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Development of Cost-Effective Method for the Production and Purification of Microbial Lipase Enzyme Using Ion Exchange Chromatography

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Abstract – This study explores the economical production and purification of microbial lipase enzymes from *Bacillus subtilis*, an enzyme with broad industrial applications in food, pharmaceuticals, and biofuels. *Bacillus subtilis* was cultured in defined media and butter waste, supplemented with various salts for enhanced production. Among all the combinations used for enzyme production, maximum lipase activity (18 U/ml) was achieved with butter waste supplemented with 1.0% ammonium nitrate salt. Partial purification was conducted by anion exchange chromatography with 20 mM sodium phosphate buffer (pH 8) as binding buffer. Elution profile showed the maximum enzyme recovery i.e., 4.22 U/ml and 5.22 U/ml in 50% and 30% of elution buffer, respectively. The collected fractions were desalted by commercial Sephadex G-25 and were further analyzed for the presence of lipase enzyme and total protein content. The purified lipase demonstrated significant activity when applied against various commercial oil samples compared to commercially available enzymes. This work highlights a cost-effective approach for the production of microbial lipase enzyme with promising industrial potential.

Key words: Microbial enzyme, Lipase enzyme, Ion exchange chromatography (IEX), Fast performance liquid chromatography, Turbidimetric method

1. Introduction

Lipase enzyme (EC.3.1.1.3) is one of the most significant classes of hydrolytic enzymes, triacylglycerol acyl-hydrolases, which hydrolyze fatty acids at the water-oil interface into triglycerides and glycerol. Since lipases catalyze the trans-esterification reaction using a variety of acyl groups as substrates, they are employed in a number of industrial applications [1,2]. In addition to hydrolysis, lipases catalyze other synthesis reactions such as amidation, alcoholysis, esterification, acidolysis, and aminolysis [3,4]. Its resilience against severe pH, temperature, organic solvents, and substrate specification are some of its other distinctive properties [5]. Numerous animal species, plants, bacteria, yeasts, and fungus naturally produce lipases. Lipases are isolated from bacteria because of their increased activity at different pH values. In addition, enhanced production of microbial lipases through genetic manipulation of bacterial cells is widely applied. The bacterial lipases, on the other hand, have limited applications due to lower specificity, reduced enantioselectivity in the production of pharmaceuticals, and incompatibility with high temperature [3,6-8]. The available enzymes in the market, frequently employed as biocatalysts, are not cost-effective due to expensive organic sources used in the production media [9]. On the other hand, production of lipase enzymes on agricultural or other renewable resources offers a cost-effective production method. However, optimization of their use is still challenging due to variable composition of substrate and lower yield of enzyme [10]. Lipases are employed in many different fields including food, paper and pulp manufacturing, medical, and as cleaning agents [3]. Therefore, it is necessary that the lipase enzyme should be purified through established purification methods for the removal of impurities such as other host proteins, nucleic acids, or cell debris which may interfere with the activity and stability of the enzyme [7,11,12].

Chromatography is an important step in the purification of biological molecules in downstream processing that enables effective and quick separation of proteins and enzymes [13,14]. There are different modes of chromatography that separate the biomolecules based on surface charge, size, affinity with specific ligand and hydrophobicity. Among these, ion exchange chromatography (IEX) is a powerful technique [15,16]. This chromatography is the most important type of chromatography used for separation of peptides, proteins, nucleic acid and related biopolymers [17,18]. Lipase enzyme is selectively separated, based on surface charge, from other proteins of similar molecular weight by adjusting ionic strength and pH of the buffer [18,19]. Furthermore, ion exchange chromatography (IEX) is cost effective approach compared to other purification techniques, specially when used a polishing or intermediate step in multi-step purification process [20].

Growing population and increasing demand for food have increased biological waste globally. The microorganisms have great

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potential to utilize renewable waste as a growth substrate to produce valuable products [21]. Globally, high-income and low-income countries generate 34% and 5% of biological waste, respectively. 39% of the waste is collected and the remaining is dumped in open areas. Biological treatment of the dumped waste is four to seven times cost effective than other treatment methods [22-24]. The consumption of solid waste for the production of valuable product is expected to increase from 1.3 billion tones to 2.2 billion by 2025 [23].

Lipase enzymes play a vital role in the global enzyme market. The value of enzyme market in 2022 was USD 13.11 billion and estimated to grow by 6.5% to 2030 [25]. The reported cost to produce 1 kg of microbial lipases is USD 4,393.96, using an optimized condition for *Bacillus sp*., while the chemically synthesized lipase can be 500 times more expensive as it includes advanced genetic engineering or precise chemical reactions [26,27]. The utility of lipase enzymes in the food industry accounts for 50-60% of the global enzyme market and grows with an annual growth of 6-8%. Lipase enzyme have broad range of applications in oil processing, food industry, detergents, and pharmaceuticals [28]. It is also reported that China produces biodiesel with the help of lipolytic enzyme and had decreased the cost of biodiesel to USD 32/t [25]. This work is an attempt to produce lipase enzyme by culturing *Bacillus Subtilis* on clarified butter mawa (bio-waste), as a substrate for the growth of microorganisms.

2. Material and Methods

2-1. Materials

Sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), calcium chloride ($CaCl_2$), and Tween 20 were obtained from Raidel de Haen (Seezle, Germany). Sodium chloride (NaCl), di-potassium hydrogen phosphate (K_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), iron sulphate ($FeSO_4$), magnesium sulphate ($MgSO_4$), sodium carbonate (Na_2CO_3), nutrient broth and manganese sulphate ($MnSO_4$) were purchased from Merk (Germany). Potassium chloride (KCl) and coomassie-brilliant Blue G-250 from AppliChem (Darmstadt, Germany), ammonium sulphate, and agar from BioChem (Germany). Tris-HCl from Bio Basic Inc. (Markham Antario, Canada). Ethanol from Sigma Aldrich (Steinheim, Germany), hydrochloric acid from Scharlau (Spain) and chromatographic resin, Macro-Prep DEAE (Diethylamino ethyl) and NGC Quest 10 Plus Chromatography system were purchased from Bio-Rad (Kabelsketal, Sachsen-Anhalt Germany). Phenolphthalein indicator was purchased from Thermofisher (Waltham, United States). Coconut Oil, mustard oil, sesame oil, clarified butter, olive oil and cooking oil were purchased from the local market.

2-2. Methods

2-2-1. Bacterial culture

Bacillus Subtilis was cultured in nutrient broth for 48 hours at 37 ℃. After incubation, the culture was centrifuged at 10000 rpm for 15 minutes to separate supernatant and bacterial cells. The crude supernatant was assayed for the presence of lipase and the cells were stored in 40% glycerol at 4 ℃ for future use.

2-2-2. Plate assay for confirmation of lipase

The qualitative analysis of lipase was done by using tween agar plate assay. The media used for this assay contains 2% of tween-20 (substrate for lipase), 0.01 g of methyl red (indicator), and 2 g of agar (solidifying agent). The final volume was adjusted to 100 mL and autoclaved at 121 ℃ for 20 minutes. The media was poured into the plates and allowed to solidify. Wells were bored in the solidified media. Different concentrations of crude enzyme were poured into each well and were incubated for 8 hours [17].

2-2-3. Waste selection

The microorganisms when grown in culture medium of different compositions has an impact on the production of target molecules. To study this effect, the renewable waste i.e., mawa (fat rich renewable and cost-effective waste) from clarified butter was selected as a growth medium to produce microbial lipase.

2-2-4. Optimization of culture medium

Bacillus Subtilis is a soil bacterium that interacts with different salts in soil, which may influence its growth. Eight different culture media were composed by the addition of different salt combinations. Microbial growth and lipase production were assessed at different combinations. The salts were added in the ratio of 0.1% to the culture medium. All the combinations of the medium used in this study are shown in Table 1.

2-2-5. Turbidimetric method for lipase Activity

The turbidimetric method was employed to quantitatively determine

Table 1. Combination of different salts with complex media

Combinations	Salts added with complex media
Combination 1	Na_2HPO_4, NaH_2PO_4, KCl and $MgSO_4$
Combination 2	KH_2PO_4, Na_2HPO_4, KCl and $MgSO_4$
Combination 3	Na_2HPO_4, NaH_2PO_4, KCl, $MgSO_4$, KCl, $FeSO_4$ and $MgSO_4$
Combination 4	KH_2PO_4, Na_2HPO_4, KCl, $MgSO_4$, KCl, $FeSO_4$ and $MnSO_4$
Combination 5	NH_4NO_3, K_2HPO_4, $MgSO_4$ and KCl
Combination 6	NH_4NO_3 and $(NH_4)_2SO_4$
Combination 7	$(NH_4)_2SO_4$
Combination 8	NH_4NO_3

the lipase enzyme. Shortly, 100 µl of crude supernatant (enzyme), 3.6 µl of tween 20 (substrate) and 300 µl of 120 mM of calcium chloride (enzyme activator) was added to a test tube and vortexed gently. The mixture was subjected to UV-spectrometer at 500 nm [29]. The absorbance value was noted and the units of lipase were calculated by using Equation 1.

$$\text{Unit (lipase)} = \frac{\text{Change in Absorbance}}{\text{Minute}} \quad (1)$$

2-2-6. Chromatographic experiments

Chromatographic separation was performed by subjecting the crude supernatant to ammonium sulphate precipitation (20% $(NH_4)_2SO_4$) and subsequent centrifugation at 13000 rpm for 10 minutes. Supernatant was discarded and pellet was resuspended in 20 mM phosphate buffer for further purification. A glass column packed with 1 ml slurry of Macro-Prep DEAE was equilibrated with 20 column volumes (CV) of binding buffer. After equilibration sample was loaded through injection loop and was eluted with linear gradient from 0% to 100% elution buffer followed by re-equilibration. The elution of proteins was observed in real time from the chromatogram during the process of fast performance liquid chromatography. The fractions were collected in 1 mL tubes through an automatic fraction collector and processed for the determination of lipase enzyme units by turbidimetric assay and total proteins concentration by Bradford assay.

2-2-7. Confirmation of lipase by full cream milk assay

Full cream milk assay was used to confirm the activity of lipase. The activity of the enzyme was observed at ambient temperature and boiling temperature. On the other hand, controls were also performed by taking distilled water instead of lipase enzyme. The reaction mixture consisted of 5 mL of full cream milk and 7 mL of sodium carbonate. Next, 5 drops of phenolphthalein solution (indicator) were added and mixed until the solution became homogeneous. Finally, 1 mL of 0.5% of lipase solution was added to the reaction mixture and incubated for 20 minutes. The activity of lipase enzyme was confirmed by color change as phenolphthalein remained colorless in acidic environment.

2-2-8. Determination of percentage of free fatty acid by titration

The lipase activity was further confirmed by the release of free fatty acid by titration method. 1 g of oil sample was added with 10 mL of 99% ethanol and kept at 40 ℃. After complete dissolution of oil, 1 mL of 0.1% phenolphthalein and lipase solution (0.5%) was added to the sample and incubated for 10 minutes. After incubation, 0.1 N sodium hydroxide (NaOH) solution was added drop by drop to the sample solution by using a burette until its color turned pink and remained stable for 40 seconds. The volume (V) of titrant was noted for each sample and percentage of free fatty acids was determined by Equation 2.

$$\%FFA = V \times N \times 28.2/W \quad (2)$$

Where, V was volume of titrant, N was normality of sodium hydroxide, W was weight of oil sample, and 28.2 was molecular mass of oleic acid divided by 10. The molecular mass of oleic acid was divided by 10 because normality of sodium hydroxide was 0.1 N.

2-2-9. Stability of lipase enzyme

The stability of the lipase enzyme was assessed by measuring the free fatty acids released during its activity, using cooking oil as the substrate, as previously described in section 2.2.9. The reaction was conducted at various temperatures (4 ℃, 25 ℃, 30 ℃, 35 ℃, 40 ℃, 45 ℃, 50 ℃, 55 ℃, 60 ℃, 65 ℃, 70 ℃, and 100 ℃) for 10 minutes, using a heated water bath. The enzyme concentration was maintained at 10 U/ml, and the substrate concentration was fixed at 1 g for each experiment to ensure consistency.

2-2-10. Effect of enzyme concentration

The effect of lipase enzyme concentration was evaluated using different enzyme concentration (10 U/mL, 20 U/mL, 30 U/mL, 40 U/mL, 50 U/mL, and 60 U/mL) while maintaining a constant substrate concentration of 1 g of cooking oil. Enzyme activity was determined by measuring the percentage of free fatty acids (%FFA) released during the hydrolysis of cooking oil for 10 minutes.

2-2-11. Statistical Analysis

To ensure reliability and validity of the statistical analysis, all the experiments were conducted in triplicate. Arithmetic means of the yields of lipase enzyme production for each experiment were calculated along with standard error and variances. To assess whether the data was symmetrical and normal, as is required for one-way analysis of variance, the study computed skewness and the Shapiro-Wilk test which provided robust results in small samples. Moreover, homogeneity of variances, another requirement of the analysis of variance, is assessed through Levene's test of homogeneity. Finally, the study employed the one-way analysis of variance and the post-hoc analysis to compare lipase enzyme production obtained from different media with the control group. Statistical significance of the tests was set at $p \leq 0.05$.

3. Results and Discussion

3-1. Plate Assay

The presence of extracellular lipase enzyme, in the supernatant, was confirmed by agar plates added with 2% of Tween-20 as substrate. The enzymatic activity of supernatant, at different volumes, as mentioned in Table 4 was determined. It was observed, as shown in Figure 1, that by increasing the volume of crude supernatant, the clear clear zone around the well increased. Distilled water was used as a control (well C) which did not show any clear zones. This method was first introduced by MYA Samad *et al*. [30].

In Figure 1, the encircled yellow zones around the wells showed lipase activity while C was taken as control. 20 µL of supernatant

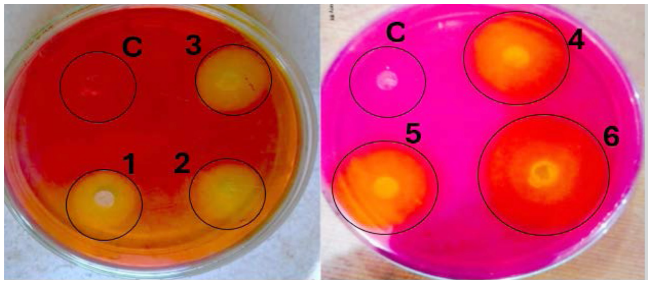

Fig. 1. Plate assay for the confirmation of lipase enzyme activity, where C represents control (distilled water only); 1 represents 20 µL, 2 represents 30 µL, 3 represents 40 µL, 5 represents 50 µL, and 6 represents 60 µL of supernatants.

was added to well 1, 30 µL to well 2, 40 µL to well 3, 50 µL to well 4, 60 µL to well 5 and 70 µL to well 6. The presence of lipase can be confirmed by the color change of phenol red from red to yellow [31,32]. A similar protocol was used in this research work with a different pH indicator, methyl red, which clearly determined the activity of lipase (Figure 1).

3-1-1. Effect of media composition on lipase production

Eight different salt combinations were made separately and added to complex media. Then bacterial cells were inoculated to the medium and cultured for 24 hours at 37 ℃. The production of lipase was observed at 500 nm (turbidimetric method), shown in Table 2.

Table 2 shows descriptive statistics of lipase enzyme production by using media of different compositions as outlined in Table 1, as well as results of the normality tests. To ensure reliable, valid, and precise results, each experiment was performed in triplicate. The results indicated that there were significant variations in the mean scores of lipase enzyme production across different media compositions, with the greatest mean production recorded for combination 8, and the lowest for the control. The very small variances and Standard Error of Means (SEM) estimates associated with each media composition implied consistency and precision of the estimated means. Likewise, the small skewness values indicated symmetry of the data points around their means, while all the Shapiro-Wilk test values being insignificant implying that the data was normally distributed.

To evaluate the effects of different media on lipase enzyme production, the One-way Analysis of Variance (ANOVA) test, as shown in Table 3, was performed. However, the analysis required homogeneity of variances across groups of combinations. The last row of Table 3 reports results of Levene's test of homogeneity of variances. The insignificant test value implied homogenous variances across groups and hence validity of the ANOVA analysis. The ANOVA results implied that variations in lipase enzyme production explained by different media were significantly larger than residual variations, leading to a significant F-value. In other words, the test results implied that different media explained significant variations in mean lipase enzyme production.

Given that variations in lipase enzyme production were significantly explained by media type, Table 4 presents the post-hoc analysis (Tukey's HSD) to statistically compare differences in production to different media types. More specifically, the analysis compared production of different media types with the control group, and the results revealed significant differences.

However, combination 8 produced the highest mean difference as compared to control (17.880) and hence it was the most effective growth media for lipase enzyme production. More specifically, the complex medium added with ammonium nitrate produced maximum

Table 2. Descriptive statistic for lipase enzyme production using various growth medium compositions

Media Compositions	Statistics					
	N	Mean	Variance	SEM	Skewness	S-W
Control	3	0.54	0.001	0.02	1.49	0.89 [0.36]
Define Media	3	1.35	0.002	0.02	-1.29	0.92 [0.46]
Complex media	3	1.76	0.000	0.01	0.94	0.96 [0.84]
Combination 1	3	3.37	0.001	0.01	-0.59	0.99 [0.78]
Combination 2	3	3.71	0.001	0.01	0.59	0.99 [0.78]
Combination 3	3	3.97	0.000	0.01	0.00	1.00 [1.00]
Combination 4	3	4.47	0.000	0.01	1.29	0.92 [0.46]
Combination 5	3	5.8	0.002	0.03	0.00	1.00 [1.00]
Combination 6	3	13.4	0.006	0.04	0.59	0.99 [0.78]
Combination 7	3	18.39	0.004	0.04	0.67	0.98 [0.75]
Combination 8	3	18.42	0.001	0.02	-1.46	0.89 [0.36]

Note: N represents the number of experiments; SEM. the Standard Error of Mean; and S-W, the Shapiro-Wilk test. Values in square parenthesis indicate the corresponding p-values.

Table 3. Homogeneity of variances and ANOVA results

	Variations	SS	MS	F-Value	Sig
ANOVA Results	Explained (Between-Groups)	1328.9	132.9	82742.1	0.001
	Residual (Within-Groups)	0.035	0.002		
Levene's Homogeneity Test (Variance)	1.67	NA	NA	NA	0.152

Note: SS represent Sum of Squares and MS represent Mean Squares.

Table 4. Post Hoc Analysis-Media Type Versus Control Group

Media type	Mean Difference	St. Error	Sig.	95% Confidential Interval	
				Lower bound	Upper bound
Define Media	0.807	0.033	<0.001	0.690	0.924
Complex Media	1.223	0.033	<0.001	1.106	1.340
Combination 1	2.827	0.033	<0.001	2.710	2.944
Combination 2	3.173	0.033	<0.001	3.056	3.290
Combination 3	3.430	0.033	<0.001	3.313	3.547
Combination 4	3.927	0.033	<0.001	3.810	4.044
Combination 5	5.260	0.033	<0.001	5.143	5.377
Combination 6	12.860	0.033	<0.001	12.743	12.977
Combination 7	17.850	0.033	<0.001	17.733	17.967
Combination 8	17.880	0.033	<0.001	17.763	17.997

units of enzyme when compared with the control. Previously, the optimization of culture medium was done by Fariha *et al*. by providing different nitrogen (beef extract, yeast extract, peptone and tryptone) and carbon sources (olive oil, glycerol, mannitol, raffinose, maltose, sucrose, dulcitol, arabinose, adonitol, mannose, salicin, fructose, sorbitol, galactose and glucose) which yielded 17.90 U/mg of lipase after 24 hours of incubation [32]. These carbon and nitrogen sources were comparatively expensive and have no significant differences with the production of lipase by using ammonium nitrate and clarified butter mawa (combination 8) as a growth medium for *Bacillus subtilis*.

3-2. Chromatographic experiments and effect of desalting on lipase activity

Ion exchange chromatography (IEX) was used for the partial purification of lipase enzyme [33]. The chromatographic experiment was performed manually and by using Fast performance liquid chromatography (FPLC). 20 mM sodium phosphate buffer was used as a binding buffer while the same buffer added with 1 M and 1.5 M sodium chloride, in stepwise gradients, was used as elution buffer for the partial purification of lipase enzyme. In both cases the maximum lipase activity was observed in the fractions containing salt concentration of 0.5 M (NaCl). The collected fractions were processed to quantify the concentration of total protein and lipase enzyme. The effect of desalting was also checked on the protein and enzyme content in the collected fraction. As shown in Figure 2, desalting with Sephadex G-25 significantly increased the lipase quantity in the fractions collected, utilizing 1 M and 1.5 M NaCl as elution buffer. This clearly shows that the presence of salt hinders the enzyme activity. Further, the enzyme activity was increased by up to three times when the fractions were subjected to desalting [34,35]. The negative impact of salt on lipase activity presented a challenge in purification strategies [36].

Manually, chromatographic experiments were also performed with the same buffer system to confirm the elution behavior of lipase enzyme. In case of 1 M NaCl, the lipase enzyme eluted in fraction 5 in which the salt concentration was almost 0.5 molar. Similarly, in case of 1.5 M NaCl, the maximum activity of lipase enzyme was observed in fraction 3 which also has the same molarity of NaCl (0.5 M). Hence, from both elution buffer systems (1 M and 1.5 M NaCl), it was confirmed that lipase enzyme was eluted at approximately 0.5 M of NaCl concentration.

Figure 2 shows the elution behavior of lipase enzyme with 1 M(A) and 1.5 M(B) NaCl in elution buffer. In case of 1 M NaCl, the maximum units of lipase enzyme (1.56) were observed in fraction 5 with total protein concentration of 1.081 mg. Furthermore, the desalting of fractions increased the activity of lipase enzyme by approximately 3-folds with maximum units of 4.21 with total protein concentration of 1.4 mg in fraction 5. The elution behavior of lipase enzymes changed with change in the concentration of NaCl. For fractions eluted with salt concentration of 1.5 M, the maximum lipase units (1.81) were observed in fraction 3 with total protein concentration of 1.04 mg by using 1.5 M sodium chloride in elution buffer. Furthermore, when the fractions were subjected to desalting, the concentration of lipase enzyme present in the fractions increased two-fold. The desalting of fractions from the process of chromatography confirmed the increase in lipase activity by 3-fold (5.22 units of lipase enzyme).

FPLC was performed to compare the purification of lipase by traditional manual method and on automated chromatographic system, which resulted in a small increase in the activity of lipase as automated chromatography has very few chances of experimental errors. The 20 mM sodium phosphate buffer was used as a binding buffer and 1 M NaCl was added to binding buffer to form elution buffer. FPLC column was equilibrated with binding buffer followed by sample loading with loading loop after equilibration, and the elution started gradient wise. Twenty-four fractions were collected, and the peaks of protein were observed at 280 nm in real time. The total protein concentration was confirmed by Bradford assay and concentration of lipase enzyme by turbidimetric method by using UV-spectrometer. The total protein concentration and lipase activity is shown in Figure 3. Esra Buyuk and Orkun Pinar used a similar method of ion exchange chromatography (IEX) for the purification of lipase enzyme, with salt (NaCl) concentration of 0.1 to 0.5 M in elution buffer. The fractions with high protein concentration were assayed for lipase activity and pooled and dialyzed against sodium acetate buffer to remove NaCl as it has a negative effect on lipase activity [34,37]. Furthermore, it also confirmed that the wild strain of *Bacillus subtilis*

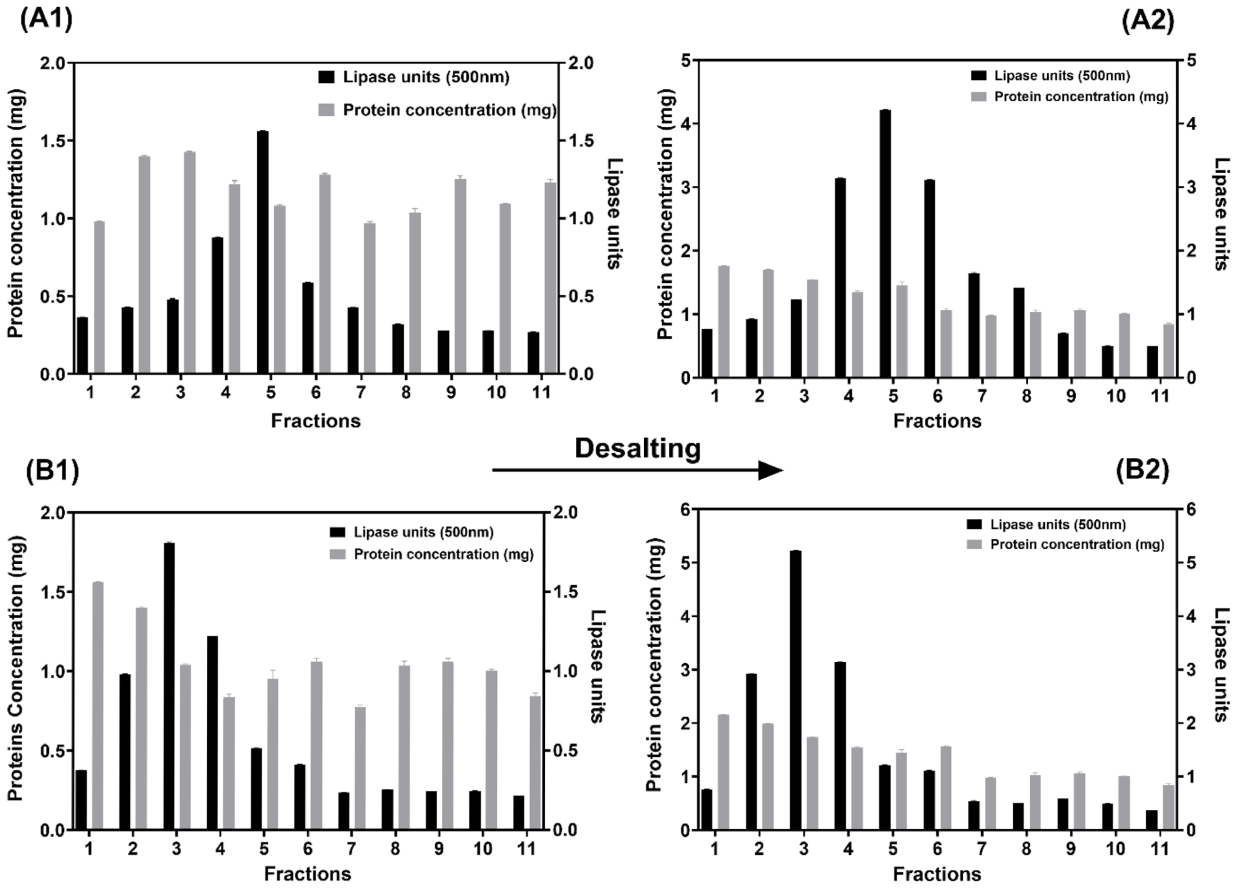

Fig. 2. Total proteins and lipase units before desalting and after desalting (with Sephadex G-25), of fractions eluted from ion exchange chromatography (IEX) in which the stationary phase was Macro-Prep DEAE, mobile phase of 20 mM phosphate buffer and elution buffer (20 mM phosphate buffer added with 1 M NaCl (A1) and 1.5 M NaCl (B1), respectively) where A2 and B2 shows the effects of desalting on lipase activity.

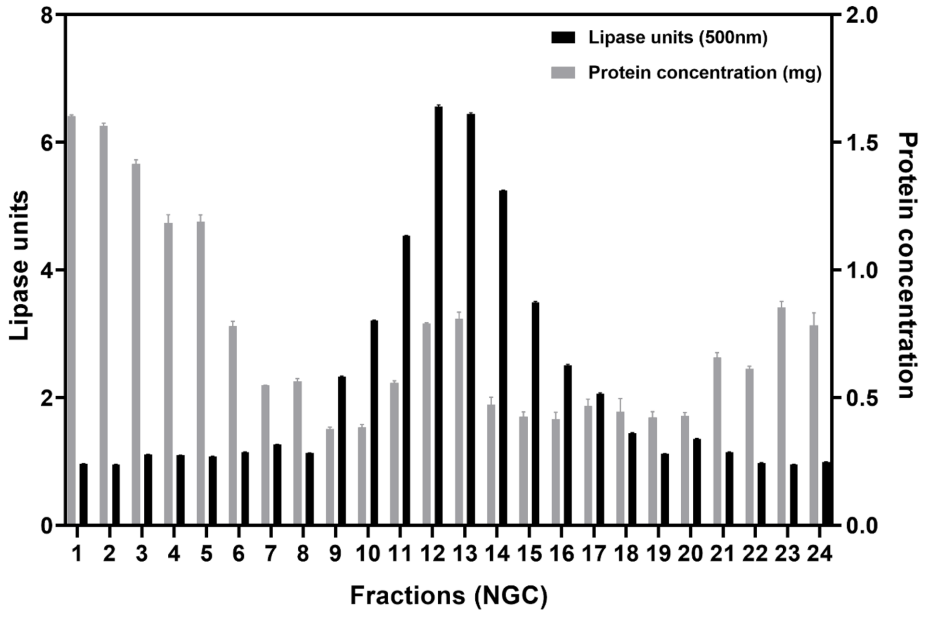

Fig. 3. Total proteins and lipase units in fractions eluted from ion exchange chromatography (IEX) in which the stationary phase was Macro-Prep DEAE, mobile phase of 20 mM phosphate buffer and elution buffer (20 mM phosphate buffer added with 1 M NaCl).

produced a variety of proteins having different elution behavior. The loosely bound proteins were eluted initially at lower concentration of NaCl, while the strongly bound proteins were eluted later with high concentration of NaCl. The results of FPLC was observed in

real time at 280 nm and the collected fractions were subjected to turbidimetric method for checking the lipase activity [20].

The detailed results of FPLC are presented in Figure 3, which shows the total protein concentration and lipase units in fractions eluted during the process of chromatography. After performing FPLC and desalting, the fractions were assayed for lipase units and total protein concentration. The maximum lipase units were eluted in fraction 12 and 13 with 6.55 and 6.44 units at 50% of elution buffer, with the total protein concentration of 0.78 and 0.80 mg.

3-3. Confirmation of lipase after purification

The purified lipase was confirmed by titration, using full cream milk. 5 mL of full cream milk was taken in a flask. 7 mL of sodium carbonate (0.05 M) was added to it, followed by the addition of 1 mL of 0.1% phenolphthalein indicator.

Figure 4 shows that samples added with lipase solution at ambient temperature turned white due to lipase activity (released fatty acids) while the control sample and sample with boiled lipase remained pink. This color change confirmed that lipase enzyme was active at ambient temperature and decreasing the pH of the solution by continuous release of free fatty acids. Normally, phenolphthalein was colorless at pH 8.5 or less than 8.5. While, with a pH higher than 8.5 it turned pink.

Fig. 4. Confirmation of purified lipase with full cream milk after purification with chromatography.

3-4. Application of lipase enzyme in the hydrolysis of different oil samples

The hydrolysis efficacy of the purified lipase enzyme was assessed by applying the enzyme on different commercial oil samples. To evaluate the efficiency of hydrolysis, the percentage of free fatty acids in oil samples before (control) and after treating with lipase, at different temperatures, were calculated.

3-4-1. Treatment of oil samples with lipase

Different commercial oil samples including coconut oil, sesame oil, cooking oil, olive oil, mustard oil, and clarified butter were taken for this study. These samples were initially treated with commercial lipase (0.5%) to find out the free fatty acid, which was taken as positive control. On the other hand, negative controls were also taken by adding distilled water instead of enzyme. Another set of oil samples was treated with purified bacterial lipases (0.5%), demonstrated from the standard curve of commercial lipases, at ambient and boiling temperature. The highest %FFA was observed in sesame oil by treating with commercial lipase.

It is clear from Figure 5 that the microbial lipases has more prominent activity at ambient temperature when compared to the commercial lipase enzyme. Furthermore, Figure 5 also shows that the lipase enzyme from our study is almost equally effective against all the commercial oil samples with highest efficacy against coconut oil. This method has already been used for the determination of %FFA in sunflower and soyabean oil samples [38].

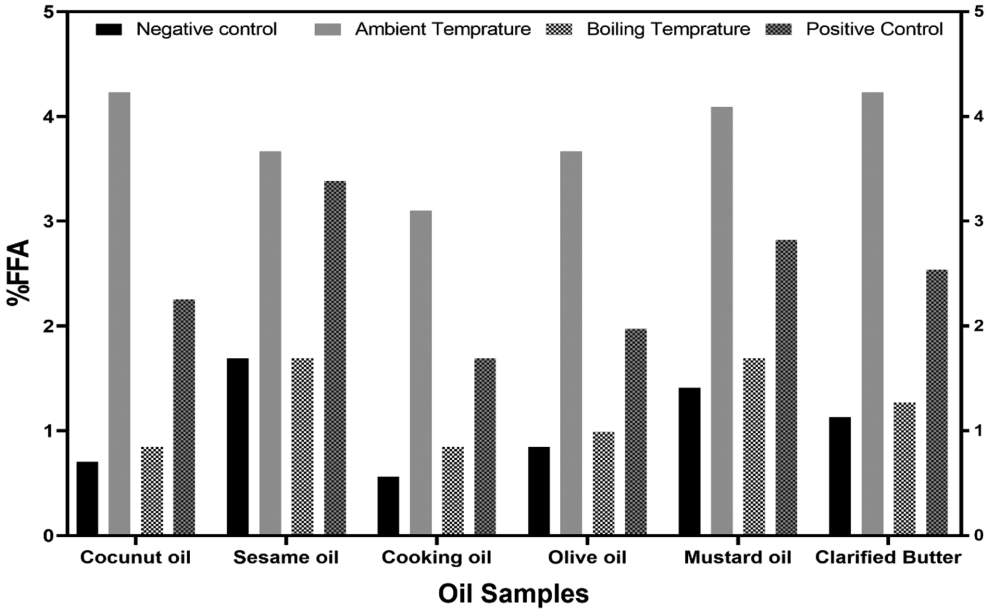

Fig. 5. Determination of the percentage of free fatty acids released from different oil samples treated with lipase.

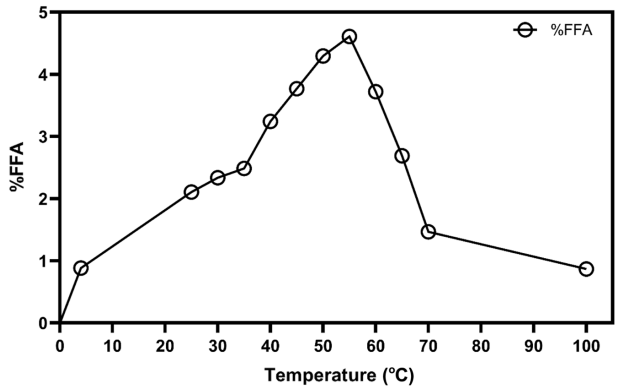

Fig. 6. Effect of temperature on lipase activity.

3-4-2. Thermal stability of lipase enzyme

Lipase activity was determined based on the amount of free fatty acids (%FFA) released by using cooking oil as a substrate. The experiment was conducted at various temperatures, at different incremental gaps (from 4 ℃ to 100 ℃).

The enzyme activity increased with the increase in temperature as shown in Figure 6. The maximum activity was observed at 55 ℃ with a %FFA of 4.61%. However, exceeding this temperature, the released %FFA decreased, indicating that the enzyme remained stable, and maximum active, up to 55 ℃. At temperature 4 ℃ and 100 ℃, the lipase enzyme was almost inactive when the activity was compared to negative control (%FFA= 0.80). This work was in agreement with the available literature [39].

3-4-3. Effect of enzyme concentration on reaction rate

The effect of enzyme concentration on the reaction rate was determined by increasing the enzyme concentration while keeping the other parameters constant. To do so, reaction rate was determined by increasing the concentration of lipase enzyme, while the substrate concentration (1 g) and temperature (55 ℃) was kept constant.

Figure 7 illustrates the effect of enzyme concentration on the reaction rate. The activity was assessed by the amount of the free fatty acids released during the reaction. At lower concentration of 10 U/mL, only 4.5% of free fatty acids (%FFA) were released. A steady increase in %FFA was observed as the enzyme concentration increased, reaching 26.03% at 40 U/mL. However, further increase in concentration

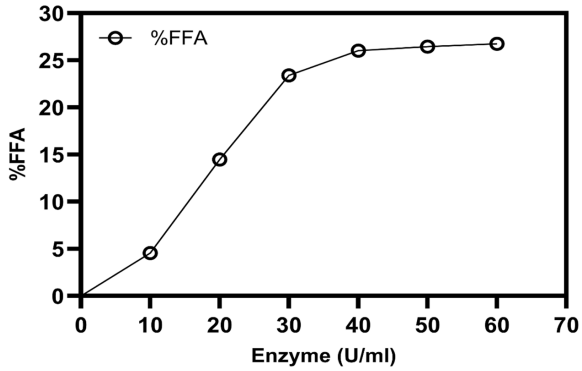

Fig. 7. Effects of enzyme concentration on %FFA.

of lipase enzyme did not resulted in a significant rise in %FFA, indicating that all the available substrates had been hydrolyzed at 40 U/ml, as reported in the literature [39].

4. Conclusion

The lipase production from *Bacillus subtilis*, partial purification, and characterization has been investigated in this study. This study suggested that the addition of ammonium nitrate salt to complex media enhanced the lipase production which highlighted the importance of optimizing nutritional parameters. The purification experiments revealed a critical interplay between salt concentration and enzyme activity, emphasizing the need for careful post-chromatographic processing. The implementation of a desalting step dramatically increased lipase activity, demonstrating the reversible nature of salt-induced activity loss. Fast performance liquid chromatography (FPLC), offering better separation, higher enzyme activities, and excellent reproducibility was used for the recovery of microbial lipases. These findings help to understand the factors influencing lipase production and purification. Optimized conditions helped to open new avenues for enhanced production of the enzyme in biofuel production, food processing, and pharmaceutical manufacturing.

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