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RAPD- PCR markers based genetic variability study of Lipase producing gene among the Aeromonas Species Isolated from Clinical Isolates

Huda Noor Hasan[1], Dr. A. Krishna Satya[2]
[1]P.G. Scholar, [2]Supervisor, Assistant Professor
[1,2] Department of Biotechnology
[1,2]Acharya Nagarjuna University, Nagarjuna Nagar, Andhra Pradesh, India
Email : [1]drose5243@gmail.com, [2]akrishnasatya78@gmail.com

Abstract :
Lipases are a group of enzymes which vary in size, substrate specificity and activity among other enzymes. They also vary with other enzymes in terms of their activators and inhibitors.

As in recent decades, interest over lipase had been drastically increased owing to its vast number of industrial applications. As such metagenomics paved new ways to identify and screen novel methods to isolate the enzyme from different sources [6]. Wide variety of lipolytic genes have been traced out from the marine environment especially from the sponges like *Aplysina aerophoba, Hyrtios erecta.* Very recently about 15 new genes which are completely lipolytic in nature were isolated and purified from south china marine sediments.

Lipases also called triacylglycerol hydrolase (EC 3.1.1.3) are known to cleave the ester bond of triacylglycerol and aids in the synthesis of esters *in vitro.* Very fine advantage of using these enzymes is that they have a great stability over the organic media and moreover they do not need the help of cofactors. They are also specialized in working over a wide variety of media and subtrates [7].

These class of enzymes especially which are having a awide variety of applications are isolated from several micororganims. Their source of finding the enzymes is vast. Yeast especially candida species are mostly used in the production [8]. Though production is achieved, novel methods need to be traced to enhance the specificity and production. Recent studies were also done on inducing the strains to produce the desired enzyme [9]. Recent studies also used agricultural wastes and products as media for fermentation to produce these enzymes. This not only increased the quality but also reduced the cost of production. One of the best application of lipases is the biodiesel industry. Since its discovery lipases were being tried for their usage in several industries. Recent trends and studies proved of the fact that they can be best used in the biodiesel industry [10]. Biodiesel is produced from renewable sources which mostly consist of alkyl esters of fatty acids which are primarily obtained by the esterification of lipids.

Keywords:- Lipase, Enzymes, Gene, Species, RAPD- PCR, Markers

INTRODUCTION

Lipolytic enzymes like Lipases (EC 3.1.1.3)) are important classes of enzymes which carry out vital and new reactions in aqueous and non aqueous environments. They are of great importance due to their vast number of industrial applications. They are used in the generation of biodiesel, food flavours, cosmetic and laundry industries. They are also widely used in the pharmaceutical industries. They belong to the third largest enzyme group, after

proteases and glucose in terms of production and market value.

Even though they are of great economical importance, study on lipases extracted from marine microbes are too low and limited. Lipases are a triad serine hydrolases, which aids in hydrolyzing the triacyl glycerol to glycerol and also aids in the synthesis of short chain (≤10) and long chain (≥10) acylglycerols [1]. Although many of the enzymes differ in their sequence homology and their relation to the activators and inhibitors, all the lipases share a common domain called as αβ hydrolase fold. This fold is made up of parallel β-pleated strands which make up the actual catalytic domain. A total of five parallel β-pleated strands are present in lipases and are usually separated by stretches of α-helix. As such the overall structure looks like super helically twisted–pleated sheet [2].

Aeromonas spp. are present in wide range in both soil and water and are responsible for the cause of diseases in fishes and amphibians. They are most commonly seen in untreated and sewage waters. Not only in the water bodies but also in beef and pork, fisheris, poultry and even in raw milk [3]. As today, *Aeromonas* genus was considered to be an important disease-causing pathogen found in fishery industries and other cold-blooded species.

They are infectious but the mode and path physiology of the infection seems to be little controversial owing to the presence of enter toxins and hemolysisns. Aeromonas species is believed to cause mild abdominal cramps with diarrheal disease in healthy individuals. Studies also reported that the bacteria also causes watery diarrhea either of chronic or acute. Aeromonas spp. is found to cause acute diarrhea in children also [4].

Molecular studies often confirms of the role of the pathogen in causing a particular disease. Isolation and purification of the strains not only allows us to identify the source of the organism but also the command on the environments the pathogen is having. Molecular characterization studies are paving new ways into finding the molecular and pathophysiologcal mechanisms which will aid in controlling the infection [5]. Epidemiological studies also aid in locating the distribution and moreover will help in determining the vastness and extent of the infection. PCR amplification and RAPD profiling will give the world a chance to find out their close relatives. This not only allows one to find out the possible cause for the spread but also to find out possible antibacterial targets. Nosocomial infections which are mostly resistant to antibiotics, need immediate attention to find out novel antibacterial targets. This will surely lead to the generation of next generation drugs. The present study was undertaken to isolate the strains from the clinical samples and to study their genetic profile by using PCR based molecular markers.

MATERIALS AND METHODS

Sample collection and bacterial strain isolation:

Cotton swabs and dressings from the human wounds were collected from hospitals and dispensaries in and around Bangalore. The 80 samples were collected aseptically and processed for the lab for isolation of the strains. For isolation of microorganisms, the swabs were suspended in about 90mL of sterile saline and incubated in a shaker at 37°C for 45min. Ten-fold serial dilution method was employed to isolate the bacterial isolates from the samples.

Isolation of bacteria:

Aeromonas bacteria were isolated using the medium called as Rimler-Shotts (RS) medium (11). The composition of the medium was indicated as below.

Table 1: Rimler-Shotts (RS) medium composition.

Chemical	Qty in gm
L-lysine-hydrochloride	5
L-ornithinehydrochloride	6.5
Maltose	3.5
Sodium thiosulfate	6.8
L-cysteine-hydrochloride	0.3
Bromothymol blue	0.03
Ferric ammonium citrate	0.8
Sodium deoxycholate	1
Novobiocin	0.005
Yeast extract	3
Sodium chloride	5
Agar	13.5

The components were dissolved as indicated in the table by constant stirring maintaining the pH at 7.0. The contents were boiled and made to 1litre with distilled water. The medium was autoclaved and poured into plates.

3.4: Screening of strains for extracellular lipase

The isolates obtained from the plates were screened for lipase activity. Rhodamine B plate assay method was used for screening the lipolytic activity. The rhodamine B plate contained the following contents.

Table 2: Rhodamine B plate medium.

Chemical	Qty in gm
Nutrient broth,	0.80%
NaCl	0.40%
0.01% Rhodamine B solution	500mL
Agar	1%
Olive oil.	8%

The contents were mixed according to the table above maintaining the pH of the medium at 7.0. An overnight fresh colony of each isolated strain was streaked onto a rhodamine B plate and incubated at 37°C for about 24hr. following incubation, the plates were exposed to UV light (350nm) for estimating the lipase activity. The presence of fluorescence on plates indicates the strain is positive for lipolytic activity. Colonies with no fluorescence have no lipase activity. The colonies with and high fluorescence were selected for further studies and subcultured.

3.5: Strains identification

Biochemical identification: Biochemical tests were done for identification of the isolated lipase positive colonies. According to the Bergey's manual of systemic bacteriology, these tests were selected and were done in triplicates.

The tests done were gram staining, Vogues-Proskauer test, oxidase, catalase and citrate Consumption test.

3.5.1: Grams Staining:

It is the technique used in microscopic techniques to visualize the clarity of the microscopic image. It is the most widely used procedure and discovered by the Danish scientist and physician **Hans Christian Joachim Gram** in 1884. It is a differential staining technique which differentiates the bacteria into gram-positive and gram-negative. The procedure is based on the ability of bacteria to retain the color of the stain used. Gram-negative bacteria are decolorized by the alcohol and as such stain red. Gram-positive bacteria are not decolorized by alcohol and will remain as purple.

1. Materials Required:

- Clean glass slides
- Inoculating loop
- Microscope
- Immersion oil
- Distilled water

1.1. Reagents:

- Primary Stain - Crystal Violet
- Mordant - Grams Iodine
- Decolourizer - Ethyl Alcohol
- Secondary Stain - Safranin

Protocol:

1. With a sterile loop, a drop of sterile water is placed on the slide. A single loop was taken from the culture plate

and dropped into the water drop. With the help of the loop spread the drop onto the slide. This makes the smear.
2. Heat fixing kills the bacteria in the smear, which adheres the smear firmly to the slide.
3. The smear is allowed to air dry.
4. Place slide with heat fixed smear on staining tray.
5. Gently apply the smear with crystal violet and leave for 1min.
6. Following incubation, rinse the smear with tap water.
7. Then add gram's iodine to the smear and allow to stand for 1min.
8. The slide is rinsed again with tap water and decolorized with 95% ethyl alcohol or acetone.
9. Following that counter stain with safranin and let stand for 45 seconds.
10. Blot dry the slide and view the smear using a light-microscope under oil-immersion.

3.5.2: Vogues Proskauer test:

Differentiation of the groups of Enterobacteriaceae can be done by testing the biochemical properties and enzymatic reactions by using specific substrates. The enteric organisms are further classified into lactose fermenters and non fermenters. *Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae* are lactose fermenters. S*almonella typhimurium, Shigella dysenteriae, Proteus vulgaris, Pseudomonas aeruginosa, Alcaligenes faecalis* etc are non lactose fermenters. The VP test determines the capacity of the bacteria to produce non acidic or neutral end products, like acetyl methyl carbinol. The reagent used is Barritt's reagent which is made of alcoholic a-naphthol and 40% potassium hydroxide solution. On reaction, a pink complex is formed giving a rose colour to the medium. Development of a deep rose colour within 15min indicates positive. The absence of rose color is a negative result.

1.1.1. Media preparation:

Glucose: 5gm
Peptone: 5gm
Dipotassium hydrogen phosphate: 5gm
Make up to 1000 ml. (pH 6.9)

Reagents: Barritt's reagents A and B.

Solution A: 6gm of a-naphtholin in 100ml of 95% ethyl alcohol.

Solution B: 16gm of potassium hydroxide in 100ml of water.

Procedure:

1. Remove the cap from the TSB tube and a single colony was removed with the help of a sterile loop.
2. Two sterile MR-VP broth tubes were taken one named Test and the other one control.
3. The tubes were inoculated with the inoculum from the TSB.
4. The tubes were incubated for 24 to 48 hours at 37°C.
5. Following incubation, 10 drops of Barritt's A reagent and 10 drops of Barritt's B reagent was added to each broth.

2. 3.5.3: Catalase test:

Bacteria which contain flavoproteins reduce oxygen (O_2) and result in the production of hydrogen peroxide (H_2O_2) and sometimes toxic superoxide (O_2–). Over accumulation of such toxic substances lead to the death of the bacteria as they powerful destroy the cell and its constituents. If a bacterium doesn't protect itself against such products it will be killed. Hence many bacteria has enzymes which can give protection against the toxic products.

3. Materials Required:

Cultures: 24-48 hour tryptic soy broth cultures of bacteria
Media: LB agar
3% hydrogen peroxide

Procedure: Slide Method:

1. A clean glass slide was divided into two sections and labeled as "test" and "control".
2. With a sterile loop, a small amount of the culture was taken and placed on the slide.
3. Add a drop of hydrogen peroxide over the smear.
4. Observe the fluid for the appearance of gas bubbles.

3.5.4: Oxidase Test

This assay is based on the identification of bacteria which produce cytochrome c oxidase. This enzyme is very critical for the electron transport chain. Almost all the microbes which are oxidase positive are aerobic organism. This means they can use oxygen as a terminal electron acceptor during respiration. Bacteria which are oxidase-negative may be anaerobic or aerobic. Such bacteria might respire using other oxidases during electron transport.

Procedure: Paper strip method

1. Whatman's No. 1 filter paper strips are pre soaked in freshly prepared 1% solution of tertramethyl-p-phenylene-diamine dihydrochloride.
2. Following incubation, the strips are drained of the reagent.
3. They are then freeze dried and stored in a dark bottle.
4. A strip is removed and placed in a petri plate.
5. The colony to be tested is picked up with a small loop and smeared over the moist area.
6. An intense deep-purple colour indicates the bacteria is positive to the test.
7. Absence of colouration indicates negative reaction.

3.5.5: Citrate utilization test:

It is mostly employed as part of a group of tests, the IMViC (Indole, Methyl Red, VP and Citrate). They are used to differentiate between the members of the Enterobacteriaceae basing on their by-products. It is also used to screen the coliforms in aquatic environment to estimate the potability of the water. Citrate utlilization test is used to determine whether the bacteria can utilize sodium citrate as its only carbon source or not.

Procedure of citrate utilization test:

1. Simmons citrate agar was added to the slants and left for 30min standing.
2. A loopful of colony was picked up by the sterile loop and inoculated onto the slant.
3. The sample was now incubated at 35^{o}C to 37^{o}C for about 18 to 24 hours.
4. Blue colour formation was observed.

Growth will be visible and will be of Prussian blue and that indicates positive.
No visible growth and absence of colouration indicates negative.

3.6: Lipase assay

The lipase assay was determined by the pNPP (pnitrophenyl palmitate) hydrolysis method, described by Ghori *et al.* (2011). It is the assay where the lipase if present hydrolyzes a triglyceride substrate to form glycerol. This product formed can thus be quantified enzymatically by spectrophotometer at 570nm.

Materials required:

1. Overnight culture
2. Polyvinyl alcohol solution
3. Isopropanol
4. Sodium hydroxide and Conc HCl.

Procedure:

1. 5ml of sample culture was added to a test tube pre soaked with 800µl of 0.25% PVA poly vinyl alcohol solution.
2. PVA is prepared in phosphate buffer and 100µl of 8mmol/l pNPP solution in isopropanol.
3. The contents are incubated for 5min at 30°C.
4. The reaction was stopped by adding 500µl of 3mol/l HCl solution.
5. The contents are mixed thoroughly and centrifuged at 5000rpm for 10min.

6. 500µl of the supernatant was added to 1mL of 2mol/l NaOH solution.
7. The absorbance was measured in a spectrophotometer at 410nm.
8. One unit of enzymatic activity (U) was defined as the release of 1µmol of pNP released per minute
9. **Lipase Activity Assay**

Lipase activity was assayed with **-nitrophenyl-palmitate (NPP) as substrate [21]. NPP was first mixed with 0.5mL of DMSO (dimethyl sulfoxide) and then diluted to about 50mM with 50mM sodium phosphate buffer (pH 7.0).

Materials required:

1. Overnight culture
2. -nitrophenol
3. Olive oil
4. Sodium tetraborate.

Procedure:

1. To 20ml of LB broth, a single colony was inoculated and incubated at 37C for overnight.
2. Following incubation, the culture was checked for turbidity.
3. The culture was centrifuged at 8000rpm for about 10min.
4. The pellet was discarded and supernatant was made into aliquots for futher use.
5. To 2ml of supernatant 1ml of -nitrophenol (NP) was added and mixed thoroughly.
6. After 5min of preincubation, 0.9mL of substrate solution (olive oil) was added.
7. The reaction was stopped at different intervals (1 and 2min) by heat shock (90^0C, 1min).
8. Following the treatment 1mL of saturated sodium tetraborate solution was added.
9. The contents were mixed thoroughly and the absorbance was measured at 405nm.
10. The activity was estimated according from the standard curve using the -nitrophenol (NP molar extinction coefficient: 1.8 × 104M−1 cm−1).
11. Sample without enzyme solution was used as negative control.
12. One unit of enzyme activity was defined as the amount of enzyme that releases 1µmol of NP per ml/min.

3.8: Shake flask culture

The culture isolated was subcultured furthered and stored on slants.

1. About 250ml of LB broth was inoculated into 500ml of Erlenmeyer flask and the contents were autoclaved.
2. 2% olive oil was added as substrate for the production of lipase.
3. The flasks were inoculated with 20µl of culture.
4. The contents were mixed thoroughly and incubated at 37 °C on orbital shaker at 220rpm for 24hrs.
5. The growth of the culture was obtained after every 8 hours and the growth curve was maintained.

3.9: Bacterial growth curve:

The total increase in the cell size or cell density during the development of the bacteria is called as growth. It is the most unique character of all the organisms. Bacteria require basic requirements for generating their energy for the cellular machinery. As such the growth is mediated by both physical and nutritional factors. Physical factors like pH, temperature and moisture play a vital role, whereas the amount of carbon, nitrogen and other elements are the nutritional factors. Bacteria are unicellular prokaryotes which once they reach a size, they start dividing by binary growth and attains a geometric progression.

To estimate the growth of such a bacteria, the living cells are inoculated into the growth medium supplemented with growth regulators and incubated at appropriate conditions. The bacteria starts feeding on the media components and start dividing in number. This in turn increases the cell biomass. The growth dynamics can be

studied by plotting the cell growth versus incubation time. The curve is mentioned as sigmoid curve and also called as standard growth curve.

The amount of growth increase can be estimated by using the Spectrophotometer. The Spectrophotometer measures the Optical density which is directly proportional to the growth o the organism.

4. Materials Required:

- Nutrient broth
- Micropipettes
- Cuvette
- Conical flask
- Sterile tips
- Culture – Overnight culture of lipase positive isolates.
- Colorimeter

5. Procedure:

1. Each isolated colony from the isolates were inoculated separately into 15ml nutrient broth and kept for overnight incubation at 37C.
2. Following incubation, after every 8 hours of time, 2ml of culture was removed aseptically from the tubes.
3. OD of this culture was measured at 630nm and recorded.
4. The samples were collected after every 30min and the experiment was done for 2 days.
5. The same protocol was followed for each of the isolates.
6. Using this OD value, a standard growth curve was plotted (Absorbance verses time).

3.10: Purification of enzyme

20ml of overnight culture was used for the purification step. All purification steps were carried out at room temperature. The experiment was done for all the ten lipase positive isolates.

3.10.1: **Cell disruption.**

Each isolate was grown for 72hrs in the following medium. The medium consists of the following components.

Chemical	Qty in gm
Olive oil	1%
NaCl	10%
Tryptone	1.50%
Soya peptone	0.50%

The components were added accordingly and the colonies inoculated into 50ml of medium.

The culture was incubated at 37C for overnight.

1. Following incubation, the cells were centrifuged at 10000g for 10min at 40C.
2. The culture supernatant was discarded
3. The pellet was washed twice in 50mM Tris-HCl buffer (pH 8.5).
4. The cells were then homogenized in by ultrasonic treatment for 30min.
5. The cell particles were removed by centrifugation at 10000g for 10min at 40C.
6. The pellet was discarded and the supernatant was collected in a fresh centrifuge tube.

3.10.2: Anion-exchange chromatography

1. The samples collected from the cell disruption were stored in refrigerator until further use.
2. The samples were applied onto sepharose column.
3. The column was equilibrated with 50mM Tris-HCl buffer (pH 8.5).
4. The column was washed with the same buffer thrice and saturated.
5. The column was now eluted with 50mM Tris-HCl buffer (pH 8.5) containing 1 M NaCl.
6. Following elution, the column was eluted with a linear gradient of sodium chloride (NaCl, 0–1M) of the same buffer.
7. The collected fractions were assayed for the protein estimation.
8. The fractions with more or less equal absorbance value were pooled.

9. The pooled fractions are then dialyzed using dialysis membranes against 20mM phosphate buffer (pH 6.0).
10. The active fractions showing lipase activity were pooled.

3.11: Effect of Initial pH on Lipase Production.

The enzyme purified was studied for the characterization with the pH. pH is very critical for the overall maintenance and functioning of the enzyme.

Substrate preparation

Buffer solution: Na_2HPO_4/NaH_2PO_4 50mM (pH=7)

Olive oil emulsion (40ml of olive oil and 60ml of gum arabic–emulsifier solution 5%, w/v)

To 50ml of olive oil emulsion 45ml of buffer solution was added and mixed thoroughly in a homogenizer. The contents are then stored in the refrigerator until further use.

Protocol:

1. The effect of initial pH on lipase production was analyzed from 3.0 to 10.0.
2. The initial medium pH was maintained and adjusted by adding 1M NaOH 1M HCl.
3. 2ml of enzyme solution was added to the contents and the pH was maintained in between 3 to 10.
4. At each pH the specific activity and total activity of the enzyme was calculated.
5. Olive oil is used as substrate.
6. Lipase enzyme (100µg/ml) was used as positive control or standard.
7. The OD values were recorded and standard graph was plotted with specific activity versus pH of the solution.

Determination of enzymatic activity:

- 1ml of crude enzyme solution was added to 9ml of substrate.
- The contents are mixed thoroughly and incubated in a shaker for 30min at T=28°C
- Following incubation the 20ml of sample was titrated with NaOH (50mM, pH=9) solution.

Specific Activity: Specific Activity of an enzyme is the amount of product formed per unit time (enzyme activity) per milligram (mg) of protein. Specific activity reveals the relative purity of the enzyme. It determines whether the enzyme purified is of pure standard or not.

Specific activity can be calculated by the given formula:

Specific Activity = amount of product formed/unit time/mg protein.

From the specific activity, the total activity can be calculated by the given formula.

Total activity = (specific activity) x (total mg protein used in reaction)

3.12: Effect of Initial temperature on Lipase Production.

The enzyme purified was studied for the characterization with the temperature. Temperature studies on the enzyme are very important for determining the overall enzyme stability.

Protocol:

1. The effect of initial temperature on lipase production was analyzed from 20^0C to 40^0C.
2. 2ml of enzyme solution was added to the contents and the temperature of the solution was maintained in between from 20^0C to 40^0C.
3. At each temperature the specific activity and total activity of the enzyme was calculated.
4. Olive oil is used as substrate.
5. Lipase enzyme (100µg/ml) was used as positive control or standard.
6. The OD values were recorded and standard graph was plotted with specific activity versus pH of the solution.

Specific Activity: Specific Activity of an enzyme is the amount of product formed per unit time (enzyme activity) per milligram (mg) of protein. Specific activity reveals the

relative purity of the enzyme. It determines whether the enzyme purified is of pure standard or not.

Specific activity can be calculated by the given formula:

Specific Activity = amount of product formed/unit time/mg protein.

From the specific activity, the total activity can be calculated by the given formula.

Total activity = (specific activity) x (total mg protein used in reaction)

3.13: Taxonomic characterization of isolated bacteria.

3.13.1: Extraction of DNA

Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl-ammonium bromide (CTAB) method.

5.1.1. Composition of Extraction buffer

Stock Solution	Buffer composition
1M Tris HCl	100mM Tris HCl
1M EDTA	100mM EDTA
4M NaCl	1.4M NaCl
	1% CTAB
	Proteinase K - 0.03µg/µl

SDS 20% w/v

Chloroform: isoamyl alcohol (24:1)

Isopropanol

Ethyl alcohol 70% v/v

Protocol:

1. 1ml bacterial culture was centrifuged at 10000rpm for 2min. at 4 °C
2. The supernatant was discarded and the pellet was washed with sterile distilled water.
3. 675µl of extraction buffer was added and incubated at 37°C for about 30min.
4. 75µl of SDS (20%) was added and incubated at 65°C for about 2 hours.
5. The contents are mixed thoroughly and centrifuged at 10000rpm for 10min at 4°C
6. The clear solution was collected in a separate microcentrifuge tube.
7. To the tube equal volume of Chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 10000rpm for 10min at 4°C.
8. The upper aqueous layer was removed and collected in a fresh microcentrifuge tube.
9. To the aqueous portion 0.6 volumes of isopropyl alcohol was added and incubated at room temperature for about 1hour and centrifuged at 10000rpm for 10min.
10. Pellet was cleaned with 500µl of 70% ethanol and centrifuged at 10000 rpm for 10 min at room temperature.
11. The pellet obtained was dried and dissolved in 20µl sterile distilled water.

QUANTITTIVE ANALYSIS OF DNA

PRINCIPLE: Nucleic acids absorb light at a wavelength of 260nm. If a 260nm light source falls on the sample, the quantity of light which passes through the sample can be quantified and the amount of light absorbed can be estimated. For double-stranded DNA, an Optical Density (OD) of 1 at 260nm correlates to a DNA concentration of 50ng/µl, so DNA concentration can be easily calculated from OD measurements. If sample is found to be pure i.e. free from contaminants such as proteins, phenols, agarose, or other nucleic acids), such a sample can be measure using spectrophotometer.

For quantitating DNA or RNA, readings should be taken at wavelengths of 260nm and 280nm respectively.

The reading at 260nm allows calculation of the concentration of nucleic acid in the sample.

- O.D. at 260nm for double-stranded DNA = 50ng/ul of dsDNA
- O.D. at 260nm for single-stranded DNA = 20-33ng/ul of ssDNA
- O.D. at 260nm for RNA molecules = 40ng/ul of RNA

The reading at 280nm gives the amount of protein in the sample.

- Pure preparations of DNA and RNA have OD260/OD280 values of 1.8 to 2.0, respectively.

If there is a contamination with protein or phenol, the ratio will be less than the values given above, and accurate quantification of the amount of nucleic acid will not be possible.

PROCEDURE:

1. To 98µl of TE buffer 2µl of DNA sample extracted was added.
2. The contents are mixed thoroughly and placed in the cuvette holder in the spectrophotometer.
3. The OD at 260nm and 280nm are recorded.
4. Similarly readings were observed for all the ten samples.
5. 100µl of sterile TE buffer was used as blank.
6. The reading obtained were used for calculating the 260/280 ratio.

For example:

dsDNA concentration = 50 µg/mL × OD_{260} × dilution factor

- dsDNA concentration = 50 µg/mL × 0.65 × 50
- dsDNA concentration = 1.63 mg/mL

QUALITATIVE ANALYSIS OF ISOLATED DNA

Agarose gel electrophoresis: Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. We will be using agarose gel electrophoresis to determine the presence and size of PCR products. Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA.

Purpose: To determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product.

Materials needed:

- Agarose
- TAE Buffer
- 6X Sample Loading Buffer
- DNA ladder standard
- Electrophoresis chamber
- Gel casting tray and combs
- DNA stain
- Staining tray
- Pipette and tips

TAE Buffer (1L)

Tris Base 4.84g
Glacial Acetic Acid 1.14ml
0.5M EDTA (pH 8.0) 2 ml
Add Tris base to ~900 ml H_2O. Add acetic acid and EDTA to solution and mix. Pour mixture into 1L graduated cylinder and add H_2O to a total volume of 1 L.

6X Sample Loading Buffer

1 ml sterile H_2O

1 ml Glycerol
Enough bromophenol blue to make the buffer deep blue (~ 0.05 mg).

PROTOCOL

1. Weigh 1.25gm agarose powder and add it to a 500ml flask.
2. Add 200ml of TAE Buffer to the flask.
3. Melt the agarose in a microwave or hot water bath until the solution becomes clear.
4. Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
5. The ends of the casting tray are covered with two layers of tape and the combs are placed in the gel casting tray.
6. The molten agarose with ethidium bromide is poured into the casting tray and cooled until it is solid (it should appear milky white).
7. The combs are pulled out carefully and the tape was removed.

Loading the gel

1. 6µl of 6X Sample Loading Buffer was added to each 25µl PCR reaction.
2. The order each sample to be loaded on the gel was noted along with controls and ladder.
3. 20µl of each sample was pipetted and added to the respective wells.
4. 10µl of the DNA ladder standard was added into the first well to calculate the size of the DNA.
5. Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected.
6. Turn on the power supply to about 100 volts. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – it should not exceed 5 volts/ cm between electrodes. Once the run is completed 3/4th, stop the power and observe the gel in the UV transilluminator.

3.13.2: PCR (POLYMERASE CHAIN REACTION)

Polymerase Chain Reaction is considered to be the most important research tool of the 20th century in molecular biology. It allows the exponential amplification of short DNA sequences. It was invented by Kary Mullis in association with Fred Faloona, Henry A. Erlich, and Randall K. Saiki in the year 1983, while he was working in Emeryville, California for Cetus Corporation.

Components: The basic components and reagents required to set up a 100µl PCR reaction are:

DNA template: The reaction solution should contain at least (100ng/µl).

Primer: These are oligonucleotides that define the sequence to be amplified. Two primer that are complementary to the 3′ ends of each of the sense and anti-sense strand of the DNA target. Primers are designed using the Primer3 software.

$MgCl_2$: It is the cofactor of the enzyme. It is beneficial to optimize the magnesium ion concentration. The magnesium ion also affects the primer annealing, strand dissociation temperatures of template and PCR product and product specificity. Taq DNA polymerase need free magnesium ions for its activity.

Deoxyneuclotide triphosphates (dNTPs): These are the DNA building blocks. dNTPs (dTTP-thymidine triphosphate), dCTP (deoxycyctidine triphosphate), dATP (deoxyadenosine triphosphate) and dGTP (deoxyguanosine triphosphate) solutions neutralized to pH 7.0. Primary stock solution are diluted to 10mM aliquoted and stored at -20^{0}C. A working stock of about 1mM is needed for active amplification. dNTP concentrations between 20 and 200µM is optimum for the reaction.

DNA polymerase: It is an enzyme that catalyzes the reaction. Taq DNA polymerase isolated from *Thermus aquaticus* growing in hot springs was chosen for the experiments since it can withstand high temperatures of about 90^{0}C. But the amplification done is 99% as the enzyme is lacking the proof reading activity.

Principle: Polymerase Chain Reaction or PCR is an *invitro* technique based on the principle of DNA polymerization reaction. It relies on the thermal cycling consisting of repeated cycles of heating and cooling of the reaction using thermostable DNA polymerase, primer sequences and dNTPs. It can amplify a specific sequence of DNA by as many as one billion times and most of the PCR methods can amplify DNA fragments of up to ~10 kilo base pairs (kb) also.

Table 3: table showing the characteristics of the primers designed for the lipase gene.

Primer	Sequences (5'-3')	GC %	Tm Value	Length	Product Size
FP	GACTCCCTCAAGGACAGCAG	60	59.99	20	188bp
RP	GTACCGAACCAGTCGGAGAA	55	60.11	20	

Protocol:

1. All the reagents used are DNase and RNase free.
2. The table below provides standard reaction conditions for PCR. Mg2+ (1.5mM) ; KCl (50mM); dNTPs (200µM); Primers (1µM); DNA polymerase (1-5 units); Template DNA (1pg to 1µg).
3. The amount of template DNA needed according to the sequence.
4. If the thermal cycler is not provided with a heated lid, overlay the mixtures with 1 drop of mineral oil.
5. The contents are mixed thoroughly and placed in the micro titer plate in the PCR machine.
6. The DNA is amplified using the denaturation, annealing, and polymerization times and temperatures listed below in the table.

To set up a 25µl reaction the concentration of the reagent are as follows for Master Mix.

PCR components	Volume (µl)
Nuclease free water	13.5
10X reaction buffer with $MgCl_2$ (1.5mM)	3
dNTP mix (2.5mM)	2
Primer I (10picomoles/µl)	2
Primer II (10picomoles/µl)	2
Taq DNA polymerase (5U)	0.5
Template DNA (50ng/µl)	2
Total volume	**25**

The PCR to amplify the lipase (*lip*) gene was run by using following conditions.

S. No	Temperature	Duration	Cycles
1	94°C	5 min.	1
2	94°C	45sec	30
3	55°C	1min.	
4	72°C	1min.	
3	72°C	10min.	1
4	4°C	Hold	1

CONFIRMATION OF PCR PRODUCTS IN AGAROSE GEL

Following the amplification of the 5 samples, withdraw 10µl of sample from the reaction mixture and the samples are analysed on the electrophoresis through an agarose gel. The amplified products were resolved by electrophoresis in 1.5% of agarose gel using 1x TAE buffer at 50volt for 2½ hours. A 100bp molecular marker was included along with the samples. Gels were visualized by staining with Ethidium bromide (1µl/10ml) and banding patterns were photographed over UV light in a GelDock.

Both strands of the PCR product of the 5 sample products were sequenced by dideoxy chain termination method (Eurofins, Bangalore).

6. 3.13.3: RAPD PCR amplification:

In this project, RAPD markers were used for the analysis of the finger print of the Aeromonas species. About 5 RAPD primers were used for amplification of the isolated Aeromonas species. Random Amplified Polymorphic DNA (RAPD) is the method of generating a biochemical fingerprint of the specific species. On comparison of the finger print, the relation between the species can be estimated. It is sued to analyse the genetic diversity of an organism by using RAPD primers.

Protocol:

Preparation of master mix for PCR:

The master mix is prepared according to the table given below.

S. No	Ingredients for PCR	Volume in µl
1	Molecular biology grade water	76µl
2	10X assay buffer	10µl
3	2.5mM dNTP each	7.5µl
4	Random primer	5µl
3	Taq DNA polymerase	2.5µl

The contents are mixed thoroughly by pipetting.

1. 20µl of the above PCR master mix was added to each of the different PCR tubes.
2. The tubes are labelled accordingly.
3. 1µl of isolated DNA was added to the PCR tubes.
4. The thermocycler parameters were maintained according to the table given below.
5. Following the run on the thermocyler the contents are removed and run on 0.8% agarose gel using TAE buffer.

S. No	Temperature	Duration	Cycles
1	94°C	5 min.	1
2	94°C	1min	35
3	36°C	1min.	
4	72°C	2min.	
3	72°C	7min.	1
4	4°C	Hold	

SNO	BANDS	Sequence
1	RAPD1	GGTGCACGTTA
2	RAPD2	CCATGCCGGAG
3	RAPD3	CTGCCTCGAGG
4	RAPD4	TTAGGGCCGCC
5	RAPD5	TGGTGACCTGA

3.13.4: Phylogenetic analysis of bacterial strain.

The amplified DNA fragments were subjected to sequencing from Eurofins, Bangalore. The deduced sequences were subjected to BLAST algorithm from the National Centre of Biotechnology, Bethesda, MD,USA (http://www.ncbi.nlm.nih.gov) to retrieve for homologous sequences in GenBank and to align them for a phylogenetic analysis. The sequences aligned were run for multiple sequence alignment program using clustal omega. Phylogenetic trees were constructed by neighbor-joining analysis.

Among these 20 isolates the strains which are having high lipase activity are selected and among them 5 of them (B2, B4, B6, B11 and B15) were screened for the genetic diversity experiments. The samples were labelled as S1, S2, S3, S4 and S5.

Results:

4.1: Sample collection and bacterial strain isolation:

Cotton swabs and dressings from the human wounds were collected from hospitals and dispensaries in and around Bangalore. The samples were collected aseptically and processed for the lab for isolation of the strains. Around 20 different colonies were isolated from the samples. All the samples were subjected to preliminary lipase activity using Rhodamine plate assay. Only 5 colonies showed highly reactiveness to the lipase assay.

The lipase producing strains were isolated from different sources. From the total of 80 samples, Rhodamine negative strains were ignored and the positive strains (20 strains) were biochemically characterized using Bergey's Manual of Systemic Bacteriology.

4.2: Strains identification

Biochemical identification: Biochemical tests were done for identification of the isolated lipase positive colonies. According to the Bergey's manual of systemic bacteriology, these tests were selected and were done in triplicates. The tests done were gram staining, Vogues-Proskauer test, oxidase, catalase and citrate Consumption test.

Table 4: table showing the results of biochemical characterization of the species identified. All the 20 isolates were found to be of same result.

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e-ISSN: 2348-6848
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Volume 06 Issue 09
August 2019

TEST	Result
Gram stain	-
VP test	+
Catalse	+
Oxidase	+
Citrate consumption	+

4.3: Lipase assay

The lipase assay was determined by the pNPP (pnitrophenyl palmitate) hydrolysis method, described by Ghori *et al.* (2011). It is the assay where the lipase if present hydrolyzes a triglyceride substrate to form glycerol. This product formed can thus be quantified enzymatically by spectrophotometer at 570nm. Only the samples which are positive on the rhodamine plate assay were selected for further experimentation.

Table 5: table showing the values of the colony froming units and the lipase activity of the 20 isolates. All the values were average of triplciates.

Strain	Cfu	Lipase activity
B1	1.13×10^4	2.39±1.2
B2	$\mathbf{2.87 \times 10^7}$	**41.24**±1.5
B3	0.95×10^5	3.67±0.24
B4	$\mathbf{4.5 \times 10^7}$	**93.24**±1.4
B5	1.89×10^5	7.88±1.4
B6	$\mathbf{3.1 \times 10^7}$	**59.11**±1.9
B7	1.03×10^6	12.32±2.2
B8	1.87×10^6	10.3±3.2
B9	1.11×10^6	11.23±1.0
B10	1.24×10^6	4.9±1.22
B11	$\mathbf{2.67 \times 10^7}$	**32.11**±2.2
B12	1.1×10^6	7.89±1.4
B13	1.5×10^4	1.78±1.1
B14	0.65×10^4	1.34±3.2
B15	$\mathbf{2.15 \times 10^7}$	**23.01**±0.2
B16	0.78×10^6	4.43±0.08
B17	0.88×10^6	4.35±0.2
B18	1.21×10^5	3.24±1.6
B19	1.26×10^6	6.23±1.8
B20	1.63×10^6	5.67±1.9

Among these 20 isolates the strains which are having high lipase activity are selected and among them 5 of them (B2, B4, B6, B11 and B15) were screened for the genetic diversity experiments. The samples were labelled as S1, S2, S3, S4 and S5.

4.4: Lipase Activity Assay.

Lipase activity was assayed with **?**-nitrophenyl-palmitate (**?**NPP) as substrate [21]. **?**NPP was first mixed with 0.5mL of DMSO (dimethyl sulfoxide) and then diluted to about 50mM with 50mM sodium phosphate buffer (pH 7.0).

Only the five samples which showed high lipase activity were selected for the remaining experiments.

Table 6: Table showing the lipase activity and specific activity values of the five isolates. All the values were average of triplicates. Values are represented as values ±s.e.

SNO	Lipase activity (U)	Specific activity
S1	32.34±4.3	3.21±1.8
S2	58.23 ±3.21	4.21±0.9
S3	45.65±4.8	4.04±1.34
S4	94.23±3.3	6.43±2.2
S5	23.45±8.2	3.01±2.3

4.5: Bacterial growth curve:

Bacterial growth was found to be significant for the five samples. Among them the sample 4 followed by sample 3 showed high amount of growth.

Table 7: Table showing the values of biomass growth in presence of substrate. all the values were average of triplciates.

	Time in hours									
Sample	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
S1	0	0.12	0.23	0.25	0.26	0.29	0.34	0.37	0.39	0.52
S2	0	0.11	0.15	0.16	0.18	0.21	0.26	0.28	0.36	0.49
S3	0.18	0.25	0.29	0.35	0.39	0.41	0.58	0.65	0.72	0.75
S4	0.13	0.24	0.33	0.36	0.39	0.52	0.65	0.77	0.87	0.89
S5	0.1	0.15	0.19	0.29	0.36	0.37	0.39	0.45	0.48	0.61

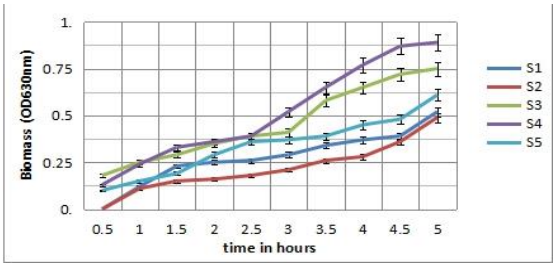

Figure 1: Time growth and biomass production in the presence of olive oil. The 5 isolates which were lipase positive were selected for the growth curve. All the values were average of triplicates.

4.6: Purification of enzyme

20ml of overnight culture was used for the purification step. All purification steps were carried out at room temperature. The experiment was done for all the five lipase positive isolates.

4.7: Effect of Initial pH on Lipase Production.

The enzyme purified was studied for the characterization with the pH. pH is very critical for the overall maintenance and functioning of the enzyme. **Specific Activity:** Specific Activity of an enzyme is the amount of product formed per unit time (enzyme activity) per milligram (mg) of protein. Specific activity reveals the relative purity of the enzyme. It determines whether the enzyme purified is of pure standard or not.

Specific activity and total activity can be calculated by the given formula:

Specific Activity = amount of product formed/unit time/mg protein.

Total activity = (specific activity) x (total mg protein used in reaction)

Table 8: table showing the specific and lipase activity of the five isolates. The effect of pH was shown on the activity of the enzyme. All the values are average of triplicates. LA: Lipase activity; SA: specific activity.

		S1		S2		S3		S4		S5	
SNO	pH	SA	LA	SA	LA	SA	LA	SA	LA	SA	LA
1	3	23.12	1.21	42.34	1.34	32.13	1.26	23.2	1.21	9.45	0.97
2	4	29.87	1.65	52.13	2.67	40.02	2.01	45.56	3.11	12.13	1.76
3	5	31.24	2.12	54.34	4.11	41.23	2.34	87.67	6.86	20.08	2.01
4	6	33.56	2.34	59.65	4.56	43.45	4.32	96.45	7.86	24.56	2.45
5	7	32.34	2.11	42.56	4.01	34.56	3.45	75.34	5.89	21.23	2.03
6	8	28.76	1.78	42.11	3.45	32.14	3.12	64.54	5.32	18.98	1.67
7	9	21.12	1.23	40.08	3.12	30.09	2.04	63.45	5.12	18.56	1.23
8	10	19.56	1.02	34.56	2.3	23.45	1.45	32.34	2.12	17.32	1.12

All the five isolates showed significant activity towards the pH. Isolate 4 was shown to have high stability in terms of pH. And the enzyme was found to be highly stable at pH 4. The specific activity was found to be 96.45, 59.65, 43.45, 33.56 and 24.56 for S4, S2, S3, S1 and S5 respectively.

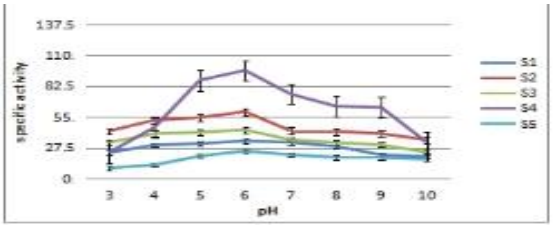

Figure 2: graph showing the effect of pH on specific activity of the enzyme isolated from the five isolates. S1, S2, S3, S4 and S5 are the isolates. All the values are average of triplicates. Values are expressed as activity ±s.e

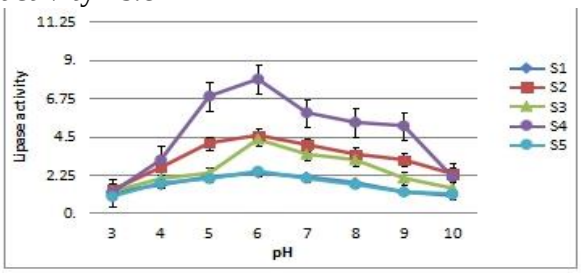

Figure 3: graph showing the effect of pH on lipase activity of the enzyme isolated from the five isolates. S1, S2, S3, S4 and S5 are the isolates. All the values are average of triplicates. Values are expressed as activity ±s.e

4.8: Effect of Initial temperature on Lipase Production.

The enzyme purified was studied for the characterization with the temperature. Temperature studies on the enzyme are very important for determining the overall enzyme stability.

Table 9: table showing the specific and lipase activity of the five isolates. The effect of temperature was shown on the activity of the enzyme. All the values are average of triplicates. LA: Lipase activity; SA: specific activity.

		S1		S2		S3		S4		S5	
SNO	Temp	SA	LA	SA	LA	SA	LA	SA	LA	SA	LA
1	20	20.12	2.01	41.23	1.78	33.45	1.87	24.56	1.11	10.21	1.21
2	22.5	31.23	2.45	54.67	2.78	41.23	2.01	42.34	2.87	11.34	1.45
3	25	32.34	2.65	59.87	4.19	45.32	2.31	89.65	5.89	19.76	2.09
4	27.5	36.78	3.56	62.34	4.8	48.9	4.56	97.89	6.78	23.45	2.87
5	30	31.23	3.12	45.67	4.53	37.89	3.55	76.87	6.8	20.23	2.12
6	32.5	28	2.13	41.11	3.9	35.43	3.11	68.79	5.45	19.99	1.56
7	35	20.1	2.08	38.79	302	31.23	2.54	60.09	5.12	18	1.11
8	37.5	17.89	1.67	35.55	2.56	21.38	2.32	56.78	4.89	16.78	1.02
9	40	16.43	1.24	32.34	2.11	20.12	2.04	56.09	4.32	15.23	0.45

All the five isolates showed significant activity towards the temperature. Isolate 4

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August 2019

was shown to have high stability in terms of temperature. And the enzyme was found to be highly stable at 27.5^{0}C. The specific activity was found to be 97.89, 62.34, 48.9, 27.5 and 23.45 for S4, S2, S3, S1 and S5 respectively.

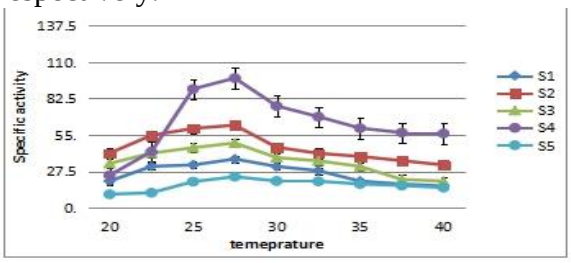


Figure 4: graph showing the effect of temperature on specific activity of the enzyme isolated from the five isolates. S1, S2, S3, S4 and S5 are the isolates. All the values are average of triplicates. Values are expressed as activity ±s.e

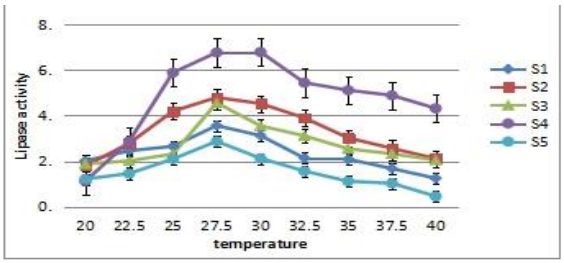


Figure 5: graph showing the effect of temperature on lipase activity of the enzyme isolated from the five isolates. S1, S2, S3, S4 and S5 are the isolates. All the values are average of triplicates. Values are expressed as activity ±s.e

4:9 Taxonomic characterization of isolated bacteria.

6.1.1. 4.9.1: Extraction of DNA

6.1.2. Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N- trimethyl- ammonium bromide (CTAB) method. The DNA was quantified using spectrophotometer and the DNA was sued for further experiments.

4.9.2: PCR (POLYMERASE CHAIN REACTION)

The primers designed using primer3 software was used for the amplification. The product length was found to be 188bp. The five samples were amplified using the primers. The amplified product was found to be 188bp approximately.

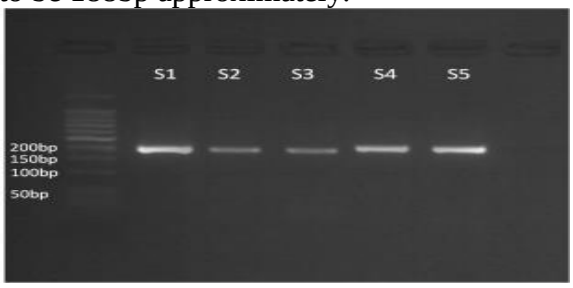


Figure 6: gel showing the amplified products of the five isolates S1, S2, S3, S4 and S5. 50bp DNA ladder was used as marker.

7. 4.9.3: RAPD PCR amplification:

In this project, RAPD markers were used for the analysis of the finger print of the *Aeromonas* species. About 5 RAPD primers were used for amplification of the isolated Aeromonas species.

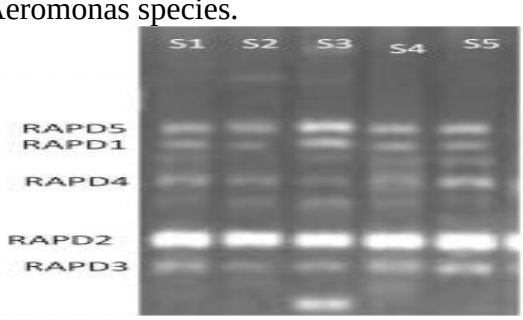


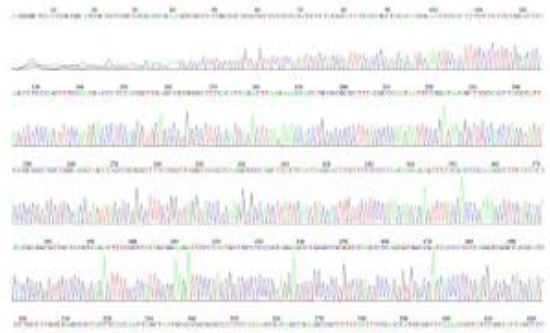
Figure 7: gel showing the RAPD bands amplified using the five primers. The bands were run on 0.8% agarose gel. All the bands were measured suing the molecular markers.

Around 7 polymorphic DNA bands were obtained using the 5 RAPD primers. All the bands amplified shows relatedness among the isolates. Although RAPD does not give authentic differentiation of closely related organisms it can be still used as preliminary screening for relatedness. All the bands amplified showed similarity among the isolates. This proves of the possible relatedness among the family.

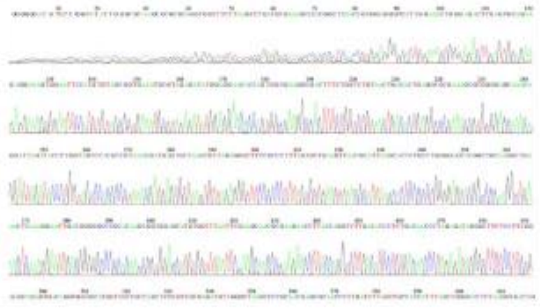
4.9.4: Sequencing results:

The sequences obtained were retrieved in FASTA format and undergone MSA using clustal omega.

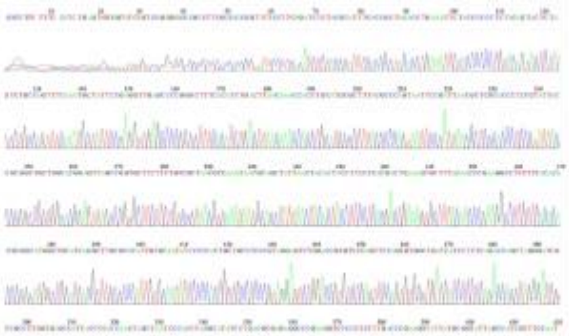
>SUQ143226.1 Sample1, partial cds



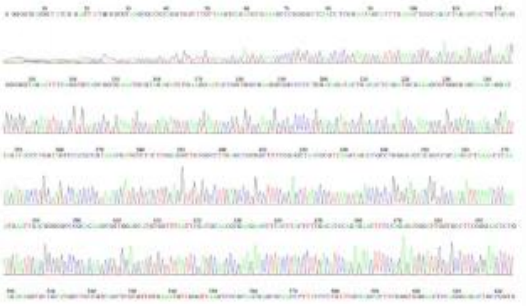
>SUQ143227.1 Sample2, partial cds



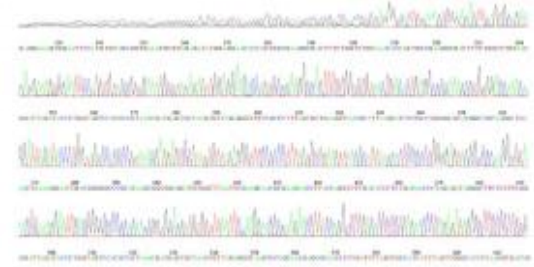
>SUQ143228.1 Sample3, partial cds



>SUQ143229.1 Sample4, partial cds



>SUQ143224.1 Sample5, partial cds



The amplified fragments were sequenced and obtained as FASTA sequences. The sequences were aligned using MSA to find the relatedness among the members.

SUQ143226.1
GCGGCCGCATCCGTGCTCAAG------
AGCCCGACCCTGGACGCTGCCGCCCT
GTGGAAG
SUQ143228.1
GCTGCGGCGTCCGTGCTGAAG------
AGCCCGACCCTGGATGCGGCCGCCCT
GTGGAAG
SUQ143229.1
GCCGCTGCATCTGTAGTG---------
TTGCAGGGGCTGGATGCCGCCAAGG
TATGGAAG
SUQ143224.1
CAGGATGCCAAAGGCAACATCAGCC
TGCCGGGCGCTTATACGATTAATGGC
CTCAATGGC
SUQ143227.1
CAGGATGCACTGGGCAACACCAACC
TGCCCGGAACCTATACCCTAGC---
CGTGACTGGC
SUQ143226.1
CAGGACGCCCTGGGCAATACCAGCC
TGCCTGGCACCTATACCCTGTC---
GGTGAGCGGC
SUQ143228.1
CAGGATGCCAAGGGCAACACCAGCC
TGCCGGGCACCTATACCCTGAG---
CGTGACCGGC
SUQ143229.1
CAGGATGCGTTGGGCAATACCAGCCT
GCCCGGCACCTATACCCTGGC---
CGTGACCGGC
SUQ143224.1
GGGGTGCCCTTCCTGACCCAGCTGGA
CAATGAGCAGTTCCTGCCGCAGGAG
CAGAAGGGT
SUQ143227.1
GGCAGCCCCTTCCTGACCCAGCTGGA
TGCCGAGAACTTCCTGCCCCAGGAGC
AGAAGGAT
SUQ143228.1

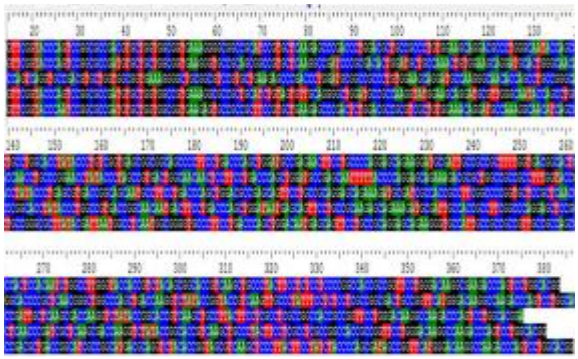


Figure 8: Picture showing the multiple sequence alignment using the BioEdit programme. SUQ143224.1,SUQ143227.1, SUQ143226.1,SUQ143228.1 and SUQ143229.1 are the legends for the sequences from the top to bottom.

Figure 9: Dendrogram generated using clustal omega. SUQ143224.1: S5; SUQ143226.1: S2; SUQ143227.1: S3; SUQ143228.1: S4; SUQ143229.1: S5.

A phylogenetic tree drawn by the neighbour joining method suggested of the relatedness among the species. Basing on the e score and the blast search score, the fragments were closely matching to the members of the *Aeromonas* family.
SUQ143224.1(S5); SUQ143226.1 (S2); SUQ143227.1 (S3); SUQ143228.1 (S4); SUQ143229.1(S5) were found to match to *Aeromonas salmonicida, Aeromonas hydrophila, Aeromonas veronii, Aeromonas enteropelogenes and Aeromonas caviae* respectively.

Discussion:

Clinical samples collected from wound in hospital environment were screened for the lipase producing organisms. Lipase producing bacteria especially *Aeromonas* species are predominant species which live on the hospital wastes. They harbour the area with the help of the lipase protein as such the gene is very critical for its pathogenesis. Such a gene would be of great interest to study as it might lead to the discovery of novel antibacterial targets.
Samples collected were processed to the lab aseptically and screened for the lipase producing organisms. Rhodamine plate assay was used for initial screening of bacteria. About 60 colonies obtained were screened and only 20 of them were found to be positive for the lipase producing organisms.

Among the 20 isolates screened all of them are lipase positive. This was confirmed on the rhodamine plate assay. Among these 20 only 5 isolates were found to be producing high lipase content. These 5 isolates only were further used for experimentation. The lipase enzyme was produced on shake flask and the enzyme was extracted in pure form. The purified enzyme thus separated was subjected to stability studies. The strain 4 was found to better in terms of lipase production. The enzyme was found to be stable at pH 4 and at temperature 27.5^{0}C.
DNA was extracted from all the five isolates and the gen lipase (*lip*) was amplified with the primers specified. The bands obtained was found to be at 188bp approximately and the bands were eluted from the gel and purified. The bands were further sequenced using Sanger's method of sequencing. The sequences obtained were run on Blastn and the phylogeentic analysis was determined to find the relatedness of the species. From the multiple sequence alignment and the e score obtained suggests that S5, S2, S3, S4 and S5 were found to match to *Aeromonas salmonicida, Aeromonas hydrophila, Aeromonas veronii, Aeromonas enteropelogenes and Aeromonas caviae* respectively.

The RAPD fingerprint obtained 7 polymorphic DNA bands using the 5 RAPD primers. These bands confirms of the relatedness among the species with a single

family. All the species belong to the same family.

Conclusion:

The present study was designed to study and isolate the enzyme lipase form the clinical wastes. The bacteria isolated were confirmed of the species *Aeromonas*. These strains produced lipase enzyme which might be of great interest for the further studies. The enzyme was isolated and purified from all the strains and was confirmed of the stability.

The PCR bands obtained were sequenced and matched to *Aeromonas* species. All the 5 species isolated belong to *Aeromonas* species. The enzyme was purified and study for its characteristics. Further the enzyme need to be purified at HPLC grade and studied for its structure. This study might pave way for the elucidation and characterization of the molecular pathogenesis associated with the lipase gene.

Patients with prolonged enteritis required treatment. A quinolone was the drug of choice, although increased occurrence of quinolone-resistant *Aeromonas* spp. strains has been reported in industrialized countries Regarding the β-lactam antibiotics, *Aeromonas* spp. strains analyzed in this study were, as expected, uniformly resistant to ampicillin, whereas third-generation cephalosporins, such as cefotaxime, showed good activity. These results are in accordance with those reported by other authors, showing that third-generation cephalosporins are active against *Aeromonas* spp. The percentage of strains with resistance to chloramphenicol, tetracycline, or trimethoprim-sulfamethoxazole ranged from 22.9% to 45%. These levels of resistance are likely related to the extensive use of these antimicrobial agents in unindustrialized countries.

Hence novel antibacterial targets can be found which might help the biologists to design effective drugs against many of the antibiotic resistant nosocomial infections.

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