Research Article

Production and Partial Purification of Lipase and Esterase from a Local Isolate of *Bacillus subtilis*

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Abstract | At present, microbial Lipases and Esterase are achieving much awareness with the fast progress of enzyme technology. Lipases and Esterase signify the most important group of biocatalysts for various industrial applications, Like fat and oleo-chemical industry, food processing, flavor development, detergent industry, medical and pharmaceutical, waste treatment, bio diesel. This study aimed to assess the optimization of lipase and Esterase enzyme produced by a local isolate *Bacillus subtilis* selected from Slaughterhouse soil in Baghdad. The submerged culture method was used to produce both lipase and esterase. Olive oil medium was used to produce lipase, which is the preferred and most widely used substrate for lipase enzyme production, which a higher activity was 37 U/ml, while modified butterfat medium was used because it contains a good percentage of fats containing short-chain fatty acids that stimulate the production of esterase. which gave the highest activity 44 U/ml, The results were more effective than expected compared to other methods and materials used. after that Lipase and Esterase enzymes were partially purified by 70% ammonium sulfate precipitation and dialysis, it is one of the most widely used methods in enzyme purification research. To evaluate its effectiveness in food applications later.

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Introduction

Enzyme production from microorganisms is a cornerstone of biotechnology, with applications spanning industries such as food processing, pharmaceuticals, biofuels, and waste management. Enhancing these production methods is critical for meeting global demand, improving efficiency, and ensuring sustainability. Enzymes are used today to make more than 700 commercial products. Enzyme-based products and solutions are used in over 40 industry sectors, from household care (e.g., detergents) and bioenergy to agriculture, animal health, and food. Food application of enzymes include baking, brewing, beverage, juice, wine, dairy, and oil/fats (Yimer and Tilahun, 2018)

The global industrial enzyme marketing was valued

at $7.42 billion in 2023 and is projected to increase at 6.3% yearly due to rising request in food, textiles, and biofuels 8. Traditional enzyme extraction from plants and animals is expensive and inactive, whereas microbial fermentation offers scalability, cost-effectiveness, and higher yields (Region and Segment, 2025).

The genus Bacillus, particularly Bacillus subtilis: Gram-positive, spore-forming, non- pathogenic, represents one of the most significant bacterial species in industrial applications (AlBadran and Alshamary, 2019; Omar and Auda, 2024). This importance from its capacity to produce diverse enzymes that maintain activity under high temperatures and wide ranges of pH, coupled with minimal production of inhibitory secondary metabolites. Notably, Bacillus species contribute to over 50% of bacterial enzymes utilized in various biotechnological industries (Abbood and Auda, 2020). Furthermore, their metabolic products are classified as Generally Recognized as Safe (GRAS), underscoring their suitability for food, pharmaceutical, and industrial applications (Su *et al*., 2020; Thwaite and Atkins, 2012).

Lipases (triacylglycerol acylhydrolases, EC. 3.1.1.3) are stimulate the division and forming enzymes that catalyze the hydrolysis of triglycerides into glycerol and free fatty acids, playing essential function in digestion, lipid metabolism, and manufacturing applications. Among microbial sources, Bacillus subtilis, has acquired considerable attention due to its capability to produce thermostable, alkaline tolerant, and organic solvent resistant lipases (Iqbal *et al*., 2015; Ali *et al*., 2022). These enzymes display good stability under tough industrial conditions, making them valuable in biotechnology, food processing, detergent formation, and biofuel production (Suci *et al*., 2018).

The lipase from *Bacillus subtilis* is usually extracellular, facilitating simple extraction and purification. Its wide substrate specificity and compatibility with surfactants consolidate its applicability in detergent manufacture, in addition, its activity in organic solvents allows for use in esterification and transesterification reactions, important for biodiesel synthesis. Given its cost-effective production, height yield, and adaptation (Kaur *et al*., 2023; Zhao *et al*., 2021; Guncheva *et al*., 2011).

Esterases (EC 3.1.1.1) are a class of hydrolases that stimulate the division and forming of ester bonds in short-chain fatty acid esters, playing essential roles in metabolic pathways and industrial biocatalysis. Among microbial esterases, these produced by *B. subtilis*—have garnered significant attention due to their strong activity, thermal stability, and ability to diverse pH conditions. diverse lipases, which prefer long and medium-chain triglycerides, esterases show higher specificity for shorter acyl-chains (<C10), make of them uniquely valuable for applications in food flavoring, pharmaceutical structure, and bioremediation (Bhardwaj *et al*., 2020; Acharya *et al*., 2016).

Esterases from numerous of the microbial sources are being used in the food, beverage and dairy industry as fragrance and increase flavor compounds. Its capability to function under alkaline conditions has led to its incorporation in detergent formularization, while its enantioselectivity supports chiral drug synthesis. Recent advances in genetic engineering have moreover enabled the overexpression and tailoring of *B. subtilis* esterases for experts' industrial needs (Soumya *et al*., 2020: Sayali *et al*., 2013).

To increase the productivity of low-yielding enzymes, work on engineering strains (such as CRISPR, metabolic pathway optimization, use of effective plasmids) that enhance yield, or improving fermentation (pH, temperature, oxygen, substrate, shaking speed) enhances productivity.

Materials and Methods

In this study, the substrate for enzyme production was changed to determine its effect on production.

Bactria activation

*Bacillus Subtilis spp***.** used for this study was obtained from dep. Food sciences /College of Agricultural Engineering Sciences/ University of Baghdad. The strain was activated in Luria-Bertani (LB) broth, pH 7.0, at 37±2˚C for 24 h, and reactivated by supplementing it with 0.1% tributyrin used for the production of lipase, and esterase.

Screening of lipase and esterase production

Screening of lipase and esterase producing *B. subtilis* was done by hole plate-based screening method. Screening was done inoculating the bacteria on tributyrin agar consisted of dissolving peptone (5

g/L), yeast-extract (3 g/L), NaCl (5 g/L) , agar (20 g/l), tributyrin (10ml/L), Tween 80 agar consisted of peptone (10 g/L, NaCl (5 g/L) , $CaCl_2$ (0.1 g/L) agar (20 g/L) tween 80 (10ml/L), phenol red agar plates consisted of peptone (5 g/L), yeast-extract (3 g/L), NaCl (5 g/L), agar (20 g/L), Olive oil (10ml/L), phenol red (0.1 g/L). and sterilized at 121 C for 15 min and cooled to 60 °C. 20 ml/L Olive oil, then the pH of all media was then adjusted to 7.2 using 0.1 M NaOH. After autoclaving and cooling the last media to 60 °C, 10 mL of olive oil and 10 mL of phenol red (1 mg/mL) were added, then media were poured into petri dish (Jaeger and Kovacic, 2014; Muhsin, 2016; Pham *et al*., 2021).

Production of lipase and esterase

Lipase production was carried out in medium which used by Kumar *et al*., (2012) with some modification containing the following: peptone-8 g/l, yeast extract 2 g/l, NaCl 5 g/L, $MgSO_4$ 0.1 g/L, $CaCl_2$ 1g/L, K_2HPO_4 0.05 g/L $FeCl_2$ 0.1 g/L, 10 ml/L Olive oil emulsified in same amount 10% agacia gum then the pH of media was then adjusted to 7.4 using 0.1 M NaOH. while Esterase production was carried out in medium which used by Bhardwaj *et al.* (2020) with some modification containing the following: peptone-8 g/L, yeast extract 1 g/L, tryptone 1 g/L g/L, NaCl 5 g/L, $MgSO_4$-0.1 g/L, $CaCl_2$ 1g/L, K_2HPO_4 0.05 g/L $FeCl_2$ 0.1 g/L, 10 ml/L butterfat emulsified in same amount 10% agacia gum then the pH of media was then adjusted to 7.4 using 0.1 M NaOH. The sterilization of the media was at 121 C for 15 min and it was cooled at room temperature and the culture suspension (10% v/v) was added and incubated for 48 h at 37 C in a shaking incubator (150 rpm). And then, the samples were taken after incubation periods and the culture broth was refrigerator centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant or crude enzymes extract were stored at 4°C till the assay for lipolytic activity is complete.

Lipase and esterase activates

The activities of the lipase and Esterase samples towards para-Nitrophenyl palmitate (p-NPP) and para-Nitrophenyl acetate were determined. The reaction mixtures of Lipase activities contained 0.1 ml p-NPP (50 mM), 2.8 mL Tris–HCl buffer (0.1 M, 0.4% Triton X-100 and 0.1% Arabic gum, pH 8.0). After heating in the water bath for 5 min at 37°C, 0.1 ml lipase crud enzyme was added, and heating was continued at the same temperature for another 10 min then the tubes were put in ice bath to stop the reaction. The absorption value was obtained thereafter using a UV -spectrophotometer at 410 nm (Li *et al*., 2018). while, estimated of esterase activity was carried out as described with some modification (kim *et al*., 2013). Esterase activity was determined using p-nitrophenyl acetate (p-NPA) and the enzyme reaction was performed in 10 mM Tris-HCl (pH 8.0) buffer, a reaction mixture composed of 10 µL of 50 mM p-NPA dissolved in methanol as substrate, 40 µL of ethanol, 950 µL of 100 mM Tri-HCl (pH 8.0) and 50 µL of esterase crud enzyme was incubated and the release of p-NP was measured at 410 nm using a spectrophotometer. and heated the tube at 37°C for 10 min then the tubes were put in ice bath to stop the reaction. Then the spontaneously released p-NP was measured as a blank and it was subtracted from the amount of enzyme-mediated released p-NP to calculate enzyme activity.

One unit of lipolytic activity was defined as the amount of enzyme needed to release 1 µg of 4-nitrophenol per min under standard assay conditions.

Protein concentration assay

Protein concentration was measured spectrophotometrically at 660 nm by the Lowry method using bovine serum albumin as protein standard.

Lipase and esterase purification

Lipase and esterase precipitation by ammonium sulphate $(NH_4)_2SO_4$

The 70% salt was added (43.6 g of Ammonium Sulphate $(NH_4)_2SO_4$) to 100 ml of crud lipase and esterase solutions. Ammonium sulphate was added very tardily with continuous stirring of the solution on a magnetic stirrer in refrigerated conditions. The solution was centrifuged at 10,000 rpm for 10 min at 4 °C. These precipitated were collected and dissolved in appropriate amount of 50mM Tris HCl solution (Duong and Gabelli, 2014).

Dialysis

The precipitated and dissolved crud enzymes was collected in 45ml of 50mm tris HCl and submitted to dialysis.

About 15 cm size of dialysis bag (10 to 14 kDa) was successively boiled in 500ml of deionized water, 1+1% sodium bicarbonate and EDTA solution and

repeat it by adding 500ml of deionized water for 10 min at 60 °C. Then the dialysis bag was cooled to room temperature. the dissolved crud enzymes were transferred to dialysis bag of one end. then the bag was tightly tied, and dialysis bag was suspended in a beaker containing cooled tris-HCl buffer 20mM. This set up was kept on magnetic stirrer in refrigerator overnight with replaced buffer solution three times (Muthusamy and Beslin, 2018).

Results and Discussion

Screening of lipase and esterase

The Figure 1(a,b,c) showed ability of *Bacillus subtilis* to produce lipase enzyme by checked with lipid hydrolysis in tributyrin agar media and tween 80 agar media and phenol red agar media. All the plates were formed the clear zone around the holes of *Bacillus subtilis*. A clear zone around the holes was observed due to degradation of fat substrates in the media. The degradation of fats due to secretion of the lipase enzyme by *B.subtilis* while a screening method for lipase/Esterase producers was using pH indicator dyes, specifically phenol red and rhodamine B dyes, this screening method that used phenol red and produced more compare and easily distinguishable hydrolysis zones (Red to Orange) when pH reduces from neutral to acidic (Kumari *et al*., 2023). In Addition, the used of Tween 80 as lipase substrates in screening procedures has been criticized because Tween 80 may be hydrolyzed by esterases, giving false-positive results for lipases. Nonetheless, Tweens are attractive because they are very readily incorporated into growth media, promoting optimal contact between cells and/or enzymes and the substrate (Bharathi and Rajalakshmi, 2019), which means *Bacillus subtilis* able to produce lipase and esterase on several media which contain oil source.

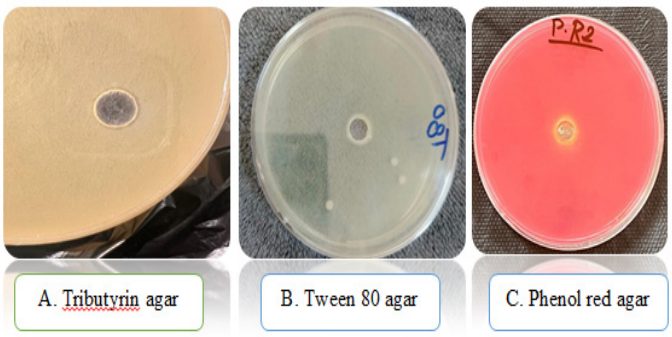

Figure 1: *The ability of bacillus subtilis to product lipase in different midea.*

Production of lipase and esterase

The results in Table 1 showed that the crude extract of lipase enzyme exhibited an enzymatic activity of 21 U/mL. These findings were consistent with the results obtained by Ma *et al*., 2017 and Mazhar *et al*., 2017 in lipase production. Meanwhile, the crude extract of esterase enzyme exhibited an enzymatic activity of 19 U/mL, a result higher than that reported by Kumar *et al*., 2012 when using various oils (coconut oil, castor oil, Tween 80, and tributyrin) as substrates for esterase production.

Olive oil is widely used as a substrate for lipase production due to several biochemical and practical characteristics, as proved by multiple studies. Here are the key reasons: High Oleic Acid Content Olive oil include ~80% oleic acid (a long-chain of fatty acid), which is a premium inducer for lipase synthesis. Lipases preferentially hydrolyze triglycerides with long-chain fatty acids, and olive oil's structure aligns with this specificity, making it a criterion carbon source in lipase production media (Sipiczki *et al*., 2024). And, Effective Interfacial Activation, Lipases work at the oil-water interface. Olive oil's hydrophobic properties differentiate this interface, triggering the "interfacial activation" mechanism of lipases, where the enzyme shifts to its active conformation. This is critical for high enzymatic activity (Panzanaro *et al*., 2010). And enhanced enzyme yield and stability, many of studies refer that olive oil significantly increases lipase production compared with other oils (Sipiczki *et al*., 2024). And last but not least, Microbial Preference, Lipase-producing yeasts (e.g., Candida, Yarrowia), molds and bacteria naturally succeed in olive oil-rich environments, as seen in olive oil-contaminated soils or freshly pressed oils (Lee *et al*., 2015).

While the Using of oils with short-chain fatty acids (SCFAs, e.g., C4–C8 (Butterfat)) for microbial esterase production offers diverse quality over long-chain triglycerides (like olive oil) due to differences in enzyme specificity, substrate approachability, and metabolic efficiency. Because: Substrate Specificity of esterases, esterases preferentially hydrolyze short-chain fatty acid esters SCFAS (≤C10), while lipases target long-chain triglycerides (≥C10). For example, *Bacillus subtilis* esterase activity was much higher with tributyrin (C4) than with olive oil (Jawed *et al*., 2016; Vasilescu *et al*., 2019; Panda *et al*., 2018). And, Enhanced Enzyme Kinetics, Faster hydrolysis: SCFAs have lower steric impediment, which allowed

Table 1: *Synopsis of partial purification of lipase and esterase*

Purification step	Size (mL)	Activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U)	Total activity (U/mg)	Number of purifications	Yield (%)
Lipase							
Crude enzyme	95	21	0.113	185.84	1995	1	100
Ammonium precipitation	41	30	0.152	197.37	1230	1.06	61.65
Dialysis	17	37	0.169	220.24	629	1.11	51.14
Esterase							
Crude enzyme	94	19	0.119	163.56	1814.2	1	100
Ammonium precipitation	34	32	0.183	178.69	1111.8	1.09	61.28
Dialysis	22	44	0.194	229.38	979	1.40	53.96

quicker arrival to the esterase's active site. and Higher solubility: SCFAs are more water-soluble, ease microbial uptake and reducing dependence on emulsification (Schönfeld and Wojtczak, 2016). Finally, Metabolic performance, Direct assimilation: SCFAs enter central metabolism (e.g., β-oxidation) transporters, saving energy, many microorganisms produce esterases efficiently with caprylic acid (C8) but struggles with stearic acid (C18) (Jawed *et al.*, 2016).

Esterase preferentially hydrolyzes short-chain fatty acid esters SCFAS (≤C10), while lipase target long-chain triglycerides (≥C10).

Purification of lipase and esterase.
Partial purification of lipase and Esterase were realized by ammonium sulphate precipitation (70%), then dialysis. The lipase was 1.11-fold partially purified, and obtained in a 51.14% yield. Specific activity was 629 U/mg. while the Esterase was 1.4-fold partially purified, and obtained in a 53.96% yield. Specific activity was 979 U/mg. the results of partial purification of lipase and Esterase from *B. subtilis* are summed up in Table 1.

The decrease in enzyme yield may be due to the presence of protease residues that may be activated at high temperatures, in addition to losses during the enzyme purification stages and high temperatures that may damage the enzyme.

May be the production of lipase and Esterase enzymes at low levels in *Bacillus subtilis* can be referred to individual biological and technically factors, as showed by research. Here are the key reasons:

Proteolytic Degradation of *B. subtilis* produced extracellular proteases that degrade heterologous proteins, including lipases, which is one of the main reasons for the decrease in the levels of produced lipase (Kaur *et al.*, 2023). As Well Native Promoter and Expression System Limitations, Although *B. subtilis* can excreted lipase normally, the extracellular production level is too low (roughly 12–25 U/mL in wealthy medium) (Wu *et al.*, 2020). And Ma *et al.*, (2018) Replacing of the original promoter with more powerful constitutive promoters (e.g., P43, PAE, or dual promoters like PhpaII-PamyQ') has significantly raise lipase yields, indicating that transcriptional organization is a major predicament. And Lipase secretion mechanism is inefficient, *B. subtilis* excretes lipases via the Sec pathway, which can become saturated due to competition with other extracellular material or proteins (Ma *et al.*, 2018). Finally. Suboptimal conditions (e.g., untrue pH or temperature) can reduce enzyme production (Ghori *et al.*, 2011).

The production of esterase enzymes at low levels in *Bacillus subtilis* can be referred to various factors. The most important, including regulatory properties Example, *B. subtilis* esterases are often subject to tight transcriptional and post-translational regulation and Some esterases, like Est55, shortage classical signal peptides and depend on non-classical secretion pathways through the stationary phase, which may limit their extracellular yield (Qiao *et al.*, 2023), mechanisms Esterases are secreted through the stationary phase via non-lytic mechanisms, but their released is slower compared to Sec-dependent proteins. This limits extracellular cumulation such as environmental conditions, like Carbon Source and Growth Conditions of Esterase production in many times induced by specific substrates (e.g., olive oil, cypermethrin and another) (Yang *et al.*,

2011). In their absence, expression residues low, and Suboptimal culture conditions (e.g., pH, temperature, aeration and shaking) can also inhibit enzyme yields, as seen in *B. subtilis* strain 1D, where cypermethrin degradation required optimized parameters (Gangola *et al*., 2018), and one of the most important reasons is competition with other *B. subtilis* hydrolases for it excretes many and various hydrolytic enzymes (e.g., proteases, xylanases, amylase and others), which may compete for transcriptional and translational resources, for instance, protease activity summits through exponential growth, potentially hydrolyze esterases or diverting resource from their synthesis (Matos *et al*., 2018).

Conclusions and Recommendations

This study identified the importance and role of *Bacillus subtilis* bacteria in enzyme production, particularly lipase and esterase, and their ability to consume different types of oil sources for enzyme production. Changing the carbon source in the production medium resulted in different enzyme production. Olive oil is considered a good source for producing lipase, which hydrolyzes long-chain oils. butterfat is also a good source for producing esterase, given its high content of short-chain fatty acids. These results are considered weak because B. subtilis may prioritize other metabolic pathways (protease, amylase and other) over lipase and esterase production under standard conditions. To increase the production of lipase and esterase enzymes, research is directed towards genetic engineering and genetic manipulation such as CRISPR technology, or increasing the number of plasmids that produce the enzymes, or increasing the overexpression of the enzymes. To increase the production of lipase and esterase enzymes, research is turning to genetic engineering and genetic manipulation such as CRISPR Technology, increasing the number of plasmids that produce the enzymes, or increasing the overexpression of Lipase/ Esterase specific chaperones.

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Novelty Statement

Olive oil medium was used to produce lipase, which is the preferred and most widely used substrate for lipase enzyme production, which a higher activity was 37 U/ml, while modified butterfat medium was used because it contains a good percentage of fats containing short-chain fatty acids that stimulate the production of esterase.

Author's Contribution

Mustafa Shakir: Collect the data and write first drift
A.H. Bayar: Editing the drift and supervision of the project.

Generative AI or AI assisted technology statement
The authors declare that no generative artificial intelligence (AI) tools or AI assisted technologies were used in the preparation, writing, data analysis or editing of this manuscript.

Conflict of interest
The authors have no conflict of interest.

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