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Original Article

Isolation, Identification of Lipase Producing Bacteria *Alcaligenes* Species from Dairy Effluent and Production Optimization of the Enzyme

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Abstract

The global market for microbial lipases is expanding rapidly, driven by the shift toward green chemistry, enzyme-based industrial processes, and the preference for microbial sources over animal or plant alternatives. Microbial lipases find applications in a wide range of industries and there is a continuous look for novel lipase producers from the relatively unexplored industrial effluents. In light of this, dairy effluents were screened for lipase producing bacteria. Five bacterial strains were isolated and identified as *Alcaligenes* species using 16S rRNA sequencing technique. Among them, the isolates, designated as DPE-3 and DPE-5, and identified as strains of *Alkaligenes faecalis*, were found to produce relatively higher amounts (38.1 and 50.8 units/mL respectively) of the enzyme lipase. These two bacterial isolates were studied further for the production optimization. The optimum pH for the isolate DPE-3 was found to be pH 7 and the optimum pH for the isolate DPE-5 was found to be pH 4 indicating the adaptability and therefore industrial usefulness of the isolates. The optimum production temperature for the isolate DPE-3 was 37^0C while the isolate DPE-5 could produce the enzyme invariably at all the tested temperatures (30^0C, 37^0C and 42^0C). Further studies revealed starch as an optimum carbon source and ammonium sulphate as an optimum nitrogen source for the enzyme production. Addition of sodium acetate as an inducer in the medium enhanced the enzyme production for both the isolates. Thus, the study brought out the potential of novel strains of *Alcaligenes faecalis* as promising cell factories for the industrially important lipase enzyme production. However, a systematic and statistical production optimization studies are a way to go further.

1. Introduction:

Microbial lipases (EC 3.1.1.3; triacylglycerol acyl hydrolases) are versatile biocatalysts with widespread application in industrial, pharmaceutical, and environmental fields. Unlike simple esterases, true lipases act preferentially at the oil-water interface (interfacial activation) to hydrolyze long-chain triacylglycerols into free fatty acids, diacylglycerols, monoacylglycerols, and glycerol [1]. Microbial sources of lipases include primarily bacteria (*Bacillus*, *Pseudomonas*), but also fungi (*Rhizopus*, *Aspergillus*, *Mucor*), and yeasts (*Candida*) [2].

Microbial lipases have received much more attention in industry because of the availability of wide range of hydrolytic and synthetic activities, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and easy cultivation of microbes on inexpensive media [3,4].

Lipolytic enzymes are highly diversified in their industrial applications. Lipases have emerged as key enzymes which are useful in food, dairy, paper, textile, leather, detergent industries, wastewater treatment, production of the chemicals, pharmaceutical and cosmetics, synthesis of surfactants, polymers, vegetable fermentation and curing of meat products [5,6]. Since lipase enzymes have numerous industrial applications, and commercial viability, therefore researchers continue to look for novel lipase producing microorganisms and characterize the enzyme with respect to functionality and production optimization.

In the present study, attempts have been made to isolate the lipase producing bacteria from dairy effluent; lipid rich environment likely to harbor lipolytic microorganisms. The isolated lipase producing bacteria were screened on the basis of their lipase producing ability on tributyrin agar plates, identified, and were further examined for the quantitative production of lipase enzyme under different parameters.

2. Materials and Methods:

2.1 Collection of sample

Dairy effluent samples, a lipid rich environment, were collected in sterile containers from selected industrial waste sites and immediately transported to the laboratory. The samples were stored at 4°C to maintain sample integrity prior to bacterial enrichment and isolation procedures.

2.2 Isolation of lipase producing bacteria

2.2.1 Enrichment

1 mL of the sample was inoculated into 50 mL of sterile minimal salt medium (procured from HiMedia laboratories) in 100 mL Erlenmeyer flask supplemented with 1% (v/v) refined olive oil as the only carbon source. The flask was incubated on a shaker at 150 rpm at room temperature for 24-48 hours [7].

2.2.2 Serial dilution and plating

Ten-fold serial dilution was carried out up to 10^{-6}. Spread plate technique was employed whereby 0.1 ml of the aliquot was taken from the last three dilutions and spread on tributyrin agar plates. The plates were incubated at room temperature for 24-48 hours. After incubation the plates were examined for discrete colonies with a zone of clearance. [8]

2.3 Identification of the lipase producing bacterial isolates

The bacterial isolates from dairy industry effluents were identified and designated as DPE-1, *Alcaligenes* sp. AMT-03 gene sequence for 16S rRNA partial sequence, DPE-2, *Alcaligenes* sp. AMT-03 gene sequence for 16S rRNA partial sequence, DPE-3, *Alcaligenes faecalis* AMT-03 gene sequence for 16S rRNA partial sequence DPE 4 and DPE-5, *Alcaligenes faecalis* strain CD234 gene sequence for 16S rRNA full length sequence [9].

2.4 Culture preservation

The productive bacterial cultures were preserved in refrigerator at 4°C on nutrient agar slants. These cultures were then utilised for enzyme assays and optimisation studies.

2.5 Bacterial isolates, culture media and growth conditions

The lipase enzyme producing bacterial isolates, isolated from dairy industry effluent were designated as DPE-1, DPE-2, DPE-3, DPE-4 and DPE-5. They were inoculated in 250 ml Erlenmeyer flasks containing 100 mL minimal salt medium and incubated at 30°C for 24 hours.

2.6 Lipase enzyme assay

After incubation, the cell free supernatant was obtained by centrifugation at 8000 rpm for 10 minutes and was used as the source of crude extracellular enzyme. Lipase activity was measured by titrimetric method using olive oil as a substrate. Olive oil (10% v/v) was emulsified with gum arabic (5% w/v) in 100 mM potassium phosphate buffer of pH 7.0. 100 µL of enzyme source was added to the emulsion and incubated for 15 minutes at 37°C. The reaction was stopped and fatty acids were extracted by addition of 1 mL of acetone and ethanol solution in the ratio of 1mL:1mL. The amount of fatty acids liberated were estimated by titrating with 0.05 M NaOH, using phenophathelin indicator, until colour changed from colourless to light pink, indicating change in the pH from acidic to alkaline condition [10]. A standard graph for the acetic acid equivalent was prepared (figure 1) by carrying out the titrations in above mentioned form in the said medium using following concentrations of the acetic acid (0.01mM-0.1 mM) [10].

2.7 Production optimization studies

Production optimization studies were carried out on the bacterial isolates DPE-3 and DPE-5 for the parameters such as pH of the medium, incubation temperature, carbon and nitrogen sources, and inducer in the medium as described below:

2.7.1 Effect of pH of the medium on lipase enzyme production

The efficient bacterial isolates were grown in sterile minimal salt medium with pH 4, pH 7 and pH 9 and incubated at room temperature (28 ± 1 ^{0}C) to optimize the pH conditions for enzyme production. The production of enzyme was determined by quantitative assay of lipase enzyme by titrimetric method using olive oil as a substrate [10].

2.7.2 Effect of temperature on lipase enzyme production

The efficient bacterial isolates were grown in sterile minimal salt medium with pH 4 and were incubated at various temperatures such as 30^{0}C, 37^{0}C and 42^{0}C. The production of enzyme was determined by quantitative assay of lipase enzyme by titrimetric method using olive oil as a substrate [10].

2.7.3 Effect of carbon sources on lipase enzyme production

The efficient bacterial isolates were grown in sterile minimal salt medium with pH 4 and incubated at 30^{0}C with various carbon sources such as glucose, sucrose and starch. The production of enzyme was determined by quantitative assay of lipase enzyme by titrimetric method using olive oil as a substrate [10].

2.7.4 Effect of nitrogen sources on lipase enzyme production

The efficient bacterial cultures were grown in sterile minimal salt medium with pH 4 and incubated at 30^{0}C with various nitrogen sources such as peptone, beef extract and ammonium sulfate. The production of enzyme was determined by quantitative assay of lipase enzyme by titrimetric method using olive oil as a substrate [10].

2.7.5 Effect of inducers on lipase enzyme production

The efficient bacterial isolates were grown in sterile minimal salt medium with pH 4 and incubated at 30^{0}C with inducers such as olive oil and sodium acetate. The production of enzyme was determined by quantitative assay of lipase enzyme by titrimetric method using olive oil as a substrate [10].

3. Result:

3.1 Isolation of lipase producing bacteria:

Lipase producing bacteria (five isolates) were successfully isolated from dairy effluent on tributyrin agar plates showing zone of clearance.

3.2 Identification of bacterial isolates:

Based on 16S rRNA sequencing, bacterial isolates (DPE1, DPE-2, and DPE-4) were identified up to genus level; *Alcaligenes*, using partial gene sequence. Bacterial isolates (DPE-3 and DPE-5) were identified as *Alcaligenes faecalis* using full length sequence.

3.3 Lipase enzyme assay as a measure of enzyme activity of bacterial isolates

The standard graph for lipase production, measured in terms of acetic acid equivalents, is reported (figure 1). Enzyme Unit: "One unit of enzyme is defined as the amount of enzyme required to hydrolyse 1 µmol of fatty acids as acetic acid equivalent."

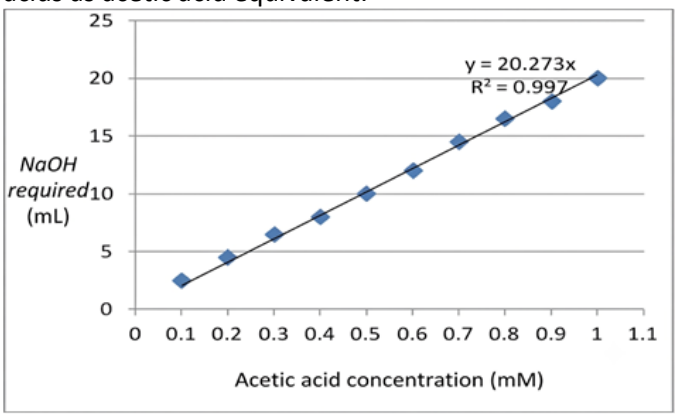

Figure 1: Standard graph for lipase enzyme activity

The enzyme activity of the five isolates DPE-1 to DPE-5 is reported (figure 2). DPE-5 produced maximum enzyme units (50.8 units/mL), followed by DPE-3 which produced 38.1 units/mL under similar experimental conditions. Thus, they were selected for the production optimization studies.

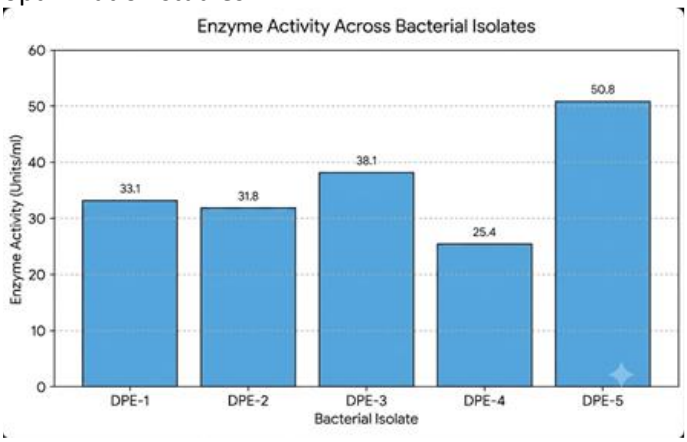

Figure 2. Quantitative assay of lipase enzyme activity across bacterial isolates.

3.4 Effect of different parameters on production of lipase enzyme

3.4.1 Effect of pH:

The optimization study on the effect of pH on lipase enzyme production indicated that the bacterial isolate DPE-3 produced lipase enzyme with 20.8 units/mL at pH 7.0 (optimum pH 7.0) whereas the bacterial isolate DPE-

5 produced lipase enzyme with 20.8 units/mL under acidic conditions at pH 4.0 (optimum pH 4.0) (figure 3).

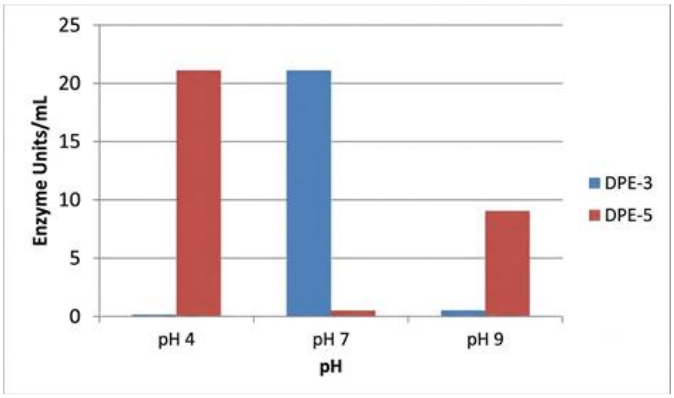

Figure 3: Effect of pH on lipase enzyme production.

3.4.2 Effect of temperature:

Study on the effect of temperature on lipase enzyme production indicated that the bacterial isolate DPE-3 produced lipase enzyme 15.9 units /mL at 37^0C (optimum temperature 37^0C) whereas the bacterial isolate DPE-5 produced lipase enzyme 12.7 units/mL at all the three temperatures tested, 30^0C, 37^0C and 42^0C (figure 4).

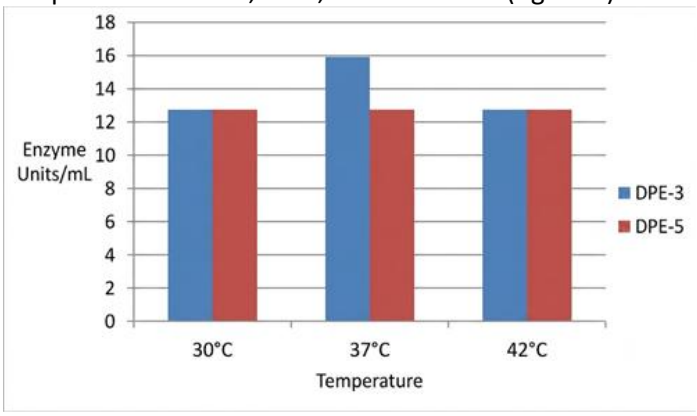

Figure 4: Effect of temperature on lipase enzyme production.

3.4.3 Effect of carbon sources:

The optimization study on the effect of carbon sources on lipase enzyme production showed that the isolate DPE-3 did not produce the enzyme lipase in the presence of carbon sources such as glucose and sucrose whereas starch elicited lipase enzyme production by 6.4 units/ml. The isolate DPE-5 produced maximum lipase enzyme activity of 12.7 units/ml in the presence of starch and an enzyme activity of 9.5 units/mL in the presence of glucose and sucrose (figure 5).

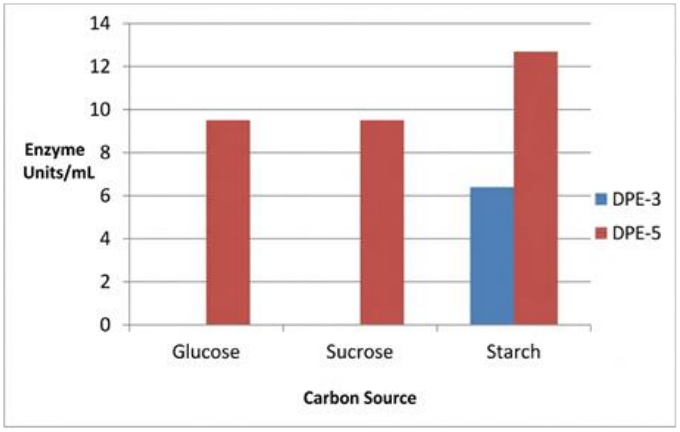

Figure 5: Effect of carbon sources on lipase enzyme production.

3.4.4 Effect of nitrogen sources:

The bacterial isolate DPE-3 produced maximum enzyme (47.6 units/ml) in presence of ammonium sulfate (inorganic nitrogen source) as compared to peptone and beef extract (organic nitrogen sources). On the other hand, the isolate DPE-5 produced 57.2 units/ml in the presence of ammonium sulfate as compared to the complex nitrogen sources; peptone and beef extract (47.6 and 31.8 units/mL, respectively) (figure 6).

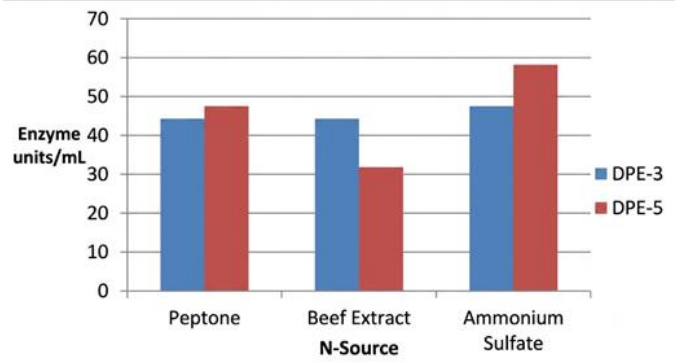

Figure 6: Effect of nitrogen sources on lipase enzyme production.

3.4.5 Effect of inducers:

The bacterial isolates (DPE-3 and DPE-5) showed maximum lipase enzyme activity in presence of sodium acetate (38.1 units/mL) as compared to olive oil (34.9 and 22.2 units/mL, respectively). The enzyme activity was significantly higher for the isolate DPE-5 in the presence of sodium acetate as compared to olive oil (figure 7).

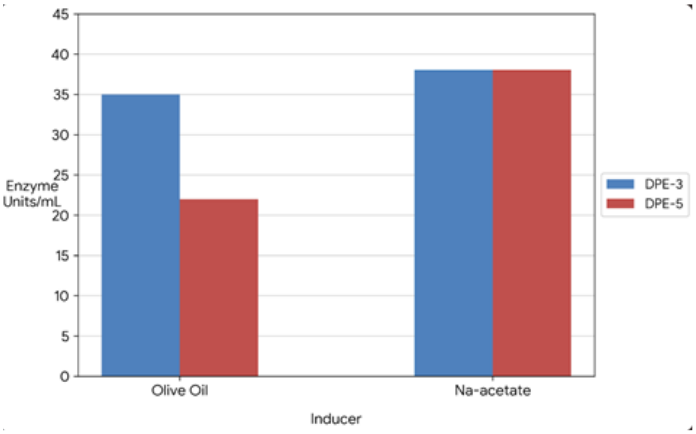

Figure 7. Effect of inducers on lipase enzyme production.

4. Discussion

Bacterial lipases have gathered immense biotechnological interest due to their high stability, chemo-, regio-, and enantioselectivity in aqueous and non-aqueous media [8,11]. In the present study, five lipase producing bacteria were successfully isolated from lipid rich source of dairy effluent. Isolation of lipolytic bacteria from such effluents offers a sustainable strategy for wastewater treatment while simultaneously providing a rich bio-resource for industrial enzymes [12]. Among the microbial diversity inhabiting dairy wastewater, members of the genus *Alcaligenes*—particularly *Alcaligenes faecalis*—frequently emerge as dominant bacterial isolates [13,14]. The abundance of *A. faecalis* in dairy effluents are attributed to its remarkable tolerance to alkaline environments, high concentrations of oxidizable organic matter, and rich nutrient loads (such as nitrates, calcium, and phosphates) [13,14]. Because dairy effluent contain milk triglycerides, *Alcaligenes* species adapt by upregulating extracellular lipase enzymes (triacylglycerol acylhydrolases, EC 3.1.1.3) to cleave hydrophobic lipids into free fatty acids and glycerol for carbon and energy uptake [8,12,15].

Bacterial isolates were then identified using partial and/or full sequence 16S rRNA sequencing. Bacterial isolates designated as DPE-1, DPE-2 and DPE-4 were identified as *Alcaligenes* species whereas isolates DPE-3 and DPE-5 were identified as strains of *Alcaligenes faecalis*. Lipases from *Alcaligenes* species are well-known in industrial biotechnology and a recent research [16] on *Alcaligenes* species—particularly *Alcaligenes faecalis*—highlights their growing importance as sources of microbial lipases.

The five bacterial isolates were evaluated for their extracellular crude lipase yield. DPE-5 exhibited the highest enzyme production, reaching 50.8 units/mL, followed by DPE-3 (38.1 units/mL), DPE-1 (33.1 units/mL), DPE-2 (31.8 units/mL), and DPE-4 (25.4 units/mL) (figure 2). The substantial variation in crude enzyme activity among isolates of the same screening series reflects inherent genomic diversity and differential regulatory mechanisms governing lipase gene expression [17]. Lipase expression in the genus *Alcaligenes* (including related pseudomonad species) is frequently controlled by specialized two-component regulatory systems (such as *LipQ-LipR*) and specific gene operons (*lipAB*) [17,18]. The high yield observed in strain DPE-5 suggests either enhanced basal expression of its lipase structural genes or superior secretion machinery relative to the lower-producing strains [15,18]. The enzyme yield obtained from isolate DPE-5 (50.8 units/mL) compares favorably with prior wild-type *Alcaligenes* and reported *Pseudomonas* strains [11,15]. Microbial lipases typically display crude activities in the range of 10 to 60 units/mL under un-optimized shake-flask conditions [4,15]. The elevated production in isolate DPE-5 makes it a promising candidate strain for further downstream processing (recovery and enzyme purification), medium optimization by Response Surface Modelling (RSM) and scale-up studies. This will help elucidate the full industrial potential of isolate DPE-5 for applications in biodiesel synthesis, detergent formulations, and organic synthesis. Conversely, the lower activity recorded for isolate DPE-4 (25.4 units/mL) may indicate repressor activity, nutrient limitations in the production medium, or lower copy numbers of functional lipase gene clusters [15,17].

Production parameters for lipase synthesis play a crucial role in maximizing enzyme yield and catalytic efficiency. Study on the effect of pH on lipase enzyme production has brought out that the bacterial isolate DPE-3 produced maximum enzyme at pH 7 (neutral conditions) whereas the isolate DPE-5 produced maximum enzyme at pH 4 (acidic conditions). In a recent study [14] alkaline lipases (optimum pH 8-10) are reported from *Alcaligenes faecalis* supporting the fact that strains of these species are typically alkaliphilic. In our study, DPE-5 isolate produced lipase under acidic conditions; a property that could expand its industrial application.

Study on the effect of temperature on lipase enzyme production revealed that the isolates DPE-3 and DPE-5 could produce the enzyme at a wide range of temperatures (30^0C, 37^0C, and 42^0C) indicating its mesophilic nature. Optimal growth and enzyme production typically occurs within the mesophilic range.

Study on the effect of the selected carbon sources revealed that the isolate DPE-3 did not produce the enzyme in the presence of glucose and sucrose but produced in the presence of starch as a carbon source. This may be due to catabolite repression caused by simple sugars reducing lipase synthesis. However, the isolate DPE-5 could produce the enzyme lipase in presence of glucose, sucrose, and starch as carbon sources; maximum in the presence of starch (figure 5).

Among nitrogen sources tested on the enzyme production, ammonium sulphate elicited highest enzyme production compared to beef extract and peptone for both the bacterial isolates DPE-3 and DPE-5. This is not as anticipated as organic sources of nitrogen such as peptone and beef extract would provide necessary amino acids and essential vitamins that boost lipase production and secretion.

Effect of inducers on the lipase enzyme production has brought out that sodium acetate was more efficient than olive oil for the induction of lipase production. This may be due to the utilization of sodium acetate as an additional carbon source rather than an inducer. In literature, vegetable oils such as olive oil has been reported as inducers for lipase production.

5. Conclusion

This study brought out the potential of strains of *Alcaligenes* species isolated from dairy effluent and identified using 16S rRNA sequencing, as novel producers of lipase enzyme with possible industrial applications. Two promising strains (DPE-3 and DPE-5) of *Alcaligenes faecalis* were identified. The enzyme yield obtained from the strain of *Alcaligenes faecalis* isolate DPE-5 (50.8 units/mL) compares favorably with prior reported wild-type *Alcaligenes* and *Pseudomonas* strains. Production optimization studies revealed starch as a favourable carbon source and ammonium sulphate as a favourable nitogen source for optimal enzyme production. Further studies on media optimization by RSM and optimization of enzyme activity, efficient recovery of the enzyme and its purification, and scale up is a way to establish it as a promising bacterial cell factory for lipase enzyme production.

Author contributions: SS: Developed the idea, wrote and verified the manuscript.

Competing interest: The author declare that no conflict of interest exists.

Ethical Statement: This work does not violate ethical guidelines.

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Appendices:

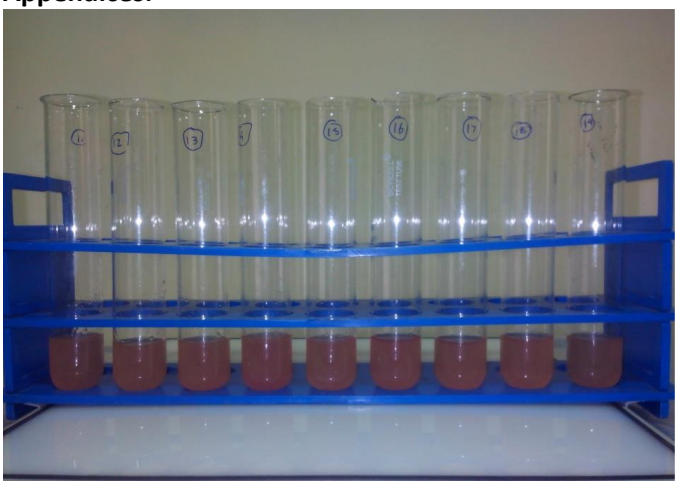

Figure 8: Quantitative Lipase Enzyme Assay by Titrimetric Method using olive oil as a substrate

Abbreviations:

DPE: Dairy Processing Effluent (DPE-1 to DPE-5)

AMT: Ammonium transporter

µg/ml: Micrograms per millilitre

rRNA: Ribosomal Ribose Nucleic Acid

M: Molar

mM: Millimolar

µmol: Micro Molar

µL: Micro Litre

NaOH: Sodium Hydroxide

Rpm: Revolutions per Minute

% v/v: Percent Volume/Volume

% w/v: Percent Weight/Volume

RSM: Response Surface Modelling or Methodology

LipQ: Lipase Q, Lipase Family Protein Involved in Lipid Catabolism

LipR: Lipase Regulator

LipAB: Lipase A-B Complex/Operon