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https://journal.uitm.edu.my/ojs/index.php/MJCET

EM·J·C·E·T

e-ISSN: 2682-8588

Malaysian Journal of Chemical Engineering & Technology 7(2) 2024, 314–321

Malaysian Journal of Chemical Engineering and Technology

Purification of *Geobacillus thermodenitrificans* thermostable lipase

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ARTICLE INFO

Article history:
Received 17 April 2024
Revised 31 October 2024
Accepted 31 October 2024
Online first
Published 31 October 2024

Keywords:
Enzyme purification
Lipase
SDS-PAGE

DOI:
10.24191/mjcet.v7i2.1194

ABSTRACT

The demand for a structured method in producing high quality lipases has tremendously increased lipase purification technology within specific range of its original function. Crude lipase enzyme produced by a locally isolated *Geobacillus thermodenitrificans* (LGT) has demonstrated a notable potential for various bio-deinking capacities and other enzymatic actions based on the credential amount used. The enzyme production was set up in an airlift fermenter system using a cultivation medium prepared from (w/v) 1.25% glucose and yeast extract, 0.75% NaCl and 0.10% olive oil. Fermentation was accomplished in 24 hours with an air flow rate of 1.00 L/min. Physical parameters were maintained at 7.0% (v/v) inoculum size and pH 6.8. The extracted extracellular crude lipase was purified to homogeneity using four-step procedures. The acetone precipitation, Sephadex G-100 filtration chromatography and double steps of DEAE Sefarose CL-6B anion with exchange chromatography has resulted in a final yield of 25.00%. The molecular weight of the purified enzyme was estimated to be 33.5 kDa using an SDS-PAGE analysis. The lipase enzyme has a high potential to be further used for many industrial purposes and has been purified with 22.1-fold protein. The above functional reported characteristics of the LGT was considered a modest quality taking into consideration of its ability to stand high temperature range.

1. LIPASE AND FUNCTION

Lipase occupies a high usage within biocatalyst by having an enormous biological function and is very unique. It appears to be a great catalyst for hydrolysing fats into various fatty acids and useable glycerol between water and lipid interface (Menalla et al., 2024) as well as it can reverse the reaction in nonaqueous media (Nik Him & Azmi, 2017). Lipases are mostly targeted for industrial applications by focusing on the development of biotechnologically significant aspects (Vardar-Yel et al., 2024). According to Grand View Research (2023) and Subroto (2020), lipase consumption in 2030 would reach USD 14.0 to 14.5 billion as its substrate would significantly be accepted as the major abundant natural molecules. Currently, commercial lipase has been extensively used in a lot of enzymatic deinking, food industries, health and medical related industries as well as the oil and gas industries (Ali et al., 2023). These commercial established lipases have been used for many other applications as well.

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https://doi.org/10.24191/mjcet.v7i2.1194

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On the other hand, even having such important functions in most industries, lipase's purification has not been focused and very limited reports on newly isolated or lab-scale produced microbial lipase purification methods have been reported. This might be due to the main interest of most researchers who aim at lipase function instead of lipase production line. Microbial lipases are produced mostly by a submerged culture or some solid-state fermentation methods but the second appears to be an interesting alternative for microbial enzyme production due to the possibility of usage of residues and by-products from agro-industries as nutrient sources and support for the microorganism development (Chandra et al., 2020). Productions must follow with standard purification methods to ensure lipase proteins are well separated from the unwanted residues or the non-protein particles. Those residues and particles are commonly collected from the substrates, cells or other material in the fermentation system. The substrates of the reaction can vary from byproducts material, or any reactant chosen for the cell growth with focus cell respiration, immobilisation and protein production. This value adds to lipase production and at the same time provides lower cost in the technology processing. Thus, purification is hoped to improve lipase function beyond limit. On the contrary, it helps to subdue enzyme shortage, reduce enzyme cost and prepare locally produce industrial enzyme. This paper will center on a purification of lipase from *Geobacillus thermodenitrificans* (LGT), a thermotolerant Malaysian isolated species. This enzyme possesses high capacity of enzymatic activities and has significantly set off comparable results to a commercial lipase (LS) from Sigma with various potential factors.

2. METHODOLOGY

2.1 Microbial source

The microbe used was a thermotolerant Gram negative *Geobacillus thermodenitrificans* nr68. This species was isolated from a hot spring in Machang Kelantan, Malaysia with temperature of the pond and the soil recorded as 48 °C. This species has been identified using APi-20E, biochemical tests, Gram staining test and 16S rRNA analysis. Fig. 1 shows rod shape Gram negative *Geobacillus thermodenitrificans* nr68. The species was maintained at −20°C in a freeze-dried powder form.

Fig 1. Gram negative-stained Geobacillus thermodenitrificans

Source: Authors' own data

2.1 Production and media development for LGT

Circular airlift fermenter system was used with optimum parameters of 65 °C, pH of 6.8, air flow rate of 1.00 L/min and inoculum size at 7.0% (v/v) (Nik Him & Azmi, 2017). The production medium contains 0.75% (w/v) glucose, 1.25% (w/v) yeast extract, 0.45% (w/v) NaCl and 0.10% (v/v) olive oil (Nik Him,

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2003). The specific growth, μ of the *Geobacillus thermodenitrificans* has been recorded highest with 1.25% (w/v) glucose and yeast extract and flow rate of 1.0 L/min (0.12 hour^{-1}). This cultivation has the lowest doubling time (td, 5.77 hours), the rate of enzyme formation, dp/dt of 1.73 UmL-1h^{-1}, a very high rate of specific enzyme production which was recorded as 11700.0 U/g of mass, the rate of glucose used, ds/dt of 0.22 gL-1h^{-1} and quite a low formation of enzyme per gram, that was 4110.0 U/g of glucose.

2.2 Enzyme and assays

Lipase from *Geobacillus thermodenitrificans* (LGT) was used. Enzyme activities were assayed by olive oil emulsion method modified from Mustranta et al. (1993). Substrate was prepared by homogenising 30 mL of olive oil with 70 mL of emulsification reagent. (Keong et al., 2013). One unit of lipase represented the release of 1 μmol of fatty acid per min under the above assay condition.

2.3 Protein content determination

Protein content in the cell-free supernatant was done using bovine serum albumin as standard according to method by Lowry et al. (1951).

2.4 Enzyme purification

Purification of crude LGT was executed using acetone precipitation to concentrate the crude lipase, Sephadex G-100 filtration chromatography and twice of DEAE Sefarose CL-6B filtration chromatography.

2.5 Acetone precipitation

Approximately, 80% (v/v) of cold acetone (4:1 analytical grade acetone to dH_2O at temperature −20°C) was added into 200 ml of crude LGT, the mixture was vortex before placing at −20°C for overnight (Nik Him, 2010). Mixture was washed in new cold acetone twice, followed by spinning at 13,000 X g for 10 min (4°C, Sorvall RC-5C). The supernatant was carefully poured off and pellet was dissolved in a phosphate buffer (5.0 ml, 0.2M, pH 6.8). Sample was vortex after washing away the supernatant. Crude LGT has shown an activity of 20.50 U/ml and 46.23 U/ml after the acetone precipitation. The enzyme was dialysis for 24h (10.0 mm dialysis tube, Spectrum Medical Industries, 10,000 D) using method by Bollag et al. (1996) and Nik Him & Azmi (2017). Enzyme activity after dialysis was recorded as 58.22 U/ml.

2.6 Gel-filtration chromatography

Sephadex G-100 (Sigma) was loaded into an 80.0 cm × 3.0 cm column after equilibration with phosphate buffer (0.2M, pH 6.8). Elution of 1.0 ml concentrated enzyme was performed using a flow rate of 20 mL/h. Fractions were collected at interval of 5 ml (Nik Him & Azmi, 2017).

2.7 DEAE Sefarose CL-6B anion exchange chromatography (1)

Fractions of 10-20, 30-45 and 55-65 with the highest activities of LGT after gel-filtration were used. They were concentrated using ultrafiltration membrane (Minitans, mv cut-off 10,000) and were eluted into the DEAE Sefarose CL-6B packed on 2.5 × 40 cm column at 16 ml/hour and fraction of 4.0 ml. The column was before prepared according to Nik Him (2010). LGT were eluted at linearity of 0.50 L from 0 to 0.5 M NaCl with the same buffer. The purity of LGT collected from fraction no 15–30 and 35–55 were studied.

2.8 DEAE Sefarose CL-6B anion exchange chromatography (2)

All LGT collected from fraction no 15–30 and 35–55 were dialysis in 0.2 M phosphate buffer (pH 6.8) over-night followed by steps in Section 2.7 but using 0 to 0.2 M NaCl. LGT activity was recorded at fractions 30–50 and one single band was seen on the SDS PAGE.

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2.9 Determination of LGT molecular weight using SDS- PAGE

The molecular mass of the purified lipase was determined by SDS-PAGE as described by Bollag et al. (1996) using denatured protein and 12.5% of acrylamide gel. Bromophenol stained; small molecular weight protein markers were used (14.4–97.0 kDA, Pharmacia Biotech). Rf was measured by length of LGT band movement over length of marker. The gel was stained with silver staining method as described by Bollag et al. (1996).

3. RESULTS AND DISCUSSION

Geobacillus thermodenitrificans n68 was compatible to the species reported by Balan et al. (2017) and was growing very well at 45–70 °C, at pH 6-7 but very greatly performing the best quality of activities at pH 6.8 (Nik Him, 2010). The growth was very much affected by the content of NaCl and the best range was between 0.25 and 0.30%. The typical cells of *Geobacillus thermodenitrificans* nr68 is shown in Fig. 1. The extracellular lipase from *Geobacillus thermodenitrificans* nr68 was purified using a Four-Steps Procedures. Acetone precipitation has yielded the amount of 89.9% proteins with LGT activity of 3768 U and has decreased to 86.4% or 3428.4 U after an overnight of dialysis process. The result was as expected; as dialysis process would allow net movement of the proteins from higher concentration to a lower concentration until the equilibrium factor was gained which then would give lower enzyme activity. Fig 2 (a) shows profile of crude LGT using the Sephadex G-100 with 3 major peaks from fractions 10–20, 30–45 and 55–65 with total activity of 2550.6 U. Foreign non-lipase protein was detected from fractions 5–10 and 30–60. Gel Sepharose CL-6B (1) shows 2 peaks equivalent to LGT enzyme protein peaks (Fig. 2 (b)) and CL-6B(2) exhibited single peak P1 by 22.1 times, with specific activity at 17.2 U/mg proteins and yield of 25.0% at fraction 35–55 (Fig. 2 (c)).

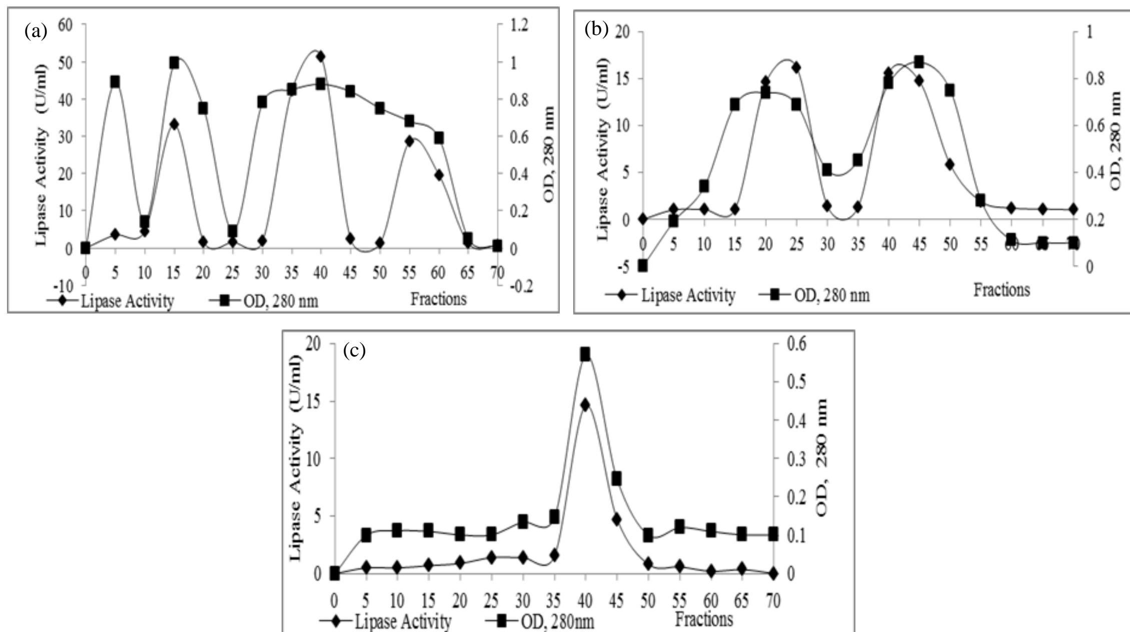

Fig 2. (a) Profile of crude LGT using Sephadex G- 100, (b) Sepharose CL-6B (1) and (c) Sepharose CL- 6B (2)

Source: Authors' own data

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The purification of LGT is summarised in Table 1. Total protein decreased significantly during the process but total activity was maintained and so the increment of the specific activity of the LGT. The same result has been shown by Vieira de Jesus et al. (2016) using lipase from *Penicillium restrictum*. From the result obtained, it is believed that there was no hydrophobic interaction involved during elution of LGT in all three columns.

Table 1. LGT purification steps

Purification Steps	Protein Total (mg)	Total LGT Activity (U)	LGT Specific Activity (U/mg)	Yield (%)	Purity in fold
Crude lipase	5744.20	3868.0	0.67	100.00	1.00
Acetone precipitation	1880.2	3868.0	2.00	89.9	2.31
LGT after 24 h dialysis	1020.2	3768.0	3.36	86.4	3.51
Sephadex G-100	745.0	3428.4	4.17	78.4	4.38
Sefarose CL6B-1	156.9	3112.8	8.49	33.5	10.88
Sefarose CL6B-2	56.89	1332.5	17.2	25.0	22.1

Source: Authors own data

In order to get the molecular weight of the protein enzyme, protein molecular standard was used in the same gel plate. Low molecular weight standard with set of markers varies in size from phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), tripsin inhibitor (20.1 kDa) and α-laktalbumin (14.4 kDa) were used. Standard and the enzyme protein was rinsed with Coomassie Brilliant Blue R-250 after gel separation followed by a destaining method for 12–14 hours. Band visualization is shown in Fig. 3.

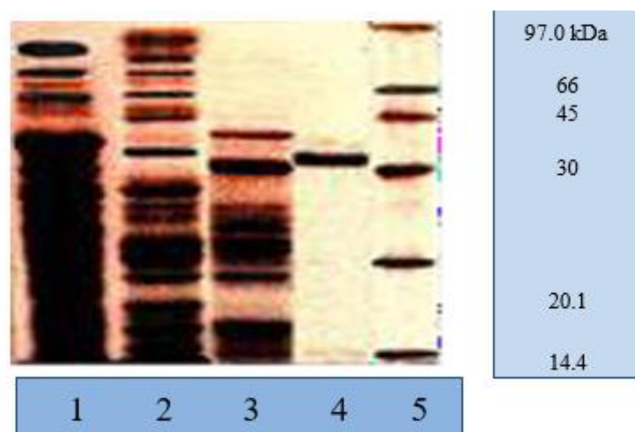

Fig 3. SDS-PAGE of thermostable LGT. Lane 1: Denatured enzyme. Lane 2: LGT on G-100. Lane 3: LGT on CL-6B(1). Lane 4: LGT on CL-6B(2), purified lipase. Lane 5: Protein markers

Source: Authors' own data

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The LGT was calculated to have a molecular weight of 33.5 kDa by using Rf method (Fig.4). When using a uniform density, the relative migration distance of a protein known as Rf, (where f is a subscript) is negatively proportional to the log of its mass, so we could estimate the unknown protein molecular weight using the plotted graph. After running the gel, determination of the relative migration distance (Rf) of the protein standards and the unknown size LGT protein was calculated using the following Eq. (1).

$$Rf = \frac{\text{Migration distance of the protein}}{\text{Migration distance of the dye front}} \quad (1)$$

Plotting the Rf will generate a linear plot for most proteins when the protein samples are fully denatured and the gel percentage is appropriate for the molecular weight range. As expected, the LGT have a moderate protein size. This result is significant because according to the researchers who are working on these microbial lipases, most reported lipase protein sizes are between 20-60 kDa (Rabbani et al., 2023). Lipase from *Bacillus* are reported to have low molecular weight of ~20 kDa (Saxena et al., 2019) whereas thermophilic lipase BTID-B from *Bacillus thermoleovorans* ID-1 purified by Keong et al. (2013) was about 43 kDa. The LGT protein size is with great advantages because they are more stable to wider temperature ranges reported in many academic writings. This characteristic is believed to be supported by the smaller changes (unfolding) in the protein's tertiary structure (Świderek et al., 2023). Interpolating the value from Fig. 4 has been giving the size of the LGT but it is important to verify the accuracy of the calculated value as this method may somehow contributed to about 3% to 4% of inaccuracy (Lan et al., 2024). This is due to the presence of polypeptides such as glycol- and lipoproteins that usually leads to erroneous results since they are not fully coated with SDS and thus, would not behave as expected.

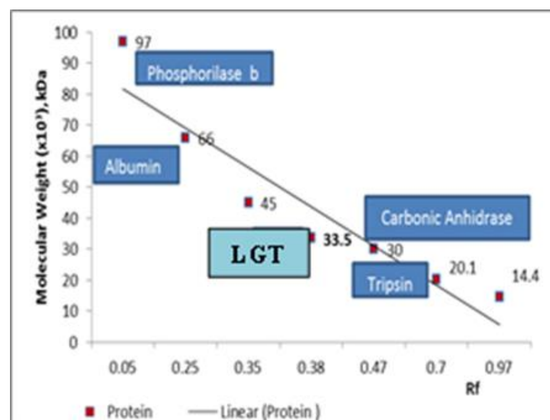

Fig 4. Rf for markers and LGT with the molecular weights. LGT have been calculated to have a moderate size molecular weight of 33.5 kDa

Source: Authors' own data

4. CONCLUSION

Lipase enzyme produced by locally isolated thermotolerant *Geobacillus thermodenitrificans* nr68 (LGT) has been purified with 22.1 fold. The molecular weight of LGT was 33.5 kDa. It was a moderate protein size but a very significant other microbial lipases as reported by other researchers.

https://doi.org/10.24191/mjcet.v7i2.1194

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ACKNOWLEDGEMENT

The authors gratefully acknowledge the Universiti Teknologi MARA (UiTM) and USM for the facilities used during the research.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS' CONTRIBUTIONS

Nik Raikhan Nik Him: Experimental, conceptualisation, validation and analysis. **Muhammad Syafiq Abu Hassan:** Conclusion, references and conceptualisation

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