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# Properties of extracellular lipase from isolated bacterial on bovine milk fats

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## Abstract

Ninety-six isolates from different sources had been isolated and their lipolytic activities investigated using phenol red agar plates. Examine the properties of the lipase enzyme that was separated from the most active isolate of bacteria (MC1) on cow's milk fat. The enzyme showed activity (23.06 U/ml) over an incubation period on 24 h. The biochemical and morphological identification of the bacterial isolate revealed that it was *Bacillus* sp. The stability of MC1 lipase under varied temperature conditions was enhanced by studying its characterization; it exhibited a maximal activity of 36.17 U/ml at 40 °C and remained stable up to 70 °C with 17% of its relative activity. The lipase enzyme was shown to be more stable at different pH levels (ranging from 5 to 10); at pH 7, the maximal lipase activity was 29.57 U/ml. Addition of metal ion like $Ca^{2+}$ enhancing the lipase activity. It is favored in biological and industrial processes for this reason.

*Keywords*: isolation, hydrolysis, lipase, enzyme activity, bovine milk.

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## 1. Introduction

The world is concerned about a clean environment, and microbial products are receiving a great deal of industrial interest. The biotechnology industry has been directed to the production and use of enzymes of microbial origin; one of the most important sources of many enzymes is fungi, yeasts, and bacteria. Microorganisms are able to hydrolysis vegetable and animal fats with a considerable amount of energy. Lipolytic microorganisms can be found in different habitats, like soils contaminated with oils, seeds, wastes of vegetable oils, and dairy product industries (Sharma *et al.,* 2001). The ideal source for many unknown functional microorganisms is soil. Lipases (Triacylglycerol hydrolases E.C. 3.1.1.3) are serine hydrolases enzymes produced by mammals, plants, fungi, and bacteria (Melani *et al.,* 2019). At the lipid-water interface, the carboxyl ester bonds in the triacylglycerol are hydrolyzed by lipases to produce fatty acids and glycerol (Fickers *et al.,* 2011; Gupta *et al.,* 2004). Lipases do not require any cofactors (Beisson *et al.,* 2000). Under natural conditions, at the interface between an unsolvable phase of substrate and an aqueous phase where the enzymes keep on liquefying, the lipases catalyze carboxyl ester bonds in the triacylglycerol. Lipases naturally catalyze triacylglycerol into diacylglycerols, monoacylglycerols, fatty acids, and glycerols (Almeida *et al.,* 2019; Pascoal *et al.,* 2018). Besides hydrolysis activity, the lipases display an important role in many reactions, like interesterification, esterification, aminolysis and alcoholysis activity, which are useful in a wide range of industries (Karadzic *et al.,* 2006; Rajendran *et al.,* 2009). Due to the great demand for lipase enzymes, scientists have been forced to look for new sources of bacteria that can grow in cheaper conditions. Optimization of production is vital in biological processes in order to achieve a better yield at a low cost (Ebrahimpour *et al.,* 2008; Haddar *et al.,* 2010; Horamnia *et al.,* 2010). Lipases from bacteria are most preferred due to their stability, ease of production, and variety of catalytic activities available at a low cost. However, they are naturally occurring in mammals, plants, and microorganisms (Bharathi *et al.,* 2019; Fatima *et al.,* 2020). After protease and carbohydrase, lipases are listed as the third largest group of commercialized enzymes due to their stability in organic solvents (Casas-Godoy *et al.,* 2012; Hasan *et al.,* 2006). Microbial lipases have many applications in industrial and biological uses, including food and pharmaceutical industries, leather, detergents, drinks, agrochemicals, cosmetics, and waste treatment (Chary and Devi, 2018; Gupta *et al.*, 2004; Sarmah *et al.*, 2018; Sharma *et al.*, 2001; Thirunavukarasu *et al.*, 2008). As people worldwide are interested in renewable energy, lipases have an important application in that field, especially in biodiesel production, which is an expanding sector (Colla *et al.,* 2010). Currently, production and use of enzymes of microbial origin are the largest sectors of catalytic enzymes in the biotechnology industry (Freedonia, 2014). This work aims to isolate strains of bacteria that are highly capable of secreting lipase under a variety of circumstances and have high activity and stability. The enzyme is

suitable for the industrial sector because of these advantageous characteristics.

## 2. Materials and methods

### *2.1 Chemicals*

*p*-Nitrophenyl palmitate (*p*-NPP) Sigma-Aldrich used as a substrate for measuring the lipase activity. All other reagents were of analytical grade.

### *2.2 Sample collection and isolation bacterial procedures*

From oil-contaminated soil around Assiut city, Egypt, lipid-rich soil samples were collected in sterile 100 mL bottles. Samples were taken to the laboratory and refrigerated at 4°C. For bacterial isolation and identification, soil samples were serially diluted, and 0.1 ml of each dilution was plated on nutrient agar plates. Based on morphological and biochemical characteristics, identification of bacterial isolates was detected (Joyruth and Gowther, 2020; Williams and Wilkins, 1986).

### *2.3 Screening of isolates bacterial for lipase producers using phenol red agar media*

Using olive oil, cow milk fat, sheep tail fat, vegetative oil and Tween-80 as lipid substrates. Visual observation and measurement of the clear zone on the agar surface in millimeters were used to identify enzymatic activity as mentioned by Singh *et al.* (2006).

#### *2.3.1 Preparation of phenol red agar plates*

Phenol red agar medium was made up according to Ilesanmi *et al.* (2020), with a slight modification of the following percentages (w/v, v/v): phenol red dye 0.01; olive oil 1.0; $CaCl_2$ 0.1; 0.5 peptone and agar 2.0. The medium's pH was raised to 7.3 by adding 1 mol/L NaOH or 1 mol/L HCl. Culture supernatant (20 µL) was added to the agar wells, and they were incubated for 48 h at 37°C. The changed of the phenol red dye from red to yellow, signifying the presence of lipolytic action. All experiments were carried out in triplicate.

### *2.4 Kinetics of growth and extracellular lipase production*

#### *2.4.1 Culture media composition*

Nutrient broth medium, which contained (g/L); peptone 5, NaCl 5, yeast extract 2, and beef extract 1, served as the pre-culture medium for the growth of bacterial isolate. The pre-culture was incubated for 16 h at 37°C with 150 rpm of shaking. The baseline culture media for lipase synthesis (g/l) are glucose (10), peptone (10), yeast extract (5), NaCl (5), $CaCl_2$ (2), $(NH_4)S_2O_4$ (2), $MgSO_4.7H_2O$ (0.2), $KH_2PO_4$ (1), $K_2HPO_4$ (0.3) and CMF (cow milk fat) 2% (v/v). The medium's pH was raised to 7.4. Five millimeters of pre-culture media were injected, and the flasks were then incubated at 37°C with 180 rpm shaking in order to measure the enzyme activity under these conditions. Following incubation for 24, 48 and 72 h,

the lipase activity was ascertained (Safoura *et al.,* 2017). Samples were then centrifuged for 10 min at 4°C at 10,000 rpm in a high-speed refrigerator centrifuge. As stated by Winkler *et al*. (1979), the lipase activity was measured using the culture supernatants that were collected (crude enzyme) with certain modifications employing *p*-NPP as a substrate for lipase activity. The quantity of enzyme that released 1 µ mol of *p*-nitrophenol per milliliter and a minute under slandered test conditions was determined as one unit (U) of lipase activity. Every experiment was carried out three times.

### *2.5 Characterization of lipase enzyme*

#### *2.5.1 Effects of temperature on lipase activity and stability*

Measurements of the enzyme activity at various temperatures within the range of 30 to 70°C at buffer pH 8.0 were made to ascertain the impact of temperature on the activity of the crude lipase enzyme. For a total period of 2 h, the temperature stability of lipase was investigated between 50 and 70°C. At pH 8.0, the activity was monitored every 15 min.

#### *2.5.2 Effects of pH on lipase activity and stability*

By tracking the enzyme's activity across a pH value range of 5.0 to 10.0, we determined the optimum pH of the extracellular lipase. The pH 4.0-5.0 acetate buffer, pH 6.0-8.0 sodium phosphate buffer and pH 9.0-10.0 glycine NaOH buffer were the buffers (50 mM) (Mazhar *et al.,* 2016).

#### *2.5.3 Impact of metal ions on lipase activity*

The effects of different metal ions were measured using $CaCl_2$, $MgCl_2$, $CoCl_2$, $Ni^{2+}$ and $Mn^{2+}$. The crude lipase was pre-incubated with these agents at 1 and 10 mM concentrations for duration of 1 h, after which the lipase activity was measured.

#### *2.5.4 Impact of EDITA and SDS inhibitors on lipase activity*

After determining the inhibitors effects using EDITA and SDS, the partially purified lipase was pre-incubated for 1 h at concentrations of one and ten mM of these agents, after which the lipase's residual activity was measured.

## 3. Results

### *3.1 Profile of isolates bacterial*

The screening of isolates from various soil sources resulted in the isolation and subculturing of 96 bacterial isolates. These isolates were further characterized based on Gram staining, catalase activity, and lipase production on phenol red agar media supplemented with Tween 80 as a fat source (Table 1). The results indicated that 11 isolates exhibited lipolytic activity, with 9 being Gram-positive and 2 Gram-negative. The lipolytic activity was

evidenced by a color change of the phenol red dye from red to yellow, signifying the release of fatty acids and a decrease in pH.

Table (2): Screening of some bacterial isolates from various soil sources.

| Source of isolation | Morphological shape | | Catalase test | | Production of lipase enzyme on phenol red agar | | Gram staining | |
|---|---|---|---|---|---|---|---|---|
| | *Bacilli* | *Cocci* | +ve | -ve | +ve | -ve | +ve | -ve |
| Soil contaminated with fats A | 17 | 7 | 20 | 4 | 2 [a] | 22 | 19 | 5 |
| Soil contaminated with fats B | 9 | 5 | 12 | 2 | 4 [a] | 10 | 12 | 2 |
| Soil without fats | 23 | 12 | 25 | 10 | 1 | 34 | 25 | 10 |
| Bovine milk | 5 | 7 | 10 | 2 | 2 ° | 10 | 10 | 2 |
| Sheep tail fats | 7 | 4 | 11 | 0 | 2 [a] | 9 | 10 | 1 |
| Total of the isolates | 61 | 35 | 78 | 18 | 11 | 85 | 76 | 20 |

[a] refers to lipolytic bacteria with positive gram (+ve), ° refers to lipolytic bacteria with negative Gram (–ve).

### *3.2 Screening on different fat sources*

The lipolytic isolates were screened for their ability to produce lipase using phenol red agar plates supplemented with various fat sources: cow milk fat (CMF), sheep tail fat (STF), vegetative oil (Veg. oil), olive oil, and Tween 80 (a synthetic fat source). The diameter of the clear zone, indicative of lipolytic activity, was measured for each isolate (Table 2). The isolate coded MC1 demonstrated the highest lipolytic activity on CMF, with a clear zone diameter of 21 mm. The color change of phenol red from red to yellow, as shown in Figure (1), further confirmed the release of fatty acids and the lipolytic action of the bacterial isolate.

Table (2): The diameter of isolates bacterial on different fat sources (millimeters).

| Isolate code | Tween 80 | CMF | STF | Veg. oil | Olive oil |
|---|---|---|---|---|---|
| MC1 | 18±0.01 | 21±0.01 | 15±0.01 | 16±0.0057 | 11±0.0057 |
| MC2 | 9±0.0057 | 11±0.005 | 8±0.005 | 10±0.005 | 3±0.01 |
| MC3 | 5±0.0057 | 6±0.005 | 2±0.02 | 3±0.01 | 3±0.05 |
| MC4 | 5±0.01 | 3±0.005 | 3±0.01 | 4±0.01 | 2±0.01 |
| MC5 | 1±0 | 0.006±0.0005 | 0 | 0 | 0 |
| MC6 | 13±0.005 | 14±0.005 | 9±0.005 | 8±0.005 | 7±0.005 |
| YC1 | 14±0.004 | 15±0.01 | 6±0.01 | 10±0.005 | 5±0.01 |
| YC2 | 4±0.01 | 6±0.01 | 3±0.01 | 2±0.01 | 3±0.005 |
| R5 | 12±0.01 | 11±0.05 | 5±0.01 | 13±0.05 | 10±0.01 |
| JS | 7±0.005 | 10±0.005 | 11±0.005 | 10±0.005 | 5±0.005 |
| SER | 3±0.005 | 2±0.01 | 3±0.01 | 2±0.005 | 4±0.005 |

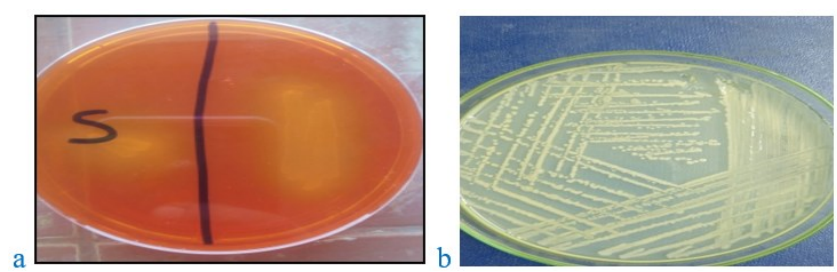


Figure (1): The lipolytic activity of MC1 on phenol red agar media supplemented with 1% CMF (a), [MC1] bacterial growth on agar media (b).

### *3.3 The quantitative screening of different isolates on cow milk fat spectrophotometric by using para-nitrophenyl palmitate (p-NPP) at 410 nm*

The lipase activity of the positive isolates on 1% CMF was quantitatively assessed using *p*-NPP as a substrate. The absorbance was measured at 410 nm after incubation for 24, 48, and 72 hours. The results, presented in Table (3), showed that the isolate MC1 exhibited the highest lipase activity (23.06 U/ml) after 24 hours of incubation. The activity decreased to 10.7 U/ml after 72 hours, possibly due to the accumulation of fatty acids (Figure 2).

Table (3): Lipase activity of different isolates is measured on the CMF using *p*-NPP assay substrate at 24, 48 and 72 h.

| Isolate code | Lipase activity after 24 h | Lipase activity after 48 h | Lipase activity after 72 h |
|---|---|---|---|
| YC1 | 22.1±0.007 | 11.85±0.01 | 8.19±0.01 |
| MC2 | 13.6±0.002 | 16.7±0.006 | 15.06±0.05 |
| MC3 | 9.39±0.005 | 4.9±0.02 | 2.24±0.04 |
| MC4 | 1.78±0.01 | 0.22±0.01 | 0.03±0.001 |
| MC5 | 2.69±0.005 | 0.49±0.01 | 0.003±0.004 |
| MC6 | 17.3±0.03 | 9.78±0.01 | 8.5±0.02 |
| MC1 | 23.06±0.003 | 19.6±0.004 | 10.7±0.04 |
| R5 | 14.8±0.003 | 11.7±0.005 | 10.9±0.05 |
| JS | 12.4±0.001 | 8.04±0.004 | 6.7±0.01 |
| SER | 0.01±0.004 | 0.006±0.001 | 0.005±0.001 |

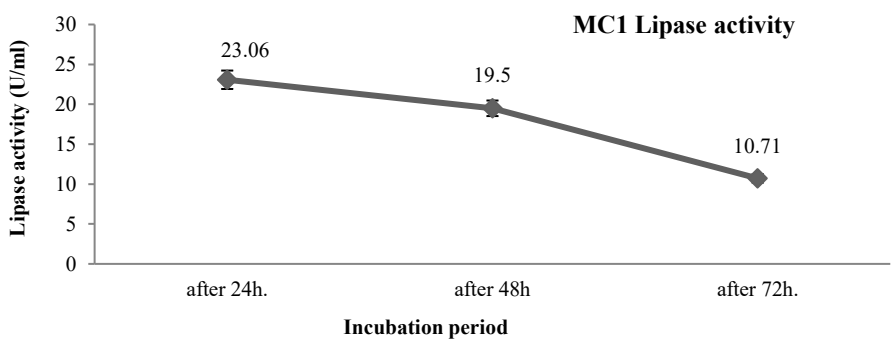


Figure (2): Lipase activity (U/ml) of the isolate MC1 on the CMF using *p*-NPP as substrate for assay.

### *3.4 Morphological and biochemical properties of bacteria coded MC1*

The morphological and biochemical characteristics of the isolate MC1 were analyzed (Table 4). The results suggested that MC1 belongs to the genus *Bacillus*. It was Gram-positive, rod-shaped, endospore-forming, and non-pigmented. The isolate was able to ferment glucose and lactose but could not hemolyze blood. It also showed a strong ability to hydrolyze starch.

Table (4): Morphological and biochemical properties of MC1.

| | |
|---|---|
| Morphological shape under light microscope | |
| Color of colony | Milky white |
| Gram staining | + |
| Shape | Rod like |
| Spore forming | Endospore |
| Pigment | Non pigmented |
| Carbohydrate fermentation | |
| Glucose | + |
| Lactose | + |
| Starch | + |
| Sucrose | + |
| Biochemical tests | |
| Indol test | - |
| VP (Voges proskauer) test | - |
| MR (methyl red) test | + |
| Urea | + |
| Nitrate reduction | - |
| Gelatin hydrolysis | - |
| Catalase test | + |
| Motility test | + |
| Blood hemolysis | - |

+ positive result, - Negative result.

### *3.5 Characterization of MC1 lipase enzyme*

#### *3.5.1 Effects of operating temperature on the lipase activity and stability assay using p-NPP*

The effect of temperature on the lipase activity of MC1 was investigated. The enzyme exhibited maximum activity at 40°C (36.17 U/ml). The activity decreased with increasing temperature, retaining only 10.94 U/ml activity at 70°C (Figure 3).

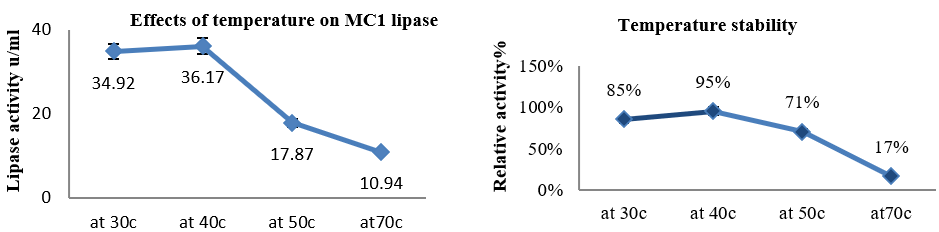


Figure (3): Effects of temperature on the activity and stability of MC1 lipase enzyme.

#### *3.5.2 Effects of operating pH on the lipase activity and stability assay using p-NPP*

The optimal pH for the lipase activity of MC1 was determined to be 7, with a maximum activity of 29.57 U/ml. The enzyme was active within a pH range of 6 to 8 (Figure 4).

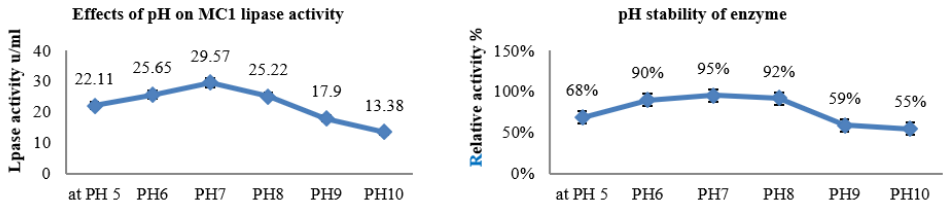


Figure (4): Effects of the pH on the activity and stability of MC1 lipase enzyme.

### *3.5.3 Effects of metal ions on the activity of (MC1) lipase enzyme*

The influence of various metal ions ($Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Ca^{2+}$, and $Mg^{2+}$) on the lipase activity of MC1 was evaluated (Table 5 and Figure 5). The results showed that $Mg^{2+}$ and $Ca^{2+}$ stimulated the lipase activity, while $Ni^{2+}$, $Mn^{2+}$, and $Co^{2+}$ inhibited it.

Table (5): Effects of metal ions ($Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Ca^{2+}$ and $Mg^{2+}$) on the relative activity of MC1 lipase enzyme.

| Effects of metal ions | 0.1 mM activity (%) |
|---|---|
| $Mn^{2+}$ | 82 |
| $Ca^{2+}$ | 126 |
| $Ni^{2+}$ | 83 |
| $Mg^{2+}$ | 104 |
| $Co^{2+}$ | 79 |

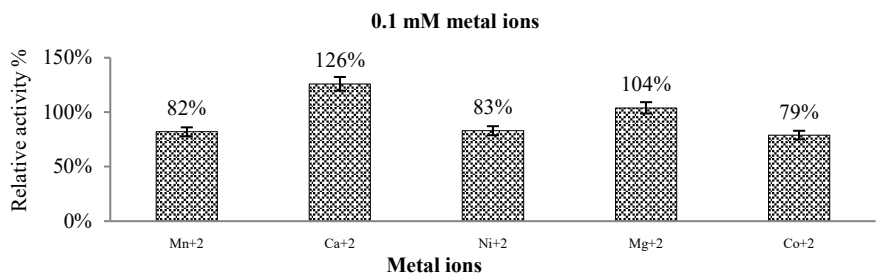


Figure (5): Effects of metal ions on the relative activity of mc1 lipase enzyme.

### *3.5.4 Effects of EDTA and SDS on MC1 lipase activity*

The effects of EDTA and SDS on the lipase activity of MC1 were also examined (Figure 6). EDTA caused a partial inhibition of the enzyme activity, while SDS significantly enhanced it.

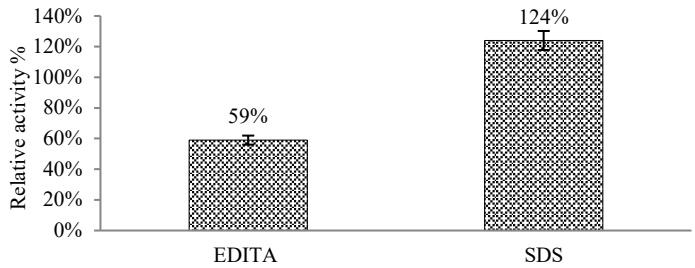


Figure (6): Effects of EDTA & SDS on MC1 lipase enzyme.

## 4. Discussion

For this study, after screening the isolates from different sources on nutrient agar, it's found that about 96 isolates are isolated, subcultured to a pure colony according to shape variation, and then frozen until further experiments. All isolates, as illustrated in Table 1, were examined for Gram staining, morphological shape, the catalase test, and lipolytic activity on phenol red agar medium. From the results, we observed that about 11 isolates improved their lipolytic action, which further investigated their lipolytic ability on different fat sources. About nine of these bacterial isolates are gram-positive, and only two are gram-negative, which further investigates their lipolytic ability on different fat sources. As illustrated in Figure 1, bacterial isolates converted the color of phenol from red to yellow as an indication for releasing the fatty acids, which decreased the pH value from neutral (red) to acidic (yellow), which improved the lipolytic action of the bacterial isolate and their ability to hydrolyze fat-forming fatty acids as mentioned by Singh *et al.* (2006). The screening and results in Table 2 showed that, isolates bacterial with codes; (MC1, MC2, MC3, MC6, YC1 and YC2) had more activity on media supplemented with cow milk fat and gave a larger clear zone as an indication of lipolytic activity. Bacterial isolates with codes; (R5 and SER) were better on media supplemented with vegetative oil and olive oil; respectively. Whilst bacterial isolates with codes JS were better on sheep fat media. Production of extracellular lipase by microorganisms is enhanced by the presence of natural fats such as cow milk fats, olive oil, and sheep tail fats (Bharathi *et al*., 2019; Fatima *et al*., 2020). All of that refers to the specificity of the lipase enzyme. From these results, after statistical analysis, we found that the bacterial isolate coded (MC1) was the best lipolytic isolate, which gave the largest clear zone as a sign of lipolytic action on cow milk fat with a clear zone diameter of 21 mm. *P*-NPP is used as a substrate for spectrophotometric assaying because lipase enzymes oxidize *P*-NPP (the colorless substrate) to produce a yellow-colored product called *p*-nitrophenol (*p*NP). The *P*NP product can be measured

spectrophotometrically at 410 nm. The amount of *P*NP produced is directly proportional to the activity of the enzyme. From the result in Table 3 and Figure 2, we observed that bacteria with code (MC1) had the highest lipolytic activity (23.06 U/ml) after incubation for 24 h, and the activity started to decrease after incubation for 72 h to 10.7 U/ml because the accumulation of fatty acids decreased lipase activity. This result agrees with Smith and Alford (1966). The results in Table 4 showed that, the morphological shape of the isolate coded MC1 is *Bacillus* sp. (+Gram). Other researchers have also reported lipase production by *Bacillus* species (Al-Dhumri and Bayoumi, 2019; Chen *et al.,* 2004; Layly *et al.,* 2021). Biochemical tests showed that it has the ability to ferment glucose and lactose. It couldn't hemolyze the blood; it has a big demand to hydrolyze the starch. Because of the unique nature of lipase enzymes in proteins, the thermostability of the lipase at high temperatures indicates its industrial applications, as Gokbulut and Arslanoğlu (2013) mentioned before. The (MC1) lipase activity reaches 95% of its relative activity at 40°C and loses up to 80% of its activity at 70°C. Similarly, the results observed in *Bacillus subtilis* by Faouzi *et al.* (2014). A decrease in the lipolytic activity was observed at temperatures above 45°C and completely ended after. Such results are similar to those reported for many bacterial species (Gilbert *et al.,* 1991). From the results in Figure 4, we observed that the MC1 lipase enzyme has the maximum activity at pH 7 (neutral nature), like many lipase enzymes in most bacterial isolates. These data are in agreement with those of Kojima and Shimizu (2003), who reported that the maximum lipase activity of *Pseudomonas fluorescens* HU 380 was at pH 7. From the results in Figure 5, we found that $Mg^{2+}$ and $Ca^{2+}$ stimulate the lipase activity, indicating the requirement of metal due to the presence of more binding sites (Ghasemi *et al.,* 2015). $Ca^{2+}$ increases the thermal stability of enzymes. An enhanced decrease in lipase activity was observed using $Ni^{2+}$, $Mn^{2+}$ and $Co^{2+}$ with about 83, 82 and 79%; respectively. Metal ions may stimulate enzyme activity by acting as a binding link between enzyme and substrate, combining with both, and so holding the substrate and the active site of the enzyme. These results recommended that, $Ca^{2+}$ and $Mg^{2+}$ stimulants were required for the stability of enzymes. In this study as found in Figure 6, EDTA produced a partial inhibition on the lipase activity of MC1 isolates, exhibiting a decrease in relative activity (59%), and SDS produced an extraordinary activation (124%). These results agree with Cristian (2002).

## 5. Conclusion

Microorganisms are an important source of commercial enzymes that are widely used in the commercial and industrial fields. These enzymes are also of great importance in the fields of renewable energy, waste recycling and environmental preservation. Analyze the characteristics

of the lipase enzyme on cow's milk fat that was isolated from the most active bacterial isolate (MC1). During the course of a 24 h incubation period, the enzyme demonstrated activity (23.06 U/ml). It was demonstrated that, the lipase enzyme was more stable at various pH values (between 5 and 10); at pH 7, the maximum lipase activity was 29.57 U/ml. Therefore, the lipase enzyme produced from isolate MC1 can be used for various industrial, commercial, and environmental purposes.

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