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Production and optimization of extracellular lipase enzyme produced by locally strain of *Geobacillus stearothermophilus*

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ABSTRACT

Thermophilic bacteria represent a precious source of highly thermostable enzymes with large features towards different applications. Among them, lipases find immense application in food, dairy, detergent, agriculture fields and pharmaceutical industries etc.

A local bacterial strain (*G. stearothermophilus*) isolated from tomato paste samples from Damascus, Syria was used to study the production and the best growth conditions for the production of the enzyme. The maximum level of the enzyme production of (71 U/ml) was recorded using an incubation temperature about 50˚C for 48 hrs and initial medium pH 9. The maximum enzyme production from *G.stearothermophilus* was also obtained glucose as carbon source and casein as a nitrogen source and the olive oil stimulated lipase in the concentration of 2%.

It was observed that lipase of this strain was influenced by different culture conditions and operational parameters. This study proposed the enzyme to be alkaline thermophilic lipase.

KEY WORDS: *Geobacillus stearothermophilus*, Thermophilic bacteria, Thermostable lipase.

1. INTRODUCTION

Enzymes are important biocatalyst for numerous reactions and employed in various areas of application. Currently, Lipolytic enzymes are attracting respectable interesting attention because of their biotechnological importance (Shariff, 2011).

Lipases or acylglycerol hydrolases (E.C.3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerols to glycerol, monoglycerides and free fatty acids (Leow, 2004). These reactions find applications in various fields such as food processing, additives in detergents and degreasing formulations, processing of fats and oils, agriculture fields, the synthesis of fine chemicals and pharmaceuticals, paper manufacturing and production of cosmetics, environmental bioremediations and in molecular biology (Zanotto, 2009; Massadeh, 2012; Mehta, 2016). Although lipases are found in plants and animals; microbial enzymes have special industrial importance because of the great variety of catalytic activities available, continuous supply because these are not seasonal fluctuations, rapid growth on inexpensive media, possible high yields and genetic manipulation. They are also more stable than animals and plants lipases and their production is more facilely and safe (Veerapagu, 2013; Alves, 2014).

The major necessity for commercial lipases is thermal stability which allows enzymatic reactions to be done at high temperatures. This has gained attention towards thermophiles in both research and industries. The advantages to choose thermostable enzymes are the intrinsic thermostability, which suggests possibilities for long time storage, increased tolerance to organic solvents, detergents, low and high pH and other denaturants, reduced risk of microbial contamination (Turner, 2007; Synowiecki, 2010).

Geobacillus stearothermophilus is a thermophilic bacterium, non-pathogenic, widely found in soil, often associated with plant food products and also can grows well in high temperature environments (Obeidat, 2012). It usually survives canning and sterilization operations where high temperatures are used (Watanabe, 2003). The growth of *G. stearothermophilus* spores results in flat sour spoilage because extracellular enzymes and acids are produced but with little or no gas generated (Oomes, 2007; Prevost, 2010). These ecological habitats of this bacterium would be a useful resource of extracellular enzymes. In the present study, we report the optimum conditions for the production, purification, and characterization of extracellular amylase from thermophilic. In this study, we mentioned the optimum conditions for the production and optimization of extracellular lipase from locally thermophilic *G. stearothermophilus* isolated from canned tomato paste.

2. MATERIALS AND METHODS

Lipase production: In a previous work, *G. stearothermophilus*, was isolated from canned tomato paste (Damascus University, Damascus/Syria) and identified according to their cultural, morphological characteristics and biochemical tests as described in Bergey's manual of systematic bacteriology (Bergy, 2009). Further, it was confirmed by PCR reaction using 16S rRNA (Cihan, 2012Al-Abras, 2015 "(In press)"). Lawrence (1967), described the ability of this organism to produce lipase enzyme by the formation of halo zones around colonies after its growth on tributyrin plates (Results not showed).

According to Lima (2004), enzyme production was done in several 250 ml Erlenmeyer flasks, each containing 150 ml of a medium consisted of (g/l): 2 casein, 3.54 KNO3; 1.0 K_2HPO_4; 0.5 $MgSO_4.7H_2O$; 0.38 NaCl; 0.01 $FeSO_4.7H_2O$, 5.0 yeast extract, 1% (v/v) olive oil and 0.6% (v/v) Tween 80 as emulsifier (Lima, 2004). The pH

was adjusted to 8.0 using 1M NaOH and HCl. Overnight cultures were suspended in 5 ml of sterile deionized water and used as the inoculums for pre-culture to adjust the turbidity of 0.5 McFarland standard and incubated at 45°C on shaking incubator at 150 rpm for 48 hrs. After incubation, each flask contents were centrifuged at 8000 rpm for 20 min at 4ºC. The cell free supernatant was collected and used as the crude extracellular enzyme.

Lipase assay: Lipase activity was detected by pNPP (p-nitrophenyl palmitate) as a substrate, method (Winkler and Stuckmann, 1979). The stock solution of the substrate (Sigma, St. Louis, MO, USA) was prepared by mixing 10 ml of isopropanol containing 30 mg pNPP with 90 ml 0.05 M sodium phosphate buffer, 2.3 mg/ml sodium deoxycholate, and 1 mg/ml gum arabic (pH 8.0).

Enzyme solution (0.3 ml) was added to 3.7 ml of the reaction solution and incubated at 45°C for 10 min. By adding 3 ml of ethanol (95%) the reaction was stopped. Lipase activity was detected by using spectrophotometer (410 nm, pH 7.0) and p-nitrophenylpalmitate (pNPP) as suggested by Winkler and Stuckmann (1979). The amount of enzyme that liberated 1 µmol of p-nitrophenol from pNPP per minute under the assay condition was defined as one unit of the lipase activity as showed by following formula,

$$\text{Lipase activity (U/mL)} = \frac{\mu mol/ml}{min}$$

Optimization of culture conditions for production of lipase from *G. stearothermophilus*:

Effect of incubation period: The tested isolate was cultured in the production medium at 45ºC for 96 hrs. The medium was harvested at 12 hourly intervals by centrifugation at 8,000 rpm for 20 min. Each supernatant collected after centrifugation was used as crude enzyme which was assayed for enzyme activity.

Effect of temperature: *G. stearothermophilus* was cultured at temperatures ranging from 15ºC to 70ºC to select the best temperature for the highest lipase production while keeping the remaining parameters constant. Each supernatant collected after centrifugation was used as the crude enzyme.

Effect of medium pH: The effect of pH of the production medium on lipase production was performed by using different initial values of pH from 1 to10 while the other parameters were constant. The pH was adjusted to the wanted value using 1N NaOH or 1N HCl.

Effect of carbon sources: Different carbon sources including glucose, lactose, mannitol, fructose, starch, glycerol and sucrose at a concentration of 1% (w/v) were added into the production medium. Each crude enzyme was studied for lipase activity after incubation at 45ºC for 48 hrs.

Effect of nitrogen sources: This effect was examined by adding the organic and inorganic nitrogen sources such as tryptone, peptone, yeast extract, urea, beef extract, soyatone, ammonium nitrate, casein, sodium nitrate, ammonium chloride, and ammonium sulphate at 1% (w/v) into the production medium.

Effect of different concentrations of olive oil: This effect was examined using different percentages of olive oil (0.2, 0.4, 0.8, 1, 2 and 4%). Each crude enzyme was tested for lipase activity after incubation at 45ºC for 48 hrs.

These data were gained using 100 mL of the production medium containing casein as nitrogen source and glucose as carbon source, at an initial pH of 9at 45ºC for 48 hrs under shaking conditions. The results were calculated from three independent cultures, and each measurement was carried out in triplicates.

3. RESULTS AND DISCUSSION

Effect of incubation time on lipase production: It was noted that the highest enzyme production was recorded after 48 hrs with 58 U/ml. Then the lipase productivity showed decreasing in the production to reach 49 U/ml after 72 hrs and 10 U/ml after 96 hrs (Fig. 1).

The maximum enzyme production started when the cell population entered into the stationary phase which means that enzyme secretion is related to the bacterial growth (Bora and Bora, 2012). Similarly, Abol Fotouh (2016), Okafor (2014) and Selvamohan, (2012) mentioned that *G. thermoleovorans*, *B. oleronius* and *Bacillus amyloliquefaciens* respectively, showed higher lipase activity after 48 hrs of incubation.

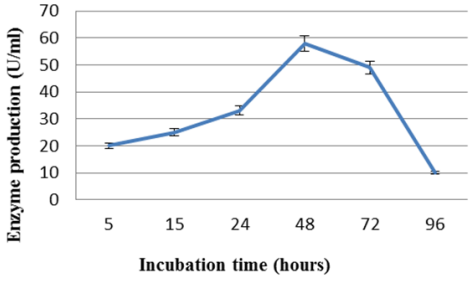

Figure.1. Effect of incubation time on lipase production. The values are mean ± SD of three replicates, error bars with 5% value

Massadeh and Sabra (2011) said that the highest lipase activity from *G. stearothermophilus*was obtained at 24 hrs of incubation, indicating that lipase was necessary for the first stages of growth. Also, Berekaa (2009) reported

that lipase by *G. stearothermophilus* showed optimum production after 30 hrs of incubation. It was suggested that the incubation time for the production of lipase is associated by the culture characteristics and is based on growth rate (Ariole and Bagshaw, 2014).

Effect of temperature on lipase production: As the temperature and pH have an important function in all the biological processes and are two significant environmental factors which influences the enzyme production (Hasan and Hameed, 2001), so the effect of temperature on lipase production was examined within a broad range of 15-70ºC while keeping all the other conditions constant. Lipase was most active in the temperature range of 37 to 50ºC, and the maximum level of lipase activity of (48 U/ml) was recorded using an incubation temperature of 50°C while increasing the temperature above 50°C led to a decrease in the lipase activity (Fig.2).

In general, studies indicated that the lipases produced by thermophilic species of *Bacillus* such as *B. stearothermophilus* are active between 50ºC and 80ºC, with good stability between 50 and 65°C (Kambourova, 2003; Lima, 2004). Sifour (2010) reported that *B. stearothermophilus* produced big yields of lipase at 55°C and 60ºC. On the other hand, Massadeh and Sabra (2011) suggested that the maximum level of lipase activity was recorded using a temperature of 45ºC.

Effect of pH on lipase production: This effect was examined in the pH range from 1 to 10. The most productivity was at pH 9 recording 55 U/ml and then it decreased at pH 10 recording 30 U/ml (Fig.3).

Jaeger (1994) refered that the enzyme activity at a high pH degree may be due to the alanine (Ala) for the first Gly-residue in the consensus sequence Gly-X-Ser-X-Gly. Massadeh and Sabra (2011) reported that *B. stearothermophilus* was able to grow and produce this enzyme at any investigated pH value and it was slightly affected by it. Abdel-Fattah (2002) pointed that the best conditions for lipase production by *Geobacillus* sp. was pH 9 and 70ºC. While Abol Fotouh (2016) mentioned that the highest production of lipase from *G. thermoleovorans* DA2 was noticed at pH 10 and after that, the enzyme activity was decreased with increasing the pH values.

Effect of carbon source on lipase production: Each lipase-producing microorganism may have its favorite carbon source for optimum production. To select the best carbon source; a variety of carbohydrate were added to the production medium.

It was noted that glucose and starch gave the highest lipase production 60 U/ml and 55.3 U/ml respectively (Fig.4). Similarly, Abdel-Fattah (2012) mentioned that the soluble starch was the best carbon source followed by D-sucrose in terms of lipase production from *Geobacillus*. And he mentioned that the main factor for the lipase expression was the carbon source, since many lipases were inducible enzymes. The same results confirmed by Ghafil and Hassan (2014) from *P. aeruginosa.*

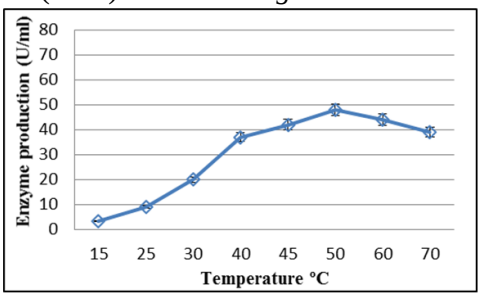

Figure.2. Effect of temperature on lipase production. The values are mean ± SD of three replicates, error bars with 5% value

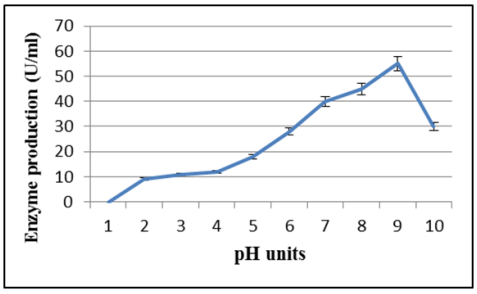

Figure.3. Effect of pH units on lipase production. The values are mean ± SD of three replicates, error bars with 5% value

On the other hand, some published experimental data have shown that lipid carbon sources (especially olive oil) stimulate lipase production, whereas carbon sources which are easy to broke down and use by bacteria play an inhibitory function (Lee, 1999; Abdel-Fattah, 2002 and Ebrahimpour, 2008).

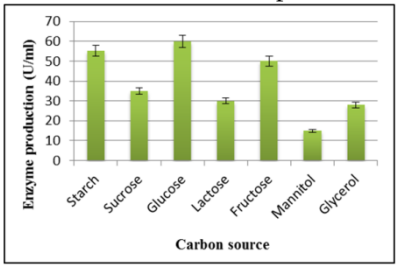

Figure.4. Effect of Carbon source on lipase production. The values are mean ± SD of three replicates, error bars with 5% value

Effect of nitrogen source on lipase production: Generally, when organic nitrogen sources are used in the production medium, microorganisms supply high yields of lipase, such as peptone, tryptone, casein and yeast extract (Sharma, 2002; Ramesh, 2014).

In this work, different nitrogen sources were supplied to the production medium. High lipase activity was obtained with casien; recording 65 U/ml. Trytone and urea have come in the second and third levels; attaining 55 and 50 U/ml respectively. While Ammonium chloride showed the poorest support for lipase production with 18 U/ml (Fig. 5).

Contrary to the current study, Bora and Bora (2012) reported that peptone was the best among all the nitrogen sources by thermophilic *Bacillus* sp. followed by tryptone, yeast extract and casien acid. While Okafor (2014) found that yeast extract showed maximum lipase activity. On the contrary, Eltaweel (2005) found that casien completely inhibited the lipase production from *Bacillus* sp. This inhibition could bethe result to the disability of the bacterium to hydrolyse it, or that the amino acids released were toxic to the bacterium, as he described. In other reports, addition of ammonium chloride and ammonium nitrate resulted in maximum lipolytic activity by with *Bacillus* species (Praveen and Sharmishtha, 2011).

Effect of different concentrations of olive oil on the lipase production: In our study, it was noted that the lipase production increased positively with increase of olive oil concentration to (2%) with an activity of (71 U/ml), then it decreased at higher concentrations of olive oil (Fig.6). Similarly, Veerapagu (2013), Amin and Bhatti (2014) and Reshma and Shanmugam (2013) reported that the highest lipase activity was obtained with 2% olive oil concentration produced by *Pseudomonas gessardii, Penicillium fellutanum* and *Aspergillus Brasiliensis* respectively.

The existence of oil in the bacterial medium stimulates bacteria to produce lipase to utilize the oil work as a nutrient source. Olive oil was employed as a carbon source for producing lipase from several bacteria such as, *Bacillus* sp. *Geobacillus* sp., *P. aeruginosa* and *Acinetobacter radioresistens* (Mehta, 2012; Narasimha, 2011 and Ghafil and Hassan, 2014). It was observed that higher concentrations of olive oil (more than 3-5%) in the culture media caused lower lipase production, this may be reason of inhibition of microbial growth at higher olive oil concentrations which lead to the minimized enzyme production levels (Mandepudi, 2012), or it could be cause of poor oxygen transfer at higher level which could modify the microbial metabolism; leading to less lipase production (Amin and Bhatti, 2014). Then the enzyme was purified and the molecular mass was detected by SDS-PAGE.

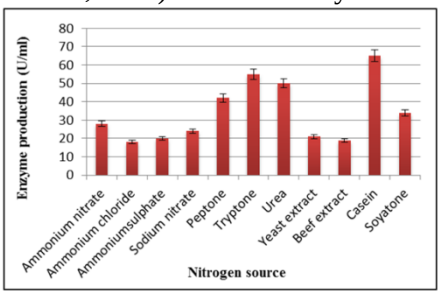

Figure.5. Effect of nitrogen source on lipase production. The values are mean ± SD of three replicates, error bars with 5% value

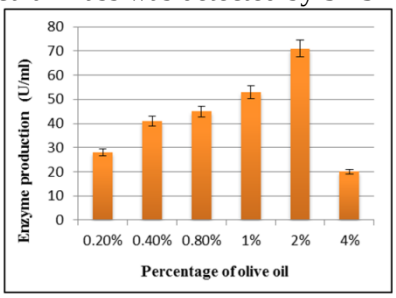

Figure.6. Effect of olive oil percentage on lipase production. The values are mean ± SD of three replicates, error bars with 5% value

4. CONCLUSION

It is evident from the above study that the culture conditions such as temperature, pH, incubation time, olive oil concentration, carbon and nitrogen sources affected lipase production significantly. This newly isolated *G. stearothermophilus* strain is a special interesting producer of an extracellular thermostable lipase enzyme especially in an inexpensive medium and its lipase is very promising and could be used for industrial purposes. However, further investigation as isolating the gene encoding this enzyme, is required to ascertain the relationship between physical and chemical effects on the crude enzyme stability and the structure of the produced lipase.

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