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# Characterisation of Some Selected Bacterial Isolates from Vegetable Oil Contaminated Soil

**B. M. Popoola[1*] and A. A. Olanbiwoninu[1]**

*[1]Ajayi Crowther University, Oyo, Oyo State. Nigeria.*

***Authors' contributions***

*This work was carried out in collaboration between both authors. Author BMP designed the study, managed the analyses of the study, managed the literature searches, wrote the protocol, and wrote the first draft of the manuscript. Author AAO performed the statistical analysis managed the analyses of the study. Both authors read and approved the final manuscript.*

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## ABSTRACT

Microbial lipases occupy a place of prominence among biocatalysts and are often used for various biotechnological applications. Because of huge variation in applications, the availability of lipases with specific characteristics is still a limiting factor. There is therefore need for extensive characterisation of lipase for various applications. This work was carried out to characterise lipases from some selected bacterial isolates.

Isolates identified as *Bacillus subtilis*, *Bacillus licheniformis, Pseudomonas cepacia Pseudomonas fluorescens, Alcaligenes* sp. *and Flavobacterium* sp. from a vegetable oil contaminated soil were characterized. Temperature, pH and ion concentration, ($NaNO_3$ and $MgSO_4$), incubation time, agitation speed, carbon sources and nitrogen sources were optimised for growth and lipase activity. Increase in microbial growth does not necessarily suggest increase in lipolytic activity as generally observed from this study. Temperature, pH, incubation time and agitation speed which had optimum enzyme activities for crude enzyme of *Pseudomonas fluorescens* (0.8 U/mL), were 27 $^{o}$C, 7.0, 24 h, and 0 rpm respectively. Growth was not generally supported by $AgN0_3$ in all the organisms selected but supported by $KNO_3$. However $MgSO_4$ generally supported lipase

**Corresponding author: E-mail: bukolamargaret@yahoo.com;*

production. Olive oil and peptone as sources of carbon and nitrogen respectively supported both growth and lipase production in the selected organisms.
These bacterial isolates characterized had lipolytic activities, hence they have high potential for various biotechnological applications.

*Keywords: Lipolytic activity; microbial growth; temperature; enzyme.*

## 1. INTRODUCTION

### 1.1 Bacterial Lipases

Bacteria produce different classes of lipolytic enzymes including carboxylesterases which hydrolyze water-soluble esters and lipases which hydrolyze long-chain triacylglycerol substrates [1]. Many bacterial species produce lipases which hydrolyse esters of glycerol with long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. With only a few exceptions, bacterial lipases are able to completely hydrolyze a triacylglycerol substrate although ester bonds are more favourable [2]. Some important lipase-producing bacterial species are *Bacillus, Pseudomonas* and *Burkholderia* [3]. Numerous lipase assay methods are available using coloured or fluorescent substrates which allow spectroscopic and fluorimetric detection of lipase activity. Another important assay is based on titration of fatty acids released from the substrate.

Bacterial lipases are mostly released outside of the cell that is called extracellular enzyme. They are influenced by nutritional and physico-chemical factors; such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, stirring conditions, dissolved oxygen concentration [1].

The major factor for the expression of lipase enzyme is carbon source. Lipases generally are produced in the presence of lipid source such as oil, triacylglycerols, fatty acids, hydrolyzable esters, the tweens and glycerols addition to carbon source, the type of nitrogen source also influence the production of lipases. Generally, organic nitrogen source is preferred by bacteria, such as peptone and yeast extract [4].

The initial pH of the growth medium is important for lipase production. Most bacteria prefer pH around 7.0 for their best growth and lipase production. The optimum temperature for lipase production is parallel with the growth temperature of the respective microorganism. It has been reported that lipases are produced in the temperature range from 20ºC to 45ºC [5]. Incubation periods change from few hours to many days until the maximum lipase production from bacteria is recorded.

The biotechnological potential of microbial lipases is relying on their ability to catalyse not only the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids. Therefore, microbial lipases have applications in different industrial processes [6]. Also the temperature stability of lipases is the most important characteristic for industrial use [7]. Moreover the extracellular bacterial lipases are commercially valuable, because their bulk production is much easier [4]. Lipase catalysed reactions are widely used especially in the manufacturing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics. Lipases are also used to accelerate the degradation of fatty wastes and polyurethane [8].

However harnessing these organisms for their potential metabolic activities is largely dependent on the culture media composition [6]. Because of huge variation in applications, the availability of lipases with specific characteristics is still a limiting factor. The production of microbial lipases is apparently important from economic and industrial standpoints. Moreover extracellular lipases can be produced at large scale under normal laboratory conditions.

It is significant to isolate microbes of high potential for various biotechnological applications. Therefore, in this study, an attempt was made to assess the bio-potentials of a few bacterial species with reference to their ability to utilize lipid from vegetable oil under laboratory conditions, the characteristics of these lipases were checked in order understand enzyme functions better and enhance enzyme production by applying suitable substrate as well as process parameters optimization.

## 2. MATERIALS AND METHODS

### 2.1 Growth studies and Production of the Enzymes

#### 2.1.1 Growth media

Isolates identified as *Bacillus subtilis*, *Bacillus licheniformis, Pseudomonas cepacia Pseudomonas fluorescens, Alcaligenes* sp. *and Flavobacterium* sp. from a vegetable oil contaminated soil were grown in a complex basal medium whose composition was a modification of the medium of Tsujisaka et al. [9] with glucose omitted. This medium contained 5% peptone, 0.1% $NaNO_3$ and 0.1% $MgSO_4$, adjusted to the desired pH, before sterilisation. Sterile olive oil (Goya) was added as carbon source.

#### 2.1.2 Lipase production

*2.1.2.1 Preparation of inoculum*

A loopfull of the pure culture of bacteria were grown overnight in nutrient broth.

*2.1.2.2 Production Procedure*

One milliliter from the above preparation was inoculated into 65 mL of sterile medium in 250mL Erlenmeyer flasks and incubated at room temperature (27ºC $\pm$ 2ºC) from 24 h to many days until the maximum lipase production was recorded. The medium was centrifuged at 30,000 g for 15 min using Himac High-speed Refrigerated Centrifuge (Hitachi model CR21GII). The supernatant of the centrifuged culture broth was then decanted leaving the cells at the bottom. The cell-free extract acted as the crude lipase enzyme.

#### 2.1.3 Growth of the isolates

Growth of the Isolates in the growth medium was examined spectrophotometrically using a Jenway 640 UV/VIS spectrophotometer at 540 nm, absorbance were measured against blank [10].

#### 2.1.4 Lipase assay

Lipase activity was measured by a modification of the assay of Parry et al. [11] using as substrate a 10 % Olive oil-gum arabic solution emulsified by sonication for 2 mins at 25watts output according to Linfield et al. [12]. One milliliter of cell-free fermentation broth prepared by centrifugation as describe above was added to 5ml of emulsion and incubated at room temperature for 1h with rapid stirring. Ethanol was added to stop the reaction and the free fatty acids produced were quantified by titration to pH 9.5 against 0.1N NaOH using a radiometer titration system. Blanks with 1ml of fermentation broth were employed in each experiment. Blanks ran with sterile or actual uninoculated broths were the same within experimental error. Samples were run in duplicate.

A unit of lipase activity was defined as the amount of sodium hydroxide (NaOH) used in the titration to bring the reaction mixture to a pH of 9.5 per min under the defined assay conditions. Alternatively, it is considered as the release of one micromole of free fatty acid (FFA)/min at room temperature.

### 2.2 Optimisation of Production Conditions

#### 2.2.1 Effect of pH on lipase production

This was carried out using a modified method of Tsujisaka et al. [9]. Growth medium was prepared in 0.2 M phosphate buffer and (0.1M citric acid mixed with 0.2 M $Na_2HPO_4$) citrate phosphate buffer of varying pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5). The lipase activity and the growth in the culture supernatant were determined using appropriate procedure.

#### 2.2.2 Effect of temperature on lipase production

The effect of temperature on lipase activity was determined using the above method. The organism was cultivated in the growth medium at different temperatures, which ranged from 20ºC, 27ºC, 40ºC, 50ºC, 60ºC to 70ºC for 24 h. The lipase activity and growth in the culture supernatant were determined.

#### 2.2.3 Effect of Metal ion on lipase production

The growth medium in which the organisms were cultivated had their metal ions varied. $ZnSO_4$, $FeSO_4$, $(NH_4)_2SO_4$, $KNO_3$, $AgNO_3$, $CaNO_3$, $Na_2CO_3$ and NaCl were used in equimolar concentration (0.1mM) instead of $MgSO_4$ and $NaNO_3$. The lipase activity and growth in the culture supernatant were then determined.

#### 2.2.4 Effect of aeration on lipase production

After inoculating the organisms into the growth medium, the flasks were continuously shaken at 27ºC for 24 h, at varied revolutions per minute (80, 100, 120 and 140) using orbital shaker

Stuart SSLI. The lipase activity and growth in the culture supernatant were then determined.

#### 2.2.5 Time course of lipase production

The organisms were cultivated in the growth medium for different periods that ranged from 24 h to 72 h. Samples were removed periodically and growth and lipase activity in the culture supernatant were determined.

#### 2.2.6 Effect of different substrates on lipase production

To determine the suitable substrate (carbon source) for the production of lipase by the organisms, substrates such as; glycerol, soy oil, olive oil and a simple sugar (glucose) were used. They were individually tested by replacing the substrate present in the growth medium at the concentration of 2 %. Thereafter, the lipase activities as well as growth in the culture supernatant were determined.

#### 2.2.7 Effect of different nitrogen sources on lipase production

The main nitrogen source in the growth medium was replaced by other nitrogen sources such as casein, urea and yeast extract at the same concentration (5%). The lipase activity and growth in the culture supernatant were then determined.

#### 2.2.8 Statistics

Statistical analyses of data were performed in Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) using the unpaired t-test using a significance level of $p<0.05$.

## 3. RESULTS

### 3.1 Optimization of Production Conditions

#### 3.1.1 Effect of pH on growth and lipase production by the isolates

The effect of pH on growth of the selected bacteria; at pH 5.5, with the exception of *Pseudomonas cepacia,* most of the bacteria did not grow, whereas majority grew best at pH 7.0-9.0 (Fig. 1). Fig. 2. shows the effect of pH on lipase production by these organisms. Lipase production was best in most of the organisms at pH 7.0-8.0.

#### 3.1.2 Effect of temperature on growth and lipase production by the selected isolates

As temperature increased from 20ºC, growth in majority of the organisms decreased (Fig. 3). While the growth of *Pseudomonas fluorescens, Pseudomonas cepacia* and *Bacillus subtilis* reached a peak at 27ºC, *Flavobacterium* sp. and *Bacillus licheniformis,* had maximal growth at 40ºC. In each instance, the peak was followed by a gradual descent to the minimum. Majority of the isolates recorded their best enzyme production at 27ºC, followed in each case by a sharp decline in production (Fig. 4).

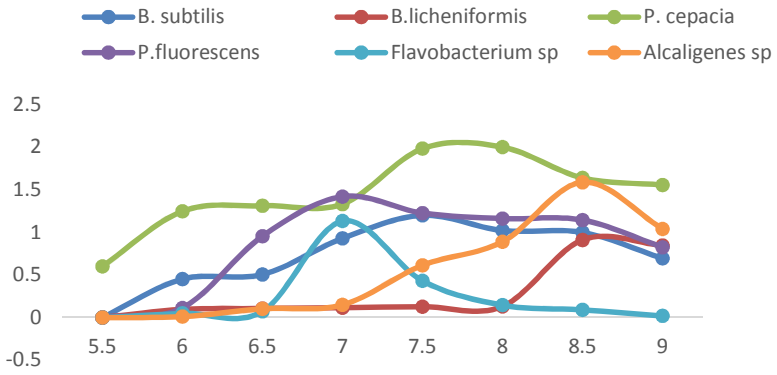


**Fig. 1. Effect of pH on growth of some selected bacterial isolates**

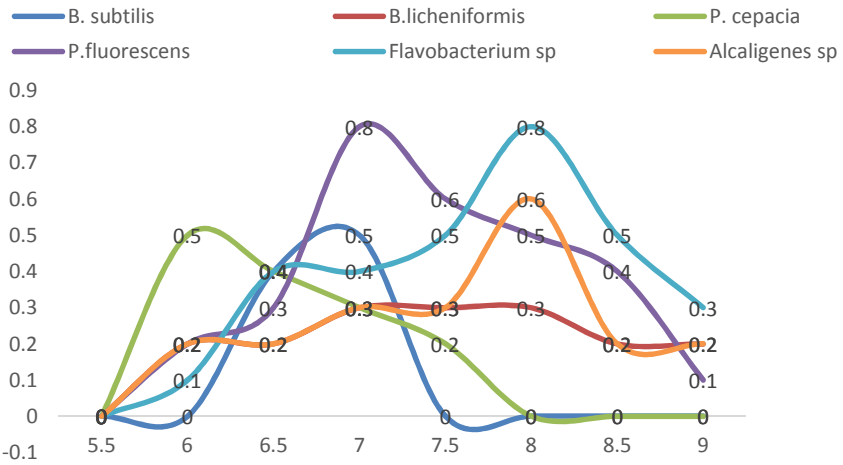


**Fig. 2. Effect of pH on lipase production by some selected bacterial isolates**

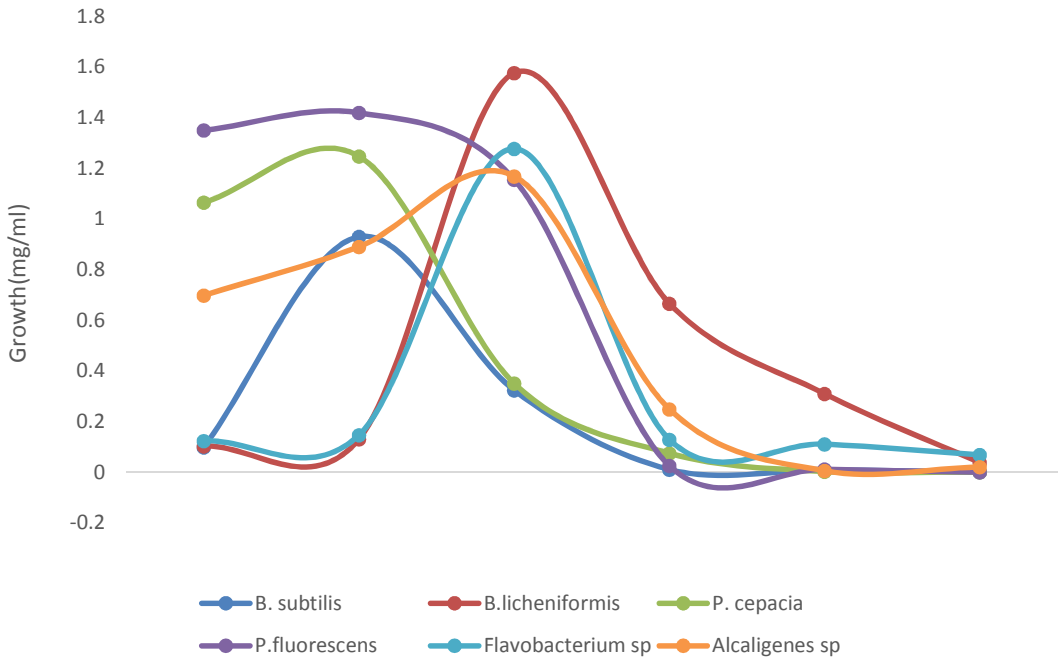


**Fig. 3. Effect of temperature on growth of some selected bacterial isolates**

### 3.1.3 Effect of metal ions on growth and lipase production of isolates

Studying the effect of cations on growth of the organisms shows that their growth was not generally supported by $AgN0_3$ but supported by $KN0_3$ (Fig. 5). Lipase production was inhibited by some cations but $MgSO_4$ generally supported lipase production (Fig. 6).

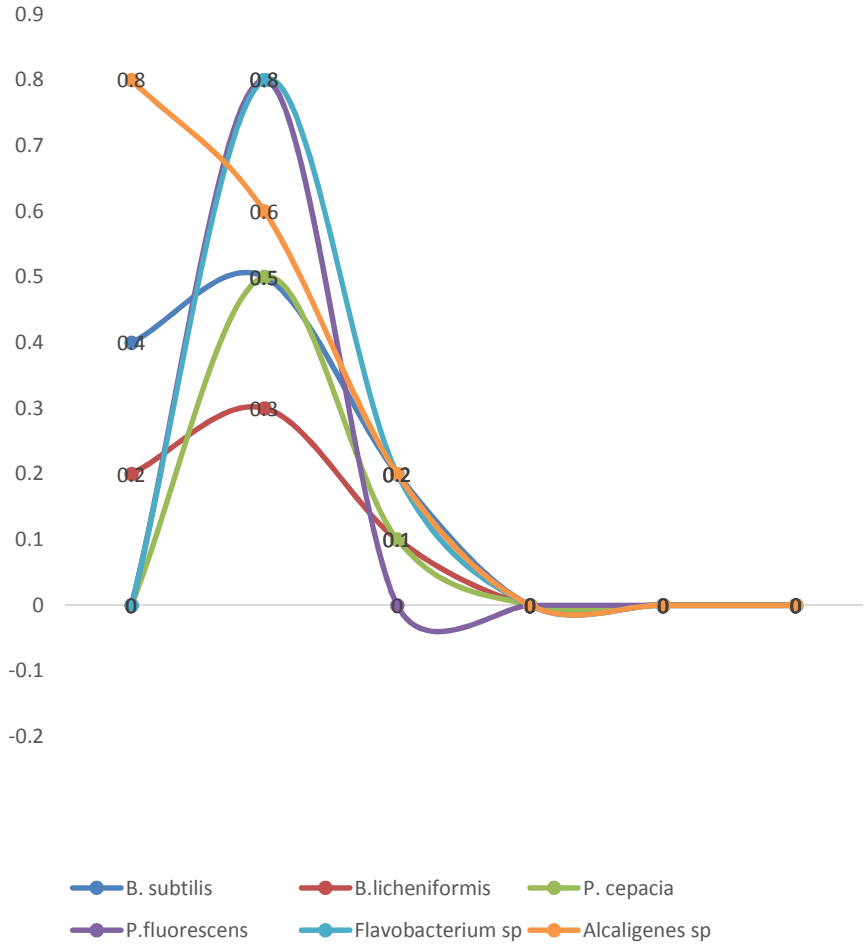


**Fig. 4. Effect of temperature on lipase production by some selected bacterial isolate**

The effect of anions on growth and enzyme activity of the different organisms are shown in Figs. 7. and 8 respectively. Lipase production was supported by $NaNO_3$ in all the organisms selected (Fig 8).

### 3.1.4 Effect of aeration on growth and lipase production of isolates

Growth increased with increase in agitation speed for *Bacillus subtilis*, *Bacillus licheniformis* and *Flavobacterium* sp. but in *Pseudomonas cepacia, Pseudomonas fluorescens* and *Alcaligens* sp. growth decreased after reaching a peak at 100 rpm (Fig 9).

Fig. 10. shows that in most of the organisms studied, agitation did not support release of lipase production except in *Bacillus licheniformis.*

### 3.1.5 Time course of growth and lipase production

Growth was supported best at 48 hours of incubation in majority of the organisms investigated, with the exception of *Bacillus licheniformis* and *Flavobacterium* sp. which grew best at 72 hours of incubation (Fig. 11). In all the organisms, lipase production was supported best at 24 hours of incubation (Fig. 12).

### 3.1.6 Effect of different carbon sources on growth and lipase production by the isolates

Fig. 13 shows the effect of different substrates on growth of organisms while Fig. 14. shows that of lipase production. While glucose supported growth slightly, lipase production in *Bacillus subtilis*, *Bacillus licheniformis* and *Pseudomonas cepacia* was nil (Fig. 14).

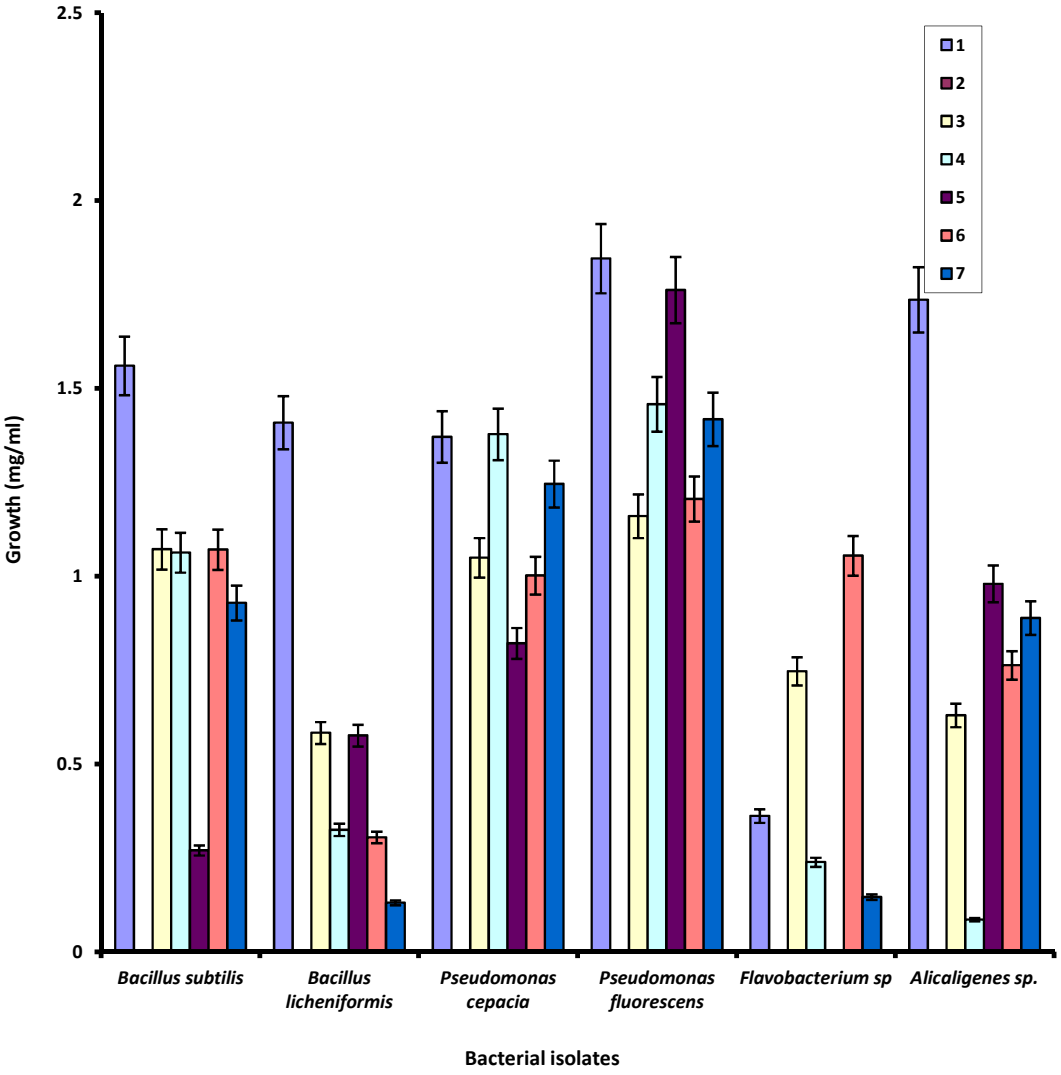


**Fig. 5. Effect of 0.1% different cations on growth of some selected bacterial isolates**

### 3.1.7 Effect of different nitrogen sources on growth and lipase production by the isolates

Fig. 15. shows the effect of different nitrogen sources on growth of the organisms. Peptone generally supported growth in all the organisms studied. Fig. 16. shows that of enzyme production, also generally supported by peptone.

## 4. DISCUSSION

The bio-potentials of a few bacterial species with reference to their ability to utilize lipid from vegetable oil under laboratory conditions was the main focus of this study, The obtained bacterial lipases were generally observed to work best in alkaline pH. For instance, *Bacillus subtilis* produced maximum lipase activity at pH 7 (0.5 U/ml). In low and high medium pH tested, the lipase activity was less. This result is consistent with the report of Mohan et al. [13], who stated that the lipase activity of *Bacillus* sp. was optimal at pH 7 during the 24 h culture period.

In this study most of the bacteria isolated had their optimum growth temperature at 40$^{o}$C, whereas their optimum lipase activity was at 27$^{o}$C. Ece Yapasan [14] reported the optimum bacterial growth temperature to be 25$^{o}$C for

*Pseudomonas* sp. with lipase enzyme showing activity above and below this temperature, although bacteria growth did not exceed 30°C, enzyme works even above 30°C. Temperature changes give rise to cleavage of hydrogen bonds between substrate and enzymes' active sites. Optimum temperature value promotes binding potential of enzyme and substrate [15]. However, in this study, temperature increase above known optimum tends most likely to denature the enzymes thereby reducing the enzyme activity.

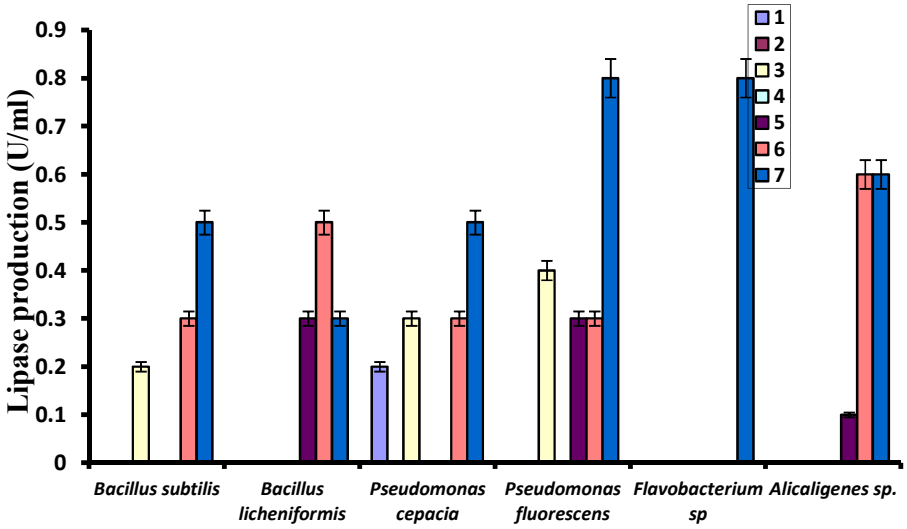


**Fig. 6. Effect of 0.1% different cations on lipase production by some selected bacterial isolate**

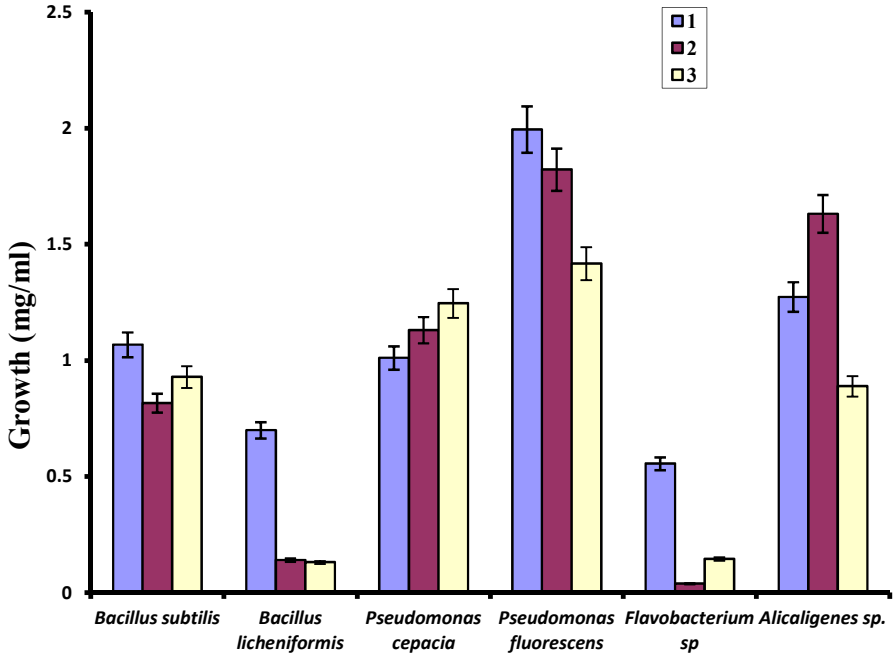


**Fig. 7. Effect of 0.1% different anions on growth of some selected bacterial isolates**

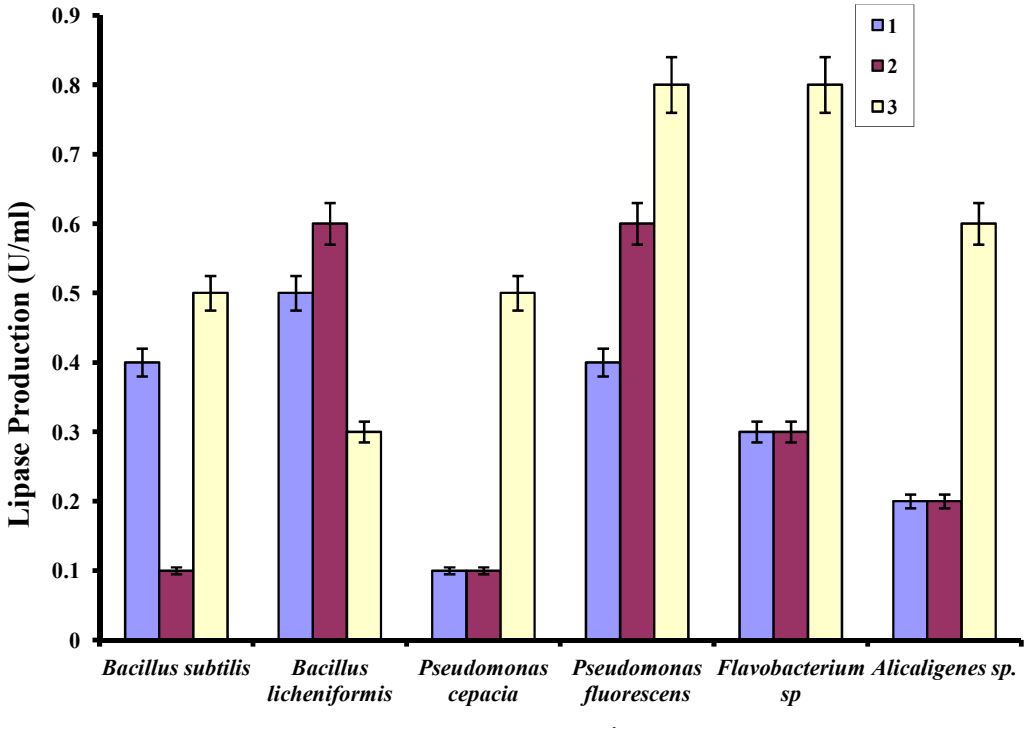


**Fig. 8. Effect of 0.1% different anions on lipase production of some selected bacterial isolates**

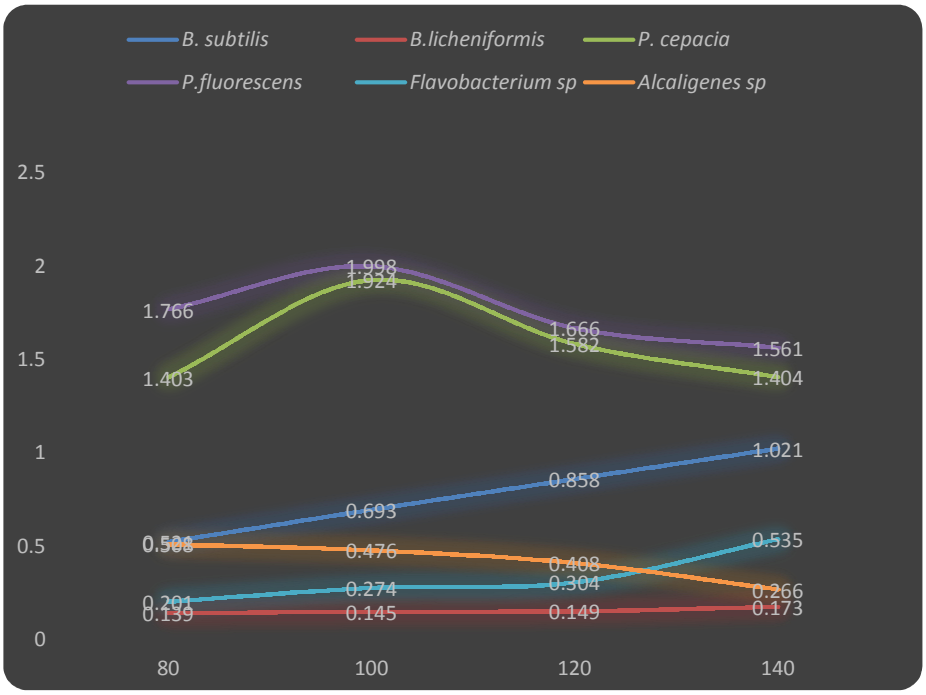


**Fig. 9. Effect of agitation on growth of some selected bacterial isolates**

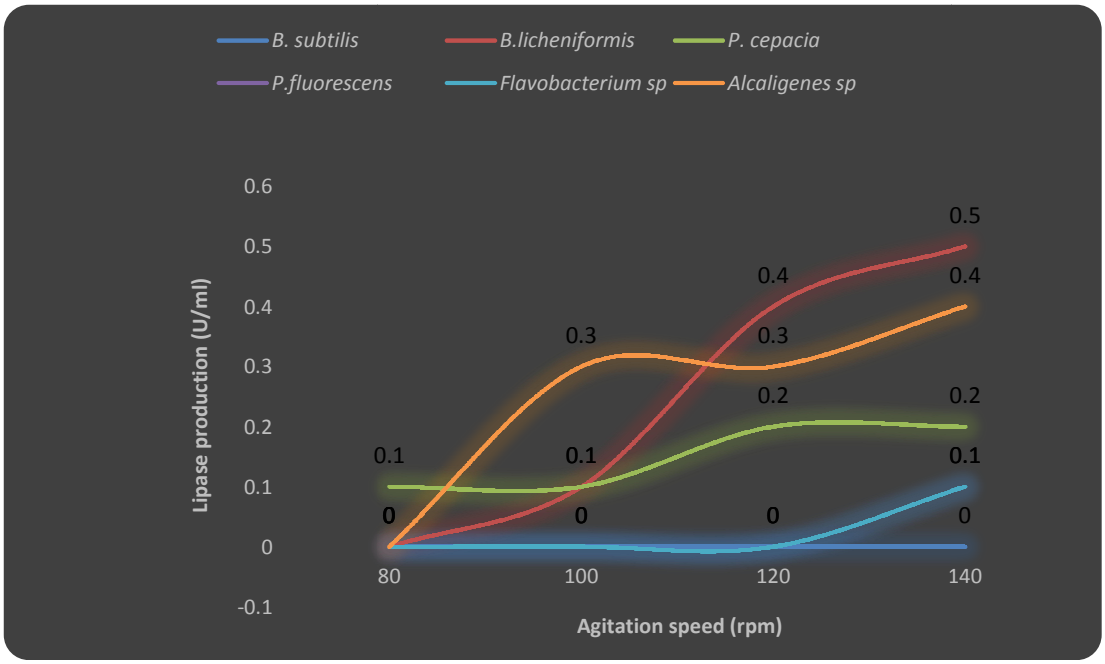


**Fig. 10. Effect of agitation on lipase production by some selected bacterial isolates**

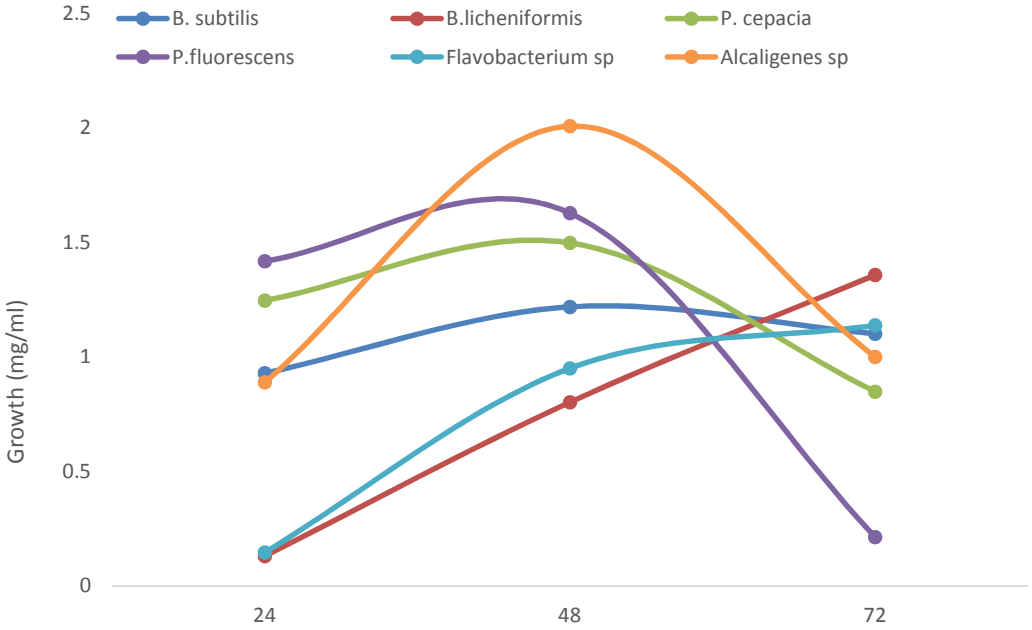


**Fig. 11. Effect of incubation time on growth of some selected bacterial isolates**

The catalytic activities of the lipases in the current study were enhanced in the presence of $Ca^{2+}$, $Mg^{2+}$ and $NH_3^+$, but inhibited by $Fe^{2+}$, $Zn^{2+}$ and $Hg^{2+}$. These results are in agreement with Chakraborty and Raj [15]. $Ca^{2+}$ ions have been known to stimulate lipase activity in varying concentrations, it has been reported that in the presence of $Ca^{2+}$, lipase activity of *Bacillus*

*licheniformis* strain H1 increased up to 120% [16] while the activity of lipase from a *Pseudomonas* sp. has been reported to be increased by 250% [17]. $Ba^{2+}$ is also known to enhance lipase activity of lipase isolated from *Burkholderia* sp. [18]. Metal ions like $Hg^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ have been reported to have inhibitory effect on *Pseudomonas* lipases [19]. *Pseudomonas* sp. lipase has also been reported to be inhibited in the presence of $Al^{3+}$, $Mn^{2+}$, $Ni^{2+}$ and $Fe^{3+}$ [17].

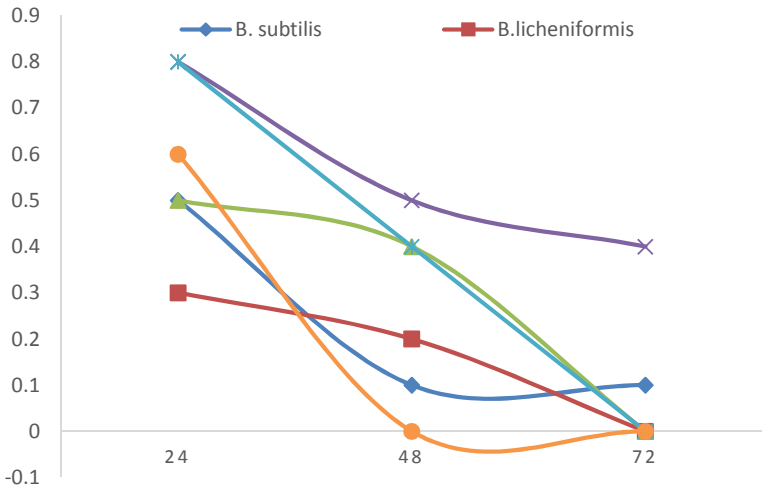


**Fig. 12. Effect of incubation time on lipase production by some selected bacterial isolates**

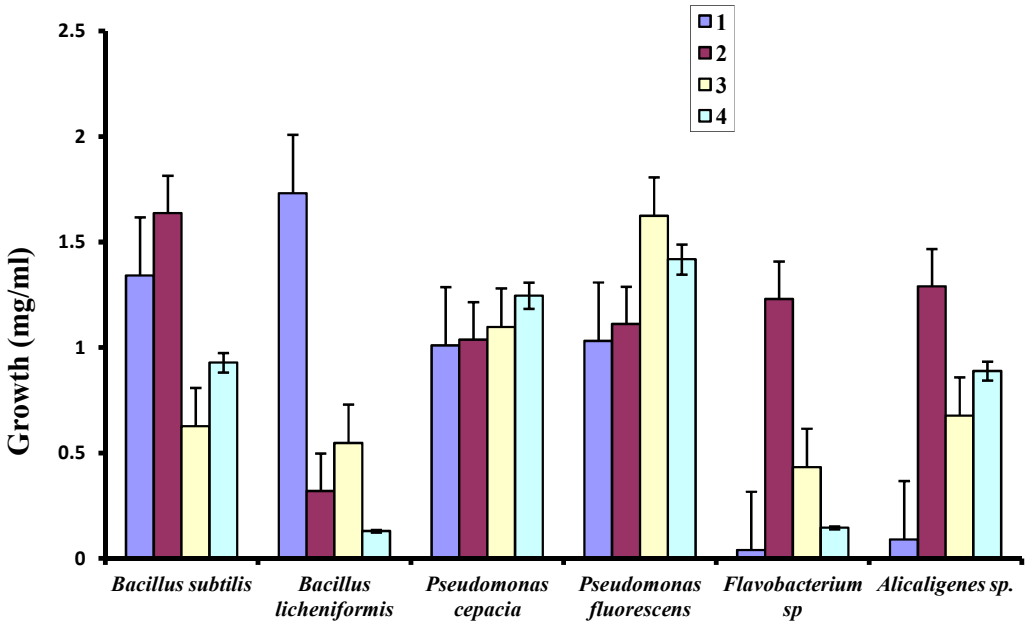


**Fig. 13. Effect of 2% different carbon sources on the growth of some selected bacterial isolates**

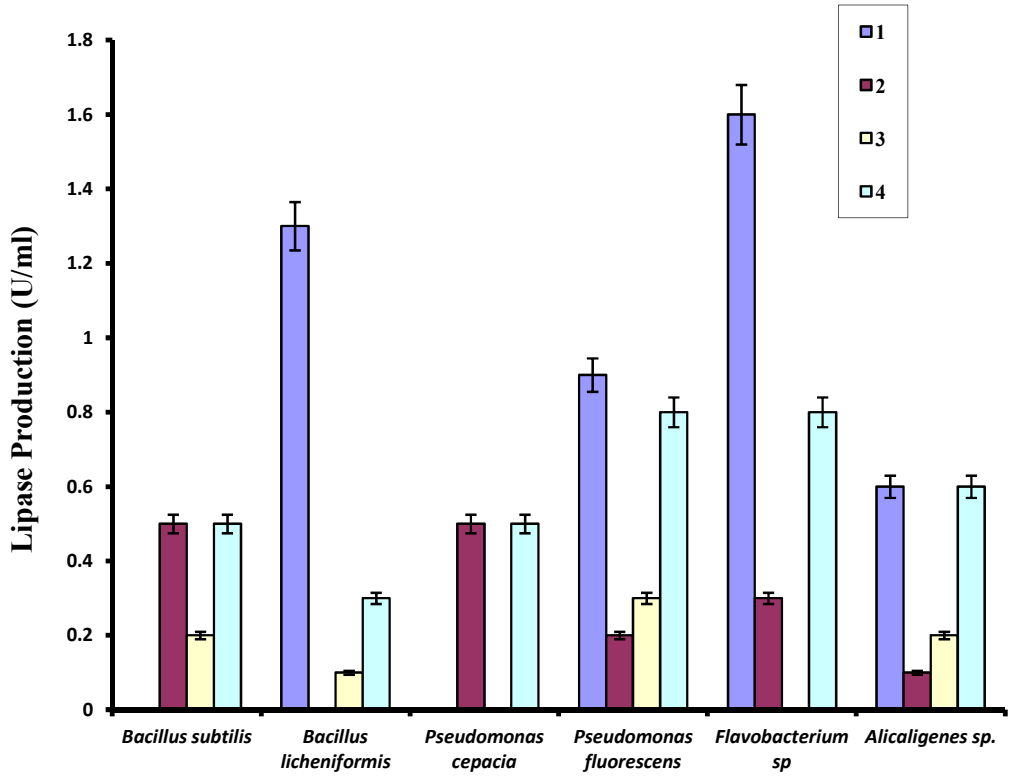


**Fig. 14. Effect of 2% different carbon sources on lipase production by some selected bacterial isolate**

In this study, agitation supported the growth of the experimental organisms, signifying that they are aerobic in nature and required large quantities of dissolved oxygen for their growth and multiplication. This is also affirmed by Chander et al. [20]. But on the contrary, Chander et al. [21] noted that agitation improved lipase production while stationary cultures produced better lipolytic activity than agitated cultures. Further, Ebrahimpour et al. [21] showed that shallow layer (static culture) where aeration is moderate produced much more lipase than shake cultures (high aeration). On the contrary *Bacillus licheniformis* and *Alcaligenes* sp., had increased lipase production at 120 rpm when compared with others. Increase in lipase production on increasing agitation could be due to increased oxygen transfer rate, increased surface area of contact with the media component or better dispersability of the carbon source. This is in agreement with Hala et al. [22] who reported that at 150 rpm, *Fusarium oxyspirum* produced highest lipase activity compared with the static culture and even at 100 rpm and 200 rpm.

As observed in this research, maximum lipase activity for enzymes studied were obtained after 24 hrs of incubation, indicating that lipase was necessary for the first stages of growth, while minimum growth for majority of the organisms were detected after 48 hrs, an observation in agreement with Ginalska et al. [23]. Further, at longer incubation periods, and for all the isolates, the lipase activity decreased which might be due to the depletion of nutrients, accumulation of toxic end-products, the change in pH of the medium, or loss of moisture. Other investigators have reported a different incubation period for optimal lipase production. Maximum lipase activity was achieved after 48 hrs of incubation by Ul – Haq et al. [24] with *Rhizopus oryzae*.

In the case of substrate hydrolysis, almost all the substrates tested were hydrolysed by the tested organisms. Most of the substrates have long carbon chains (olive oil has C18:1) which may take time to dissolve. Since lipases hydrolyse esters in emulsion and usually water-insoluble substrates, the organisms take up the substrate at different concentration, form and time.

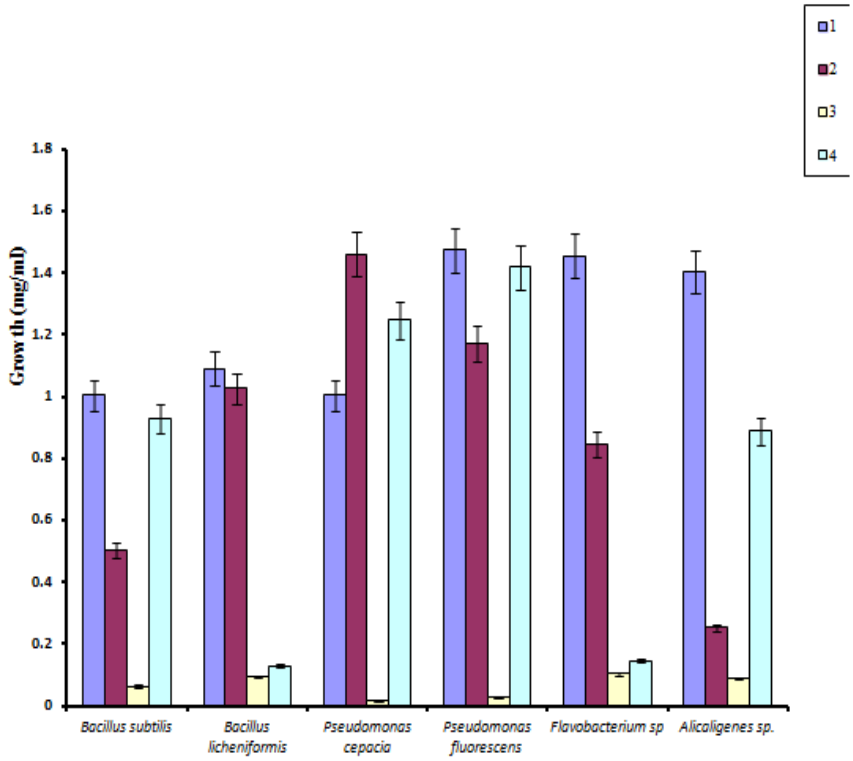


**Fig. 15. Effect of 5% different nitrogen sources on growth of some selected bacterial isolates**

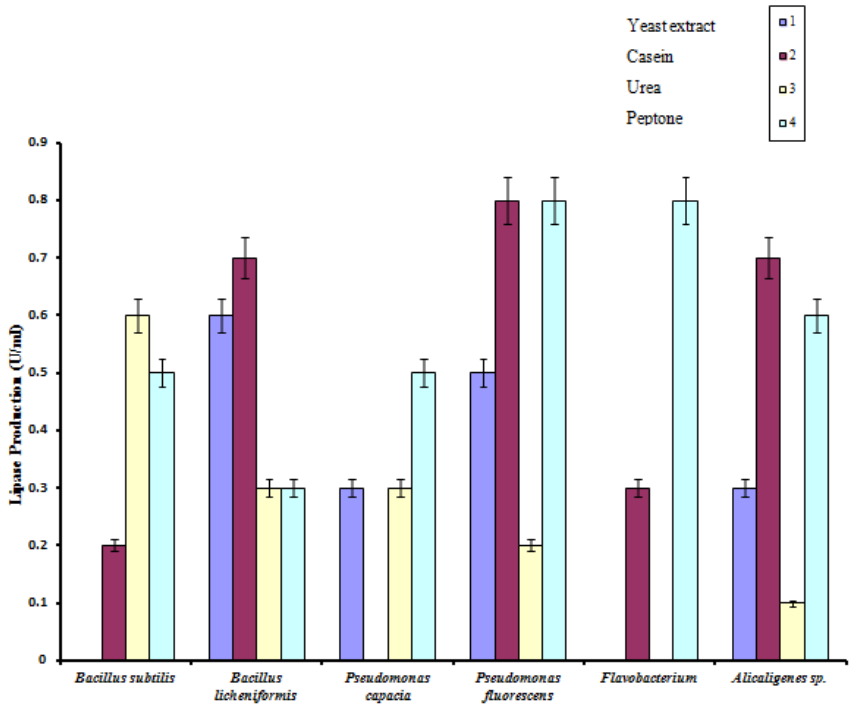


**Fig. 16. Effect of 5% different nitrogen sources on lipase production by some selected bacterial isolates**

Typically, triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyse "simple" esters and usually only tryglycerides bearing fatty acids shorter than six carbon chains (C6). Thus, these results strongly suggest that the enzymes used in this study show lipase activity. On the other hand, findings of Pogaku et al. [25], olive oil supported good growth and increased lipase activity significantly. Olive oil in this case acted as an inducer of lipase production, for all the strains used, hence lipase production has been shown to be induced remarkably in the presence of lipidic carbon sources like oils and fatty acids. Glucose, however, was found to act as a repressor of lipase production because it caused a great repression though it supported growth.

Generally, peptone stimulated lipase production better than other nitrogen sources experimented with in the scope of this study. This is in agreement with the work of Tembhurkar et al. [26]. It is also in agreement with the finding of Sirisha et al. [27] who recorded better lipase production by *Staphylococcus* when peptone was used in place of yeast extract and tryptone as nitrogen source. Gupta et al. [4] generally observed that organic nitrogen source such as peptone and yeast extract is preferred by bacteria. Also, peptone and yeast extract has been used as nitrogen source for lipase production by various *Bacillus* spp., various *Pseudomonads* and *Staphylococcus haemolyticus,* respectively [16,28,29,30]. Inorganic nitrogen sources such as ammonium chloride and di-ammonium hydrogen phosphate have also been reported to be effective in some microbes [31,32,18].

However, it is what noting that lipase production could also be influenced by the type and concentration of carbon, nitrogen, metal ion sources, the culture pH, the growth temperature, and the dissolved oxygen concentration [33].

## 5. CONCLUSION

Bacterial lipases have been studied extensively lately, however, in order to have a wider application of these lipases with relevance to our world today, there is need to extensively characterize these enzymes. Certain environmental factors such as pH, temperature, incubation time and aeration rate were observed to play a major role during the enzyme production and metabolic activities. Such studies are aimed at assessing the potential bacterial isolates which could be helpful in various biotechnological applications. There is also need to produce these lipases with improved properties by protein engineering to further enhance the usefulness of these enzymes.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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