow (acidic) and the medium in the slant remains red (alkaline), than it concludes that the organism being tested only ferments dextrose (glucose) whereas a yellow (acidic) colour in the slant and butt indicates that the organism tested ferments dextrose, lactose and/or sucrose. A red (alkaline) colour in the slant and butt indicates that the organism being tested is a non-fermenter. Hydrogen sulphide production results in a black precipitate in the butt of the tube. Gas production is indicated by splitting and cracking of the medium.

Ammonia Production: All potential isolates were inoculated into peptone nitrate broth (0.3 gm meat extract, 0.5 gm peptone, 0.1 gm potassium nitrate, 100 ml distilled water and pH 7.4), red litmus paper was placed on top of tube and incubated at 37⁰C for 24hours. Following incubation, turning of red litmus strip turn into blue colour indicates production of ammonia.

Eosin Methylene Blue (EMB) Agar plate:

Heavy streaking of all potential isolates were done on the surface of EMB agar plate (10 gm peptone, 10 gm lactose, 2 gm dipotassium-mono-hydrogenphosphate, 0.065 gm methylene blue, 0.4 gm eosin y, 15 gm agar, 1000 ml distilled and pH 7) and incubated at 37⁰C for 24hours. Appearance of green metallic sheen colonies are considered as indicative of positive result on EMB.

Macconkey Agar plate: Heavy streaking of all potential isolates were done on the surface of Macconkey agar plate (10 gm lactose, 5 gm NaCl, 3 gm peptone, 1.5 gm bile salt, 0.03 gm neutral red, 0.001 gm crystal violet, 13 gm agar, 1000 ml distilled water and pH 7) and incubate at 37⁰C for 24 hours. Appearance of pink colour colonies was considered as an indicative of positive result i.e. lactose fermenter bacteria

Casein Hydrolysis: All potential isolates were cultured on the skim milk agar plate (5 gm casein, 2.5 gm yeast extract, 1 gm skim milk powder (no inhibitors), 1 gm glucose, 15.5 gm agar, 1000 ml distilled water and pH 7) as spot or line and incubated at 37⁰C for 48-72 hours. Following incubation, appearance of clear colourless zone around growth is indicative of positive test.

Blood Agar Plate: All potential isolates were cultured on the blood agar plate (3 gm beef extract, 5 gm peptone, 10 ml blood, 15 gm agar 10, 1000ml distilled water and pH 7) as spot or line and incubated at 37⁰C for 48-72 hours. Following incubation, appearance of clear zone of haemolysis around growth is indicative of positive test.

Oxidase: Heavy streaking of all potential isolates on surface of nutrient agar slant (3 gm beef extract, 5 gm peptone 5 g, 15 gm agar, 1000 ml distilled water and pH 7) and incubated at 37⁰C for 24 hours. Following

incubation, a colony was picked up and a smear was made on a filter paper moistened with 1% tetraethyl phenylenediamine dihydrochloride solution. Formations of violet colour within 45-60 seconds were considered as indicative positive result.

Characterization of Lipase for Optimum Activity

Effect of pH on lipase activity

Studies were done to evaluate the effect of pH on lipase activity. The crude enzyme used for assay was the culture broth after separation of cells and particles. The enzyme was normally stored at 4°C until used. Optimal pH for enzyme activity was determined by incubating the reaction mixture at 37°C in buffer solutions of pH values ranging from 6 to 9, keeping other parameters same. Effect of pH on lipase action was analyzed by substituting the buffer in reaction mixture with the different buffers for different pH (Phosphate Buffer (0.1M) for pH – 6, 6.5, 7, 7.5, 8, 8.5; Ammonia Buffer (0.1M) for pH – 9) (Tembhurkar *et al.*, 2012).

Optimization of temperature for lipase production

Studies were done to evaluate the effect of temperature on lipase activity. Optimal temperature for enzyme activity was determined by incubating the reaction mixture at varying temperature in range 25 to 55°C keeping other parameters same. (Tembhurkar *et al.*, 2012).

Results and Discussion

Sample Collection and Primary Screening for Lipase Producing Bacteria

A total of 3 samples were collected from 3 different sites with vegetable oil spillage and total of 26 different isolates producing lipase were obtained on TBA plate. An isolates

showing clear zone of hydrolysis on TBA plate was considered as an indicative of lipase producer (Lawrence 1967). Table 2, shows the number of isolates showing zone of hydrolysis after 24 hours of incubation on TBA plate obtained from respective sampling site and the specific codes designated to all isolates obtained. Figure 3, shows the zone of hydrolysis obtained around isolate B4 on TBA plate. Diameter of zone of hydrolysis was measured, and the isolates with high diameter of zone of hydrolysis (more than 1 mm) were subjected for further studies considering them as potential isolates. Table 3, shows the list of potential isolates selected for further studies selected on basis of their hydrolysis zone diameter.

Secondary Screening to Determine Enzyme Units by Shake Flask Study

The potential isolates selected on basis of high diameter of zone of hydrolysis on TBA plate from primary screening were further evaluated for the determining the activity of lipase they produce under fermentative conditions. Enzyme Units were determined by the slight modification of method described by Arima *et al.*, (1972). The assay was carried out at 37°C and pH 7.5. Results showed that EU of lipase produced by isolate A2 was 12.2 EU/mL, of B4 was 13.2 EU/mL, of C1 was 11.9 EU/mL and for C2 it was 12.2 EU/mL. It was evident from the result that all isolates subjected to study were producing lipase enzyme more than 11 EU/ml. Figure 4, shows the graphical representation of EU/ml of lipase produced by potential isolates.

Characterization of Potential Isolates

Morphological and biochemical characterization of potential isolates were studied for partial identification of isolates. Bergey's Manual of Systematic Bacteriology (2nd edition) was used as a

reference for identification based on the result of various biochemical tests. B4 and C1 isolates from 4 selected isolates were Gram positive while remaining A2 and C2 were Gram negative. The Gram positive isolates were found to show big thin rods and typical colony of *Bacillus*. These isolates were endospore forming. The isolates A2 and C2 were Gram negative short rods. Isolate B4 and C1 were identified as belongs to genus *Bacillus*, while other isolates required further characterization for their identification. Further, as describe by Feng *et al.*, (2011) 16s rRNA gene sequencing is necessary for specific identification of isolates. Table 4, shows the morphological characteristics of potential

isolates and Table 5, shows the biochemical characteristics of the same.

Characterization of Lipase For Optimum Activity

Effect of pH on lipase activity

Lipases produced by potential isolates were characterized for its optimum activity. Lipases were found to give better activity at slightly alkaline pH. The optimum activity given by lipase produced by isolates A2, B4, C1 & C2 was at pH 7.5, 8, 8 and 7.5 respectively. Figure 5, shows the graphical representation of effect of pH on enzyme activity.

Table.1 Analytical methods for determining lipases (Schmid and Verger, 1998).

Methods	Principle	Advantages	Disadvantages
pH salt	Potentiometric determination of fatty acids liberated upon hydrolysis	Continuous kinetics methods for the determination of initial rates	-Expensive equipment -Added emulsifiers often modify reaction conditions -Not applicable under acidic pH conditions
Back titration	Titration of liberated fatty acids after defined exposure to lipase action	Simple	-No continuous kinetic data
Colorimetry	Liberation of a reporter group from a synthetic ester (e.g. <i>p</i> -nitrophenyl palmitate or dilauryl glycerol resorufin ester)	Fast and automatic kinetic measurements	-Unnatural substrates
Determining the hydrolysis of lipid films spread at air/water interface	Hydrolysis of a lipid monolayer at constant surface pressure with continuous supply of substrate (zero order trough)	Kinetic measurements in the absence of added emulsifier and under controlled “interfacial quality”	-Expensive equipment -Time consuming -Trained experimentalist necessary

Table.2 Number of lipase producing bacteria isolated from different sampling site.

Sr. No	Sampling Site	Number of isolates obtained	Codes designated to isolates
1	Gokul Fafda Centre, Buhari	09	A1, A2,A3, A4, A5, A6, A7, A8, A9
2	Jalaram Khichdi, Bardoli	07	B1, B2, B3, B4, B5, B6, B7
3	Hari-om Hotel, Bardoli	10	C1, C2, C3, C4, C5, C6, C7, C8, C9, C10

Table.3 List of potential lipase producing bacteria.

Sr. No	Isolate	Diameter of zone of hydrolysis (mm)
1	A2	1.4
2	B4	1.6
3	C1	1.3
4	C2	1.2

Table.4 Morphological characteristics of potential isolates

Characteristics	Isolates			
	A2	B4	C1	C2
Size	Small	Large	Large	Large
Shape	Round	Round	Round	Irregular
Margine	Even	Even	Even	Undulate
Elevation	Convex	Convex	Flate	Convex
Texture	Smooth	Smooth	Smooth	Smooth
Consistency	Moist	Moist	Moist	Moist
Opacity	Opaque	Opaque	Opaque	Opaque
Gram's nature	Gram –ve	Gram +ve	Gram +ve	Gram –ve

Table.5 Biochemical characteristics of potential isolates

Biochemical tests	Isolates			
	A2	B4	C1	C2
Glucose utilization test	+	+	+	+
Maltose utilization test	+	+	+	+
Sucrose utilization test	+	+	+	+
Lactose utilization test	-	-	-	-
Xylose utilization test	-	-	-	-
Mannitol utilization test	-	-	-	-
Indol production test	-	-	-	-
Methyl red test	-	-	-	-
Voges - proskuer test	+	-	+	-
Citrate utilization test	-	-	-	-
Nitrate reduction test	+	-	+	+
Gelatine hydrolysis test	-	-	-	-
Urea hydrolysis test	-	-	-	-
Phenylalanine Deamination test	-	-	-	-
Catalase test	+	+	+	+
Oxidase test	+	+	+	+
TSI test: -Butt	Acidic	Acidic	Alkaline	Acidic
-Slant	Acidic	Acidic	Acidic	Alkaline
-H ₂ S	-	-	-	-
-Gas	-	-	-	-
Cetrimide agar plate	-	-	-	-

Key: '+' indicates positive test and '-' indicates negative test.

Fig.1 Mechanism of Lipase action (Source: Jaeger and Reetz, 1998).

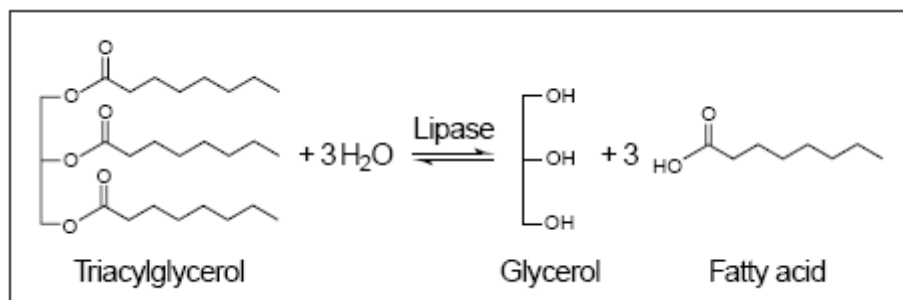


Fig.2 Structure of lipase from *Pseudomonas aeruginosa* (Source: Jaeger and Reetz, 1998).

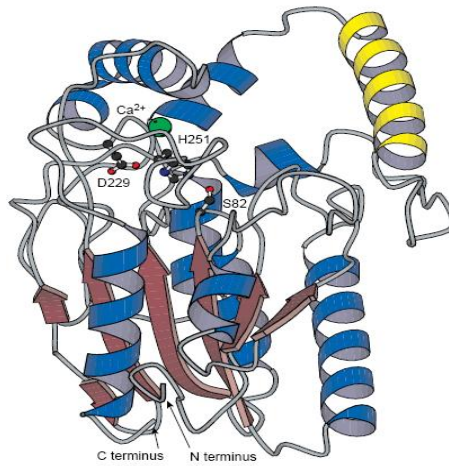


Fig.3 Shows the hydrolytic activity of lipase producer (isolate B4) on TBA plate.

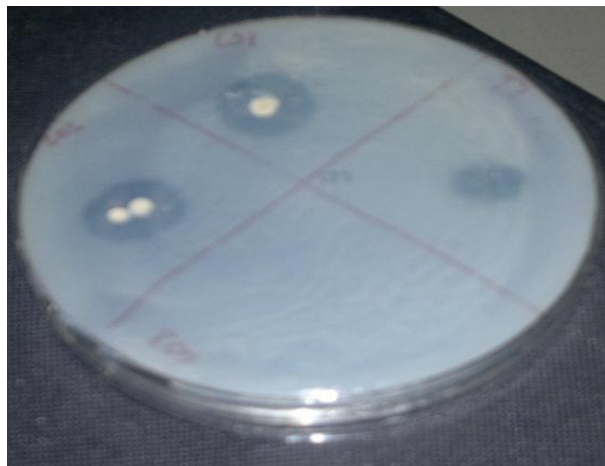


Fig.4 Eu/ml of lipase produced by potential isolates.

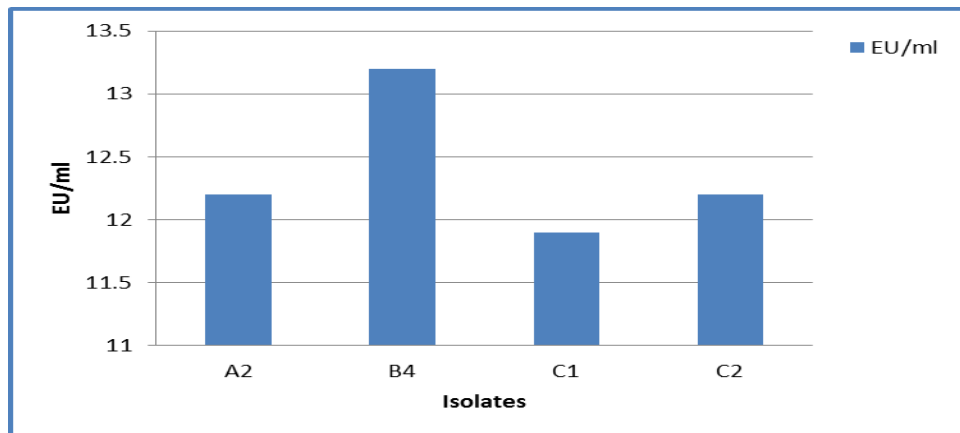


Fig.5 Effect of pH on lipase activity.

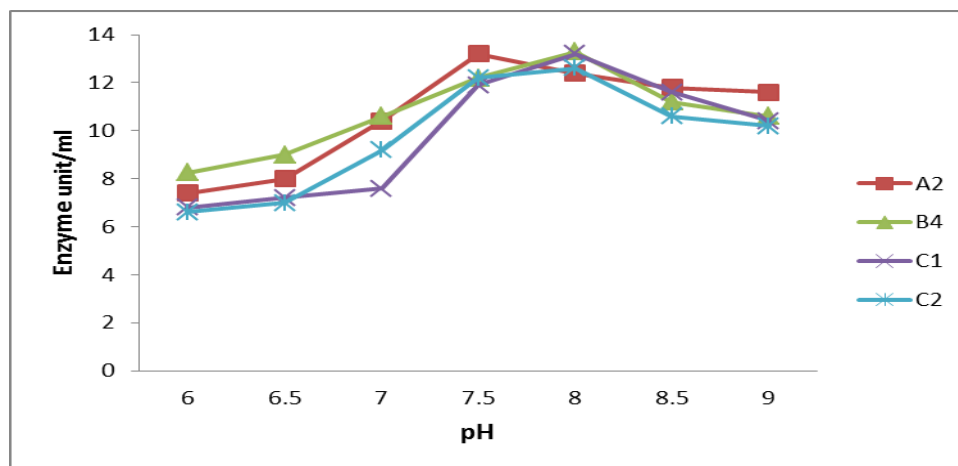
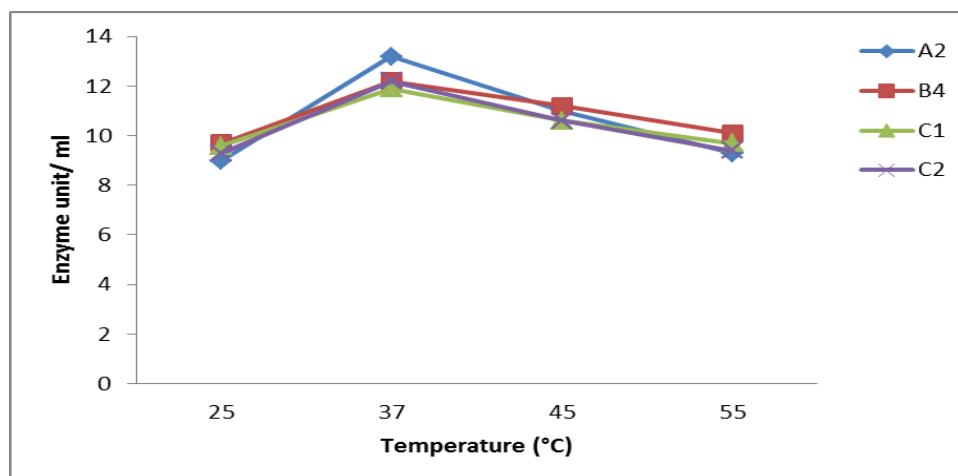


Fig.6 Effect of temperature on lipase activity.



Effect of temperature on lipase activity

Lipases produced by potential isolates were also analyzed for its optimum activity at different temperatures. Lipases were found to give better activity at 37⁰C temperature. The optimum activity given by lipase produced by isolates A2, B4, C1 and C2 was at 37⁰C respectively. Figure 5, shows the graphical representation of effect of temperature on enzyme activity.

The existence of lipase producing microorganisms in diverse environment such as industrial wastes, vegetable oil processing

factories and spillage sites, dairies, soil contaminated with oil, oilseeds, and decaying food, compost heaps, coal tips, and hot springs has been studied (Wang *et al.*, 1995).

Morphological and physiological-biochemical characterization like Gram stain, oxidase test, catalase test, Voges-Proskauer test, methyl red test, indole test, glucose fermentation test, hydrogen sulfide production, and hydrolysis of starch etc. are performed for partial identification of bacteria. Bergey's Manual of systematic bacteriology is use as a reference for

comparing the characteristics (Feng *et al.*, 2011). Further, as describe by Feng *et al.*, (2011) 16s rRNA gene sequencing is necessary for specific identification of isolates.

Generally, bacterial lipases have neutral or alkaline pH optima (Gupta *et al.*, 2004) with the exception of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 (Andersson *et al.*, 1979). Lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range 3–12 (Bradoo *et al.*, 1999). Bacterial lipases possess stability over a wide range, from pH 4 to pH 11 (Wang *et al.*, 1995).

Also bacterial lipases generally have temperature optima in the range 30–60°C (Wang *et al.*, 1995). However, reports exist on bacterial lipases with optima in both lower and higher ranges. Thermal stability data are available only for species of *Bacillus*, *Chromobacterium*, *Pseudomonas* and *Staphylococcus* (Gupta *et al.*, 2004).

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