مجلة جامعة الزاوية للعلوم الطبيعية
University of Zawia Journal of Natural Sciences *(UZJNS)*
https://journals.zu.edu.ly/index.php/UZJNS
UZJNS

Production of a Cold-Active Lipase by Fusarium Solani

Joheni M. Jwely[a], **Mohamed A. Al-Ryani**[b]

[a,b]*Department of Botany, Faculty of Science, Zintan University, Zintan, Libya.*
**Corresponding author:*
E-mail address: profdrnani@uoz.edu.ly

Received 07 Feb 2024 | Accepted 28 April 2024 | Available online 15 Sep 2024 | DOI: 10.26629/uzjns.2024.01

A B S T R A C T

The current study aimed to the production and partial purification of a cold-active lipase by some fungi isolated from the olive oil processing wastes in Al-Gabal Al-Gharby, Libya. 31 fungal species from 12 genera were isolated. *F. solani* was the most prevalent comprising 94% of total *Fusarium* and 28.7% of total fungi, 102 fungal isolates were tested for their lipolytic activity on lipase production agar medium at 10 and 20°C. The most active isolates were *Alternaria* (2 isolates), *Fusarium*, and *Penicillium* (1isolate for each one). Molecular identification of the most active four isolates was carried out by their sequencing (ITS). The four powerful fungal strains' production of cold-active lipase was maximized by optimizing some nutritional and environmental factors. *F. solani* AUMC 16063 was able to produce the maximum amount of lipase activity (46.66U/mL/min) with specific activity (202.8U/mg), utilizing ammonium sulphate as a nitrogen source after 8 days of incubation at pH 3.0 and 15°C. However, at same condition after 6 days when yeast extract was employed as a nitrogen source, the generated cold-active lipase displayed the highest specific activity of (1550U/mg) and lipase activity (36.74U/ml/min). This is the first study in which the production, partial purification, maximized and characterization of a cold-active lipase enzyme by *Fusarium solani*.

Keywords: *cold active enzymes, Lipase, Fusarium solani, lipolytic activity, specific activity.*

إنتاج الليباز البارد – النشط بواسطة فيوزاريوم سولاني

جهينة محمد جويلي[a] ، *و محمد أحمد الرياني*[b]

[a,b]*قسم علم النبات، كلية العلوم، جامعة الزنتان- الزنتان- ليبيا.*

الملخص

هدفت الدراسة الحالية إلى إنتاج وتنقية جزئية لليباز البارد النشط بواسطة بعض الفطريات المعزولة من مخلفات معالجة زيت الزيتون في الجبل الغربي- ليبيا، تم عزل 31 نوعاً فطريً تنتمي إلى 12 جنساً، فيوزاريوم سولاني هو الأكثر انتشارا حيث شكل 94% من مجموع أنواع الفيوزاريوم، و 28.7% من إجمالي الفطريات، اختبرت 102 عزلة فطرية لفعاليتها في التحلل الدهني على وسط أجار إنتاج الليباز عند 10 و 20 درجة مئوية، وكانت العزلات الأكثر نشاطا؛ الترناريا (عزلتين)، فيوزاريوم، والبنسيليوم (عزلة لكل واحد)، تم إجراء التحديد الجزيئي للعزلات الأربعة الأكثر نشاطا من خلال تسلسلها (ITS). عظم إنتاج السلالات الفطرية الأربعة الأقوى إنتاجً لليباز البارد النشط من خلال تحسين بعض العوامل الغذائية والبيئية، فطر فيوزاريوم سولاني *F. Solani* AUMC 16063 كان قادرًا على إنتاج أقصى قدر من نشاط الليباز (46.66 وحدة/مل/دقيقة) مع نشاط نوعي (202.8 وحدة/مجم)، باستخدام كبريتات الأمونيوم كمصدر للنيتروجين بعد 8 أيام من الحضانة عند درجة حموضة 3.0 و15 درجة مئوية، وفي نفس الظروف بعد 6 أيام عندما استخدم مستخلص الخميرة كمصدر للنيتروجين، أظهر الليباز النشط البارد المتولد أعلى نشاط نوعي قدره (1550 وحدة/مجم) وكان نشاط الليباز (36.74 وحدة/مل/دقيقة)، تعد هذه الدراسة الأولى التي تم فيها إنتاج وتنقية جزئية وتعظيم وتوصيف إنزيم الليباز النشط البارد بواسطة الفطر فيوزاريوم سولاني.

الكلمات المفتاحية: الإنزيمات النشطة الباردة، الليباز، فيوزاريوم سولاني، نشاط التحلل الدهني، نشاط نوعي.

1 Introduction

Microbial enzymes are frequently more useful than enzymes derived from plants or animals. In addition to being safer and more convenient to produce, microbial enzymes are also more stable than their respective plant and animal enzymes [1]. Triacylglycerol acyl hydrolases (EC 3.1.1.3) are also known as lipases and are recognized as a group of potential industrial enzymes, responsible for catalyzing the hydrolysis or breakdown of insoluble fats and oils (triglycerides), and they can release monoglycerides, diglycerides, glycerol, and free fatty acids over an oil–water interface [2]. They are adaptable and enable a variety of bioconversion reactions in both unicellular and multicellular organisms, including hydrolysis, alcoholysis, acidolysis, aminolysis, esterification, and interesterification. Triacylglycerols (TAG) must be bioconverted both inside and between organisms, and lipases are crucial for this process [3-5]. Additionally, lipases are serine hydrolase family members that are carboxylic acid esterases and do not need a cofactor to catalyze events [6]. After proteases and carbohydrates, lipases are the largest family of digestive enzymes. In the realm of biotechnology, they represent the main class of biocatalysts [7, 8].

Furthermore, lipases have selective characteristics and catalyze the formation of esters, the hydrolysis of other esters, and their transesterification [9]. In the food, detergent, chemical, and pharmaceutical industries, lipases are becoming more and more preferred due to their capacity to carry out very specific chemical transformations [10]. Lipases have great stability throughout a wide range of temperatures, pH levels, and even organic solvents, making them highly useful catalysts for reactions in both aqueous and non-aqueous conditions. Lipases have a hydrophobic lid that is essential for their interfacial activity [11-14].

Among the numerous types of microbes, fungi and yeast are recognized as possible sources of fungal lipase. Fungi produce extracellular lipolytic enzymes that are simple to extract and purify, which lowers production costs and makes them the preferred source over bacterial lipases. Fungal lipases are substrate-specific and stable under a wide range of chemical and physical conditions [15, 12]. The most common fungal strains today produce commercial lipases in their culture medium, including *Candida rugosa*, *Rhizopus oryzae*, *Mucor miehei*, *Rhizopus japonicus*, *Rhizopus arrhizus*, *Rhizopus delemar*, *Rhizopus niveus*, *Aspergillus niger*, and *Thermomyces lanuginosus*. Commercial lipases derived from fungi are used in a variety of industrial fields, including the production of detergents, food and dairy products, pharmaceuticals and medicine, biodiesel, oleochemicals, the leather industry, wastewater bioremediation, cosmetics and perfumeries, ester synthesis, paper manufacturing, and bioremediation [16, 2].

The presence of various inducers, such as carbon sources, nitrogen sources, pH, and temperature, are the main parameters taken into account. These factors substantially influence the development and catalytic activity of lipases. Lipases are present everywhere in nature and are active in a variety of temperatures. The temperature range of 0-30°C is favourable for the cold-active lipases' good activity [17]. It is well known that many microbes, including bacteria, yeast, and fungi, are capable of producing cold active lipases [18]. In extreme cold environments like Antarctica, deep sea environments, and refrigerated food samples, psychotropic and psychrophilic microorganisms are found to have a wide variety of cold-adapted lipases [19-21]. *Bacillus*, *Pseudomonas*, and *Burkholderia* are the three bacterial genera with the majority of significant lipase-producing organisms, while *Aspergillus*, *Penicillium*, *Rhizopus*, and *Candida* are the four fungal genera. Seven different genera, including *Zygosaccharomyces*, *Candida*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Lachancea*, and *Torulaspora*, are home to some of the lipase-producing yeasts [22].

The current study aimed to production and partial purification of a cold-active lipase from *Fusarium solani* of olive oil processing wastes (Fatura) in area of Al-Gabal Al-Gharby, Libya.

2 Materials and methods

2.1. Collection of Fatura samples and isolation of fungi

In Libya during 2022, seven composite samples of the waste products from the manufacturing of olive oil (Fatura) were gathered from seven cities in Al-Gabal Al-Gharby. In order to isolate fungus, samples were put in sterile plastic bags and brought right to the mycological lab. The dilution plate technique [23], was used for isolation of fungi. And Czapek's Dox agar was used as an isolation medium.

2.2. Phenotypic identification of fungi

The morphological identification of the isolated fungal genera and species was based on macroscopic and

microscopic features following the keys and descriptions of: Ellis (1976) for Dematiaceous Hyphomycetes [24], Pitt (1979) for *Penicillium* and its teleomorphs [25], Raper and Fennell (1965) for *Aspergillus* species[26], Booth (1971) and Leslie and Summerell (2006) for *Fusarium* species[27, 28], Moubasher (1993) and Domsch *et al.* (2007) for fungi in general[29, 30]. All the isolated fungal strains were preserved in the culture collection of the Assiut University Mycological Centre (AUMC).

2.3. Preliminary screening of lipolytic activity

The medium described by [31] was used for screening the lipolytic activity of the isolated fungi. The most active fungal strains were selected for the optimization of lipase production conditions.

2.4. Optimization of cold-Active lipase production conditions

2.4.1. Optimization of pH and temperature

By changing the parameters using the two factors at a time (TFAT) strategy [32] for pH (3-10) each at 10, 15, and 20°C, it was possible to maximize the synthesis of cold-active lipase by *Alternaria alstroemeriae* AUMC 16060, *Penicillium crustosum* AUMC 16061, *Alternaria angustiovoidea* AUMC 16062, and *F. solani* AUMC 16063. A 50mL of the fermentation medium (Sucrose-free Czapek's broth) was added to each 250mL Erlenmeyer flask used for the tests, along with 1.0% tween 80 as the only carbon source. Separate flasks were inoculated with 1.0% (v/v) of spore suspension taken from a 7-day-old cultures of the tested fungi, the flasks were then incubated for 10 days.

2.4.2. Optimization of nitrogen source and fermentation time

Two factors at a time (TFAT) strategy [32], nitrogen supply (peptone, yeast extract, beef extract, sodium nitrate, ammonium sulfate, and ammonium chloride; each at 0.2%) each at a fermentation duration of 1–10 days, were varied for the maximization of the cold-active lipase by *F. solani* AUMC 16063. A 50 mL of the fermentation medium (Sucrose-free Czapek's broth) was added to each 250mL Erlenmeyer flask used for the tests, along with 1.0% tween 80 as the only carbon source. Separate flasks were inoculated with 1.0% (v/v) of spore suspension taken from a culture of *F. solani* AUMC 16063 that had been growing for seven days, the flasks were then incubated under various operating conditions.

2.5. Extraction and assay of the cold-active lipase

After fermentation duration, the cell-free supernatant was obtained through 10,000 rpm centrifugation at 4°C for 10 min, and used as the source of the cold-active lipase [33]. For quantitative determination of the lipolytic activity, 3mL of 1.0% tween 80 was combined with 2.5mL of deionized water and 1mL of buffer solution respective to the desired pH tested. The mixture was maintained at 5, 10, 15, and 20°C for overnight. Afterwards, 1.0mL of the supernatant was added to the mixture to start the reaction for 60min. The reaction was then terminated by introducing 3mL of 96% ethyl alcohol, and the mixture was titrated against 0.05M NaOH solution in the presence of 4 drops of phenolphthalein solution (0.1g dissolved in 50mL of 1:1ethanol: distilled water), until the appearance of the pink color. The lipase activity was calculated according to Equation:

$$\text{Lipase activity} = \frac{\mathbf{V1 \times M \times 1000 \times DF}}{\mathbf{V2 \times T}} \text{U/mL/min}$$

Where; V1 = Volume of NaOH (mL); M = Molarity of NaOH (mM); 1000 = Conversion factor from milli-equivalent to micro-equivalent; DF = Dilution factor of the enzyme; V2 = Volume of lipase used in the reaction; and T = Time of the reaction (min).

Total protein estimation. The protein content was estimated according the Lowry *et al.* (1951) method[34].

2.6. Production of cold-active lipase from Fatura

According the method described by [35], *F. solani* AUMC 16063 was utilized to generate a cold-active lipase employing the Fatura waste as substrate under ideal production circumstances, which included an 8-day incubation period at pH 3 and 15°C with ammonium sulphate as a nitrogen supply. A 300g of Fatura were added to 3 Fernbach fermentation flasks (100g each), and each flask was moistened with 50mL of Czapek's broth, which was free of sucrose and enriched with 1g/L pectin derived from citrus peel. A 5 mL spore suspension from a 7-day-old culture of *F. solani* AUMC 16063 was used to individually inoculate each flask after sterilization. The flasks were then kept in a static environment for 8 days at 15°C. Following the incubation period, the contents of the flasks were collected using 1500mL of citrate buffer (pH 3.0). After centrifugation (10,000 rpm at 4°C for 10 min), the cell-free supernatant was used for the enzyme precipitation.

2.7. Partial purification of the cold-active lipase

Ammonium sulfate precipitation and dialysis:

Total protein was precipitated at 4°C with a 70%

saturated ammonium sulphate [36]. The precipitated protein was separated and lyophilized using a freeze dryer (VirTis, model #6KBTES-55, NY, USA). To remove salts and other small molecules, lyophilized protein was dissolved in citrate buffer (pH 4.0), dialyzed twice for two hours at room temperature (cut offs: 12-14 kD), and then cooled overnight at 4°C. Later, enzyme characterization tests were conducted using the partially purified cold-active lipase from the dialyzed protein.

2.8. Impact of pH, temperature and some ions and inhibitors on the activity of the cold-active lipase

On pure lipase activity, the effects of pH (3.0–11.0) at 5–20°C have been examined [37]. The reaction was started by adding 0.01g of enzyme powder dissolved in 1.0mL buffer solution of the desired pH values (3.0-11.0), along with 3.0mL of 1.0% tween 80, 2.5mL of de-ionized water, and 1.0 mL of buffer solution. After 60 min, the reaction was terminated by introducing 3.0mL ethyl alcohol (96%), and the flask contents were titrated against 0.05M NaOH. Additionally, ions such as Na^+, K^+, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} were tested by adding them to a solution at a concentration of 5mM/mL as NaCl, KCl, $CaCl_2$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, and $ZnSO_4$. An enzyme inhibitor was also tested using sodium dodecyl sulphate (SDS) and ethylenediamine tetra-acetic acid (EDTA), both of which had a 5mM/mL concentration. Under the standard reaction conditions of pH 4.0 and 10°C, the cold-active lipase's activity without the presence of metal ions, EDTA, or SDS was assessed to determine the residual activity.

2.9. Effect of carbon source on the *lipase* activity

The activity of the pure cold-active lipase generated in this investigation was assessed using 1.0% (w/v) of each of tween 20, tween 40, tween 80, maize oil, olive oil, sesame oil, and sunflower oil [38]. A 0.01g lipase powder was dissolved in 1.0mL citrate buffer (pH 4.0) and added to the reaction mixture, which included 3.0mL of 1% substrate, 2.5 mL deionized water, and 1mL citrate buffer (pH 4.0), for 60 minutes at 10°C. After stopping the reaction with 3.0mL of 96% ethyl alcohol, the flask content was titrated against a 0.05 M NaOH solution. The lipase activity was determined as previously stated.

2.10. Statistical analysis

The mean and standard deviation (SD) of the tentative study performed in triplicate were used to express all data. Analysis of the statistical significance was conducted according to [39]. It was deemed significant at $p<0.05$.

3 Results

3.1 Fungi recovered from Fatura

31 fungal species from 12 genera were isolated from these samples. *Fusarium* was the most common genus comprising 30.51% of total fungi. It was represented by two species, *F. oxysporum* and *F. solani*. *F. solani* was the most prevalent comprising 94% of total *Fusarium* and 28.7% of total fungi.

3.2. Preliminary screening of lipolytic activity

On lipase production agar medium at two temperatures, 10 and 20°C, 102 fungal isolates were tested for their lipolytic activity. The most active isolates were *Alternaria* (2 isolates), *Fusarium*, and *Penicillium* (1 isolate for each one). Molecular identification of the most active four isolates was carried out by sequencing their internal transcribed spacer region (ITS). Pure cultures of the four fungal isolates were deposited in the culture collection of Assiut University Mycological Centre as *Alternaria* sp. AUMC 16060, *Alternaria* sp. AUMC 16062, *Fusarium* sp. AUMC 16063, and *Penicillium* sp. AUMC 16061.

3.3. Effect of medium's pH and incubation temperature on lipase production

3.3.1. At 10°C: The four powerful fungal strains' production of cold-active lipase was maximized by optimizing some nutritional and environmental factors. For *Alternaria alstroemeriae*, *A. angustiovoidea*, and *Fusarium solani*, pH 3 produced cold-active lipase enzyme activity of 32.66, 31.5, and 33.83 U/mL/min, respectively, at 10°C. *Penicillium crustosum* performed best at pH 5, yielding 9.91 U/mL/min. The most potent strain was *Fusarium solani* when compared to the others. It generated the highest levels of specific activity (512.5 U/mg protein) and cold-active lipase activity (33.83 U/mL/min) (Table 1).

3.3.2. At 15°C: At 15°C, pH 3 produced the best cold-active lipase enzyme activity for *Alternaria alstroemeriae*, *A. angustiovoidea*, and *Fusarium solani*, with values of 33.83, 39.66, and 45.49 U/mL/min, respectively. At pH 7, *Penicillium crustosum* produced 10.49 U/mL/min, which was its best output. Comparing the tested strains, *Fusarium solani* was the most

Table 1. Effect of medium's pH on the activity of lipase produced by the highest producing fungi at 10°C

pH	*A. alstroemeriae*AUMC 16060		*A. angustiovoidea*AUMC 16062	
	Activity U/mL/min	Specific activity U/mg protein	Activity U/mL/min	Specific activity U/mg protein
3	32.66±4^{a}	269.9±36.36^{a}	31.5±1.6^{a}	85.36±4.33^{a}
4	24.5±1.2^{b}	81.21±4^{b}	26.24±1.8^{b}	83.5±5.73^{b}
5	12.24±1.5^{c}	57.5±7^{c}	13.10±1.5^{c}	57.45±6.58^{c}
6	4.08±0.8^{f}	13.03±2.55^{e}	6.41±0.8^{d}	21.01±2.66^{e}
7	6.41±0.5^{d}	24.3±1.9^{d}	6.10±0.76^{d}	28.24±3.52^{d}
8	0^{g}	0^{g}	0^{f}	0^{h}
9	2.91±0.3^{f}	25.1±2.58^{d}	1.74±0.2^{e}	14.26±1.64^{f}
10	0.583±0.2^{g}	4.3±1.47^{f}	0.58±0.1^{f}	4.83±0.83^{g}

pH	*F. solani* AUMC 16063		*P.crustosum* AUMC 16061	
	Activity U/mL/min	Specific activity U/mg protein	Activity U/mL/min	Specific activity U/mg protein
3	33.83±1.4^{a}	512.5±21.2^{a}	4.7±0.82^{c}	18.50±3.23^{e}
4	23.33±1.1^{b}	85.45±4^{c}	4.7±0.67^{c}	45.19±6.44^{c}
5	4.1±0.6d	25±3.66^{f}	9.91±0.73^{a}	86.92±6.4^{a}
6	0^{f}	0^{g}	2.33±0.5^{d}	14.29±3^{f}
7	5.24±0.42^{c}	93.57±7.5^{b}	8.74±1^{b}	53.29±9.52^{b}
8	0^{f}	0^{g}	5.24±0.92^{c}	22.88±4^{d}
9	4.1±0.5^{d}	53.94±6.58^{d}	5.24±0.71^{c}	11.54±1.56^{g}
10	2.33±0.2^{e}	38.19±3.27^{e}	2.91±0.3^{d}	4.58±0.47^{h}

Figures in table are mean of three replicates (n=3) ±SD. At the 0.05 level of probability, means in a column with the same letters are not statistically different.

Table 2. Effect of pH on the activity of lipase produced by the highest producing fungi at 15 °C

pH	*A. alstroemeriae* AUMC 16060		*A. angustiovoidea* AUMC 16062	
	Activity U/mL/min	Specific activity U/mg protein	Activity U/mL/min	Specific activity U/mg protein
3	33.83±3.2^{a}	70.04±6.62^{b}	39.66±4.2^{a}	60.73±6.43^{b}
4	18.66±1.2^{b}	97.69±6.28^{a}	26.83±2.8^{b}	53.76±5.6^{c}
5	11.08±0.84^{c}	43.28±3.28^{c}	25.08±2.2^{c}	87.38±7.66^{a}
6	9.91±0.65^{c}	16.08±1.055^{e}	13.41±1.2^{d}	20.03±1.79^{d}
7	10.49±1^{c}	17.02±1.62^{e}	12.83±0.8^{d}	19.95±1.24^{d}
8	11.08±1.1^{c}	18.62±1.85^{d}	8.16±0.6^{e}	10.96±0.8^{f}
9	5.24±0.5^{d}	10.52±1^{f}	8.16±0.64^{e}	13.97±1.1^{e}
10	11.08±0.83^{c}	17.58±1.3^{de}	5.24±0.46^{f}	7.47±0.66^{g}

pH	*F. solani* AUMC 16063		*P.crustosum* AUMC 16061	
	Activity U/mL/min	Specific activity U/mg protein	Activity U/mL/min	Specific activity U/mg protein
3	45.49±4.5^{a}	385.5±38.13^{a}	4.08±0.5^{c}	9.27±1.13^{c}
4	11.08±0.6^{b}	85.89±4.65^{b}	4.08±0.6^{c}	17.14±2.52^{b}
5	4.66±0.3^{e}	23.77±1.53^{d}	2.33±0.8^{d}	7.61±2.66^{b}
6	3.49±0.11^{f}	15.37±0.484^{g}	0^{e}	0^{d}
7	5.83±0.2^{d}	26.99±0.926^{c}	10.49±1.2^{a}	23.36±2.67^{a}
8	4.66±0.2^{e}	21.98±0.94^{e}	5.83±0.7^{b}	16.75±2^{b}
9	5.24±0.3d^{e}	17.29±1^{f}	5.83±0.62^{b}	8.95±0.95^{c}
10				

Figures in Table are mean of three replicates $(n = 3) \pm SD$. At the 0.05 level of probability, means in a column with the same

letters are not statistically different.

Table 3. Effect of pH on the activity of lipase produced by the highest producing fungi at 20 °C

pH	*A. alstroemeriae* AUMC 16060		*A. angustiovoidea* AUMC 16062	
	Activity U/mL/min	Specific activity U/mg protein	Activity U/mL/min	Specific activity U/mg protein
3	34.41±3.5^{a}	296.63±30.17^{a}	9.91±1.1^{c}	42.33±4.7^{d}
4	6.41±0.52^{c}	31.42±2.6^{c}	22.16±3.6^{a}	107.05±18^{a}
5	7.58±0.86^{c}	53.75±6.1^{b}	16.33±2^{b}	101.42±12.42^{b}
6	12.24±1.2^{b}	53.21±5.2^{b}	1.74±0.8^{g}	7.63±3.5^{g}
7	7.58±1^{c}	32.81±4.33^{c}	7.58±0.76^{d}	78.95±7.9^{c}
8	4.66±0.5^{d}	21.98±2.36^{d}	4.66±0.24^{e}	9.15±0.48^{g}
9	6.99±0.8^{c}	18.29±2.1^{e}	4.66±0.2^{e}	27.57±1.18^{e}
10	2.33±0.3^{e}	14.74±1.9^{f}	3.49±0.3^{f}	21.41±1.84^{f}

pH	*F. solani* AUMC 16063		*P.crustosum* AUMC 16061	
	Activity U/mL/min	Specific activity U/mg protein	Activity U/mL/min	Specific activity U/mg protein
3	36.74±4^{a}	854.4±93^{a}	2.33±0.4^{de}	9.58±1.64^{f}
4	1.74±0.5^{f}	14.5±4.16^{g}	3.49±0.3^{d}	19.94±1.7^{e}
5	6.99±0.85^{cd}	69.20±8.5^{d}	1.74±0.2^{e}	9.61±1.1^{f}
6	4.66±0.32^{e}	51.77±3.55^{e}	6.99±0.8^{bc}	43.14±4.94^{d}
7	8.16±1^{c}	83.26±10.2^{b}	33.24±4.6^{a}	170.46±23.6^{a}
8	2.91±0.22^{f}	18.30±1.38^{f}	8.16±1^{b}	48±5.88^{b}
9	14.58±1.4^{b}	72.9±7^{c}	8.16±0.9^{b}	43.17±4.76^{cd}
10	5.83±0.92^{de}	11.18±1.77^{h}	6.41±0.75^{c}	44.51±5.2^{c}

Figures in Table are mean of three replicates $(n = 3) \pm SD$. At the 0.05 level of probability, means in a column with the same letters are not statistically

powerful. It produced the highest levels of both cold-active lipase activity (45.49 U/mL/min) and specific activity (385.5 U/mg protein) (Table 2).

3.3.3. At 20°C At pH 3.0, *Alternaria alstroemeriae* and *Fusarium solani* exhibited their highest levels of cold-active lipase activity (34.41 and 36.74 U/mL/min, respectively) and specific activity (296.63 and 854.4 U/mg protein, respectively). The cold-active lipase activity (22.16 U/mL/min) and specific activity (107.05 U/mg protein) of *Alternaria angustiovoidea* were at their highest levels at pH 4, respectively. The maximum cold-active lipase activity was, however, produced by *Penicillium crustosum* at pH 7.0, with 33.24 U/mL/min and 170.46 U/mL/min, respectively (Table 3). It had been found that the most active strain of *Fusarium solani* AUMC 16063 produced the highest levels of cold-active lipase at low pH (3.0) and temperature (15°C). To increase the synthesis of lipase, the nitrogen source and incubation period for this strain were optimized.

4 Optimization of nitrogen source and incubation time

F. solani AUMC 16063 was able to produce the maximum amount of lipase activity (46.66 U/mL/min) utilizing ammonium sulphate as a nitrogen source by solid state fermentation after 8 days of incubation at pH 3.0 and 15°C. However, when yeast extract was employed as a nitrogen source, the generated cold-active lipase displayed the highest specific activity of 1550 U/mg protein after 6 days of incubation at the same condition (Table 4).

5 Determination of the optimum pH and temperature

The activity of the enzyme was evaluated at various pHs (3-11) and temperatures (5-20°C) in order to determine the optimum pH and temperature for the produced cold-active lipase. According to the current findings, lipase specific activity was at its highest (1052.6 U/mg) at pH 4.0 and 10°C (Figure 1).

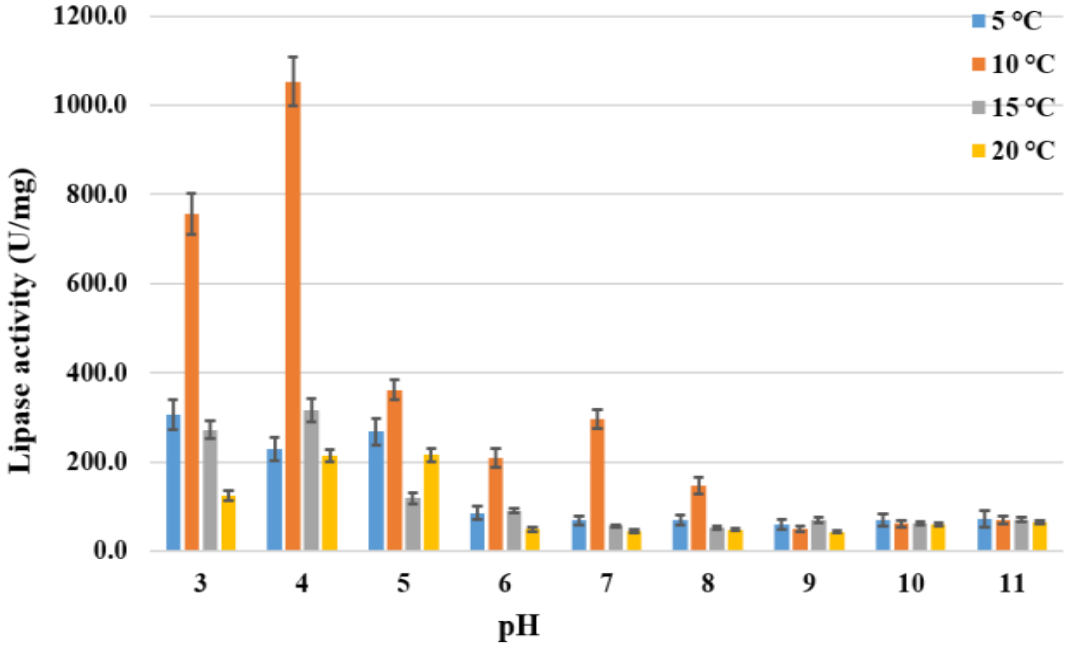

Figure 1. Effect of pH and temperature on the specific activity of the cold-active lipase produced by *F. solani* AUMC 16063.

Table 4. Optimization of Nitrogen source and incubation time of lipase production by *Fusarium solani* AUMC 16063

Nitrogen Sources	1 day		2 days		3 days	
	Lipase Activity (U/ml/min)	Specific Activity (U/mg)	Lipase Activity (U/ml/min)	Specific Activity (U/mg)	Lipase Activity (U/ml/min)	Specific Activity (U/mg)
NH_4Cl	32.08±3	1188.1±111	30.91±2	936.6±60.6	34.91±3.1	354.7±31.6
$(NH_4)_2SO_4$	33.24±2.8	831±70	32.66±2.2	640.3±43.1	33.83±3	286.6±16.6
$NaNO_3$	31.49±2.5	266.8±21.2	31.49±2	443.5±28.1	32.66±2.5	205.4±15.7
Beef extract	32.08±2.2	668.3±45.8	30.91±1.9	686.8±42.2	30.91±2.1	134.3±9.13
Peptone	30.33±2	459±30.3	28.58±2	468.5±32.8	32.08±2	163.6±10.2
Yeast extract	28.58±1.65	697.1±40.2	29.16±2	494.2±33.9	33.83±2.4	167.4±12

Nitrogen Sources	4 days		5 days		6 days	
	Lipase Activity (U/ml/min)	Specific Activity (U/mg)	Lipase Activity (U/ml/min)	Specific Activity (U/mg)	Lipase Activity (U/ml/min)	Specific Activity (U/mg)
NH_4Cl	36.47±3	835±69.77	39.66±3.3	574.7±47.8	36.74±3.2	524.8±45.7
$(NH_4)_2SO_4$	37.33±3.4	191.4±17.4	40.24±3.6	241.4±21.7	37.33±3.5	301.0±28
$NaNO_3$	37.33±3.2	289.3±24.8	36.74±3	213.6±17.4	37.33±3.6	380.9±36.7
Beef extract	36.16±3.3	206.6±18.8	39.66±4	198.3±20	36.16±3	309.6±25.8
Peptone	36.74±2.8	346.6±28	32.66±3	178.4±16.4	36.74±3	270.1±22
Yeast extract	34.99±3	188.1±16.1	36.74±3	1550.2±130	35.58±3.4	323.5±30.9

Nitrogen Sources	7 days		8 days		9 days	
	Lipase Activity (U/ml/min)	Specific Activity (U/mg)	Lipase Activity (U/ml/min)	Specific Activity (U/mg)	Lipase Activity (U/ml/min)	Specific Activity (U/mg)
NH_4Cl	41.99±3.9	617.5±57.3	43.16±4	644.1±59.7	41.99±4	542.8±51.9
$(NH_4)_2SO_4$	40.24±4	180.4±17.9	46.66±4.2	202.8±18.2	40.24±3.7	113.9±10.5
$NaNO_3$	40.83±4	491.9±48.2	37.91±3.5	375.3±35	41.99±3.9	419. 9±39
Beef extract	40.24±4.1	129.8±13.2	40.24±3.8	143.2±13.5	38.49±3	100.1±7.8
Peptone	43.74±4.2	260.3±25	43.74±4	160.2±14.6	42.58±4	121.3±11.4
Yeast extract	39.66±3.6	179.4±16.3	36.16±3.2	140.1±12.4	34.41±2.8	78.74±6.4

Nitrogen Sources	10 days	
	Lipase Activity (U/ml/min)	Specific Activity (U/mg)
NH_4Cl	40.83±3.4	729.1±60.7
$(NH_4)_2SO_4$	39.08±3.1	186.9±14.5
$NaNO_3$	40.83±3.8	295.8±27.5
Beef extract	37.91±3.5	112.4±10.4
Peptone	39.66±4	123.5±12.4
Yeast extract	38.49±3.8	154.5±25.7

6 Effect of carbon source on lipase specific activity

In this investigation, the optimal substrate for the generated cold-active lipase was investigated using several oily substrates and tween. When tested at the optimum pH (4.0) and temperature (10°C) for the enzyme, tween 80 was shown to be the best, providing the peak (7400 U/g protein) of lipase activity (Figure 2).

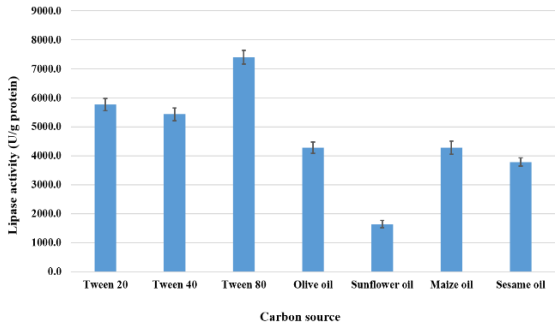

Figure 2. Effect of different oily substrates (carbon source) on the specific activity of the cold-active lipase (produced by *F. solani* AUMC 16063) at pH 4.0 and 10 °C.

7 Effect of some ions and inhibitors on the lipase specific activity

The impact of certain ions was assessed using tween 80 as a substrate at pH 4 and 10°C. In this investigation, the cold-active lipase was activated by each of the examined ions. When compared to the control, $CaCl_2$ had the highest activation impact (180%), while KCl had the lowest (102.3%) (Table5).

Table 5. Effect of some ions and inhibitors on the lipase specific activity.

Ions and inhibitors	Specific activity (U/g protein)	Residual activity (%)
Control	**7400±242**	**100±3.27**
KCl	7570±300	102.3±4
$CuSO_4$	11180±528	151±7.1
NaCl	8880±500	120±6.75
$FeSO_4$	10030±560	135.54±7.56
$NiCl_2$	12010±680	162.3±9.2
$ZnSO_4$	10030±600	135.54±8.1
$MgSO_4$	9050±420	122.3±5.67
$CaCl_2$	13320±820	180±11.1
$CoCl_2$	8220±530	111±7.16
$MnSO_4$	9870±480	133.4±6.48
EDTA	9540±360	128.92±4.86
SDS	8220±300	111±4

8 Discussion

On lipase production medium at two temperatures, 10 and 20°C, 102 fungal isolates were tested for their lipolytic activity. The majority of fungi could produce lipase activity at 20°C, where 98 out of 102 isolates (96% of all isolates tested), were able to do so, compared to 73 isolates (71.56% of all isolates) at 10°C. The most active isolates were *Alternaria* (2 isolates), *Fusarium* (1), and *Penicillium* (1). The understanding of cold-active

lipases is expanding quickly, however research on cold active lipases is fragmented and lacking. No initiatives have been made to date to arrange this data. As a result, from the material found in the literature, an overview of this crucial enzyme for biotechnology and industry as well as its traits has been collected. It is evident from the scant reports on cold active lipases that have been published that the majority of research on these enzymes has been devoted to their isolation, purification, and characterization.

Physicochemical and nutritional parameters like pH, temperature, nitrogen source, and carbon source, have a significant impact on cold-active lipases production, which are primarily extracellular. The most active isolates in this study were *Alternaria alstroemeriae*, *Alternaria angustiovoidea*, *Fusarium solani*, and *Penicillium crustosum*. The four powerful fungal strains' production of cold-active lipase was maximized by optimizing some nutritional and environmental factors. *Fusarium solani* was found to be the best strain producing the most cold-active lipase. In literature, it was demonstrated that the majority of cold-active lipases are found in bacteria that can endure temperatures as low as 5°C. Even though there are several sources of lipase, only a few numbers of bacteria and yeast were used to produce cold-active lipases. From time to time, efforts have been made to isolate these microbes' highly active lipases from cold environments.

In literature, no reports about production of cold-active lipase by *F. solani* were found. However, some other *Fusarium* species were used to produce cold-active lipases. Regarding this, [40] purify and characterize lipase from *Fusarium* sp. YM-30. [41] produced lipase enzyme from *F. solani* FS1. [42] produced lipase enzyme from *F. heterosporum*. [43] produced lipase enzyme from *F. oxysporum*. [44] developed lipase enzyme from *F. verticillioides* P24. The most popular technique for producing cold-active lipase is submerged fermentation [19, 1]. Microorganisms that are suited to the cold typically develop quickly when the temperature is low. According to [45], cold-active lipase synthesis is temperature-dependent and thermolabile.

In this study, *F. solani* AUMC 16063 utilized tween 80 and produce cold-active lipase at 15°C. Regarding this, tween 80 and Tributyrin induced production of cold active lipases at 4°C with an optimum pH 7.6 [46]. *A. nidulans* WG312 produced cold active lipase by utilizing olive oil as an inducer at 30°C [33]. Soybean oil induced the production of cold active lipases from *Acinetobacter* sp. strain no. 6 at 4 °C within four days [47]. *Aeromonas* sp. LPB 4 produced lipase at 10 °C in eight days-time durations by using tryptone and yeast extract as carbon and nitrogen source and trybutylin as an inducer [48]. *Serratia marcescens* produced cold active lipase in presence of skim milk as energy source at 6 °C in 6 days of incubation. Tween 80 and Tween 20 were the best inducers for cold-adapted lipase production with yeast extract as carbon source in 14 days at 25 °C for *Psychrobacter* sp. wp37. Another isolate of *Pseudoalteromonas* sp. wp27 produced lipases at 25 °C in 14 days with yeast extract as carbon source and olive oil and Tween 80 as inducers [49].

The investigations on cold active lipases from *Psychrobacter immoblis* B10 were perused on semi-purified preparations, the nucleotide sequence of which is also available [50]. Lipase purity is evaluated after each purification step by measuring the overall activity and specific activity. The purification efficiency is determined by total yield and purification factor [51]. For industrial applications the purification step should be economical, rapid, high yielding and easy to produce in large scale operations [52]. Prepurifaction step involves concentration of the protein containing lipases by ammonium sulphate precipitation, ultrafiltration or extraction with organic solvents.

9 Acknowledgements

The authors are deeply indebted to head, Prof Dr. Ahmed M. Moharram and staff members of Assiut University Mycological Centre. Much gratitude also goes to Dr. Osama Al- Bedak, for facilities provided during this research.

References

[1]Liu, X., Kokare, C., (2023). Microbial enzymes of use in industry. Biotechnology of microbial enzymes. Elsevier, pp. 405-444.

[2]Kumar, A., Verma, V., Dubey, V.K., Srivastava, A., Garg, S.K., Singh, V.P., Arora, P.K., (2023). Industrial applications of fungal lipases: A review. Frontiers in Microbiology 14, 1142536.

[3]Geoffry, K.,and Achur, R.N., (2018). Screening and production of lipase from fungal organisms. Biocatalysis and agricultural biotechnology14, 241-253.

[4]Patel, G.,and Shah, K., (2020). Isolation, screening and identification of lipase producing fungi from cotton seed soapstock. Indian Journal of Science and Technology13 (36), 3762-3771.

[5]Ali, S., Khan, S.A., Hamayun, M., Lee, I. J., (2023). The recent advances in the utility of microbial lipases: A review. Microorganisms11 (2), 510.

[6]Basheer, S.M., Chellappan, S., Beena, P., Sukumaran, R., Elyas, K., Chandrasekaran, M., (2011). Lipase from marine *Aspergillus awamori* btmfw032: Production, partial purification and application in oil effluent treatment. New Biotechnology28 (6), 627-638.

[7]Carvajal Barriga, E.J., and Portero Barahona, P., (2019). Production, extraction and characterization of lipases from the antarctic yeast *Guehomyces pullulans*.

[8]Lima, L.G.R., Gonçalves, M.M.M., Couri, S., Melo, V.F., Sant'ana, G.C.F., Costa, A.C.a.D., (2019). Lipase production by *Aspergillus niger* by submerged fermentation. Brazilian Archives of Biology and Technology62.

[9]Gupta, N., Sahai, V., Gupta, R., (2007). Alkaline lipase from a novel strain *Burkholderia multivorans*: Statistical medium optimization and production in a bioreactor. Process Biochemistry42 (4), 518-526.

[10]Grbavčić, S., Dimitrijević-Branković, S., Bezbradica, D., Šiler-Marinković, S., Knežević, Z., (2007). Effect of fermentation conditions on lipase production by *Candida utilis*. Journal of the Serbian Chemical Society72 (8-9), 757-765.

.[11]Khan, F.I., Lan, D., Durrani, R., Huan, W., Zhao, Z., Wang, Y., (2017). The lid domain in lipases: Structural and functional determinant of enzymatic properties. Frontiers in bioengineering and biotechnology 5, 16.

[12]Mehta, A., Bodh, U., Gupta, R., (2017a). Fungal lipases: A review. Journal of Biotech Research8.

[13]Tan, J.S., Abbasiliasi, S., Ariff, A.B., Ng, H.S., Bakar, M.H.A., Chow, Y.H., (2018). Extractive purification of recombinant thermostable lipase from fermentation broth of *Escherichia coli* using an aqueous polyethylene glycol impregnated resin system. 3 Biotech8, 1-7.

[14]Bharathi, D., and Rajalakshmi, G., (2019). Microbial lipases: An overview of screening, production and purification. Biocatalysis and Agricultural Biotechnology 22, 101368.

[15]Tan, T., Zhang, M., Wang, B., Ying, C., Deng, L., (2003). Screening of high lipase producing *Candida* sp. And production of lipase by fermentation. Process Biochemistry39 (4), 459-465.

[16]Chandra, P., Enespa, Singh, R., Arora, P.K., (2020). Microbial lipases and their industrial applications: A comprehensive review. Microbial cell factories19, 1-42.

[17]Cai, Y., Wang, L., Liao, X., Ding, Y., Sun, J., (2009). Purification and partial characterization of two new cold-adapted lipases from mesophilic *Geotrichum* sp. Sybc wu-3. Process Biochemistry44 (7), 786-790.

[18]Abada, E. M., (2008). Production and characterization of a mesophilic lipase isolated from *Bacillus stearothermophilus* ab-1. Pakistan Journal of Biological Sciences11 (8), 1100-1106.

[19]Dieckelmann, M., Johnson, L., Beacham, I., (1998). The diversity of lipases from psychrotrophic strains of *Pseudomonas*: A novel lipase from a highly lipolytic strain of *Pseudomonas fluorescens*. Journal of Applied Microbiology85 (3), 527-536.

[20]Zeng, X., Xiao, X., Wang, P., Wang, R., (2004). Screening and characterization of psychrotrophic, lipolytic bacteria from deep-sea sediments. Journal of microbiology and biotechnology14 (5), 952-958.

[21]Dominguez De Maria, P., Carboni-Oerlemans, C., Tuin, B., Bargeman, G., Van Der Meer, A., Van Gemert, R., (2005). Biotechnological applications of *Candida antarctica* lipase a: State-of-the-art. Journal of molecular catalysis. B: Enzymatic37 (1-6), 36-46.

[22]Romo-Sánchez, S., Alves-Baffi, M., Arévalo-Villena, M., Úbeda-Iranzo, J., Briones-Pérez, A., (2010). Yeast biodiversity from oleic ecosystems: Study of their biotechnological properties. Food microbiology27 (4), 487-492.

[23]Warcup, J., (1950). The soil-plate method for isolation of fungi from soil. Nature166 (4211), 117-118.

[24]Ellis, M., (1976). More dematiaceous hyphomycetes (p. 507). Kew, Surrey, England: Commonwealth Mycological Institute.

[25]Pitt, J.I., (1979). The genus *Penicillium* and its teleomorphic states *Eupenicillium* and talaromyces. Academic Press.

[26]Raper, K.B., and Fennell, D.I., (1965). The genus *Aspergillus*. Williams and Wilkins, Baltimore, U.S. A.

[27]Booth, C., (1971). The genus *Fusarium* commonwealth mycological institute. Kew, Surrey237.

[28]Leslie, J., and Summerell, B., (2006). The *Fusarium* laboratory manual blackwell publishing. Ames, Iowa.

[29]Moubasher, A., (1993). Soil fungi in Qatar and other Arab countries The Centre for Scientific and Applied Research, University of Qatar.

[30]Domsch, K., Gams, W., Anderson, T., (2007). Compendium of soil fungi. 1–672. IHW-Verlag, Eching, Germany.

[31]Ullman, U., and Blasins, G., (1974). A simple medium for the detection of different lipolytic activity of microorganisms. Zbl. Bakt. Hyg., II Abt. Orig. A229, 264-267.

[32]Moharram, A.M., Zohri, A.-N.A., Hesham, A.E.-L., Abdel-Raheam, H.E., Al-Ameen Maher, M., Al-Bedak, O.a.-H., (2022). Production of cold-active pectinases by three novel *Cladosporium* species isolated from egypt and application of the most active enzyme. Scientific Reports12 (1), 1-17.

[33]Mayordomo, I., Randez-Gil, F., Prieto, J.A., (2000). Isolation, purification, and characterization of a cold-active lipase from *Aspergillus nidulans*. Journal of Agricultural and Food Chemistry48 (1), 105-109.

[34]Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., (1951). Protein measurement with the folin phenol reagent. Journal of biological chemistry193, 265-275.

[35]Joseph, B., Upadhyaya, S., Ramteke, P., (2011). Production of cold-active bacterial lipases through semisolid state fermentation using oil cakes. Enzyme research2011.

[36]Al-Bedak, O.a.-H.M., Moharram, A.M., Hussein, N.a.-G., Taha, D.M., Stephenson, S.L., Ameen, F., (2023). Microbial exploitation of feather wastes for sustainable production of keratinase and collagenase enzymes by *Didymella keratinophila* AUMC 15399 in submerged fermentation. Fermentation9 (6), 507.

[37]Ji, X., Li, S., Wang, B., Zhang, Q., Lin, L., Dong, Z., Wei, Y., (2015). Expression, purification and characterization of a functional, recombinant, cold-active lipase (lipa) from psychrotrophic *Yersinia enterocolitica*. Protein Expression and Purification115, 125-131.

[38] Dalmau, E., Montesinos, J., Lotti, M., Casas, C., 2000. Effect of different carbon sources on lipase production by *Candida rugosa*. Enzyme and microbial technology26 (9-10), 657-663.

[39]Stahle, L., Wold, S., (1989). Analysis of variance (anova). Chemometrics and intelligent laboratory systems 6 (4), 259-272.

[40]Mase, T., Matsumiya, Y., Akiba, T., (1995). Purification and characterization of a new lipase from *Fusarium* sp. Ym-30. Bioscience, biotechnology, and biochemistry 59 (9), 1771-1772.

[41]Knight, K., Pimentel, M.D.C.B., Morais, M.M.C.D., Ledingham, W.M., Lima Filho, J.L.D., Maia, M.D., (2000). Immobilization of lipase from *Fusarium solani* fs1. Brazilian journal of microbiology 31, 219-221.

[42]Quayson, E., Amoah, J., Hama, S., Kondo, A., Ogino, C., (2020a). Immobilized lipases for biodiesel production: Current and future greening opportunities. Renewable and Sustainable Energy Reviews134, 110355.

[43]Rifaat, H.M., El-Mahalawy, A.A., El-Menofy, H.A., Donia, S.A., (2010). Production, optimization and partial purification of lipase from *Fusarium oxysporum*. Journal of Applied Sciences in Environmental Sanitation5 (1).

[44]Borges, J.P., Quilles Junior, J.C., Ohe, T.H.K., Ferrarezi, A.L., Nunes, C.D.C.C., Boscolo, M., Gomes, E., Bocchini, D.A., Da Silva, R., (2021). Free and substrate-immobilised lipases from *Fusarium verticillioides* p24 as a biocatalyst for hydrolysis and transesterification reactions. Applied Biochemistry and Biotechnology193, 33-51.

[45]Rashid, N., Shimada, Y., Ezaki, S., Atomi, H., Imanaka, T., (2001). Low-temperature lipase from psychrotrophic *Pseudomonas* sp. Strain kb700a. Applied and Environmental Microbiology67 (9), 4064-4069.

[46]Choo, D.-W., Kurihara, T., Suzuki, T., Soda, K., Esaki, N., (1998). A cold-adapted lipase of an alaskan psychrotroph, *Pseudomonas* sp. Strain b11-1: Gene cloning and enzyme purification and characterization. Applied and Environmental Microbiology64 (2), 486-491.

[47]Suzuki, T., Nakayama, T., Kurihara, T., Nishino, T., Esaki, N., (2001). Cold-active lipolytic activity of psychrotrophic *Acinetobacter* sp. Strain no. 6. Journal of bioscience and bioengineering92 (2), 144-148.

[48]Lee, H.-K., Ahn, M.-J., Kwak, S.-H., Song, W.-H., Jeong, B.-C., (2003). Purification and characterization of cold active lipase from psychrotrophic *Aeromonas* sp. Lpb 4. Journal of microbiology41 (1), 22-27.

[49]Xiao, X., Wang, P., Wang, F., (2004). Screening and characterization of psychrotrophic, lipolytic bacteria from deep-sea sediments. Journal of microbiology and biotechnology 14 (5), 952-958.

[50]Arpigny, J.L., Feller, G., Gerday, C., (1993). Cloning, sequence and structural features of a lipase from the antarctic facultative psychrophile *Psychrobacter immobilis* b10. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression1171 (3), 331-333.

[51] Joseph, B., Ramteke, P.W., Thomas, G., 2008. Cold active microbial lipases: Some hot issues and recent developments. Biotechnology advances26 (5), 457-470.

[52]Gupta, R., Gupta, N., Rathi, P., 2004. Bacterial lipases: An overview of production, purification and biochemical properties. Applied microbiology and biotechnology64, 763-781.