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Optimization, Purification and Characterization of Lipase from *Streptomyces* sp. A3301, with Application of Crude Lipase for Cooking Oily Wastewater Treatment

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Abstract

Streptomyces sp. A3301, which produces lipase isolated by Panyachanakul *et al.* [1]. This optimization was done for the subsequent purification and characterization of the biological lipase produced by the isolate. The results showed that the strain produced lipase with a maximum activity of 321 U/mL using the optimal medium and conditions (1.5 % (w/v) xylose and 2 % (w/v) yeast extract, pH 7.0 at 30 °C, 150 rpm for 3 days). The specific activity of the purified lipase was 27,000 U/mg, which was 544 times the pre-purification level, based on hydrophobic chromatography. After ion-exchange chromatography, the specific activity was 5,600 U/mg, which was 113 times the pre-purification level, with a single-peak purification profile. The purified lipase had a single band based on SDS-PAGE analysis and the molecular mass was 45 kDa. The optimum temperature and thermo-stability of A3301 lipase were 60 and 30 - 55 °C, respectively. The optimum pH of the purified enzyme was pH 9.0, and the enzyme was stable in the pH range of 8.0 - 9.0. The purified lipase was stable in acetone, chloroform and toluene, with a high relative activity of 63 - 76 %. The immobilized strain was then applied to oily-wastewater treatment. The strain can remove oil and grease in synthetic wastewater containing oil at 1 - 3 % (v/v), with removal rates of 100, 99.82 and 99.68 %, respectively, after incubation for 6 days. It was then applied to oily-wastewater treatment from a restaurant, achieving the highest degradation rates of 98.53 % after treatment for 6 days. In addition, it also affected the Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) values decreasing.

Keywords: Lipase, *Streptomyces* sp. A3301, Optimization, Lipase production, Purification and characterization of lipase, Oily wastewater treatment, Immobilization

Introduction

Lipases are among the most versatile biocatalysts due to their ability to synthesize a wide range of substrates with high stereospecificity and enantioselectivity. These enzymes, classified as triacylglycerol ester hydrolases (EC 3.1.1.3), possess a remarkable capacity to catalyze both hydrolytic and synthetic

reactions. Lipase is a crucial biocatalyst primarily because it hydrolyzes carboxylic ester bonds and can act as a catalyst in esterification, interesterification and transesterification reactions. Typically, lipases are derived from microorganisms, animals and plants. Microbial lipases are particularly interesting due to their high yield, diverse enzymatic properties and varied substrate specificities. Lipases find applications in various industries, including food, paper, textile, detergent, wastewater treatment, fine chemicals, pharmaceuticals and cosmetics [2]. Many different bacterial strains, both Gram-positive and Gram-negative, are known for their ability to produce lipase enzymes. Among the most commercially significant lipase-producing bacteria are those belonging to the genus *Bacillus*. This genus includes species such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus alcalophilus*, *Bacillus coagulans* and *Bacillus stearothermophilus*. Additionally, other bacterial strains like those from the genera *Pseudomonas*, *Burkholderia*, *Staphylococcus* and *Streptomyces* have also been reported as efficient producers of bacterial lipases [3].

Microbial lipases are generally produced as extracellular enzymes, with their production influenced by the composition of the medium and physicochemical factors such as temperature, pH, agitation speed and dissolved oxygen levels. Major factors affecting lipase activity include medium components such as carbon sources, which can induce lipase production. In addition to carbon sources, nitrogen sources and essential micronutrients are crucial for microbial growth and enzyme production. These nutritional requirements can be met by various media containing components such as sugars, oils, peptone, yeast extract, malt extract and agro-industrial residues. A mix of different media compositions can be used to optimize lipase production [1,4]. Several efforts have been made to utilize inexpensive waste materials, such as palm oil mill effluent, olive mill wastewater, waste cooking oil and agro-industrial waste, as components of fermentation media to produce microbial lipases. This alternative strategy has the potential to lower production costs while also reducing environmental pollution problems. Submerged fermentation is extensively used in industrial lipase production due to the ease of monitoring variables and enzyme isolation Hydrophobic carbon sources, such as olive, palm, coconut, sunflower, castor and soybean oils, are the most frequently used to stimulate lipase production in producer microorganisms [5].

The purification process is crucial for producing commercial enzymes. Most commercial enzymes need to be highly pure, especially for applications in fine chemicals, pharmaceuticals and cosmetics. An ideal purification system for industrial applications should be cost-effective, rapid, high-yielding, scalable and capable of continuous product recovery with high selectivity. Typical purification steps include concentrating the culture supernatant containing the enzyme by precipitation with ammonium sulfate, ultrafiltration or organic solvent extraction. Precipitation often yields higher results. Given that lipases are hydrophobic and have large hydrophobic surfaces around their active sites, affinity chromatography, such as hydrophobic interaction chromatography, can be used for purification. However, due to the high cost of hydrophobic materials, alternative methods like ion exchange and gel filtration chromatography are usually employed after precipitation [4,6,7].

Post-purification, the properties of lipases from various microorganisms are extensively studied. These properties include optimal temperature and pH, stability, molecular weight and tolerance to metal ions and inhibitors. Typically, bacterial lipases have a neutral or alkaline optimal pH and an optimal temperature range of 30 - 60 °C. They exhibit high stability in several organic solvents, such as acetone, ethanol, methanol and toluene, making them suitable for synthesis reactions. Although cofactors are generally not required for lipase activity, divalent cations like calcium can stimulate activity [4,7].

Microbial lipases undeniably play a crucial role as biocatalysts, given their ability to facilitate a wide range of catalytic reactions in both aqueous and non-aqueous media. Due to their high specificity and stability, lipases are often favored over chemical catalysts. One critical application of lipase is in wastewater

treatment, particularly for removing fat, oil and grease contaminants. These pollutants originate from households, restaurants, dairy industries and food processing units, causing issues with oxygen transfer and organic matter degradation in water bodies. Additionally, oily waste accumulation can lead to pipe and drain clogs [8]. Lipases play a crucial role in the degradation of waste oil and fats. Their catalytic action occurs at the interface between oil and aqueous phases, where they excel in breaking down and converting complex, long-chain triglycerides - typically insoluble in water - into simpler free fatty acids [9]. Microbial enzymes, particularly lipases, play a crucial role in bioremediation efforts, which are increasingly important worldwide for mitigating environmental pollution. For instance, Lesny *et al.* [10] isolated *Pseudomonas* sp. from petroleum oil-contaminated sites and demonstrated its high lipase production capacity under conditions of 30 - 37 °C and pH 4 - 7. They found that supplementing the growth medium with sucrose and yeast extract enhanced lipase synthesis. *Pseudomonas* sp. as a promising candidate for breaking down petroleum oil pollutants in soil, suggesting its potential utility in bioremediation strategies for oil spills. Similarly, Blandón *et al.* [11] identified 56 lipolytic enzyme-producing bacteria in deep-sea sediments of the Colombian Caribbean. Their findings underscored the bioremediation potential of these bacteria, which produce lipases capable of degrading hydrocarbons. This ability makes them valuable for addressing environmental challenges associated with oil contamination, further emphasizing the significance of microbial enzymes in environmental remediation efforts [12]. *Streptomyces variabilis* NGP 3, known for its lipase production, was employed in wastewater treatment. The initial degradation percentage of fat/oil in the effluent by *S. variabilis* NGP 3 was 69.33 %. By the fifth day, this percentage had significantly increased to 97.08 %. This demonstrates the effective capability of S. variabilis NGP 3 in degrading fat/oil present in the effluent over a short period [13].

The objectives of this study are optimization of lipase production by *Streptomyces* sp. A3301, purification and characterization of lipase produced by this isolated and exploration of the application of the immobilized *Streptomyces* sp. A3301 in the removal of oil and grease from restaurant wastewater.

Materials and methods

Bacterial strain

The lipase-producing actinomycete, *Streptomyces* sp. A3301, was isolated from a soil sample collected at the Sakaerat Environmental Research Station (SERS) in Pakthongchai District, Nakhon Ratchasima Province, Thailand [1]. The strain was cultured on International Streptomyces Project-2 Medium (ISP-2) for 7 days. It was then transferred to ISP-2 broth and incubated at 30 °C and 150 rpm for 2 days. Following this incubation, the strain was preserved using a 20 % glycerol (v/v) solution and stored at −80 °C until further use.

Optimization of lipase production

Crude lipase preparation

The isolated from the glycerol stock was reactivated on ISP-2 agar plates, which were incubated at 30 °C for 7 days. Subsequently, the isolated strain was cultivated in ISP-2 broth and incubated at 30 °C with agitation at 150 rpm for 2 days. Following this incubation period, the cellular biomass was collected by centrifugation at 10,000×g for 10 min. The harvested cells were washed twice with a 0.85 % (w/v) NaCl solution. The initial cell density of the inoculum was adjusted to an optical density (OD) of 0.9 at 600 nm. A 10 % (v/v) of the inoculum was then introduced into 45 mL of the production medium. This culture was incubated at 30 °C with continuous agitation at 150 rpm for 3 days. After incubation, the culture broth was centrifuged at 10,000× g and 4 °C for 30 min. The resulting supernatant was used as the crude enzyme for

subsequent enzyme activity assays [1]. The experimental design for the optimization of lipase production was conducted using a 1-factor-at-a-time approach and conducted the experiment 3 times.

Effect of incubation period on lipase production

A 10 % (v/v) of the inoculum was inoculated into a production medium formulated according to Cho *et al.* [14], this medium consisted of 10 g of glucose, 10 g of soybean, 0.1 g of Na_2HPO_4, and distilled water (DW) to a total volume of 1 L. The culture was maintained at 30 °C with continuous agitation at 150 rpm. Over a period of 7 days, samples of the culture broth were collected at 24-hour intervals. These samples were subjected to centrifugation at 10,000 rpm and 4 °C for 30 min. The resulting supernatant was used as the crude enzyme solution for subsequent enzyme activity assays.

Effect of carbon sources on lipase production

In the initial production medium, glucose was used as the designated carbon source for lipase production. Subsequently, various alternative carbon sources, including xylose, mannose, galactose, maltose and lactose, were introduced as replacements. These alternatives were utilized at a final concentration of 1 % (w/v), while the remaining parameters were kept constant.

Effect of carbon concentrations on lipase production

To determine the optimal concentration of carbon sources for effective lipase production, various concentrations of the carbon source (ranging from 0 to 3 % (w/v)) were introduced into the production medium. Throughout this investigation, the remaining parameters were kept constant.

Effect of nitrogen sources on lipase production

To explore the impact of nitrogen sources on lipase production, the soybean component in the production medium was substituted with various nitrogen sources. These sources included malt extract, tryptone, peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3, each at a final concentration of 1 % (w/v) [14]. All other parameters were maintained constant during these experiments.

Effect of nitrogen concentration on lipase production

To determine the optimal concentration of nitrogen sources for enhanced lipase production, various nitrogen source concentrations (ranging from 0 to 3 % w/v) were introduced into the production medium. Throughout these experiments, all other parameters were kept constant.

Effect of temperature on lipase production

The optimal temperature for lipase production was determined by conducting experiments at different temperatures ranging from 30 to 40 °C. All other parameters were maintained at constant levels during these investigations.

Effect of initial pH on lipase production

To ascertain the ideal pH range for optimal lipase production, experiments were performed at varying pH levels ranging from 5.0 to 10.0. Throughout these trials, all other parameters were held constant.

Enzymatic activity assay

The lipase activity was determined using a spectrophotometric assay with p-nitrophenyl palmitate (*p*NPP) as the substrate. The reaction mixture comprised 0.2 mL of enzyme solution and 0.2 mL of 0.3 mg/mL substrate (prepared by dissolving 3 mg of *p*NPP in 1 mL of isopropanol and 9 mL of 0.1 M phosphate buffer, pH 7.0). The reaction mixture was incubated at 37 °C for 10 min and then boiled in water for 5 min. Subsequently, 1.6 mL of distilled water was added to stop the reaction, and the optical density at 410 nm was determined. p-Nitrophenol was used as a standard over a concentration range of 20 - 200 µM/mL. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µM of *p*-nitrophenol per minute under assay conditions [1].

$$\text{Relative activity} = \frac{\text{Enzyme activity of sample} \times 100}{\text{Enzyme activity of control}}$$

Purification of thermo-solvent tolerant lipase

The purification process began by directly applying the enzyme solution to a Butyl Toyopearl 650 M column. The column was initially equilibrated with 100 mM Tris-HCl pH 9.0 containing 20 % saturation of $(NH_4)_2SO_4$ at a flow rate of 2 mL/min. Subsequently, a decreasing gradient elution was performed using 100 mM Tris-HCl containing 20 - 0 % saturation of $(NH_4)_2SO_4$. All fractions were assessed for protein content using Lowry's method and lipase activity. Active fractions were pooled and then applied to a DEAE 650 M anion exchange chromatography column. This column was equilibrated with 100 mM Tris-HCl pH 9.0 at a flow rate of 2 mL/min, followed by an increasing gradient elution using 100 mM Tris-HCl containing 0 - 2 M NaCl. Protein content and lipase activity were determined for all fractions. The fraction exhibiting the highest lipase activity was concentrated using 10 kDa Amicon Ultra-4 Centrifugal Filters (Merck KGaA, Darmstadt, Germany) via ultracentrifugation at 3,000 g and 4 °C for 3 h. The supernatant from each step was analyzed for protein molecular mass using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of molecular mass of protein

The molecular mass of the purified enzyme was examined using SDS-PAGE following the method described by Laemmli. A 4 % (w/v) stacking gel and a 14 % (w/v) polyacrylamide separating gel were prepared. Subsequently, the gel was stained with Coomassie Brilliant Blue (CBB) R-250 and destained. A low-range protein marker kit with a molecular weight range of 17 - 250 kDa was used for mass calibration.

Characterization of thermo-solvent tolerant lipase

Effect of temperature on lipase activity and stability

To assess the effect of temperature on lipase activity, the optimal temperature was determined by incubating the reaction mixture with purified enzyme at different temperatures ranging from 30 to 70 °C in 0.1 M phosphate buffer pH 8.0. The activity was measured using the method described below. Additionally, the thermostability of the purified enzyme was examined by pre-incubating the enzyme at different temperatures within the range of 30 to 70 °C for 1 h. Subsequently, the pre-incubated enzyme was assayed for lipase activity under optimal conditions. The maximum lipase activity observed was set as 100 % relative activity.

Effect of pH on lipase activity and stability

The effect of pH on lipase activity was assessed using different pH ranges: 5.0 - 10.0, with buffers including 0.1 M acetate buffer pH 3.0 - 5.0, 0.1 M phosphate buffer pH 6.0 - 8.0 and 0.1 M glycine-NaOH buffer pH 9.0 - 10.0. The optimal pH was determined by incubating the reaction mixture with purified enzyme at different pH values within the range of 5.0 - 10.0 under optimal temperature and incubation time. To evaluate pH stability, the purified enzyme was pre-incubated at various pH values (5.0 - 10.0) at 30 °C for 1 h. Subsequently, the pre-incubated enzyme was assayed for residual lipase activity under optimal conditions. The maximum lipase activity observed was set as 100 % relative activity.

Effect of metal ions, surfactants and enzyme inhibitors on lipase stability

The effect of metal ions (Li^+, Zn^{2+}, Mg^{2+}, $Ca^{2+,}$ Co^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+} and Ba^{2+}), surfactants (SDS, Tween 85 and Triton X-100), and enzyme inhibitors (EDTA) on lipase stability was determined. The purified enzyme was pre-incubated with 1 mM metal ions, 1 mM enzyme inhibitor, and 0.05 % surfactant at 30 °C for 1 h. Subsequently, the pre-incubated enzyme was assayed for enzyme activity under optimal conditions. A controlled reaction without the addition of metal ions, surfactants, and inhibitors was used as 100 % relative activity.

Effect of organic solvent on lipase stability

The effect of various organic solvents including acetone, chloroform, toluene, 1-butanol, isopropanol, methanol, ethanol, acetonitrile, ethyl acetate and DMSO on lipase stability was assayed. The purified enzyme was incubated with 25 % of each organic solvent at 30 °C for 1 h. Subsequently, the incubated enzyme was assayed for activity using the standard method described below. A controlled reaction without the addition of organic solvent was used as 100 % relative activity.

Application of immobilized *Streptomyces* sp. A3301 for oily cooing oil wastewater treatment

Oil and Grease removal in synthetic medium by immobilized Streptomyces sp. A3301

The immobilized of *Streptomyces* sp. A3301 on a scrub pad were utilized for the removal of oil and grease in a synthetic medium containing 1 to 3 % cooking oil. The synthetic medium composition included 2 g/L of yeast extract, 0.1 g/L of urea, 0.1 g/L of Na_2HPO_4, 0.03 g/L of NaCl, 0.014 g/L of $CaCl_2 \cdot H_2O$, 0.014 g/L of KCl, 0.01 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 mL of Tween 80 and cooking oil at concentrations of 1-3 % (v/v), with the volume adjusted to 1 L using distilled water modified from Blanchet *et al.* [15].

To initiate the process, 1 g of immobilized cells on a scrub pad was added to the synthetic medium, followed by incubation at 150 rpm and 37 °C. Sampling was performed at intervals of every 2 days for a total duration of 10 days. After incubation, the immobilized cells were removed by filtration using Whatman No.1 filter paper. Subsequently, the supernatant was analyzed for oil and grease concentration using the Partition-Gravimetric Method.

Application of immobilized Streptomyces sp. A3301 for oil and grease removal in restaurant wastewater

The *Streptomyces* sp. A3301, immobilized on a scrub pad, was employed for the treatment of wastewater from restaurants. In the wastewater treatment process, 1 g of the immobilized cells on a scrub pad was inoculated into 100 mL of the restaurant wastewater. The mixture was then incubated at 150 rpm and 37 °C. Sampling was conducted at intervals of every 2 days over a period of 10 days. After the incubation period, the immobilized cells were separated from the wastewater by filtration using Whatman No.1 filter paper. Subsequently, the supernatant was subjected to analysis for oil and grease

concentration using the Partition-Gravimetric Method. Additionally, the wastewater was analyzed for Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) to assess its pollution level and organic content.

Analysis methods

Oil and grease concentrations were analyzed by Partition - Gravimetric Method, Chemical oxygen demand (COD) was analyzed by Oxidation with potassium permanganate in Alkaline and Visual Colorimetric Method and Biochemical oxygen demand (BOD) was analyzed by Azide Modification of Iodometric Method.

$$\text{Oil and grease } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{Weight of sample (g)} \times 10^6}{\text{Volume of sample (L)}}$$

Results and discussion

Optimization of thermo-solvent tolerant lipase production by *Streptomyces* sp. A3301

The first experiment, lipase production from the *Streptomyces* sp. A3301 was investigated in production medium was designed by Cho *et al.* [14] containing (w/v) glucose, 10 g; soybean, 10 g; Na_2HPO_4, 0.1 g and adjusted to 1 L with DW. The optimal condition for lipase production was determined. We investigated the time course for 7 days. The maximum lipase production was 160 U/mL after incubation at 30 °C, 150 rpm for 3 days as shown in **Figure 1**. Afterwards, the effect of carbon sources on lipase production was studied. Various carbon sources were investigated including dextrose, xylose, mannose, galactose, maltose and lactose. Xylose is a pentose sugar, while sugars like glucose (dextrose), mannose and galactose are hexoses. Additionally, maltose and lactose are disaccharides. The ability of a strain to utilize both pentose and hexose sugars indicates its versatility in metabolizing different types of sugars. The highest lipase activity was 306 U/mL in a production medium containing xylose as carbon source as shown in **Figure 2**. After that, the effect of xylose concentration on lipase production was investigated at 0 - 3 % in production medium. The maximum lipase production was 313 U/mL at the 1.5 % of xylose as shown in **Figure 3**. Subsequently, the effect of nitrogen sources on lipase activity was studied including malt extract, tryptone, peptone, soy peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3. Yeast extract was the best nitrogen source for lipase production with lipase activity of 240 U/mL. However, this lipase could be produced by using organic nitrogen sources such as malt extract, tryptone, peptone, soy peptone and yeast extract. While strain A3301 could not use the inorganic nitrogen sources such as $NaNo_3$, $(NH_4)_2SO_4$ and KNO_3 for lipase production as shown in **Figure 4**. After that, the effect of yeast extract concentration on lipase production was investigated between 0 - 3 % (w/v). The optimum concentration of yeast extract as nitrogen source for lipase production was 2 % (w/v) with lipase activity of 255 U/mL as shown in **Figure 5**. Subsequently, physicochemical factors (temperature and initial pH) of production medium were studied. The effect of initial pH of production medium was investigated at different initial pH between 5.0 - 10.0. The maximum lipase production was 239 U/mL at the initial pH at 7.0 as shown in **Figure 6**. Moreover, lipase production was demonstrated at various temperatures between 25 - 40 °C. The maximum lipase production was 321 U/mL when incubated at 30 °C as shown in **Figure 7**. In conclusion, the optimum condition for lipase production by isolated A3301 was 1.5 % (w/v) xylose, 2 % (w/v) yeast extract at initial pH 7.0, incubated at 30 °C, 150 rpm for 3 days. The maximum lipase production was 321 U/mL, which is higher than the primary production medium by approximately 2 folds.

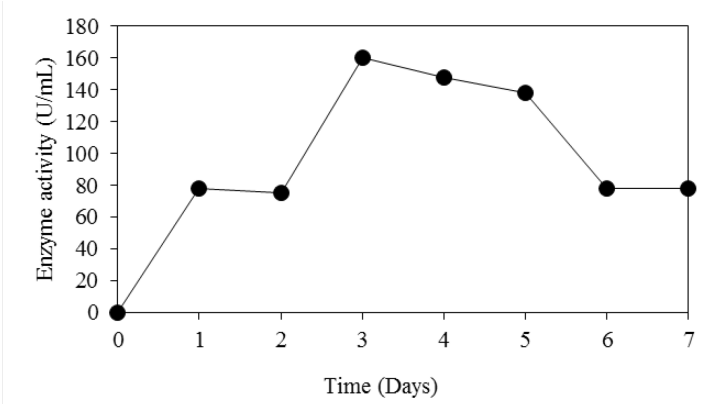

Figure 1 Time course of lipase production by *Streptomyces* sp. A3301 in 250 mL Erlenmeyer flasks, incubated at 30 °C, 150 rpm for 7 days.

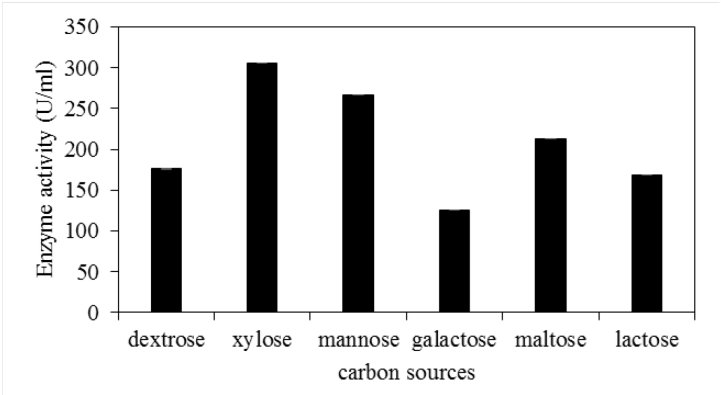

Figure 2 The effect of carbon sources on lipase production by Streptomyces sp. A3301 in 250-mL Erlenmeyer flasks, incubated at 30 °C, 150 rpm for 3 days.

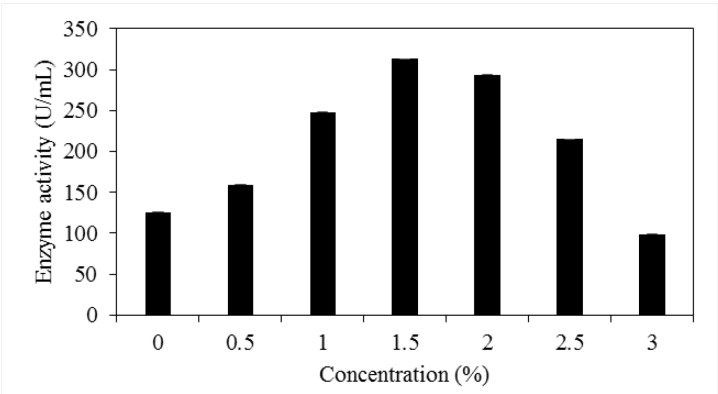

Figure 3 The effect of concentration of xylose on lipase production by *Streptomyces* sp. A3301 in 250-mL Erlenmeyer flasks, incubated at 30 °C, 150 rpm for 3 days.

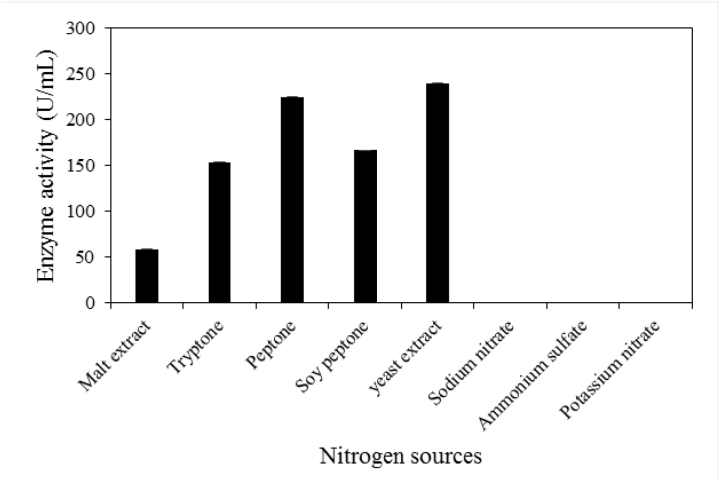

Figure 4 The effect of nitrogen sources on lipase production by *Streptomyces* sp. A3301 in 250 mL Erlenmeyer flasks, incubated at 30 °C, 150 rpm for 3 days.

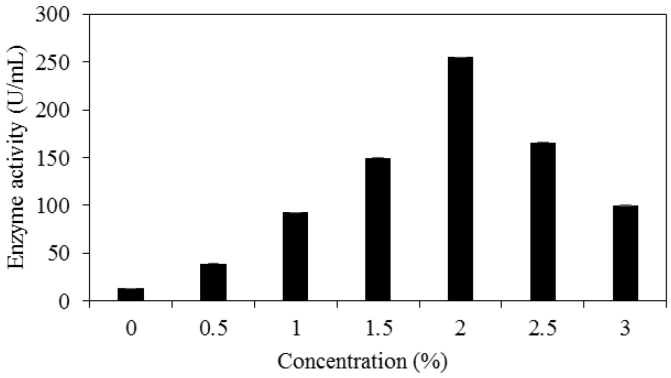

Figure 5 The effect of concentration of yeast extract on lipase production by *Streptomyces* sp. A3301 in 250-mL Erlenmeyer flasks, incubated at 30 °C, 150 rpm for 3 days.

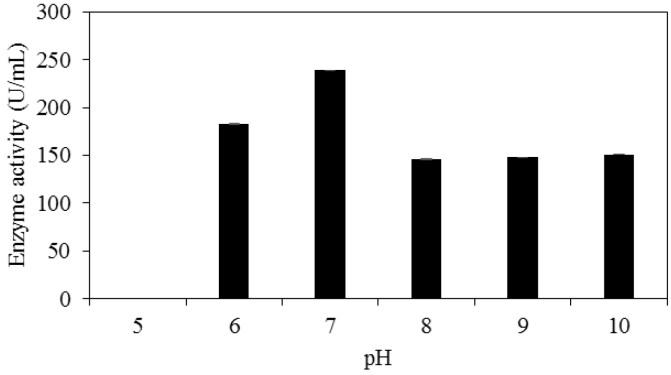

Figure 6 The effect of initial pH on lipase production by *Streptomyces* sp. A3301 in 250-mL Erlenmeyer flasks, incubated at 30 °C, 150 rpm for 3 days.

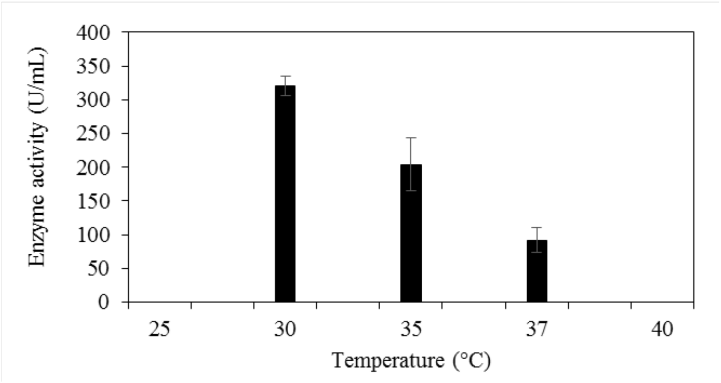

Figure 7 The effect of temperature on lipase production by *Streptomyces* sp. A3301 in 250-mL Erlenmeyer flasks, incubated at 30 °C, 150 rpm for 3 days.

The previous report showed the best lipase producing actinomyces was *Streptomyces* sp. A3301. The isolated produced maximum lipase activity was 108 U/mL in production medium [1]. Therefore, in this work, for improving the medium component and condition for maximum lipase production by A3301.

Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils [16]. However, a few authors have produced good yields in the absence of fats and oils [16]. Various effects on lipase production were optimized such as time course, carbon sources, concentration of carbon, nitrogen sources, concentration of nitrogen, pH and temperature. The time course of lipase production by isolated A3301 was determined at different incubation times of lipase production by cultivating isolated A3301 for 7 days. These results indicated that the period of lipase production was performed depends on the cell growth rate of each bacterial strain. Afterwards, the effect of carbon sources on lipase production were tested using dextrose, xylose, mannose, galactose, maltose and lactose. Production of lipase was the highest lipase activity in production medium containing xylose as carbon source. After that, optimization of xylose concentration on the enzyme production was performed at 0 - 3 % (w/v) in production medium. The concentration of xylose with 1.5 % (w/v) exhibited was the best concentration for lipase production by isolated A3301. Lipase production was decreased when increasing concentration of xylose. Hence, the effect of nitrogen sources was investigated such as malt extract, tryptone, peptone, soy peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3 in production medium. The isolated A3301 produced lipase with the maximum lipase activity in production medium containing yeast extract as nitrogen sources. After that, the effect of yeast extract concentration on lipase production was studied at the concentration of 0 - 3 % in production medium. Two % of yeast extract was the optimized concentration of nitrogen source for lipase production. This result indicated that lipase production is significantly induced by carbon sources such as sugars, oils and other nitrogen sources. In previous report, xylose is the best carbon source for lipase production by *Bacillus cereus* strain MSU AS with yielded 418.86 U/mL [17], lipase production from *Rhizopus oligosporus* with the maximum activity in 1 % xylose as carbon source [18]. In the previous report, yeast extract was found to be the most suitable nitrogen source for lipase production from *Bacillus* sp. LBN4 with enzyme activity of 3.4 U/mL [4]. Moreover, yeast extract was the best organic nitrogen source for lipase production by *Burkholderia multivorans* AU2 with the total activity of 4.70 U/mL. The organic nitrogen source plays an important role in both bacterial growth and lipase production [19]. It had been reported that *Bacillus* sp. SP5 produced the maximum lipase activity in the medium containing 5 % yeast extract as a nitrogen source [20]. Besides, *Bacillus subtilis* PCSIRNL-39 showed maximum production of lipase by using peptone as nitrogen source [21]. Moreover, organic solvent-tolerant alkaline lipase was produced from *Streptomyces*

sp. by using an optimized medium containing soybean as a nitrogen source [22]. Whereas inorganic nitrogen sources were not suitable for lipase production by isolated A3301. Similarly, peptone, yeast extract and tryptone have been reported as suitable for high extracellular lipase production by *Pseudomonas* sp., on the other hand, inorganic nitrogen compounds cannot trigger lipase synthesis [23]. In general microorganisms utilize organic nitrogen sources such as peptone and yeast extract for lipase production with high yields of lipase. In addition, organic and inorganic nitrogen sources played an important role in enzymes synthesis. Inorganic sources could be used quickly, whine organic nitrogen sources could supply cells with growth factors and amino acid required for cell metabolism and enzyme synthesis [24]. The effect of concentration of yeast extract on lipase production was determined. The result showed the optimum concentration of yeast extract was 2 % yeast extract while increasing concentration of nitrogen reduced the lipase production. This result indicated that the increase in concentration of nitrogen source produced higher biomass but reduced the lipase activity [20]. Subsequently, physicochemical factors on the enzyme production (temperature and initial pH) were studied. The effect of initial pH of production medium was investigated by adjusting production medium at different initial pH levels between pH 5.0 - 10.0. The maximum lipase production at the initial pH of production medium was 7.0. Bacteria prefer pH around 7.0 for cell growth and lipase production [4]. In more detail, the stability of enzyme catalyzed reactions is reached at specified intracellular pH values. Therefore, the changes of extracellular pH might change intracellular pH of bacterial cells that effect on destabilize enzyme synthetic network as a result, this will change cell growth and enzyme production [19]. The last factor is temperature, the lipase production was demonstrated at various temperatures between 25 - 40 °C. The maximum of lipase production was 321 U/mL when incubated at 30 °C. Temperature affected to lipase production since the cultivation at either lower or higher than the optimum temperature may lead to the decrease of bacterial growth and enzyme production. The high temperature can cause inactivation of the enzyme metabolic pathway, while low temperature may not permit flow of nutrients across cell membrane [19].

Purification of lipase from *Streptomyces* sp. A3301

A summary of the purification of lipase from *Streptomyces* sp. A3301 was shown in **Table 1**. In the first step, the enzyme was purified by Hydrophobic chromatography (Toyopearl Butyl-650M). The result showed the specific activity of 27,000 U/mg and 544 purification fold. After that, the active fractions were applied into ion-exchange chromatography (Toyopear DEAE-650M). The specific activity was 5,600 U/mg and 113 of purification fold, which resulted in single peak of purification profile. The purified lipase showed a single band on a 14 % SDS-PAGE analysis. The molecular mass was found to be 45 kDa was visualized by Coomessie brilliant blue staining as shown in **Figure 8**.

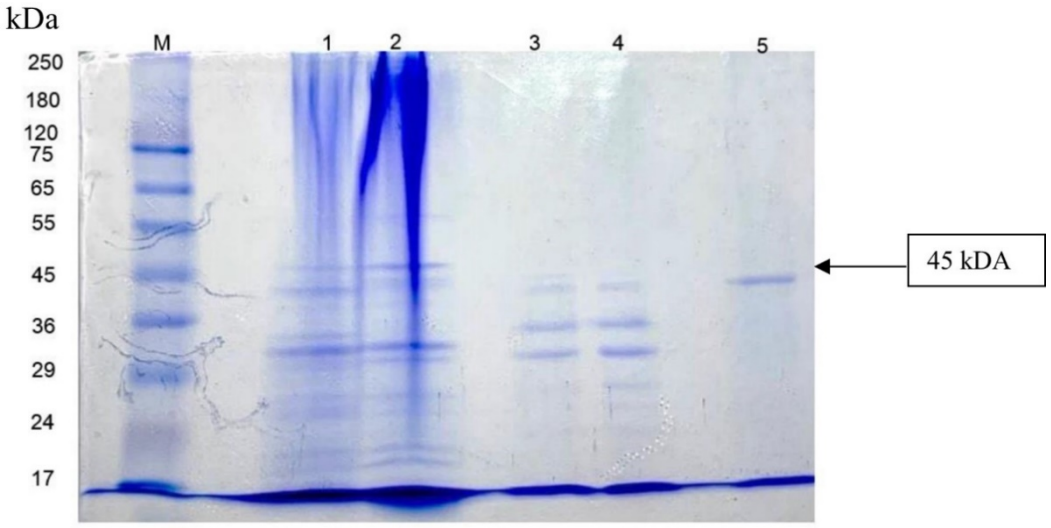

Figure 8 SDS-PAGE analysis of purification step of lipase from *Streptomyces* sp. A3301. Lane M, molecular marker; Lane 1 - 2, crude enzyme; Lane 3 - 4, active fraction from Butyl toyopeal 650M chromatography; Lane 5, active fraction from DEAE toyopeal 650 chromatography.

Table 1 Purification of thermo-solvent tolerant lipase from *Streptomyces* sp. A3301.

Purification step	Volume (mL)	Enzyme activity (U/mL)	Total activity (U)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery yield (%)
Crude enzyme	300	119	35,700	2.4	720	49.6	1	100
Butyl-650M	100	81	8,100	0.003	0.3	27,000	544	4
DEAE-650M	5	56	280	0.01	0.05	5,600	113	0.78

Characterization of purified lipase

Lipases are highly diversified in their catalytic properties such as the effect of pH, temperature, metal ions, inhibitors, surfactants and organic solvents on lipase activity. The effect of temperature on lipase activity was studied. The optimum temperature and the thermo-stability of A3301 lipase exhibited at 60 and 30 - 55 °C, respectively as shown in **Figures 9(A)** and **9(B)**. The optimum pH of lipase activity was determined. The purified enzyme exhibited the optimum pH at pH 9.0 and the stable at a pH range of 8.0 - 9.0 (**Figures 10(A)** and **10(B)**). A summary of the effect of organic solvents on lipase activity was demonstrated. The purified enzyme activity was inhibited by 1-butanol, isopropanol, methanol, ethanol, acetonitrile and ethyl acetate with relative activity of 10, 44, 35, 40, 46 and 8 %, respectively. While the organic solvents had no effect on lipase activity of crude enzyme. In addition, DMSO was found to enhance the enzyme activity with relative activity of 147 %. The effect of 1 mM of metal ions, all of metal ions were promoted the enzyme activity but only Ba^{2+} ion inhibited the enzyme activity of purified enzyme as shown in **Table 2**. The effect of 1 mM of inhibitors and 0.05 % of surfactant, the purified enzyme was inhibited by tween85 with relative activity of 14 %. While the crude enzyme was inhibited by tween 85, triton-x100 and EDTA of 26, 59 and 78 % relative activity as shown in **Table 3**.

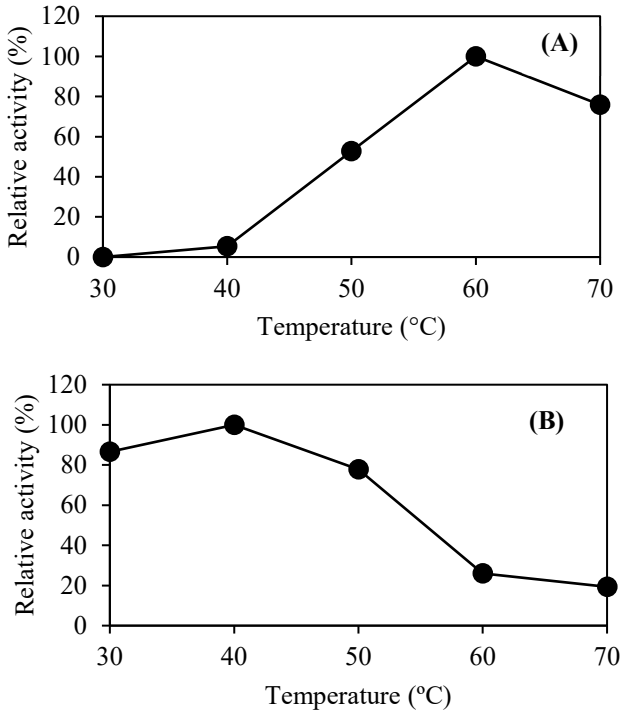

Figure 9 The effect of temperature on lipase activity of purified lipase from *Streptomyces* sp. A3301. (A) optimum temperature and (B) thermo-stability of lipase.

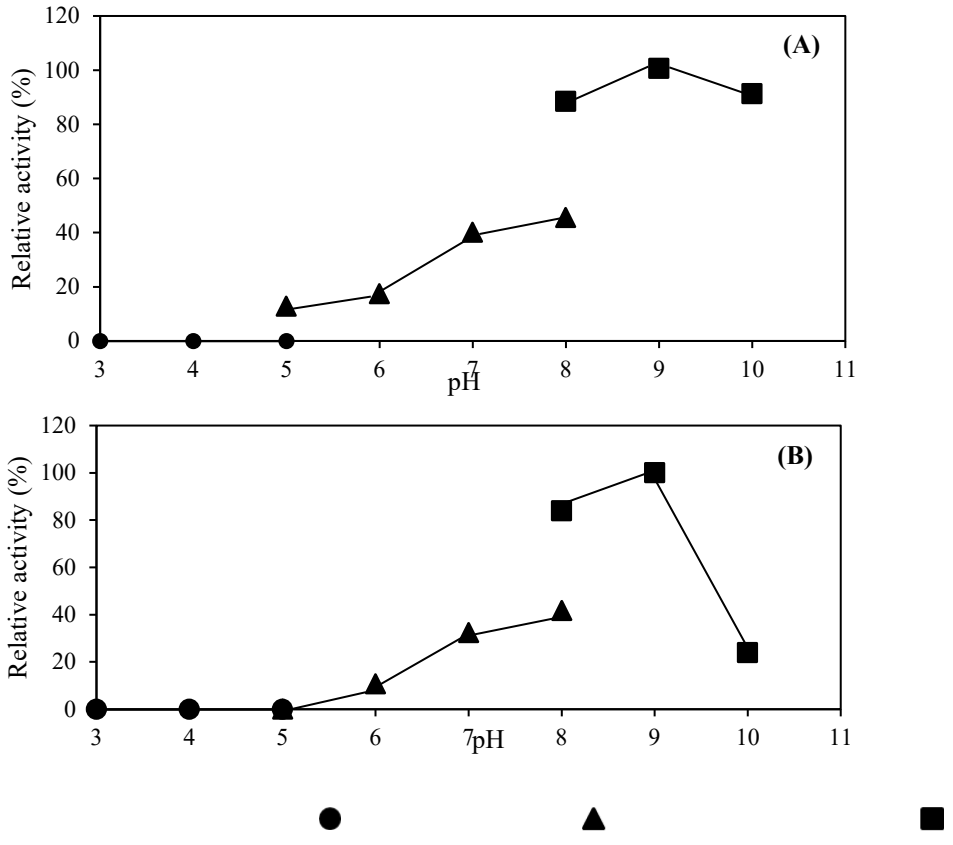

Table 2 The effect of organic solvent on the purified *Streptomyces* sp. A3301 lipase activity.

Organic solvents	Relative activity (%)
Control	100 ± 0.05
Acetone	76 ± 0.02
Chloroform	63 ± 0.03
Toluene	68 ± 0.01
1-butanol	10 ± 0.05
Isopropanal	44 ± 0.00
Methanol	35 ± 0.01
Ethanol	40 ± 0.00
Acetonitrite	46 ± 0.01
Ethyl acetate	8 ± 0.06
DMSO	147 ± 0.02

Table 3 The effect of metal ions, cheating agent and surfactant on *Streptomyces* sp. A3301 lipase activity.

Effectors	Relative activity (%)
Control	100 ± 0.02
1 mM of metal ions	
Li^{+}	137 ± 0.01
Zn^{2+}	120 ± 0.00
Mg^{2+}	101 ± 0.00
Ca^{2+}	119 ± 0.01
Co^{2+}	124 ± 0.00
Fe^{2+}	101 ± 0.02
Cu^{2+}	123 ± 0.05
Ni^{2+}	135 ± 0.00
Ba^{2+}	75 ± 0.01
1 mM of cheating agent	
EDTA	100 ± 0.02
0.05 % of surfactants	
SDS	139 ± 0.06
Tween85	14 ± 0.02
Triton X-100	104 ± 0.01

The properties of thermo-solvent tolerant lipase produced by isolated A3301 was determined by purification and characterization. The purification of thermo-solvent tolerant lipase produced by isolated A3301 was purified by loading the crude enzyme into hydrophobic chromatography and anion exchange chromatography. A summary of purification of lipase from isolated A3301, the lipase was purified with a specific activity of 27,000 U/mg-protein and this purification procedure resulted in 544-fold purification

of lipase. The molecular weight of purified enzyme was 45 kDa on SDS PAGE. In a previous study, Lipase from *Streptomyces* sp. (Loyola Lipase 1), *Geobacillus* sp. EPT9, *Spirulina platensis* and *Janibacter* sp. R02 had the molecular mass of purified enzyme about 45 kDa as same as the purified enzyme from strain A3301 [7]. The properties of purified lipase were the optimum temperature at 60 °C, thermo-stability at 30 - 55 °C. The optimum temperature and thermo-stability of lipase produced by the strain like the lipase from *Geobacillus thermodenitrificans* strain AV-5 showed the optimum temperature and pH at 65 °C and thermo-stability at 70 °C [25]. Moreover, the lipase produced by *Chromohalobacter* sp. LY7-8 was investigated with the optimum temperature at 60 °C [18]. Furthermore, optimum pH and pH stability of lipase produced by strain A3301 were demonstrated. The highest lipase activity was performed at pH 9.0 and could stable at pH range from 8.0 - 9.0. Similarly, the lipase from *Geobacillus thermodenitrificans* strain AV-5 was performed the optimum pH at pH 9.0 and pH stability at pH 10.0 [25]. The optimum pH of lipase produced from *Chromohalobacter* sp. LY7-8 showed at pH 9.0 [26]. The effect of organic solvents on lipase activity was determined by incubating the enzyme with several organic solvents such as acetone, chloroform, toluene, 1-butanol, isopropanol, methanol, ethanol, acetonitrile, ethyl acetate and DMSO. The lipase activity of purified lipase was inhibited by 1-butanol and ethyl acetate. Similarly, lipase from *Pseudomonas stutzeri* ZS04 was inhibited by n-butanol, which may be caused by structural disorders [27]. Generally, lipases are stable in organic solvents, with some exception stimulation or inhibition. Non-polar solvents shift the equilibrium from close to open form, increasing the activity of lipase. On the other hand, polar organic solvents have more harmful effects on lipase, since this type of solvent can remove some essential water layers of enzyme, changing the native conformation of lipase, through disturbing hydrogen bonding and hydrophobic interactions [23]. The effect of 1 mM of metal ions, the lipase from strain A3301 was resistance to the presence of Li^{+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+} and Ba^{2+}. Likewise, lipase from *Pseudomonas* sp. had resistance of Na^{2+}, Ca^{2+}, Li^{+} or Sr^{2+} [23]. The effect of 1 mM of inhibitors and 0.05 % of surfactant, the purified A3301 lipase was inhibited by tween85. While the A3301 activity increased when adding Triton X-100. Lipase from *Pseudomonas aeruginosa* appeared to be strongly inhibited by adding Tween 80 and Triton WR 1339 and Triton X-100 was able to increase the lipase activity [23]. The performance of lipases in the presence of detergents depends on some factors, such as ionic strength, pH and composition of the detergent. Triton X-100, Tween 20 and Tween 80 are nonionic polyoxymethylene detergents since they have a hydrophobic and hydrophilic part. The hydrophobic part usually consists of an alkyl chain (branched or unbranched), while the hydrophilic part is composed of uncharged ethylene oxide units. Generally, lipases have more positive effects in presence of nonionic surfactants than in presence of anionic or cationic surfactants, which can be explained by the type of binding between surfactant and lipase. Nonionic surfactants interact with lipase through hydrophobic interactions, while ionic surfactants can bind to lipases by a combination of electrostatic and hydrophobic interactions. Therefore, these combinations of interactions can promote conformational changes on the structure of lipase, decreasing the enzymatic activity [23].

Efficiency of oil and grease degradation of the immobilized cell in synthetic medium

Oil and Grease degradation was investigated by using the immobilized cell, *Streptomyces* sp. A3301 in the synthetic medium of 1 - 3 % concentration of oil (6,170 - 15,620 mg/L) and incubated at 150 rpm, 37 °C, and interval sampling every 2 days for 10 days. The result showed the stain could remove oil and grease in a synthetic medium containing 1 - 3 % of the oil was 100, 99.82 and 99.68 % of degradation rates, respectively after incubated for 6 days as shown in **Figure 11**.

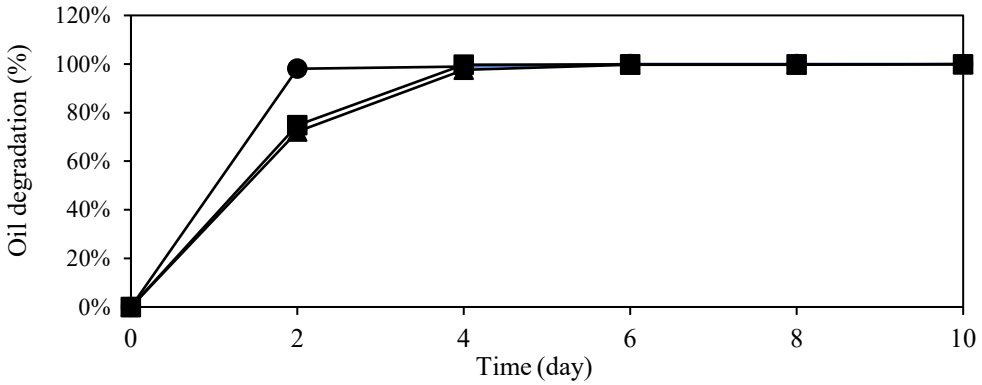

Figure 11 Effect of incubation time on oil degradation (%) at various oil concentrations in synthetic medium. ▲; oil concentrations 1 %, ●; oil concentrations 2 % and ■ ; oil concentrations 3 %.

In a previous study, the percentage of crude oil degradation in a mineral salt medium by lipase from *B. subtilis* and *P. aeruginosa* was evaluated over a period of 28 days. The lipase from *B. subtilis* was able to degrade 8.11 ± 0.70 % of the crude oil, while *P. aeruginosa* degraded 15.69 ± 0.03 % of the crude oil. That higher than the control about 3.32 and 6.43 times [28]. In another report, *P. aeruginosa* UKHL1 and its lipase for the biodegradation of oily wastewater. The culture was allowed to grow under optimized media and conditions for 24 h, followed by the addition of synthetic oily wastewater containing 1 % oil. The produced lipase and the growing culture in combination degraded 9, 21 and 37 % of the oil from the synthetic wastewater after 24, 48 and 72 h, respectively, at 37 °C and 150 rpm [29].

Oil and grease in restaurant wastewater treatment

Subsequently, the immobilized cell was applied to remove oil and grease in wastewater from restaurants in the cafeteria. Wastewater from restaurants has 410 - 1,100 mg/L of oil and grease concentration. The highest degradation rates were 98.53 % after treatment for 6 days. So, the result indicated that the immobilized cell could improve to remove oil and grease in wastewater from restaurants as shown in **Figure 12**. In addition, oil and grease removal also affected the Biochemical oxygen demand (BOD) was decreased from 100 to 60 mg/L and Chemical oxygen demand (COD) was decreased from 250 to 120 mg/L.

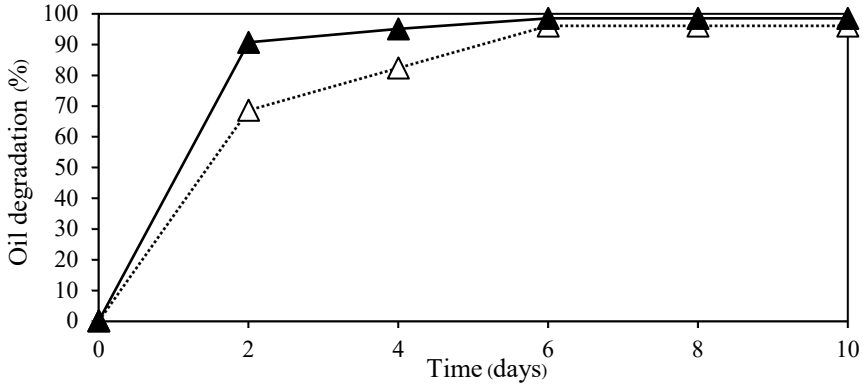

Figure ▲ treatment with *Streptomyces* sp. A3301 and △; control condition (without *Streptomyces* sp. A3301).

In the previous study, *Bacillus cereus* HSS lipase demonstrated effectiveness in treating oily wastewater. It achieved removal efficiencies of 87.63 % for biological oxygen demand, 90 % for total suspended solids, and 94.7 % for oil and grease. These results suggest its potential application in waste cooking oil (WCO) bio-hydrolysis [30]. Other lipases have also shown promise in degrading oils. For instance, *A. baumannii* RMUTT3S8-2 successfully digested oil in poultry processing wastewater, achieving an oil hydrolysis degree of 41.94 ± 4.98 % over 12 days. Additionally, *Flavobacterium* and *Acinetobacter* strains isolated from compost fertilizer removed oil by 30 and 47 %, respectively [31]. The results indicated that *Streptomyces* sp. A3301 is efficient for oily wastewater treatment.

Conclusions

Streptomyces sp. A3301 exhibited remarkable lipase production capabilities, reaching a maximum activity of 321 U/mL in an optimized production medium consisting of 1.5 % (w/v) xylose and 2 % (w/v) yeast extract, with an initial pH of 7.0. The optimal conditions for production involved incubation at 30 °C with agitation at 150 rpm for 3 days. Furthermore, the purified lipase displayed impressive specific activity of 5,600 U/mg and a purification fold of 113 after undergoing hydrophobic and ion-exchange chromatography, with a single peak observed in the purification profile. SDS-PAGE analysis revealed a single band with a molecular mass of 45 kDa. The purified lipase exhibited optimal temperature and thermostability at 60 and 30 - 55 °C, respectively. Its optimum pH was found to be pH 9.0, with stability observed in the pH range of 8.0 - 9.0. Moreover, the enzyme demonstrated stability in acetone, chloroform and toluene, with relative activities ranging from 63 to 76 %. DMSO was found to enhance enzyme activity. Additionally, the effect of 1 mM metal ions on enzyme activity revealed that all metal ions promoted enzyme activity, except for $Ba^{2+,}$ which inhibited it. Tween85 also inhibited enzyme activity.

In terms of wastewater treatment capabilities, the strain effectively removed oil and grease from synthetic wastewater, achieving removal rates of 100, 99.82 and 99.68 % for wastewater containing 1, 2 and 3 % oil (v/v), respectively, after 6 days of incubation. Furthermore, successful application of the strain in treating oily wastewater from a restaurant was demonstrated. The highest degradation rates were 98.53 % after treatment for 6 days. In addition, oil and grease removal also affected the Biochemical oxygen demand (BOD) was decreased. The results indicated the stain is efficient for wastewater treatment.

Acknowledgements

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