e-ISSN No.: 2582-4228

Journal of Indian Association for Environmental Management

Journal homepage: www.http://op.niscair.res.in/index/php/JIAEM/index

Exploration, Molecular Characterization and Optimization of Novel Lipase-producing Microalgae*Chlorella Sorokiniana* LGS2 Isolated from Lake Water

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Submitted:03 April2025Revised:21April2025Accepted: 22April2025

***Abstract*:This study investigates the production and optimization of lipase enzymes from microalgae isolated from lake water. Various microalgal strains were isolated, cultured under controlled conditions, and evaluated for lipase activity using qualitative assays such as tributyrin agar, Rhodamine B, and bromothymol blue plate methods. Molecular identification via 16S rRNA chloroplast sequencing revealed *Chlorella sorokiniana* LGS2 as the most efficient strain, with its sequence deposited in GenBank under accession number PQ583068. Lipase production was induced in BG-11 medium supplemented with olive oil, and enzymatic activity was assessed through the hydrolysis of p-nitrophenyl phosphate. Optimization of physicochemical parameters, including light intensity, temperature, pH, agitation, and substrate concentration, was conducted to maximize lipase production. This research highlights *C. sorokiniana* LGS2 as a novel and promising source for lipase production, providing a valuable foundation for future biotechnological and industrial applications.**

***Author Keywords*: *Chlorella sorokiniana* LGS2, Lipase, Tributyrin agar, Rhodamine B, and Bromothymol blue plate, BG-11 medium, p-nitrophenyl phosphate.**

I. INTRODUCTION

Microalgae present unique advantages for industrial enzyme production due to their ability to thrive on minimal resources such as carbon dioxide, water, nitrogen, salts, and light (both natural and artificial), which helps reduce production costs. Despite their potential, research on lipase enzymes derived from algae and photosynthetic cyanobacteria remains limited, with few studies exploring novel lipolytic enzymes of biotechnological significance. A 2011 study on lipase biodiversity found that algal lipases make up only 3% of known lipase sources, in contrast to bacterial (45%), fungal (21%), animal (18%), and plant (11%) lipases. Algae's ability to produce lipases is supported by their well-established capacity to synthesize lipids and fats (Patil et al., 2011). According to Yong et al. (2016), microalgae offer a promising avenue for obtaining lipases with high bioactivity. However, the synthesis of these enzymes presents challenges, such as longer cultivation periods compared to bacteria, yeast, or fungi, and the need for additional downstream processing steps to recover intracellular enzymes. Moreover, many biomolecules in microalgae are produced intracellularly in limited quantities, complicating analysis and large-scale industrial applications. Recent advancements in cell lysis techniques have significantly improved the extraction efficiency of microalgal metabolites, helping to overcome some of these limitations (Brasil et al., 2017). This study employs both qualitative and quantitative techniques to evaluate the lipase production capacity of *Chlorella sorokiniana* LGS2, while also exploring optimization methods to enhance lipase yield, thus contributing to the growing understanding of microalgal enzyme potential.

II. MATERIALS AND METHODS

Sample Collection

Water samples were collectedduring October and November of 2023 from Sungam Lake in Coimbatore, Indiaat Latitude N 10° 59' 25.8252" and Longitude E 76° 58' 21.0009". For the purpose of preventing contamination, the

samples were meticulously collected in sterile containers. To maintain the microalgae's viability after collecting, the samples were transported to the lab under carefully monitored conditions. At each sampling site, water samples were collected 0.15 meters (15cm) below the surface and then stored in cold boxes for transportation to the laboratory (Backer et al,2008).

Isolation and morphological identification of microalgae

BG-11 media (Allen, 1968) was used to inoculate microalgal colonies in a controlled setting. Cool white fluorescent lamps with anconstant light intensityused of continuous illuminatefor approximately nine days while they were being grown at a constant temperature of 25°C.Togetunialgal cultures, enriched samples weresubmittedto serialdilution andthenexaminedunderthephase-contrast microscopeofMagnus, Magcam DC Plus,100timesmagnification. Until the individual colonies were clearly separated, the diluted samples were streaked on BG-11 agar media. After the purified colonies were inoculated into new liquid BG-11 media, four distinct microalgal strains were successfully isolated and morphologically identified as *Neochlorisvigensis*, *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Asterarcysquadricellulare*at the National Repository for Microalgae and Cyanobacteria – Freshwaterat Bharathidasan University, Tiruchirappalli(Duong, 2016).

Production of lipase through enrichment

By growing the four isolated microalgae in BG-11 medium enriched with 2% (v/v) olive oil as a triglyceride source, lipase synthesis was stimulated. The cultures were maintained at 25^{o}C under cool white fluorescent lamps with anconstant light intensity continuous illumination. The extracellular enzyme was separated by centrifuging the crude enzyme for 10 minutes at 10,000 rpm after the incubation time. After then, the crude lipase enzyme-containing supernatant was collected for further analysis (Duong, 2016).

Qualitative analysis

The four cultivated unialgal isolates were subjected to qualitative analysis to screen for lipase production.

Tributyrin agar plate assay

The tributyrin assay, which was modified from the technique outlined by Safdar et al., (2024) was used to measure lipase activity by tracking the hydrolysis of tributyrin. The medium used to construct the test plates contained 15 g/L agar, 10 mL/L tributyrin, and 0.01% phenol red in distilled water that had been pH 7.5 adjusted. The hardened agar was divided into well that were 6 mm in diameter. The plates were incubated for 24 hours after the four microalgal isolates' crude lipase extract (10 µL) was added to each well.Lipase activity was demonstrated by the development of yellow halos surrounding the wells, which were caused by the release of fatty acids that decreased pH.(Figure 1)

Rhodamine b plate assay

The Rhodamine B assay was utilized to evaluate lipase activity by visualizing fluorescence under UV light, following the method of Stemler et al. (2024), with modifications. The assay plates were prepared with a medium containing 0.01% (w/v) Rhodamine B solution, 1% (w/v) olive oil emulsion, and 15 g/L agar, adjusted to pH 7. Wells with a diameter of 6 mm cm were created in the solidified agar, and 10 µL of crude lipase extract obtained from the four microalgal isolates was added to each well. After 24 hours of incubation, the plates were exposed to UV light at 350 nm. The appearance of orange fluorescence surrounding the wells served as evidence of lipase activity (Figure 2).

Bromothymol blue assay

The bromothymol blue assay was employed to evaluate lipase activity by detecting the release of fatty acids during lipid hydrolysis, following the method of Kumari et al. (2023). The assay plates were prepared using a medium containing 0.01% (w/v) bromothymol blue, 1% (w/v) olive oil, and 15 g/L agar, with the pH adjusted to 7. Wells of 6mm diameter were created in the solidified agar, and 10 µL of crude lipase extract obtained from the four microalgal isolates was added to each well. After incubation, yellow halos around the wells indicated lipase activity, attributed to the release of fatty acids that reduced the pH of the medium (Figure 3).

Hydrolysis around the well indicates the presence of lipase enzyme in the crude enzyme. The zones diameters were measured in mm and results are recorded. The enzymatic index (EI) was calculated by using below mentioned formula.

$$\text{Enzymatic index (EI)} = \frac{\text{Hydrolysis zone diameter}}{\text{well size}} \quad (1)$$

Molecular characterization

The taxonomic classification of the isolates was verified using 16S rRNA gene sequencing, as described by Burja et al. (2001). A 700–1000 bp region of the 16S rRNA gene from the chloroplast genome was sequenced and analyzed to confirm the identity of the microalgal strains examined in this study. The primers employed were 5'-GTGTGTGGTGATGTGTGTGGT-3' and 5'-GGTCCGTGTTGATCCG-3'.

Quantitative lipase assay

Using a spectrophotometric test with p-nitrophenyl phosphate (p-NPP) as the substrate, lipase activity was measured using a p-nitrophenol reference curve. 0.1 mL of culture supernatant, 0.8 mL of 0.1 M sodium phosphate buffer (pH 7), and 0.1 mL of 0.01 M p-NPA dissolved in isopropyl alcohol made up the reaction mixture. After 30 minutes of maintaining the mixture at 30 ± 1°C, 0.25 mL of 0.1 M

sodium carbonate was added to stop the reaction. Identification of the p-nitrophenol release was done by measuring the absorbance of the reaction mixture at 410 nm. The quantity of enzyme needed to release one micromole of p-nitrophenol per minute under the specified test conditions was known as one unit of lipase activity (U) (Barik et al., 2022).

$$\text{Lipase activity (U/mL)} = \frac{(\text{p-NP formed in } \mu g/mL) * 1000}{(\text{Volume of enzyme (mL)} * \text{Incubation time in min})} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

Optimization conditions for lipase production

Effect of light intensity

The microalgae were cultivated at 25°C with different light intensities (20,40,60,80,100 and 120 µmol photons m^{-2} s^{-1}). The medium was adjusted to a pH of 7.0, and the cultures were kept under continuous illumination.

Effect of the growth period

The effect of the growth period (4, 7, 10, and 12 days) was assessed under optimal light intensity of 100µmol photons m^{-2} s^{-1}.The Temperature, pH, and photoperiod were maintained at 25°C, pH 7.0, andcontinuous illumination, respectively.

Effect of photoperiod

The light periods that were investigated under ideal growth duration and light intensity were 24L, 12L:12D, 16L:8D, 8L:16D, and 24D. The Temperature and pH were maintained at 25°C and pH 7.0respectively, with optimal light intensity of 100µmol photons m^{-2} s^{-1}.

Effect of different types of oil

Optimized light intensity, growth period, and photoperiod were used to study the effects of 2% olive oil, sesame oil, maize oil, sunflower oil, and coconut oil. Temperature was maintained at 25°C, and pH at 7.0, with continuous illumination of optimal light intensity of 100µmol photons m^{-2} s^{-1}.

Effect of olive oil concentration

The concentration of olive oil (0.5%, 1%, 1.5%, 2%, and 2.5% v/v) was tested for lipase production to short down the best substrate level. The experiments were done under optimized conditions, maintaining a temperature of 25°C, pH at 7.0, and continuous illumination.

Effect of pH

The effects of several pH values (6, 7, 8, 9, and 10) on lipase production were evaluated. The pH was measured using a HANNA HI98107 Portable pH Meter. Temperature was maintained at 25°C, and pH at 7.0, with continuous illumination of optimal light intensity of 100µmol photons m^{-2} s^{-1}.

Effect of temperature

The effects of temperature on lipase synthesis were evaluated with temperature variations of 15, 20, 25, 30, 35, 40, and 45°C. The pH at 7.0, with continuous illumination at 100 µmol photons m^{-2} s^{-1}.

Effect of agitation

The varying levels of speed of agitation (125, 150, 175, and 200 rpm) were evaluated under optimized conditions of continuous illumination for their effect on lipase production.

Effect of salinity

The effects of variable concentration of NaCl (0.5M,1M,1.5M,2M,2.5M) on lipase production were assessed under optimized growth conditions in continuous illumination.

Effect of nitrogen source

To determine which of the following nitrogen sources may provide the maximum enzymatic activity, their effects on lipase synthesis were examined: NH_4NO_3, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, NH_4Cl, and KNO_3.

Effect of $(NH_4)_2SO_4$ concentration

The impact of varying concentrations of $(NH_4)_2SO_4$ (0.5,1,1.5,2,2.5 1 % (w/v)) on lipase production was assessed under optimized conditions to identify the optimal concentration for maximizing enzymatic activity.

Effect of phosphorus source

Different phosphorus sources (K_2HPO_4, KH_2PO_4 and Na_2HPO_4) were evaluated under optimized conditions for their influence on lipase production, in order to decide which is the most effective source for the maximum enzymatic activity.

Effect of K_2HPO_4 concentration

Different concentrations of K_2HPO_4 (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 g/L) were assessed for their effect on lipase production under optimized conditions, to determine the optimum concentration for maximizing enzyme activity.

Statistical analysis

Three separate experiment's mean ± standard deviation is used to describe the results. The significance level for statistical comparisons was set at $P \leq 0.05$, and a one-way ANOVA approach was used. The Origin 2024b trial version was used for data processing and graphing. Using the fitted curve a linear regression model was built to precisely identify unknown concentrations.

$$y = \beta 0 + \beta 1 x + \epsilon \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

Where y is the dependent variable, x is the independent variable, β_0 is the intercept, and β_1 is the slope are unknown constants.

III. Results and Discussion

Isolation and morphological identification

Four axenic algal cultures were obtained and morphologically identified as *Neochlorisvigensis*, *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Asterarcysquadricellulare*.

TABLE 1
Morphological characteristics of selected algal species, including cell shape, size, and distinct features

S. No	Algal Species	Cell Shape & Size	Distinct Features
1	*Chlorella sorokiniana*	Small, spherical, 2–10 µm	Smooth, rigid cell walls
2	*Neochlorisvigensis*	Spherical or oval, 4–10 µm	Visible pyrenoids
3	*Chlorella vulgaris*	Spherical, 2–10 µm	Smooth, rigid cell walls
4	*Asterarcysquadricellulare*	Non-motile, coenobial arrangement	Three-dimensional structure, enclosed within a spherical mucilage

Qualitative analysis and enzymatic index

Four microalgal species, *Chlorella sorokiniana*, *Neochlorisvigensis*, *Chlorella vulgaris*, and *Asterarcysquadricellulare*, were evaluated for enzymatic activity using the tributyrin agar assay, rhodamine B assay, and bromothymol blue assay. The hydrolysis zones were measured, and the enzymatic index was calculated for all the tested species. Among them,*Chlorella sorokiniana* exhibited the most pronounced results, highlighting its enzymatic potential. Based on EI values, *Chlorella sorokiniana* was chosen for molecular characterization to ensure accurate identification of the selected microalgal strains (Table 2) (Figure 4).

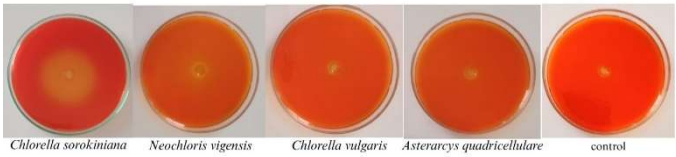

Figure 1: Tributyrin agar plate assay

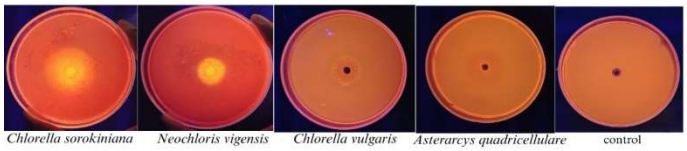

Figure 2: Rhodamine b plate assay

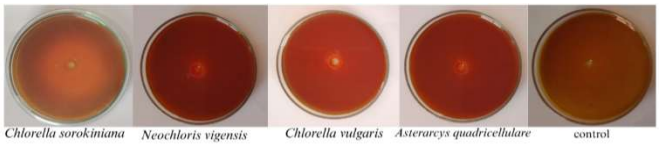

Figure 3: Bromothymol blue assay

TABLE 2
Enzymatic activity was evaluated based on the enzymatic index (EI), where EI ≥ 2.0 indicates good enzyme production. Lipase activity of microalgae strains was assessed using Tributyrin, Rhodamine B, and Bromothymol Blue assays. Data are presented as mean ± standard deviation (n = 3)

Microalgae Strains	Zone of clearance/ Enzyme Index	Tributyrin Assay	Rhodamine B Assay	Bromothymol Blue Assay
Chlorella sorokiniana	Mm	33±0.11	30±0.12	35±0.1
	EI	5.5±0.20	5±0.10	5.83±0.20
Neochlorisvigensis	Mm	11±0.03	21±0.02	11±0.02
	EI	1.83±0.03	3.5±0.02	1.83±0.02
Chlorella vulgaris	Mm	8±0.05	10±0.05	10±0.03
	EI	1.3±0.05	1.6±0.05	1.6±0.03
Asterarcysquadricellulare	Mm	8±0.01	9±0.10	9±0.02
	EI	1.3±0.01	1.5±0.02	1.5±0.02

Molecular characterization

Chlorella sorokiniana LGS2 was identified based on 16S rRNA chloroplast sequencing analysis. The sequence was analyzed using BLAST software for homologous comparison and subsequently deposited in GenBank under the accession number PQ583068.(Figure_4).

Optimization for lipase production

The optimization of factors influencing lipase production in *Chlorella sorokiniana* LGS2 was conducted using a one-factor-at-a-time approach while maintaining other parameters at their optimal levels. The unknown lipase enzyme units in *Chlorella sorokiniana* LGS2 during each optimization step were determined using a standard linear regression equation.

Optimization conditions for lipase production

Lipase production from *Chlorella sorokiniana* LGS2 was optimized using various parameters, and the results were tabulated (Table 3). The presented data highlights the significant potential of the novel microalgae *Chlorella sorokiniana* LGS2 as a robust producer of lipase, consistently

demonstrating superior activity compared to several other microbial sources under various tested conditions.

The data robustly positions the novel microalgae *Chlorella sorokiniana* LGS2 as a superior lipase producer compared to several other microbial sources across various conditions. Notably, LGS2 exhibited significantly higher lipase activity than bacteria like *Salinivibrio* sp. SA-2 and *Bacillus sonorensis* 4R, demonstrating its inherent capacity for efficient enzyme production even under saline stress and with optimized nitrogen supplementation.

Comparisons with other microorganisms, including *Exiguobacterium* sp. BBXS-7, *Aspergillus flavus*, and *Penicillium* spp., further harden LGS2's advantageous position as a potentially more efficient biocatalyst for industrial applications. The study also highlighted the crucial impact of culture conditions. Light intensity optimization, aligning with findings in other microalgae, revealed the importance of controlled light regimes to avoid photoinhibition while leveraging the benefits of continuous illumination for sustained energy supply and enhanced lipase production, contrasting with observations in *Nannochloropsisoceanica*. The significantly lower activity in *N. oceanica* and *Botryococcussudeticus* further emphasizes LGS2's potential.

Nutrient optimization, particularly of nitrogen and phosphorus, underscored the necessity of maintaining a balanced nutrient supply for maximal lipase yields in LGS2. While waste cooking oil showed promising lipase induction in other organisms, LGS2's response under the same phosphate conditions suggests specific substrate preferences warranting further investigation. The observed reduction in enzyme activity with prolonged growth and high agitation rates emphasizes the importance of optimizing harvest time and bioreactor conditions to maximize lipase recovery and prevent cell damage.

IV. Conclusion

Chlorella sorokiniana LGS2 presents compelling evidence as a promising lipase source due to its consistently superior activity and the successful optimization of key culture parameters. Future research should focus on elucidating the underlying molecular mechanisms, optimizing growth on cost-effective substrates like waste cooking oil, and exploring its potential for industrial scale-up in diverse fields, particularly within the Coimbatore region. The findings not only enhance our understanding of the enzymatic capabilities of microalgae but also open new avenues for future research aimed at harnessing these enzymes for industrial processes, such as biodiesel production and bioremediation. This groundbreaking work lays the foundation for sustainable practices in biotechnology, emphasizing the importance of innovative approaches to enzyme production.

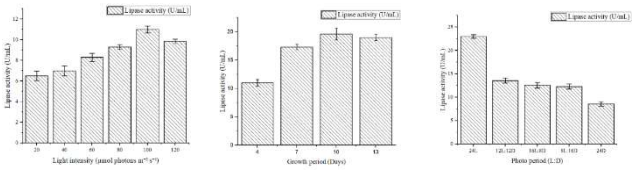

Figure 5: shows the experimental setup, capturing the conditions of light intensity, photoperiod and growth period in this study.

TABLE 3

Optimization of *Chlorella sorokiniana* LGS2 for lipase activity (U/mL). Values are expressed as the mean ± standard deviation from replicate experiments (n = 3).

Optimizing parameters	Lipase activity (U/ml)
Light intensity	11 ± 0.33 U/mL
Growth period	19.6 ± 0.21 U/mL
Photo period	23 ± 0.4 U/mL
Different types of oil	25 ± 0.61 U/mL
Olive oil concentration	31.6 ± 0.43 U/mL
pH	37 ± 1.42 U/mL
Temperature	41.3 ± 0.66 U/mL
Salinity	43.3 ± 0.64 U/mL
Agitation	48.3 ± 0.49 U/mL
Nitrogen source	52 ± 2.77 U/mL
Effect of $(NH_4)_2SO_4$ concentration	54 ± 1.31 U/mL
Effect of phosphorus source	57.6 ± 0.78 U/mL
Effect of KH_2PO_4 concentration	58.6 ± 0.80 U/mL

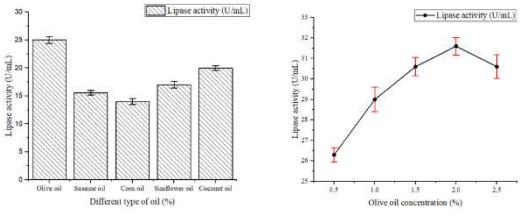

Figure 6: shows the experimental setup, capturing the c123onditions of type of oil and olive oil concentration (%).

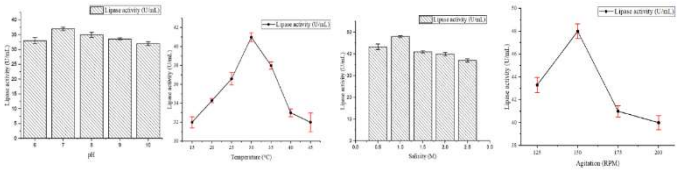

Figure 7: shows the experimental setup, capturing the conditions of pH, Temperature(°C), salinity (M), agitation (RPM) used in this study.

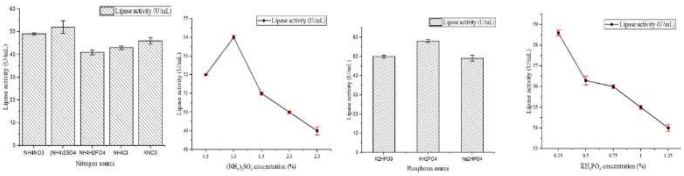

Figure 7: shows the experimental setup, capturing the conditions of Nitrogen source, $(NH_4)_2SO_4$ concentration, phosphorus source, KH_2PO_4 concentrationused in this study.

Acknowledgment

We extend our sincere gratitude to management of our Institution for providing the essential facilities and resources required to conduct this research.

V. References

Helal, S. E., Abdelhady, H. M., Abou-Taleb, K. A., Hassan, M. G., & Amer, M. M. (2017). Evaluation of factors affecting the fungal lipase production using one factor at a time approach and response surface methodology. *Egyptian Journal of Microbiology*, *52*(1), 1-16. https://doi.org/10.21608/ejm.2017.602.1012

Patil, K. J., Chopda, M. Z., & Mahajan, R. T. (2011). Lipase biodiversity. *Indian Journal of Science and Technology, 4*(8), 971–982. https://doi.org/10.17485/ijst/2011/v4i8.30

Yong, S. K., Lim, B. H., Saleh, S., & Tey, L. H. (2016). Optimisation, purification and characterisation of extracellular lipase from *Botryococcussudeticus* (UTEX 2629). *Journal of Molecular Catalysis B: Enzymatic*, *126*, 99-105. http://dx.doi.org/10.1016/j.molcatb.2016.02.004

Demir, B. S., &Tükel, S. S. (2010). Purification and characterization of lipase from *Spirulina platensis*. *Journal of Molecular Catalysis B: Enzymatic*, *64*(3-4), 123-128. https://doi.org/10.1016/j.molcatb.2009.09.011

Ben Hlima, H., Dammak, M., Karray, A., Drira, M., Michaud, P., Fendri, I., &Abdelkafi, S. (2021). Molecular and structural characterizations of lipases from Chlorella by functional genomics. *Marine drugs*, *19*(2), 70.

Savvidou, M. G., Sotiroudis, T. G., &Kolisis, F. N. (2016). Cell surface and cellular debris-associated heat-stable lipolytic enzyme activities of the marine alga *Nannochloropsisoceanica*. *Biocatalysis and Biotransformation*, *34*(1), 24-32. https://doi.org/10.1080/10242422.2016.1212843

Backer, L. C., Carmichael, W., Kirkpatrick, B., Williams, C., Irvin, M., Zhou, Y., ... & Cheng, Y. S. (2008). Recreational exposure to low concentrations of microcystins during an algal bloom in a small lake. *Marine drugs*, *6*(2), 389-406. https://doi.org/10.3390/md6020389

Allen, M. M., & Stanier, R. Y. (1968). Selective isolation of blue-green algae from water and soil. *Microbiology*, *51*(2), 203-209.

Duong VT. 2016. Isolation and evaluation of microalgae strains from The Northern Territory and Queensland - Australia that have adapted to accumulate triacylglycerides and protein as storage [Doctoral disseertation, The University of Queensland]

Safdar, A., Ismail, F., & Imran, M. (2024). Biodegradation of synthetic plastics by the extracellular lipase of Aspergillus niger. *Environmental Advances*, *17*, 100563. https://doi.org/10.1016/j.envadv.2024.100563

Stemler, C. D., Lorenz, K. K., & Scherf, K. A. (2024). Rhodamine B-based lipase activity assay with natural substrates—development and application. *International Journal of Food Science and Technology*, *59*(2), 1063-1070. https://doi.org/10.1111/ijfs.16875.

Kumari, A., Mihooliya, K. N., Sahoo, D. K., Bhattacharyya, M. S., Prasad, G. S., &Pinnaka, A. K. (2023). Description of lipase producing novel yeast species Debaryomycesapis fa, sp. nov. and a modified pH indicator dye-based method for the screening of lipase producing microorganisms. *Scientific Reports*, *13*(1), 11819. https://doi.org/10.1038/s41598-023-38241-3.

Burja, A. M., Tamagnini, P., Bustard, M. T., & Wright, P. C. (2001). Identification of the green alga, *Chlorella vulgaris* (SDC1) using cyanobacteria derived 16S rDNA primers: targeting the chloroplast. *FEMS microbiology letters*, *202*(2), 195-203. https://doi.org/10.1111/j.1574-6968.2001.tb10803.x

Barik, A., Sen, S. K., Rajhans, G., & Raut, S. (2022). Purification and optimization of extracellular lipase from a novel strain *Kocuria flava* Y4. *International Journal of Analytical Chemistry*, *2022*(1), 6403090. https://doi.org/10.1155/2022/6403090

Indrayani, I., Moheimani, N. R., &Borowitzka, M. A. (2019). Long-term reliable culture of a halophilic diatom, Amphora sp. MUR258, in outdoor raceway ponds. *Journal of Applied Phycology*, *31*, 2771-2778.https://doi.org/10.1007/s10811-019-01803-y

Ali, C. H., Zhang, J. J., Mbadinga, S. M., Liu, J. F., Yang, S. Z., Gu, J. D., & Mu, B. Z. (2015). Screening, isolation and optimization of an extracellular lipase producing Exiguobacterium sp. BBXS-7 segregated from waste

cooking oil contaminated sites. *Wulfenia J*, *2*, 185-201. https://www.researchgate.net/publication/277306558.

Colla, L. M., Ficanha, A. M., Rizzardi, J., Bertolin, T. E., Reinehr, C. O., & Costa, J. A. V. (2015). Production and characterization of lipases by two new isolates of Aspergillus through solid-state and submerged fermentation. *BioMed research international*, *2015*(1), 725959. https://doi.org/10.1155/2015/725959

Pandey, N., Dhakar, K., Jain, R., & Pandey, A. (2016). Temperature dependent lipase production from cold and pH tolerant species of Penicillium. *Mycosphere*, *7*(10), 1533-1545. https://doi.org/10.5943/mycosphere/si/3b/5

Amoozegar, M. A., Salehghamari, E., Khajeh, K., Kabiri, M., & Naddaf, S. (2008). Production of an extracellular thermohalophilic lipase from a moderately halophilic bacterium, Salinivibrio sp. strain SA-2. *Journal of basic microbiology*, *48*(3), 160-167. https://doi.org/10.1002/jobm.200700361

A'yuni, H., & Ilmi, M. (2021, May). Lipase production from Aspergillus aculeatus Ms. 11 in broth medium with variation of agitation speed. In AIP Conference Proceedings (Vol. 2353, No. 1). AIP Publishing. https://doi.org/10.1063/5.0052540.

Bhosale, H., Uzma, S., & Kadam, T. (2019). Optimization of Process Parameters for Improved Lipase Production by Hyperthermophilic Bacillus sonorensis 4R. *J. Adv. Biol. Biotechnol*, *11*, 1-10. https://doi.org/10.9734/JABB/2017/29166.

Amenaghawon, A. N., Odika, P., &Aiwekhoe, S. E. (2022). Optimization of nutrient medium composition for the production of lipase from waste cooking oil using response surface methodology and artificial neural networks. *Chemical Engineering Communications*, *209*(11), 1531-1541. https://doi.org/10.1080/00986445.2021.1980395.