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A Study on Lipase Producing Organisms Isolated from Oil Polluted Area of Erode District

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Abstract

Lipase is an enzyme produced by hydrolyzing the triglycerides, diglycerides and monoglycerides into fatty acids and glycerol. Lipase are carboxyl esterase that catalyze the hydrolysis of acyl glycerol containing fatty acid chains greater than 10 carbon atoms in length. Lipases are ubiquitous enzyme. It can also catalyze reverse reactions of fatty acid breakdown, such as esterification and trans-esterification in non-aqueous environments. Lipase have been identified and isolated from bacteria, fungi, plants, and animals. The novel biotechnological applications have been successfully established using lipase for the synthesis of biopolymers, biodiesel and some flavor compounds. Microorganisms having ability to produce lipase enzyme. For example, *Bacillus Prodigiosus, B. pyocyaneus*, *B. fluorescens, B. subtilis, B. pumilus* and *B. subtilis* etc. The objective of the present study is isolated and screened the lipase producing organism from oil contaminated Soil and the production of lipase by titrimetric method.

Keywords

Lipase, oil pollution, enzyme, biotechnological application.

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INTRODUCTION:

Lipase was first discovered by Claude Bernard in 1856. It is an enzyme produced by hydrolyzing the triglycerides, diglycerides and monoglycerides into fatty acids and glycerol (Peterson and Drablos, 1994). Lipase are carboxyl esterase that catalyze the hydrolysis of acyl glycerol containing fatty acid chains greater than 10 carbon atoms in length. Lipases are ubiquitous enzyme. It can also catalyze reverse reactions of fatty acid breakdown, such as esterification and trans-esterification in non-aqueous environments. These enzymes are also demonstrating

enantioselectivity[5]. The lipase plays an essential role in the digestion, transport and processing of dietary lipids. Lipase is an important enzyme, it performs various functions in food, dairy, detergent and pharmaceutical industries. The lactic acid bacteria are involved in the development of flavour and aroma in fermented food products. Partial purification of lipase was obtained by ammonium sulphate precipitation and dialysis. Production of lipase was optimized and characterized. Animal lipases are found in different organs, such as pancreas and digestive tract[9].

Lipase have been identified and isolated from bacteria, fungi, plants, and animals. The novel biotechnological applications have been successfully established using lipase for the synthesis of biopolymers, biodiesel and some flavor compounds[2]. Microorganisms having ability to produce lipase enzyme. For example, *Bacillus Prodigiosus, B. pyocyaneus*, *B. fluorescens, B. subtilis, B. pumilus* and *B. subtilis* etc. In some themophilic *Bacillus* sp. was produced from lipase enzyme. Not only that *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* to produce this enzyme[3]. The present study isolation and production of lipase from bacteria the following objectives to screen and isolate the lipase producing organism from oil contaminated Soil, Assay the production of lipase by titrimetric method, optimize the lipase production by various parameters and identify the isolate by biochemical and molecular characterization.

MATERIALS AND METHODS

Sample collection

Soil samples were collected at 15 - 20 cm depth with the help of sterile spatula in a sterile plastic bag from oil contaminated area in Erode (Dt). After the soil samples were brought to laboratory[4].

Isolation of lipase producing organisms

Serial dilution method was performed for isolation of lipolytic bacteria from collected soil sample. One gm of soil samples were transferred to 10 ml of 0.85% sterile saline water. Serial dilution was performed by transferring one ml of aliquot from each of the samples to nine ml of 0.85% saline water up to 10^{-6} dilutions were prepared.

From the diluted samples 0.1 ml of 10^{-4} and 10^{-5} was plated on nutrient agar and Tributyrin agar. Plates were incubated for 48-72 hours at 37°C (Davender Kumar *et al.*, 2012). Colonies with zone of clearance was picked and stored in to TBA slant for further studies.

Identification of isolated strains

The following identification tests were undertaken for the identification of isolated organisms. Preliminary tests -Gram staining, Endospore staining Motility test and biochemical test.

Identification of Lipase producing Bacteria

Lipolytic bacteria were identified by performing the following tests, Biochemical test described[6].IMViC Test - Indole production Test, Methyl red test, Voge's Proskauer Test**,** Citrate utilization test, Catalase test**,** Oxidase test, Starch hydrolysis test and Carbohydrate fermentation test

Enzyme assay

Titrimetric method

Lipase activity was measured by titrimetric method using olive oil as a substrate with some modification. Olive oil (10% v/v) emulsified with gum Arabic (5% w/v) in 100mM potassium phosphate buffer (pH 7.0). One ml of broth culture was added to the emulsion. Then incubate 15 minutes at 37 °C. The reaction was stopped, and fatty acid was extracted by adding 1 ml of acetone: ethanol (1:1). Titrated with 0.5M sodium hydroxide with phenolphthalein as indicator. During the titration process the emulsion white colour change in to pink colour. At the time volume measure to the burette containing NaOH solution.

Calculation

$$\text{Activity} = \frac{(\text{Vs} - \text{VB})\text{N} \times 1000}{\text{S}}$$

Vs - The volume of 0.5M NaOH solution (burette) consumed by the enzyme substrate olive oil (conical flask) (ml).

V_B - The volume of 0.5M NaOH solution consumed (burette) by the substrate (control) only olive oil (ml).

N - The molar strength of the NaOH solution used for titration (0.5M).

S - The volume of substrate olive oil (1ml).

One unit (U) of lipase enzyme is defined as the amount of enzyme required to liberate 1µmole of fatty acids from triglycerides.

Optimization of lipase

Effect of pH

The effect of pH on lipase production was determined by incubating the bacterial culture flask with different pH values (5.5- 8.0) and prepare Tributyrin (TBA) broth containing (Peptone-5gm, Beef extract-3gm &Tributyrin- 10 ml per liter) using the standard lipase

assay method. The pH was adjusted with 1N NaOH in broth (Citrate phosphate buffer for pH 5.5 to 6.5; Phosphate buffer for pH 7.0 to 8.0).

Effect of temperature

The effect of temperature was determined by incubating the culture flasks with different temperature (30°C- 55°C) in Tris – HCL buffer (pH 6.0) using (Peptone-5gm, Beef extract-3gm & Tributyrin- 10 ml per liter) determine the lipase production.

Effect of carbon sources

Impact of various carbon sources on lipase production was investigated by cultivating the bacterial strain. Carbon sources like glucose, mannitol, lactose, starch and sucrose. To select mannitol as a good carbon source of lipase production, for preparing TBA broth instead of using beef extract, mannitol can be used (Peptone-5gm, Mannitol-3gm, Nacl-5gm per litre) and check the lipase production using mannitol as a carbon source.

Effect of nitrogen sources

Nitrogen sources on lipase production very important to the cultivating of bacterial strain. The nitrogen sources like yeast extract, beef extract, tryptone and peptone. Choosing very specific for yeast extract and tryptone (beef extract- 3gm, yeast extract/tryptone- 3gm Tributyrin- 10 ml per liter) as nitrogen sources of lipase production. Lipase activity check by using titrimetric method.

Genomic DNA extraction

1.5ml bacterial culture transferred to sterile Centrifuge tube. Centrifuged at 10000 rpm for six mins. The supernatant was discarded and drained it. The bacterial pellets were resuspended in 1.5ml of 1M Sodium chloride and add 250 µl of 10% SDS. The content was incubating at 70 ºC for 15 mins. The sample was thoroughly extracted using 200 µl of Phenol: Chloroform: Isoamyl alcohol. Then it was centrifuged at 10000 rpm for 10 mins. And the aqueous layer was transferred to another sterile centrifuge tubes and 2.5 volume of ice cold 100% ethanol was added to the content. The DNA was recovered by centrifuging at 10000 rpm for 10 mins. After that the supernatant was discarded and the pellet was air dried. Finally, the DNA was resuspended in TE buffer of 40 µl volume. After the DNA visualized in 0.8% agarose gel electrophoresis[6].

Polymerase Chain Reaction (PCR)

The genomic DNA was subjected to Polymerase Chain Reaction for amplification of 16S rRNA sequences by using the universal primer. Forward 8F (5'-AGAGTTTGATCCTGATCCTGGCTC-3') reverse 1392R (3'-ACGGGCGGTGTGTAC-5') primer. The total reaction volume was set up to 50 µl. To the sterile PCR tubes Red dye master mix – 25 µl, Genomic DNA 6 µl, forward primer 1 µl(10picomoles) reverse primer 1 µl (10 picomoles) and distilled water - 17 µl was added. And each tube was transferred to PCR machine. Denaturation 94 ºC for 5 mins, annealing 55 °C for 1 min, Extension 72 °C for 1.5 mins and it was set up to 32 cycle.

The product of the PCR amplification was analyzed by agarose gel electrophoresis. 5 µl PCR products were mixed with 1 µl of gel loading buffer. The mixture was loaded on agarose gels (1% w/v) in TBE buffer (pH 8.3). The separation was carried out at 90V for 40 min in TBE buffer. The resulting DNA patterns were examined with UV light and photographed then analyzed by gel documentation system[1].

RESULTS:

Isolation of bacteria

The collected samples are serially diluted were plated to nutrient agar and Tributyrin plates and kept for incubation at 37 °C for 24-48 hours.

Fig.1. The Plates showing the lipolytic halos present colonies by lipase producing bacteria isolated from the soil samples.

Lipase activity

After incubation, the zone formation was observed around the colonies. A clear zone indicates the production of lipase by the microbes (Fig.2). Lipase enzyme producing organism having ability to degrade lipid into glycerol and free fatty acid.

The isolated lipase enzyme producing colonies were named as KL1, KL2, KL3, KL4, KL5 and KL6 respectively.

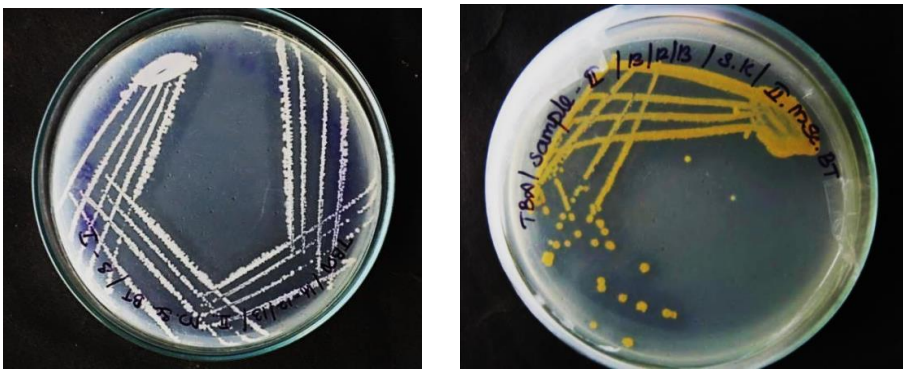

Fig.2. The plates showing lipolytic activity in Tributyrin agar plates G and H indicates KL1 and KL2.

Identification and characterization of bacteria

The morphological, biochemical and physiological features of the KL1, KL2, KL3, KL4, KL5 and KL6 isolates are performed.

Gram staining

Under the microscopic view, violet colour gram-positive Rod-shaped bacteria (KL1, KL2 and KL4) was observed. Then violet colour gram positive cocci shaped bacteria (KL 3, KL5 and KL6) were observed.

Spore staining

Among the six isolates, KL1 has the ability ability to form the spores, the spores are found in terminal region. The remaining five isolates (KL 2, KL 3, KL4, KL5 and KL6) are found to non-spore forming bacteria.

Table.1 Morphological identification of isolates

S. No	Techniques	KL1	KL2	KL3	KL4	KL5	KL6
1.	Gram staining	+	+	+	+	+	+
2.	Shape	Rod	Rod	Cocci	Rod	Cocci	Cocci
3.	Motility	+	+	+	+	+	+
4.	Spore staining	+	-	-	-	-	-

+ indicates positive result and – indicates negative result.

Indole test

The indole test showing the six (KL1, KL2, KL3, KL4, KL5 and KL6) isolates not changing the red layer in the top of the broth, it indicates absence of enterobactericae.so, the isolates are not utilizing the tryptophan amino acid.

Methyl red test

The six (KL1, KL2, KL3, KL4, KL5 and KL6) isolates was found to be negativr, it indicates glucose was not fermented by the cell, thereby no acid production was found.

Vogesprosker test

The isolates KL1, KL2, KL3, KL4, KL5 and KL6 showed negative results. The tubes remained yellow in colour. If acetone was produced, then the tube turns into red colour indicating a positive result. It indicates absence of enterobactericeae.

Citrate utilization test

Sometime citrate acts as a carbon source and also involved major role of Krebs's cycle. KL2 utilize the citrate as a carbon source and it give positive results. Colour change of medium from green to deep Prussian blue was observed. There is no colour change in other five isolates. Indicating that, the citrate was not utilized as a carbon source. The remaining isolates were negative to Citrate utilization test.

Oxidase test

In this test KL2 and KL4 given positive reaction were shown by the development of deep purple colour within 20 seconds. Other four (KL1, KL3, KL5 and KL6) isolates given negative result. Development of purple colour indicates cytochrome oxidase production and represents positive results. If the colour is not changed it indicates negative results.

Catalase test

During the aerobic respiration in the presence of oxygen, the isolates to produce hydrogen peroxide which is lethal to the cell. The enzyme catalase present in all isolates and breaks down hydrogen peroxide to hydrogen and oxygen to form bubble.In this test, the six (KL1, KL2, KL3, KL4, KL5 and KL6) isolates released air bubbles observed within 10 seconds, it indicates the production of catalase enzyme.

Starch hydrolysis test

The (KL1, KL2, KL3, KL4 and KL6) five isolates showed a zone of clearance around the colony. It shows that the culture produce amylase, the remaining portion of the plates appeared in blue colour.

Table.2 Biochemical test for isolated strain

S. No	Test	KL1	KL2	KL3	KL4	KL5	KL6
1.	Indole	-	-	-	-	-	-
2.	Methyl red	-	-	-	-	-	-
3.	Vogesprosker	-	-	-	-	-	-
4.	Citrate utilization	-	+	-	-	-	-
5.	Oxidase	-	+	-	+	-	-
6.	Catalase	+	+	+	+	+	+
7.	Starch hydrolysis	+	+	+	-	+	+

+ indicates positive result and – indicates negative result.

Carbohydrate fermentation test

The six (KL1, KL2, KL3, KL4, KL5 and KL6) isolates were subjected to different type of sugar like Dextrose, Fructose, Lactose and Sucrose. Sugar fermentation was not found in dextrose and lactose medium.

While in the fructose tube KL1, KL4, KL5 and KL6 fermented the fructose and produced acid, it gives yellow colour in the medium.

While in sucrose medium KL2 unable to fermenting the sugar but other five (KL1, KL3, KL4, LK5 and KL6) isolates are capable of fermenting the sucrose and producing the gas.

Table.3 Carbohydrate fermentation test

S.No	Sugar (1%)	KL1	KL2	KL3	KL4	KL5	KL6
1.	Dextrose	-	-	-	-	-	-
2.	Fructose	+	-	-	+	+	+
3.	Lactose	-	-	-	-	-	-
4.	Sucrose	+	-	+	+	+	+

+ indicates positive result and – indicates negative result

Lipase assay

Titrimetric method

Lipase activity was measured by titrimetric method using olive oil as a substrate. One unit (U) of lipase enzyme is defined as the amount of enzyme required to liberate 1µmole of fatty acids from triglycerides.

Optimization of lipase

Effect of pH

The isolates (KL1, KL2, KL3, KL4, KL5 and KL6) were tested with various pH from 5.5 – 8.0 at constant temperature 37 °C the enzyme production was found to be around 350 to 750U/ml, maximum enzyme production was recorded at pH 7. Gradual increase in the production was noticed from pH 5.5 to 7. When compared to other isolates KL1 to produced high amount of lipase 750U/ml at 5.5 pH. The enzyme production started decreasing from pH 7.5.

The present study revealed that the production of lipase by the isolates requires neutral pH. Most of the earlier studies also revealed the optimum pH range between 6.0 to 7.0 for the growth of bacterial strains and enzyme production.

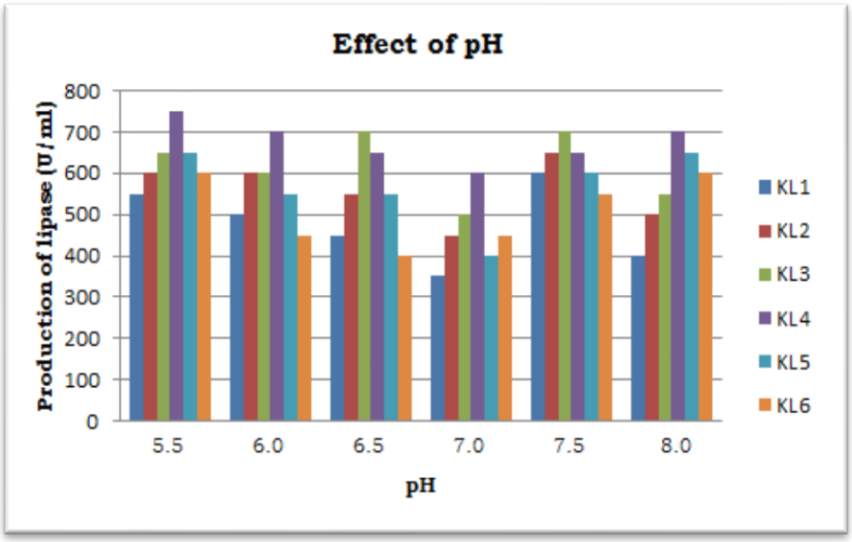

Fig. 3. Production of lipase by using different pH.

Effect of Temperature

Effect of temperature was studied at various temperatures ranging from 30 °C – 55 °C at constant pH of 7.0. While using different temperature the KL1 strain more amount of lipase production was recorded at 30 °C. When temperature increase the enzyme, production started decreasing 750U to 350U/ml with respect to the temperature 30 °C – 40 °C.

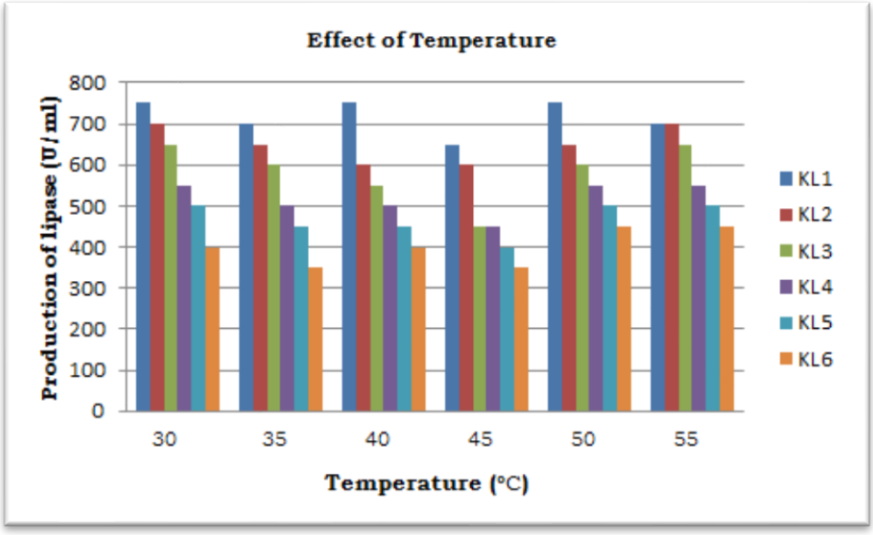

Fig. 4. Production of lipase by using different Temperature.

Effect of Carbon sources

In the present study among the various carbon sources tested mannitol was yielded minimum lipase production compared to other carbon sources. Lipase production was influenced by concentration of mannitol present in the medium. Maximum lipase production was obtained LK 1 produced 750 U/ml beef extract compared to the mannitol. The amount of carbon source in culture medium is important for the growth and production of extra cellular enzyme in bacteria. The present study resulted that the beef extract was one of the best carbon sources.

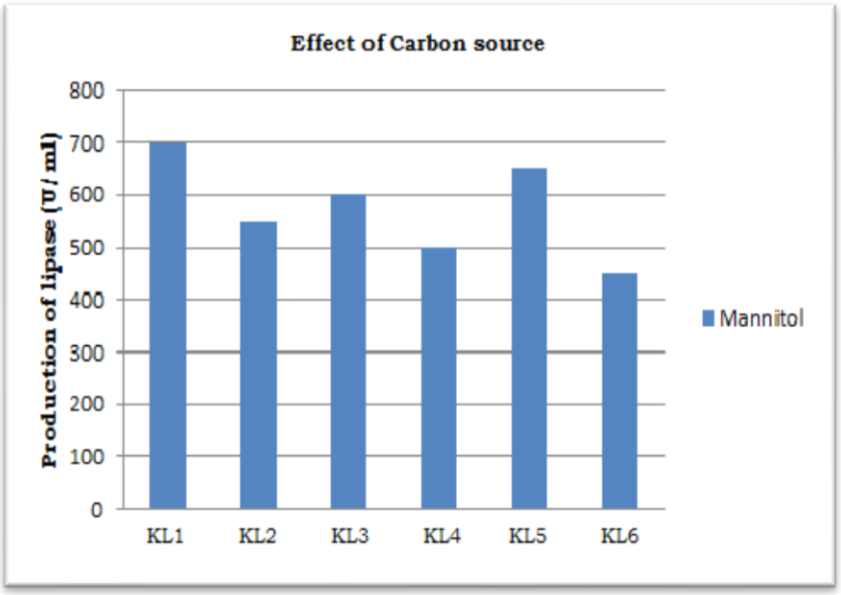

Fig. 5. Production of lipase by using Carbon source.

Effect of Nitrogen sources

Various nitrogen sources such as yeast extract, beef extract, tryptone, peptone and gelatin used to produce lipase enzyme. Comparing to other isolates, KL1 and KL3 produced maximum of lipase production was obtained 700U/ml of tryptone and yeast extract.

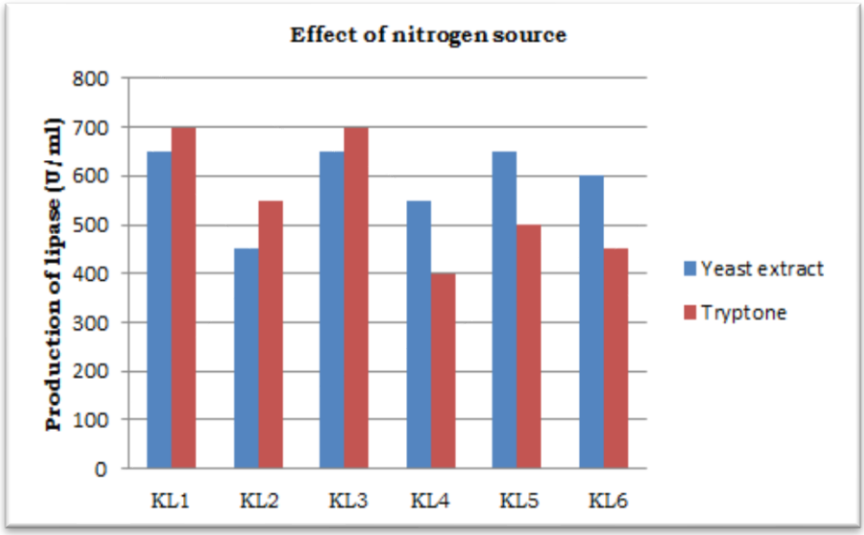

Fig. 6. Production of lipase by using Nitrogen sources.

Genomic DNA extraction and Polymerase chain reaction

The genomic DNA was extracted from the six (KL1, KL2, KL3, KL4, KL5 and KL6) isolates by using standard method. And the band was observed under UV trans illuminator. The genomic DNA was amplified by using 16s rRNA primer 8F and 1392R primer for molecular characterization. The amplified PCR product was run in 1.5% agarose and viewed in agarose gel electrophoresis. The orange colour band were observed in lane (3, 4, 5, 6, 7 and 8) indicating the amplification of 16s rRNA. The product was sent to sequencing for further analysis.

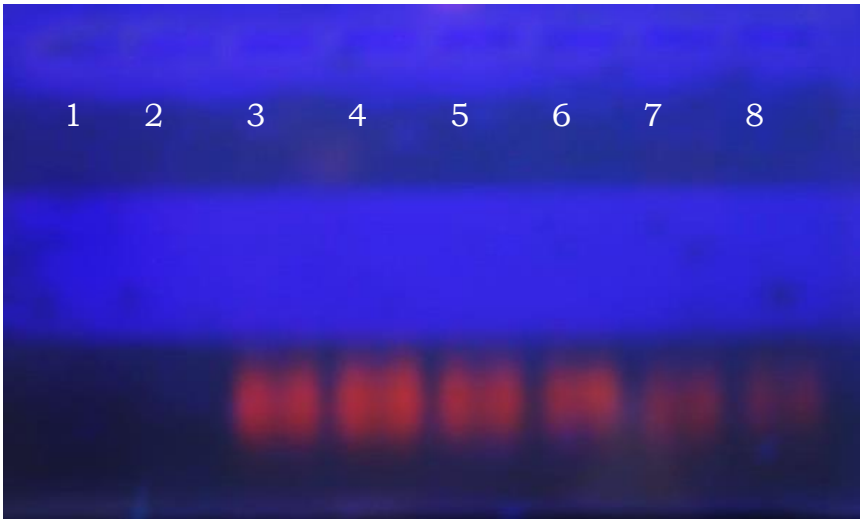

Fig.7. The PCR product the lane 3 – 8 indicates KL1 – KL6.

DISCUSSION:

Soil sample were subjected to serial dilution. Serially diluted samples were plated to nutrient agar plates and kept at 37°C for 24-48 hours and the plates were observed for growth. Lipase enzyme producing organisms was isolated and screened. The isolated organisms are named as KL1, KL2, KL3, KL4, KL5 and KL6. Morphological, Biochemical and Physiological features (Genomic DNA, PCR) can be used to identifying the organisms.

Using titrimetric method, lipase assay can be performed. Optimization can be done using various parameters such as pH (5.5 - 8.0), temperature (30 ºC - 40ºC), different carbon sources (peptone, mannitol) and different nitrogen sources (tryptone, yeast extract). From this analysis, KL1 and KL3 produced more amount of lipase enzyme was produced at 30 – 35 ºC at the pH 5.5 - 7.0. Peptone showed maximum growth of lipase production. Yeast extract was the best carbon source utilized by the bacteria and produced more amount of lipase. From this analysis, the organism can be identified as KL1 - *Bacillus* sp, KL2 - *Lactobacillus* sp, KL3 – *Micrococcus* sp, KL4 - *Lactobacillus* sp, KL5 - *Staphylococcus* sp and KL6 - *Staphylococcus* sp. Molecular characterization of genomic DNA extraction and PCR product obtained all isolates. The product was sent to sequencing for further analysis.

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