Bacillus subtilis C2 producing lipase isolated from bulk shrimp paste in Samarinda East Kalimantan

Y S Soeka1* and Sulistiani1

1Research Center for Biology - Indonesian Institute of Sciences, Cibinong Science Center, Jl. Raya Jakarta - Bogor, Km 46 Cibinong 16911

*E-mail: ceuceu_lip@yahoo.com

Abstract. Lipase is an enzyme that catalyzes the hydrolysis reaction of lipid (triacylglycerol) to glycerol and free fatty acids which has been used in various industrial applications such as food, cosmetics, detergents and pharmaceuticals. Bacillus subtilis C2 isolated from bulk shrimp paste (terasi) in Samarinda East Kalimantan, it showed producing lipase, and able to degrade fat. Lipase activity was determined titrimetrically. The ability of the strain to degrade fatty substrates was investigated in the medium 0.1% of olive oil, virgin coconut oil (VCO), cooking oil, tween 80, and Schleicheroleosa oil. The activities of lipase treated based on the period of incubation, temperature, and pH. The results showed that the optimum activity of lipase was 1.09 U/mL after five days incubation, 1.43 μmol/mL at 30°C and 1.96 U/mL at pH 9.0. The enzyme gave the highest lipase activity 1.76 μmol/mL used olive oil as a substrate. The physiology analysis showed the bacterium B. subtilis C2-lipolytic was thermotolerant, halotolerant and strictly aerobic.

1. Introduction
Lipases (EC.3.1.1.3, triacylglycerol acylhydrolases) are a group of enzymes, which have the ability to hydrolyze triacylglycerols at an oil-water interface to release free fatty acids and glycerol [1]. Lipases are produced by plants, animals, and microbes but only microbial lipases are found to be industrially important since they are diversified in their enzymatic properties and substrate specificity [2,3]. Microorganisms such as bacteria, yeast and fungi and their enzymes are widely used in several food preparations for improving the taste and texture and they offer huge economic benefits to industries [4]. Microbial lipases are more widely applied in industries due to their shorter production time, ease for bulk production, which is further enhanced with the advancement in fermentation technologies and ease to manipulation, either genetically or environmentally [5]. Lipases are occupying a place of prominence among biocatalysts who have the ability to catalyze a wide variety of reactions and are an important group of biotechnologically relevant enzymes and they find massive applications [6]. Lipases have become a part of modern food industry. The major applications for industrial enzymes include food and beverages (dairy, bakery, fruit juices, beer, wine), applications account for 55–60% of the global enzymes market [7]. The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easy [1,8]. Accordance with Aravindan et al. [9] the tremendous potential of lipases in food and allied technology applications shows the need to develop novel cost-effective technologies for production, scaling up of this versatile enzyme. A large number
of hydrolytic applications, like flavour development in dairy products (cheese, butter and margarine), alcoholic beverages, milk chocolate, etc., is a promising field of lipase enzyme. Production of diet control foodstuff, meat technology and the processing of sausages are some areas in the food industry with commercial potential. Generally, bacterial lipases are glycoproteins but some extracellular bacterial lipases are lipoproteins [10,11]. Many species of bacteria, yeast and molds are found to produce lipases [12]. Accordance with Ertugrul et al. [14] that Bacillus sp. isolated from olive mill wastewater (OMW) exhibited a high lipase activity. The purpose of this study was to determine the lipase activity of Bacillus subtilis C₂ from bulk shrimp paste.

2. Materials and Methods
2.1 Microorganism and Media
Bacteria strains used in this experiment Bacillus subtilis C₂ isolated from bulk shrimp paste from traditional markets in Samarinda East Kalimantan. Identification of the isolate by 16s r DNA sequencing [15]. Bacterium was cultivated in nutrient agar (NA) slant media.

2.2. Screening of lipase producing bacteria
Lipase producing microorganisms produced a clear zone (hydrolysis) when their appropriate dilutions were spread on the olive oil and glyceryltributyratge agar medium respectively. Agar medium containing per liter of 0.5% beef extract, 0.5% peptone, 0.3% yeast extract, 0.25% NaCl, 0.1% polyvinyl alcohol, and 1.0% olive oil and glyceritriburin respectively, each sterilized at 121°C for 15 minutes, and then the sterilized media was poured into petriplate. Isolated strain was streaked on the glyceryltributyrate agar plate and the olive agar plate, and incubated at 37°C for 24 h to observe zone [16].

2.3. Enzyme production
Positive bacterial strains were cultivated in lipase producing medium for enzyme production. Production medium was prepared containing g/L: peptone 5.0, yeast extract 10.0, NaCl 5.0 and olive oil (1% w/v) as inducer. The initial pH of the medium was adjusted to 7.0. In Erlenmeyer flasks (500 mL) containing 100 mL of production medium, inoculum culture (1% w/v) was added and gently swirled. The inoculated flasks were incubated at 37°C on a rotary shaker at 120 rpm for 1-8 days [17]. Daily sampling of culture filtrate was taken from samples to determine daily enzyme activity. The culture was precipitated by centrifuge (2270 x g) for 5 min at 4°C and the cell free culture supernatant fluid was used as the sources of extracellular enzyme. The lipase activity in the supernatant was determined by the titrimetry method.

2.4. Lipase assays
Lipase enzymes can be quantitated using the titrimetry method [16]. Substrate (1%) is titrated against 0.05 NaOH. Lipase Assay B. subtilis C₂ was assayed for extracellular lipase production using a titrimetric method using olive oil as a substrate. To 1 mL of olive oil, 0.5 mL CaCl₂, 4.5 mL of glycine-NaOH buffer 0.05 N (pH 7.0). The reaction mixture was incubated at 40°C for 10 minutes [15]. then 0.6 mL crude lipase enzyme was added. Reincubated at 40°C by shaking at 120 rpm for 30 min. The reaction was stopped by the addition of 10 mL of an acetone-ethanol 95% (1:1) mixture. The free fatty acids produced was titrated with 0.05N NaOH to a perceptible pink colour using phenolphthalein solution as an indicator. Simultaneously the blank was run with 0.6 mL of distilled water in place of enzyme extract. Activity measurements were carried out in duplicate. Determine the NaOH (N) molar by titration with oxalic acid using the Phenolphthalein indicator (NaOH vs. oxalic acid).

One unit of lipase activity can be defined as the amount of enzyme that liberates 1 µmol of fatty acid per minute at 40°C at pH under the assay conditions.

\[
\text{Lipase activity (µmol/mL)} = \frac{(A-B) \text{ NaOH consumed (mL)} \times \text{Molarity of NaOH (N)} \times 1000}{\text{Volume of enzyme (mL)} \times 30(\text{min})}
\]  
(1)
A = mL NaOH for sample titration
B = mL NaOH for blank titration
1000 = conversion from mmol to µmol
VE = enzyme volume
30 = reaction time

The effects of various factors such as incubation time, temperature, pH, and various types of different oils as carbon source on lipase production were studied to optimize enzyme activity.

2.5. Characterization of lipase enzyme
The effect of incubation time against enzyme activity was carried out by reacting enzyme supernatant in a period of time, i.e. one to eight days. The effect of temperature against enzyme activity was carried out by reacting enzyme solution with the concentration of olive oil 1% in a buffer solution with the obtained optimum pH from the previous analysis at various temperature, i.e. 30, 40, 50, 60 and 70°C at the obtained optimum incubation time. The effect of pH against enzyme activity was carried out by reacting enzyme solution with the concentration of olive oil 1%, then incubated in glycine-NaOH buffer 0.05 N at various pH. The tested pH was 6.0, 7.0, 8.0, 9.0, 10, and 11 were adjusted by adding Na2CO3 on a pH meter. The effect of different oils as carbon source 1% on enzyme activity was carried out by reacting enzyme solution with the olive oil, virgin coconut oil (VCO), cooking oil, tween 80, Schleichera oleosa oil at the obtained optimum temperature and optimum pH.

2.6. Physiology Analysis of Bacillus subtilis C2-Lipolytic Bacteria
Biochemical characterization, the isolate was subjected to a series of biochemical tests which included growth at different pH ranges, NaCl concentrations, and temperature. The effect of pH on bacterial growth was determined by growing the isolate in nutrient broth medium with different pH (2.5, 6.8, 8, 9). The effect of temperature was determined by incubating the culture at different temperatures (8, 37, and 45°C). Similarly, effect of salinity (NaCl) was determined using different NaCl concentrations (4, 6.5, 10% (w/v)) in the culture media. The oxygen requirement was determined by stab the culture in nutrient agar containing agar 0.3%. After 48 h of incubation, the bacterial growth was observed [18,19]. The survival of cells under heat treatments was determined by the culture was heated at 65°C for 10 minutes and the cell viability was observed by spreading culture on nutrient agar medium after 48 h the colony was observed.

3. Results and Discussion
Shrimp paste (terasi) is either dark brown, gray or red and has a strong aroma, and normally consumed as a condiment [20,21]. Fermented shrimp products are widely consumed in Southeast Asian countries. They mainly categorized into shrimp sauces, shrimp pastes, and fermented products [22].

Lipase reaction occurs at the interface between the aqueous and the oil phases, because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic) [23]. Lipases produced from microbes and specifically bacterial lipases play a vital role in commercial ventures [24]. Bacillus subtilis C2 can degrade olive oil and glycerin tributirate by forming a clear zone around the colony after 2 days incubated at 37°C, figure 1A and B. It shows that a clear zone around the colony on the plate so that olive oil and figure 1B screening for lipase activity by Tributyrin Clearing Zone (TCZ) are considered a positive colony for the production of the lipase enzyme. Hundred bacteria were isolated from different environmental sources as shown in table 1 and screened on tributyrin agar plates and rhodamine-olive agar plates. Twenty-six colonies were screened on the basis of appearance of transparent zones of lipolytic activity. Bacillus subtilis C2 lipase selection derived from the largest clear zone of the qualitative selection of the amylase enzyme in the study [15] consisted of C2, C3, C4 and C5 isolated from bulk shrimp paste from traditional markets in Samarinda, East Kalimantan.

The level of lipase enzyme activity can be evaluated by observing the presence of clear zones around the colony at 37°C [25,26]. Sources of lipid substrates that can be used in screening lipase
producers include tributyrin, triolein, Tween 20, Tween 80, olive oil, etc [27,28]. Olive oil incorporated into the agar medium became a good choice for screening lipase-positive colonies [25,29].

Figure 1. (A) A clear zone, B. subtilis C_2 degrades olive oil; (B) A clear zone, B. subtilis C_2 degrades glyceryl tributyrate (TBA)

Titrimetric methods are the most widely used quantitative methods for screening lipase producers, popular and are more sensitive than volumetric methods [27,30]. These methods mainly involve incubation of substrate with the enzyme followed by end point alkali titration of the released free fatty acids [31]. The most commonly used alkali is NaOH and substrates used in these methods tributyrin and olive oil [32]. Volumetric methods are based on the titrimetric estimation of liberated free fatty acids from triacylglycerols by the catalytic action of lipases [27]. According to [33] addition of CaCl_2 in the medium for quenching the fatty acids. The research on the identification and isolation of new lipase produced in microorganisms has been intensified in recent years [34,35,36]. The commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications [37]. The majority of the enzymes in current industrial use are of microbial origin and are produced in conventional aerobic submerged fermentation, which allows greater control of the conditions of growth than solid-state fermentation [38]. Bacterial lipases are mostly extracellular and are greatly influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and incubation time [39,40]. Currently, bacterial lipases are a great demand because of potential industrial applications [41]. The increased demand for microbial originated industrial enzymes especially lipases which have received least priority till last one and half decades and gained lots of importance in recent days owing to their applications in wide variety of fields such as food, dairy, pharmaceutical, detergent, textile, oleo-chemical, perfume and cosmetic industries etc., has lead to the identification of novel enzymes from new sources with unique properties [27].

Optimization of process variables effect of incubation period: lipase production by Bacillus subtilis C_2. The experiment revealed that the best incubation time for lipase production was 5 days at 30°C. The optimum enzyme activity (1.09 µmol/mL) was obtained by 5 days of incubation. The activity gradually decreased after 5 days (figure 2). Incubation periods change from a few hours to many days until the maximum lipase production from bacteria is recorded [30]. Generally, the organic nitrogen source is preferred by bacteria, such as peptone and yeast extract [1].
Temperature also plays an important role in the metabolic processes of a microorganism [42]. The study of effects for temperature optimization on lipase production shows that bacteria produce lipases in a temperature range from 30°C to 70°C. The optimal temperature of the lipase activity was measured at five days incubation using olive oil as substrate at 30°C, 1.43 U/mL (figure 3), and production enzymes decreased after an increase in temperature above 40°C to 70°C but the decrease was not significantly different. The temperature dependence of the activity of enzymes resembles in some respect the pH dependence: increasing with rising temperature, passing a maximum, followed by a decrease [43]. The reports Bhosale et al. [44] on lipase production by Bacillus sp. under thermo-alkalophilic conditions are scanty. The optimum temperature for lipase production is in parallel with the growth temperature of the respective microorganism [30]. The results of this study differ from those of [45] Hasan et al. (2006) enzymes have significant activity at temperatures ranging from 47°C to 60°C and differ significantly from the results of their activity and begin to lose their activity after 60°C. The extracellular lipases from B. subtilis have a maximum production obtained after 72 hours at 50 °C, pH 8. The results showed that the use of olive oil strongly induces the lipase production from Bacillus subtilis [46]. Most lipases can act in a wide range of temperatures [47]. The results of the study of [48] Suci et al. (2018) the optimal temperature for the production of B. subtilis is 30°C. Lipases are being developed to carry out the transformations without the extreme temperature and pressure conditions which are essential for the traditional industrial processes [49].

As the pH played an important role in all the biological processes, lipase production was tested within a broad range of pH (6, 7, 8, 9, 10 and 11). The enzyme production varied considerably from 0.39 to 1.96 µmol/mL, and it was found to have an optimum activity at alkaline pH condition, and display the optimum activity at pH 9 (1.96 µmol/mL) then followed by pH 10 and 11 (1.53 µmol/mL),
pH 6 and 8 (0.51 $\mu$mol/mL) and minimum production at pH 7 (0.39 $\mu$mol/mL) (figure 4). However, it was noted that the lipase production was declined with an increase in pH from pH 10.0 to pH 11.0 but was able to produce lipase towards alkaline pH which shows its alkalo-tolerant nature [50]. The activity of enzymes depends strictly on the pH in the assay mixture and the pH-value of the maximum of the pH-activity curve is the pH optimum [43]. Generally, bacterial lipases have neutral or alkaline pH values and show activity in a broad pH range (pH 4 to pH 11) [1]. The data is in agreement with literature information suggesting that Bacillus lipases generally have a pH optimal of 7.0-9.0 [51]. Previously described in Shah and Bhatt [52] and [46] B. subtilis lipases remain active in the pH range of 6.0 to 10.0. According to [53] thermostable lipase from Bacillus sp. those active in alkaline conditions (pH 9.0-10.0) are very few. Lipases active at alkaline pH are of immense importance in food, dairy, detergent industry [54, 55]. Most lipases can act in a wide range of pH, though alkaline bacterial lipases are more common [47]. Alkaliphiles have also made a great impact on industrial applications [45].

![Figure 4. Effect of pH on lipase activity.](image)

The major factor for the expression of lipase enzyme is a carbon source. Lipases generally produced in the presence of lipid sources such as oil, triacylglycerols, fatty acids, hydrolyzable esters, tweens and glycerols addition to carbon source [30]. Olive oil, virgin coconut oil (VCO), cooking oil from palm oil and Kosambi oil (Schleichera oleosa) as natural nutrients. Tween 80 as nonionic surfactants. In figure 5 the addition of various sources of olive oil, VCO, cooking oil, tween 80 and Kosambi (Schleichera oleosa) oil with a concentration of 1% each for the fifth day incubation enzyme, at 30°C, at pH 9. Optimum lipase activity was obtained in olive oil (1.76 $\mu$mol/mL), followed by tween 80 (1.29 $\mu$mol/mL), VCO (1.2 $\mu$mol/mL), Schleichera oleosa oil (0.73 $\mu$mol/mL), cooking oil (0 $\mu$mol/mL). The production of lipase was more significant in culture medium added with lipids as the carbon source than in the culture medium without lipids. It was demonstrated that the lipase activity is induced by the presence of lipid substrates in the medium. Extracellular lipase production by different microorganisms on lipids has been extensively reported [56]. Kosambi (Schleichera oleosa) is the name of a kind of dry area tree, a rambutan relative of the Sapindaceae tribe. Some regional names Family: Sapindaceae, Kingdom: Plantae, Species: S. oleosa, Genus: Schleichera. Oil extracted from the seed, called 'kosambi oil', is a valuable component of true Macassar oil used in hairdressing; it is also used for culinary and lighting purposes and in traditional medicine, it is applied to cure itching, acne and other skin afflictions. Distribution occurs naturally from the foothills of the Himalayas and the western Deccan to Sri Lanka and Indo-China. It was probably introduced to Malaysia and has naturalized in Indonesia (Java, the Lesser Sunda Islands (Bali and Nusa Tenggara), Sulawesi, the Moluccas, Ceram and the Kai Islands). It is occasionally cultivated throughout the tropics, especially in India [57].
The most suitable sources for lipase production are microbes including bacteria, fungi and yeast. These microorganisms can produce high quality lipases at a lower cost and shorter time [36]. The research on the identification and isolation of new lipase produced in microorganisms has been intensified in recent years [34, 35, 36]. The B. subtilis group cells of these organisms are less than 1μm wide, sporangia are not swollen, and spores are ellipsoidal. They are in general mesophilic with regard to temperature and while often being tolerant to higher pH levels [58]. Attractive properties of Bacillus subtilis like its capability to secrete homologous and heterologous proteins in appreciable quantities into the growth medium and classified as generally regarded as a safe organism by US Food and Drug Administration have made it an important expression host to produce proteins of commercial interest [59]. Lipases have many applications and benefits in the food and agroindustries, where they have quantitative and/or qualitative impacts [60]. Lipases have been also used for addition to food to modify flavor by the synthesis of esters of short chain fatty acids and alcohols, which are known flavor and fragrance compounds [61]. They are desirable for the production of flavors in cheese and for the interesterification of fats and oils. The lipase also accelerates the ripening of cheese and lipoysis of butter, fats, and cream. Lipases facilitate the removal of fat from meat and fish products [62]. Bacillus sp., a family of Gram-positive bacteria, has been extensively investigated and used in a number of fields [63,64]. Several members of this genus are non-pathogenic and easy to cultivate; they secrete key extracellular hydrolytic enzymes such as proteases, amylases, and lipases with remarkable thermostability and alkaline stability [65]. Of particular significance are the lipases, which attract a great deal of attention because of their unique protein sequences and rare biochemical properties [66,67]. The commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications [37]. There are still many enzymes derived from microbes that have not been explored and there are many opportunities to find broader industrial applications of microbial enzymes, especially in the food sector[4].

Biochemical tests show Bacillus subtilis C2 able to grow well at pH 6.8-9, NaCl up to 6.5%, and temperature 37-45°C, the isolate was strictly aerobic (figure 6) and heat tolerance at 65°C for 10 min. Thermophilic/thermotolerant organisms have an advantage in that they withstand higher temperatures during processing and storage. They have a better chance of remaining viable during the drying process required for prolonged storage and they lead to a distinctly effective product [68]. In addition, growth and enzyme production based on characteristics may be conducted under different physical factors, i.e., pH, temperature and salinity.

Figure 5. Effect of carbon lipids source on lipase activity
Bacillus subtilis C2 has diverse characteristics that were useful in various industrial applications. The isolate was subjected to different stress factors. The isolate showed resistance to the high temperature (45°C 24 h and 60°C 10 min) and high osmolarity (up to 6.5% NaCl) that might allow isolating with stand with various conditions during industrial fermentation (table 1).

Table 1. Effect different pH ranges, NaCl concentrations, temperature and heat treatment on the growth of Bacillus subtilis C2.

| Isolate C2 | pH  | NaCl (%) | Temperature | Heat treatment |
|------------|-----|----------|-------------|---------------|
|            | 2.5 | 6.8      | 8           | 9             | 4              | 6.5          | 10           | 8°C          | 37°C         | 45°C         | 65°C 10m     |
| a          | -   | +        | +           | +            | +             | +            | -            | +            | +            | +             | +             |
| b          | -   | +        | +           | +            | +             | +            | -            | +            | +            | +             | +             |
| c          | -   | +        | +           | +            | +             | +            | -            | +            | +            | +             | +             |

+: grow, +_: slightly grow, -: not grow, (a, b, c: replication)

4. Conclusion

Bacillus subtilis C2 isolated from bulk shrimp paste (terasi) in Samarinda East Kalimantan showed producing lipase, able to degrade fat. The ability of the strain to degrade fatty substrates was investigated in the medium 0.1% of olive oil, virgin coconut oil (VCO), cooking oil, tween 80, and Schleichera oleosa oil. The optimum activity of Bacillus subtilis C2 lipase was 1.09 U/mL after five days incubation, 1.43 μmol/mL at 30°C and 1.96 U/mL at pH 9.0. The enzyme gave the highest lipase activity 1.76 μmol/mL used olive oil as a substrate. B. subtilis C2-lipolytic was thermotolerant, halotolerant and strictly aerobic.

Acknowledgments

This research was funded by DIPA of thematic of Research Center for Biology, Indonesian Institute of Sciences. Authors acknowledge Ninuk Setiantingrum and Khairunnisa S.Si. for the assistance in the laboratory

5. References

[1] Gupta R, Gupta N and Rathi P 2004 Bacterial lipases: an overview of production, purification and biochemical properties Appl. Microbiol. Biotechnol 64: 763-781
[2] Ma J, Zhang Z, Wang B, Kong X, Wang Y, Cao S and Feng Y 2006 Over expression and characterization of a lipase from Bacillus subtilis Protein Expr. Purif 45(1): 22-29
[3] Ramakrishnan V, Goveas L C, Narayan B and Halami P M 2013 Comparison of lipase production by Enterococcus faecium MTCC, 5695 and Pediococcus acidilactici MTCC, 11361 using fish waste as substrate: optimization of culture conditions by response surface methodology ISRN Biotechnol Article ID 980562, 9 p
[4] Raveendran S, Parameswaran B, Ummalyma S B, Abraham A, Mathew A K, Madhavan A, Rebello S and Pandey A 2018 Applications of Microbial Enzymes in Food Industry Food Technol. Biotechnol. 56(1): 16-30

[5] Kumar M D J, Rejitha R, Devika S, Balakumaran M D, Rebecca A I N and Kalaichelvan P T 2012 Production, optimization and purification of lipase from Bacillus sp. MPTK 912 isolated from oil mill effluent Adv. Appl. Sci. Res. 3(2): 930-38

[6] Mohammed H J 2013 Physicochemical factors affected the partial purified lipase activity of Acinetobacterbaumannii “local isolates” Iraqi J. Pharm. Sci. 22(1): 82-9

[7] Guerrand D 2017 Lipases industrial applications: Focus on food and agroindustries. OCL. 24: 403

[8] Jianrong L, Yu D, Haiming J, Lili C, Xin Z and Yumni T 2011 Production, purification and characterization of lipase from Serratia sp. L-11 J. Biotechnol 29: 120-121

[9] Aravindan R, Anbumathi P and Viruthagiri T 2007 Lipase applications in food industry Indian J. Biotechnol 6: 141-158

[10] Abdel-Hameed A M, Kither M K and Farmman M S 2013 Production and purification of lipase from Pseudomonas ecepacia and study some effected conditions on production. Diyala Agric. Sci. J 5(2): 436-450

[11] Sagar K, Bashir Y, Phukan M M and Konwar B K 2013 Isolation of lipolytic bacteria from waste contaminated soil: a study with regard to process optimization for lipase. Int. J. Sci. Technol. Res 2(10): 214-218

[12] Liu Z, Chi Z, Wang L and Li J 2008 Production, purification and characterization of an extracellular lipase from Aureobasidium pullulans HN2.3 with potential application for the hydrolysis of edible oils. Biochem. Eng. J 40: 445-51

[13] Svendsen A 2000 Lipase protein engineering: Review Biochem. Biophys. Acta 1543(2): 223-238

[14] Ertugrul S, Donmez G, Takac S 2007 Isolation of lipase producing Bacillus sp. from olive mill waste water and improving its enzyme activity. J. Hazard Mater 149(3): 720-724

[15] Soeka YS 2016. Karakterisasi Bakteri Penghasil α-Amilase dan Identifikasi Isolat C2 yang Diiisolasi dari Terasi Curalah. Berita Biologi 15(2): 185-193

[16] Iqbal SA and Rehman A 2015 Characterization of Lipase from Bacillus subtilis I-4 and Its Potential Use in Oil Contaminated Wastewater Braz. Arch. Biol. Technol. 58(5): 789-797

[17] Bharathi D, Rajalakshmi G, S. Komathi S 2019 Optimization and production of lipase enzyme from bacterial strains isolated from petrol spilled soil Journal of King Saud University – Science 31: 898–901

[18] Parvathi A, Krishna K, Jose J, Joseph N and Nair S 2009 Biochemical and molecular characterization of Bacillus pumilus isolated from coastal environment in Cochin, India. Braz. J. Microbiol 40(2): 269-275

[19] Ali N, Ullah N, Qasim M, Rahman H, Khan S N, Sadiq A and Adnan M 2016 Molecular characterization and growth optimization of halotolerant protease producing Bacillus Subtilis Strain BLK-1.5 isolated from salt mines of Karak, Pakistan. Extremophiles 20(4): 395-402.

[20] Aryanta WR 2000 Traditional fermented food in Indonesia Jpn. J. Lab 10(2): 90-99

[21] Kobayashi T, Kajiwara M, Wahyuni M, Kitakado T, Hamada-Sato N, Imada C and Watanabe E 2003 Isolation and characterization of halophilic lactic acid bacteria isolated from terasi shrimp paste: A traditional fermented seafood product in Indonesia J. Gen. Appl. Microbiol 49(5): 279-286

[22] Hajep P and Jinap S 2012 Fermented Shrimp Products as Source of Umami in Southeast Asia. J. Nutr Food Sci. S10 006. 5 page

[23] Reis P, Holmberg K, Watzke H, Leser M E and Miller R 2009 Lipases at interfaces: A review. Adv. Colloid Interface Sci 147-148: 237-250

[24] Gupta R, Rathi P, Gupta N and Bradoo S 2003 Lipase assay for conventional and molecular screening: an overview Biotechnol. Appl. Biochem. 37: 63-71

[25] Kim E K, Jang W H, Ko J H, Kang J S, Noh M J and Yoo O J 2001 Lipase and its modulator from Pseudomonas sp. strain KFCC 10818: Proline-to-glutamine substitution at position 112
induces formation of enzymatically active lipase in the absence of the modulator. *J. Bacteriol* **183**(20): 5937-5941

[26] Mazhar H, Abbas N, Hussain Z, Sohail A and Ali SS 2016 Extracellular lipase production from *Bacillus subtilis* using agro-industrial waste and fruit peels *Punjab Univ. J. Zool* **31**(2): 261-267

[27] Lanka S and Latha J N L 2015 A short review on various screening methods to isolate potential lipase producers: lipases-the present and future enzymes of biotech industry. *Int. J. Biol. Chem* **9**(5): 207-219

[28] Tripathia R, Singha J, Bhartia RK and Thakura IS 2014 Isolation, Purification and Characterization of lipase from *Microbacterium* sp. and its application in biodiesel production *Energy Procedia* **54**: 518-529

[29] Hube B, Stehr F, Bossenz M, Mazur A, Kretschmar M and Schafer W 2000 Secreted lipases of *Candida albicans*: Cloning, characterisation and expression analysis of a new gene family with at least ten members *Arch. Microbiol* **174**: 362-374

[30] Yapasaran E 2008 Partial Purification and Characterization of Lipase Enzyme from a *Pseudomonas* Strain. A Thesis Submitted to the Graduate School of Engineering and Sciences ofİzmir Institute of Technology in Partial Fulfillment of the Requirements for the Degree of Master of Science Chemistry

[31] Cherry I S and Crandall Jr L A 1932 The specificity of pancreatic lipase; its appearance in the blood after pancreatic injury *Am. J. Physiol* **100**(2): 266-73

[32] Parry Jr R M, Chandan R C, and Shahani K M 1966 Rapid and sensitive assay for milk lipase. *J. Dairy Sci* **49**(4): 356-60

[33] Singh R, Gupta N, Goswami V K and Gupta R 2006 A simple activity staining protocol for lipases and esterases. *Appl. Microbiol. Biotechnol* **70**: 679-682

[34] Hasan F, Shah A A and Hameed A 2006 Industrial Applications of Microbial Lipases *Enzyme Microb. Technol* **39**(2): 235-251

[35] Shu Z Y, Jiang H, Lin R F, Jiang Y M, Lin L and Huang J Z 2010 Technical methods to improve yield, activity and stability in the development of microbial lipases *J. Mol. Catal B: Enzymatic* **62**: 1-8

[36] Treiche H, Oliveira D, Mazutti M A, Luccio M D and Oliveira JV 2010 A review on microbial lipases production *Food Bioprocess Technol* **3**(2): 182-96

[37] Pratush A, Gupta A, Vyas G and Sharma P 2013 Bacterial Lipases: Production Strategies and Industrial Applications. Microbiology Application.In book: Microbiology Application, Edition: 2013, Publisher: Bhalla publishers, Dehradun. India, Editors: ChandiCharanRath64-83 https://www.researchgate.net/publication/303973493

[38] Nigam P S and Pandey A. Chapter 10 2009 Solid-state fermentation technology for bioconversion of biomass and agricultural residues. Solid state fermentation for the production of industrial enzymes pp197-221

[39] Elibol M and Ozer D 2000 Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*. *Process Biochem* **36**: 325-39

[40] Mazhar H, Abbas N, Ali S, Sohail A, Hussain Z and Ali S S 2017 Optimized production of lipase from *Bacillus subtilis* PCSIRNL-39 *Afr. J. Biotechnol* **16**(19): 1106-1115

[41] Sirisha E, Rajasekar N and Narasu L M 2010 Isolation and optimization of lipase producing bacteria from oil contaminated soils *Adv. Biol. Res* **45**: 249-52

[42] Nigam P S 2013 Review microbial enzymes with special characteristics for biotechnological applications. *Biomolecules* **3**: 597-611

[43] Bisswanger H 2014 Review Enzyme Assays. *Perspect. Sci* **1**: 41-5.5

[44] Bhosale H J, Uzma S Z and Bismile P C 2015 Optimization of lipase production by thermo-alkalophilic *Bacillus* sp. *8C Res. J. Microbiol* **10**(11): 523-532

[45] Hasan F, Shah A A and Hameed A 2006 Influence of culture conditions on lipase production by *Bacillus* sp. FH5 *Ann. Microbiol* **56**(3): 247-52
[46] Laachari F, El Bergadi F, Bahafid W, Sayari A, Elabed S, Mohammed I and Ibnsouda S K 2014 Biochemical study of lipases from *Bacillus subtilis*. *Moroccan J. Biol* 11: 1-9

[47] Hasan F, Shah A A and Hameed A 2007 Purification and characterization of a mesophilic lipase from *Bacillus subtilis* FH5 stable at high temperature and pH. *Acta Biol. Hung* 58(1): 115-32

[48] Suci M, Arbianti R and Hermansyah H 2018 Lipase production from Bacillus subtilis with submerged fermentation using waste cooking oil. IOP Conf. Series: Earth and Environmental Science 105

[49] Kumar A, Dhar K, Kanwar S S and Arora P K 2016 Lipase catalysis in organic solvents: advantages and applications *Biol. Proced Online* 182

[50] Golani M, Hajela K and PandeyGP 2016 Screening, Identification, Characterization and Production of Bacterial Lipase from Oil Spilled Soil *Int. J. Curr. Microbiol. App. Sci* 5(3): 745-63

[51] Nawani N and Kaur J 2007 Study on lipolytic isoenzymes from a thermophilic *Bacillus* sp.: production, purification and biochemical characterization *Enzyme Microb. Tech* 40: 881-87

[52] Shah K R and Bhatt S A 2011 Purification and characterization of lipase from *Bacillus subtilis* Pa2 J. *Biochem. Tech* 3(3): 292-295

[53] Kumar S, Kikon K, Upadhyay A, Kanwar S S and Gupta R 2005 Production, purification and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. *Protein Expres. Purif* 41: 38-44

[54] Bora L and Bora M 2012 Optimization of extracellular thermophilic highly alkaline lipase from thermophilic *Bacillus* sp. isolated from hot spring of Arunachal Pradesh, India *Braz. J. Microbiol* 43: 30-42

[55] Bhosale H, Shaheen U and Kadam T 2016 Characterization of a Hyperthermostable Alkaline Lipase from *Bacillus sonorensis* 4R. *Enzyme Res*. Volume 2016, Article ID 4170684, 11 pages

[56] Mahadik N D, Puntambekar U S, Bastawde K B, Khire J M and Gokhale D V 2002 Production of acidic lipase by *Aspergillus niger* in solid state fermentation *Process Biochem* 38: 715-21

[57] Anonymous, *Schleicheræoleosæ* (Lour.) Oken, Allg. Naturgesch. Bot. 2: 1341 (1841) [www.asianplant.net › Sapindaceae › Schleicheræa](https://assets.publishing.service.gov.uk › file › ID)

[58] UK Standards for Microbiology Investigations 2018.Identification of *Bacillus* species. Bacteriology – Identification. Issued by the Standards Unit, Public Health England 3.1 27. [https://assets.publishing.service.gov.uk › file › ID](https://www.asianplant.net › Sapindaceae › Schleicheræa)

[59] Eggert T, Poudroyeny G, Pencrac’e G, Douchet I, Verger R, Dijsktra W B and Jaeger K E 2002 Biochemical properties and three-dimensional structures of two extracellular lipolytic enzymes from *Bacillus subtilis* *Colloids Surf. B Biointerfaces* 26: 37-46.

[60] Singh R, Kumar M, Mittal A and Mehta P K 2016 Microbial enzymes: industrial progress in 21st century. 3*Biotech 6*: 174

[61] Macedo G A, Lozano M M S and Pastore G M 2003 Enzymatic synthesis of short chain citronellyl esters by a new lipase from *Rhizopus* sp. *Electron J. Biotechnol* 6(1) fulltext-2

[62] Ray A 2012 Application of lipase in industry *Asian J. Pharm. Tech* 2: 33-7

[63] Hirose K, Sano I, Shioda M, Kumano M, Nakamura K, and Yamane K 2000 Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study *Microbiology* 146: 65-75

[64] Tjalsma H, Bolhuis A, Jongbloed J D, Bron S, and van Dijl J M 2000 Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome *Microbiol. Mol. Biol. Rev* 64(3): 515-547

[65] Kanmani P, Kumaresan K and Aravind J 2015 Gene cloning, expression, and characterization of the *Bacillus amylyoliquefaciens* PS3 lipase *Braz. J. Microbiol* 46(4): 1235-43

[66] Chen L, Coolbear T and Daniela R M 2004 Characteristics of proteinases and lipases produced by seven *Bacillus* sp. isolated from milk powder production lines *Int. Dairy J* 14: 495-504

[67] Olusesan A K, Azura L K, Forghani B, Bakar F A, Mohamed A K, Radu S, Manap M Y, and Saari N 2011 Purification, characterization and thermal inactivation kinetics of a
nonregioselective thermostable lipase from a genotypically identified extremophilic *Bacillus subtilis* NS 8. *N. Biotechnol* 28: 738-745

[68] Kosin B and Rakshit S K 2006 Microbial and processing criteria for production of probiotics: A Review *Food Technol. Biotechnol* 44(3): 371-379