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AN *IN VITRO* STUDY ON ENZYMATIC TRANSESTERIFICATION OF MICROALGAL OIL FROM *CHLORELLA VULGARIS* FOR BIODIESEL SYNTHESIS USING IMMOBILIZED *STEPTOMYCES* Sp. LIPASE

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Abstract:

Biodiesel is advised for use as an alternative fuel for conventional petroleum-based diesel because it is a renewable, with an environmentally friendly emission profile and is readily biodegradable. The effect of various growing conditions on biodiesel properties produced from Chlorella vulgaris has been investigated. The Chlorella vulgaris grown at different initial pH (4.0, 5.0, 6.0, 7.0 and 8.0) and Ammonia sources namely ammonium nitrate, ammonium sulphate, ammonium chlorite, ammonium tartrate and urea in laboratory condition. Chlorella vulgaris showed a gradual increase in cell number from pH 5.0 to 7.0. Maximum growth biomass of 6.6 mg/mL and Protein content 68.51 mg/mL was recorded at pH 7.0 on 7th day. Among the ammonia sources the maximum growth biomass of 7.8 mg/mL and maximum total protein content of 70 mg/mL was recorded in the presence of urea on 7th day. For lipase production, Streptomyces was inoculated in to the modified large production medium at different pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0), carbon source (glucose, sucrose, lactose and maltose) and nitrogen sources (ammonium sulphate, ammonium nitrate yeast extract and peptone). The Streptomyces Sp. was able to release a maximum protein content of 67mg/ml at pH 8.0 after 66 h and lipase of 77 U/ml at pH 8.0 after 36 h. The maximum extra cellular protein content of 71 mg/ml in the existence of Sucrose at 36 h and maximum enzyme activity of 91 U/ml at 96 h compared to the rest of sugars. Whereas, the occurrence of nitrogen sources the yeast extract supported a maximum extracellular protein content of 74 mg/ml at 36 h and lipase production of 94U/ml at 36 h.

Key words: *Chlorella vulgaris, Biodiesel, Streptomyces, lipase, protein, biomass*

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INTRODUCTION:

Algae are organisms that raise in aquatic environments and use light and carbon dioxide (CO_2) to create biomass. There are two classifications of algae: macroalgae and microalgae. Macroalgae are the large (measured in inches), multi-cellular algae. On the other hand, are tiny (measured in micrometers), unicellular algae that normally grow in suspension within a body of water is called microalgae. In addition to producing biofuel, algae can also be explored for a variety of other uses, such as fertilizer, pollution control, and human nutrition. Certain species of algae can be land-applied for use as an organic fertilizer, either in its raw or semi-decomposed form [1].

Algae can also be used to generate energy in several ways. One of the most efficient ways is through utilization of the algal oils to produce biodiesel. Some algae can even produce hydrogen gas under specialized growth conditions. The biomass from algae can also be burned, similar to wood, to generate heat and electricity. Algal biomass contains three main components: carbohydrates, proteins, and lipids/natural oils. Because the bulk of the natural oil made by microalgae is in the form of TAGs which is the right kind of oil for producing biodiesel-microalgae are the exclusive focus in the algae-to-biofuel arena. Microalgae grow very quickly compared to terrestrial crops. They commonly double in size every 24 hours. During the peak growth phase, some microalgae can double every 3.5 hours [2]. Oil content of microalgae is usually between 20 percent and 50 percent, while some strains can reach as high as 80 percent [3].

The process of isolation and selection of algae strains needs to consider the requirements of algal oil suitable for biodiesel production. Algal lipids occur in cells predominantly as either polar lipids (mostly in membranes) or lipid bodies, typically in the form of triacylglycerides. The latter are accumulated in large amounts during photosynthesis as a mechanism to endure adverse environmental conditions. Polar lipids usually contain polyunsaturated fatty acids which are long-chained, but have good fluidity properties. TAG in lipid storage bodies typically contain mostly saturated fatty acids which have a high energy contents, but, depending on the fatty acid profile of the algae strain, may lack fluidity under cold conditions. Provided the algal oil is low enough in moisture and free fatty acids, biodiesel is typically produced from TAG with methanol using base-catalyzed transesterification [4].

The interest in microbial lipase production has increased due to many useful features such as broad substrate specificity, the versatility of the molecular structure, and stability in organic solvents. Lipases secreted into the culture medium by many fungi and bacteria recently have attracted considerable attention owing to their biotechnological potential. Bacterial lipases were observed in the strains of *Serratia marescens, Pseudomonas aeruginosa* [5] and *Bacillus* species [6]. Other genera like *Acinetobacter, Staphylococcus, Streptococcus, Burkholderia, Achromobacter, Arthrobacter, Alcaligenes, Chromobacterium* and *Streptomyces* have been studied as lipase producer [7].

In this study has been designed to address the issues associated with the use of microalgae (using *C. vulgaris* as a model) for fuel production. The effect of various growing conditions on biodiesel properties produced from *C. vulgaris* has been investigated. Finally, efficient in using immobilized lipase from *Streptomyces* sp. for the inter esterification process, since the method was cost-effective and eco-friendly, no solvent was involved and the enzyme was encapsulated in a natural polymer.

MATERIALS AND METHODS:

Cultures

The *Chlorella vulgaris* strain used was provided by Gloris Biomed Research Centre, K.K. Nagar, Chennai-600 078, Tamil nadu, India. In the laboratory, it was cultured in half-strength Zarrouk's media [8].

The *Actinobacteria* culture *Streptomyces* was procured from CAS in Botany University of Madras, Chennai 600 025. The fungal culture was maintained in Potato Dextrose Agar Medium (PDA) and also these organisms were maintained at 4^{o} C in slants as a mother culture.

Optimization parameters and growth conditions of *Chlorella* sp.

The *Chlorella* sp. was grown at different initial pH: 4.0, 5.0, 6.0, 7.0 and 8.0; light intensities: Further, the alga, grown kept under different light periods: 12/12 Light/Dark, continuous light and dark. Further, the alga was also grown at different ammonia sources namely ammonium nitrate, ammonium sulphate, ammonium chlorite, ammonium tartrate and urea. This experiment was conducted for a period of 8 days. At every 24 hours interval the following parameters: i) the total biomass [9] and total protein [10] were recorded.

Biomass Assay

Algal growth was monitored using the optical density of the culture at 450 nm (OD450) according to Hsieh

and Wu and by determination of algal cellular dry weight (CDW, g l 1). The mean growth rate (R`) was calculated according to Robert.

Gas Chromatography–Mass Spectrometry (GC–MS) and Gas Chromatography Analysis
The GC–MS analysis was carried out in an Agilent 6890 N gas chromatograph equipped with an Agilent 5975B mass selective detector.

Lipase production Media
For lipase production, the composition of the basal medium with an initial pH value of 7.2 consisted of glucose 0.2% (w/v), peptone 0.5% (w/v), $MgSO_2$ 0.01% (w/v) and K_2HPO_2 0.1% (w/v), supplied with olive oil 2% (v/v).

Effect of pH and temperature on Lipase production
Streptomyces was inoculated in to the modified large production medium at different pH viz., 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. The cultures were incubated at 30 ± 2 º C temperatures at pH 7.5. The experiments were conducted for a period of 120 h. Every 24 h interval, the culture filtrates were collected after centrifugation and used for the estimation of Lipase activity and protein content.

Effect of Different Carbon and Nitrogen source on Lipase production
Different carbon source namely glucose, sucrose, lactose and maltose were amended separately at the concentration of 2% at pH 6.0 and nitrogen sources namely ammonium sulphate, ammonium nitrate yeast extract and peptone were amended at the concentration of 1% separately in the production medium. Then the experimental flasks were inoculated with *Streptomyces* and incubation at 30 ± 2 º C for 120 h. The culture filtrate was collected by centrifugation at every 24 h interval and the supernatant was used for the estimation Lipase activity and protein content.

Lipase activity
Lipase activity was also measured using various *p*-nitrophenyl esters as described by Kordel *et al*. (1991) with some modifications [11].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-Poly acrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of (Laemmli, 1970) [12].

Determination of molecular mass
The molecular mass of the purified laccase was determined on SDS-PAGE. Purified protein samples were run on SDS-PAGE with concurrent run of standard protein markers consist of Phosphorylase b (97.4), Bovine Serum Albumin (66.0), kDa purchased from SRL difco Chemical Ltd. After separation, the gels were stained with silver nitrate as described by Blum *et al*. (1987) [13].

Extraction and treatment of algal oil
The pulverized algae after dehumidification were subjected to ultrasonication at 24 kHz with constant temperature (50 °C ± 1) and for a time interval of 5 minutes. Ultrasonication enhanced the net amount of oil obtained. The algae after ultrasonication was directly loaded into the Soxhlet extractor and the solvent extraction was done for each batch using hexane for 3 hours. The amount of solvent was maintained to five times of the amount of the algal mass. The solvent mixture was removed from the sample extracts using the rotary vacuum evaporator and the excess solvents were reused for following batches. The algal oil was filtered using Whatman filter paper No.42 for removing crude impurities and the filtrate was stirred with 1% of 85 % phosphoric acid for 10 minutes using a magnetic stirrer at 80°C, followed by mixing with sterile distilled water for 30 minutes. The mixture was then allowed to settle in a separating funnel and the oil was separated from other activated constituents. The obtained oil was then treated with activated charcoal to remove coloured impurities.
The mass percentage of the total lipid extracted was calculated from the difference in the flask weight at the start and end of the extraction process. The quantity of lipid extracted from the sample was calculated using the formula,

Oil extraction Yield (%) =Weight of Oil extracted (g) / Weight of algae biomass (g) ×100

Transesterification
The oil extracted from FWM-CV was converted to biodiesel using transesterification. The transesterification was conducted by heating the lipid to 48 °C. At the same time 0.45 g of NaOH was added to 11 mL of methanol and shaken. Due to low lipid weight, the amount of this mixture was increased to be around 20 times greater than the lipid weight and then added to the lipid samples for 40 min. After 10 h, the oil phase was separated to another flask and centrifuged to remove the glycerin.

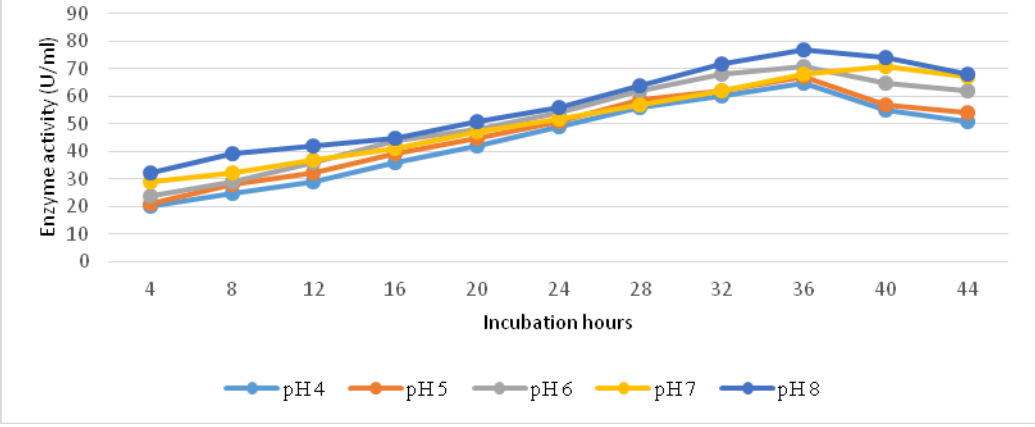
RESULTS AND DISCUSSION:
Biodiesel production from microalgae is an emerging technology considered as very promising source of

energy, mainly because of its higher oil productivity and reduced competition for land. Algae oil extraction is very similar to soybean oil extraction and then the oil has to be transesterfied with an alcohol to form biodiesel [14]. Biodiesel (monoalkyl esters of long chain fatty acids) is a potential renewable biofuel and it is biodegradable, nontoxic, no net carbon dioxide and free from sulfur [15]. Biodiesel which is biodegradable and nontoxic has low discharge profile and environmentally advantageous [16]. Transesterification reaction can be conducted by lipases as enzymatic biocatalyst.

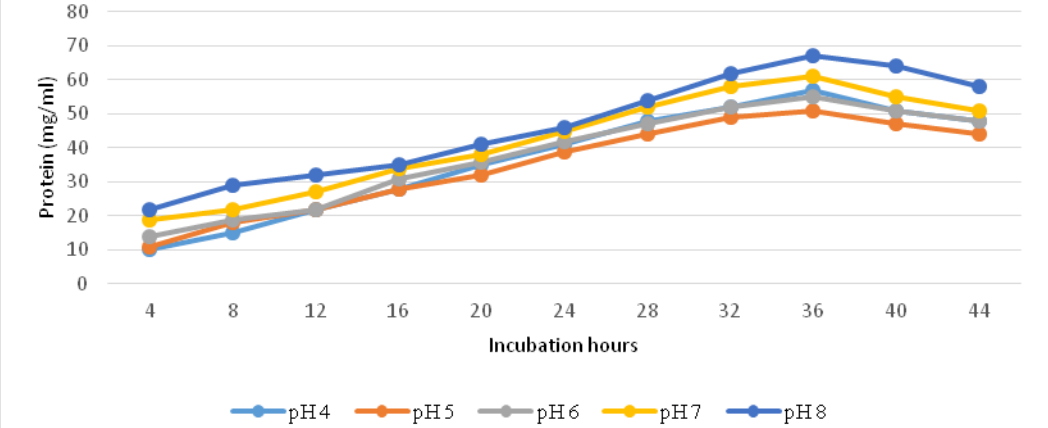
Optimization pH on lipase production by *Streptomyces* Sp. using submerged fermentation

It was evident that the pH significantly influenced the extracellular protein content and lipase activity in *Streptomyces* Sp. The *Streptomyces* Sp. was able to release a maximum protein content of 67mg/ml at pH 8.0 after 66 h and lipase of 77 U/ml at pH 8.0 after 36 h (Graph-1 and 2).

Graph-1. Optimization of different pH for production of lipase enzyme from *Streptomyces* Sp.

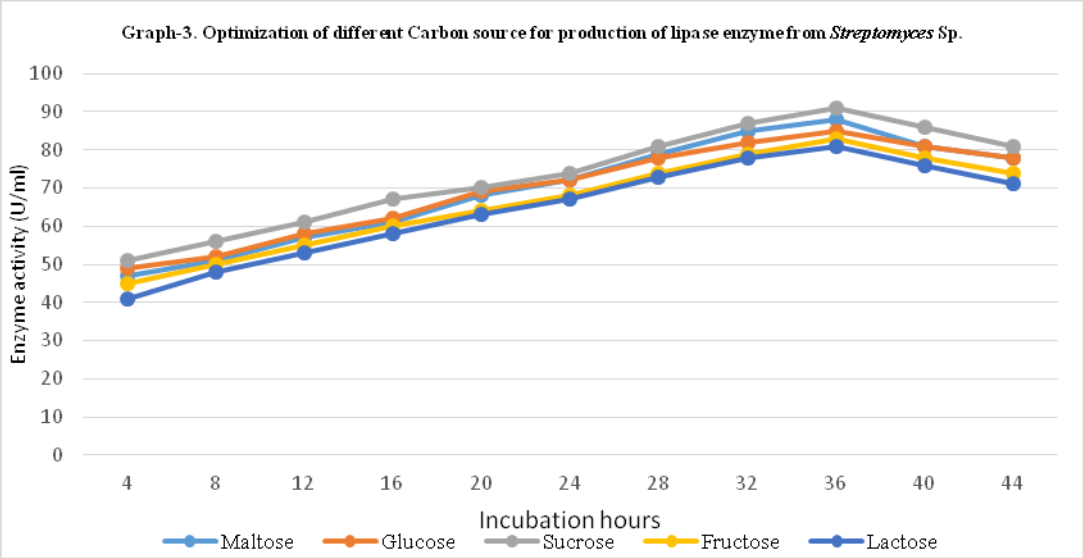


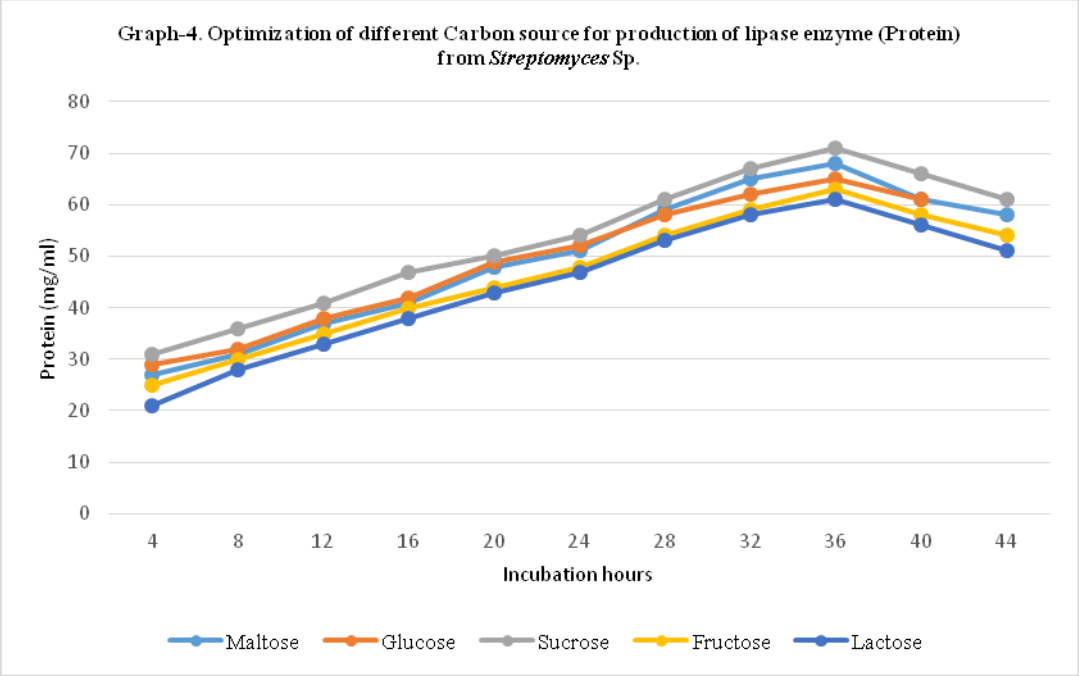
Graph-2. Optimization of different pH for production of lipase enzyme (Protein) from *Streptomyces* Sp.



Optimization carbon source on lipase production by *Streptomyces* Sp. using submerged fermentation

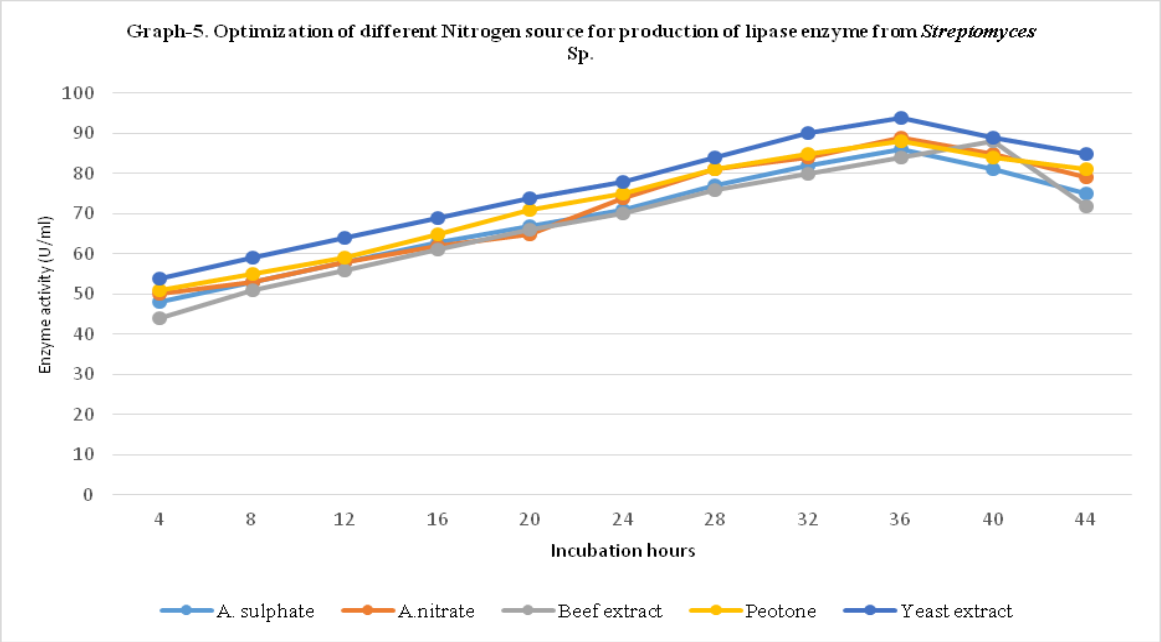
Among the different concentrations Maltose, Lactose, Glucose, Fructose and Sucrose tested, *Streptomyces* produced a maximum extra cellular protein content of 71 mg/ml in Sucrose at 36 h and maximum enzyme activity of 91 U/ml at 96 h compared to the rest of sugars (Graph-3 and 4). An excellent extracellular lipase of *Hendersonula toruloidea* was produced in shake-flask cultures contained 0.2%; olive oil, at an initial culture pH of 6·0 during the late logarithmic phase after 120 h [17]. The lipase from newly isolated *Streptomyces* sp. showed the highest activity pH 7.0. 77 U/ml. On the contrary, lipase production from the newly isolated strain of *Geotrichum* sp. was at maximum at pH 5.0 [18].

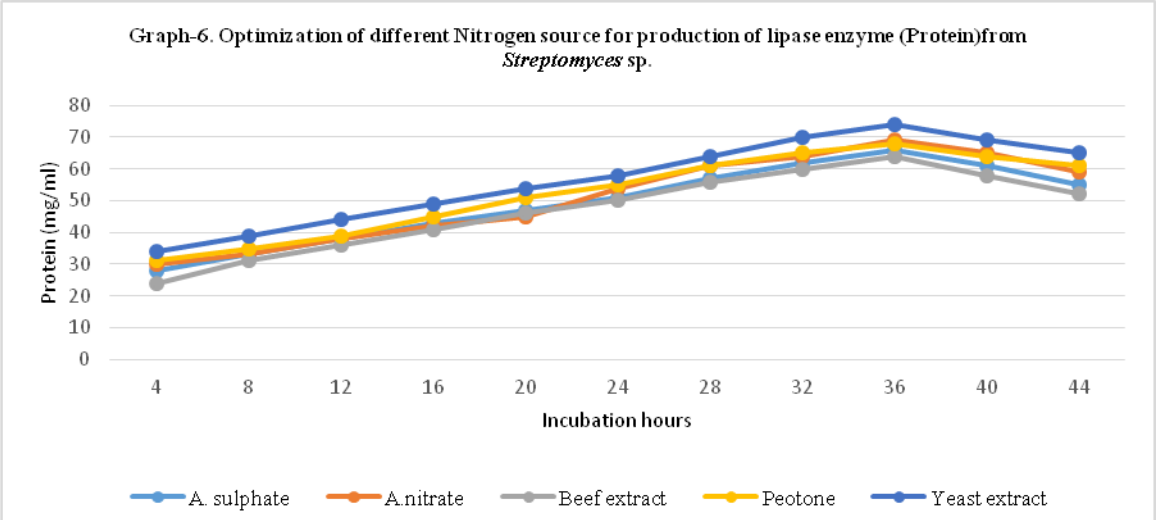




Optimization nitrogen source on lipase production by *Streptomyces* Sp. using submerged fermentation

Different nitrogen source peptone, ammonium nitrate, ammonium sulphste, beef extract and yeast extract was tested for extracellular protein and amylase production in *Streptomyces*. Among them, yeast extract supported a maximum extracellular protein content of 74 mg/ml at 36 h and lipase production of 94U/ml at 36 h (Graph-5 and 6).



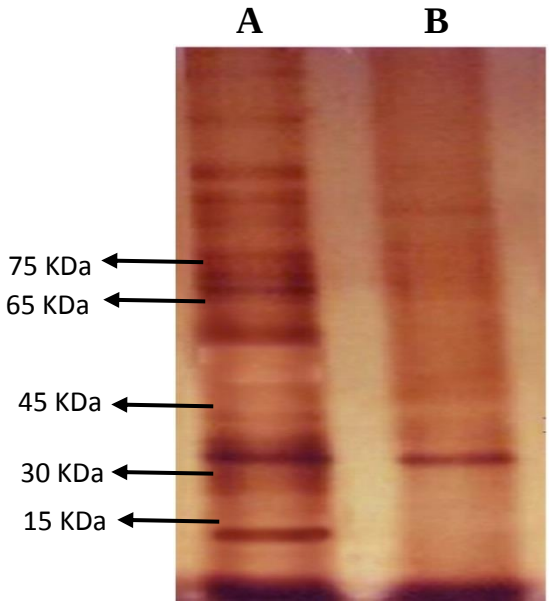


Molecular mass determination of partial purified lipase by SDS-PAGE

The partial purified lipase (3.0µg) was analyzed on SDS-PAGE (10%w/v) and stained with silver nitrate. On sodium dodecyl sulfate-poly acrylamide gel electrophoresis the purified Lipase showed a single band indicting that it was electrophoretic homogeneous. The molecular mass of the purified lipase was determined as 42 kDa by comparing with relative mobility of the molecular mass of protein ladder (Fig-1). Abdul Hamid *et al*. (2003) purified extracellular lipases with approximately 67 kDa using ultra filtration of cell-free culture supernatant of *Bacillus* sp. [19]. Lower molecular weight of 50 kDa lipase was purified from the culture filtrate of *Streptomyces cinnamomeus* [20].

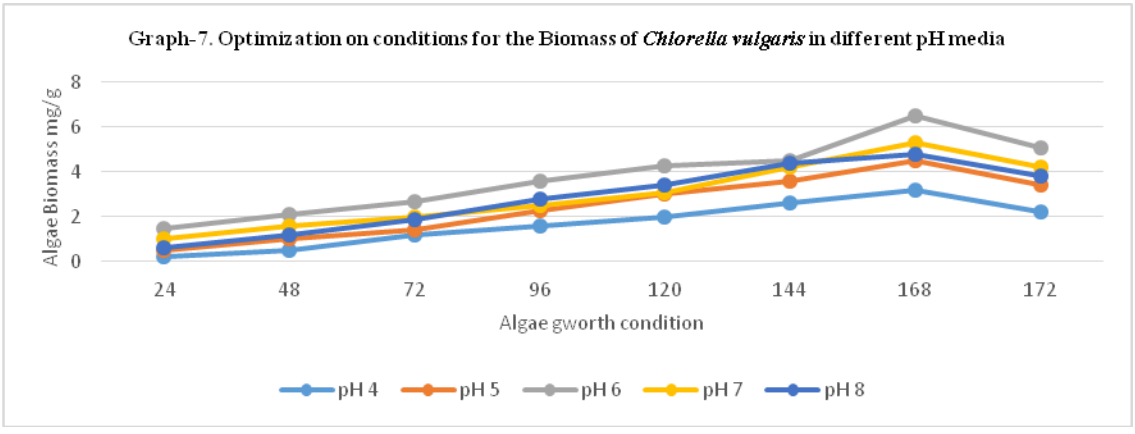
Optimization of conditions for the growth of *Chlorella vulgaris* in different pH media

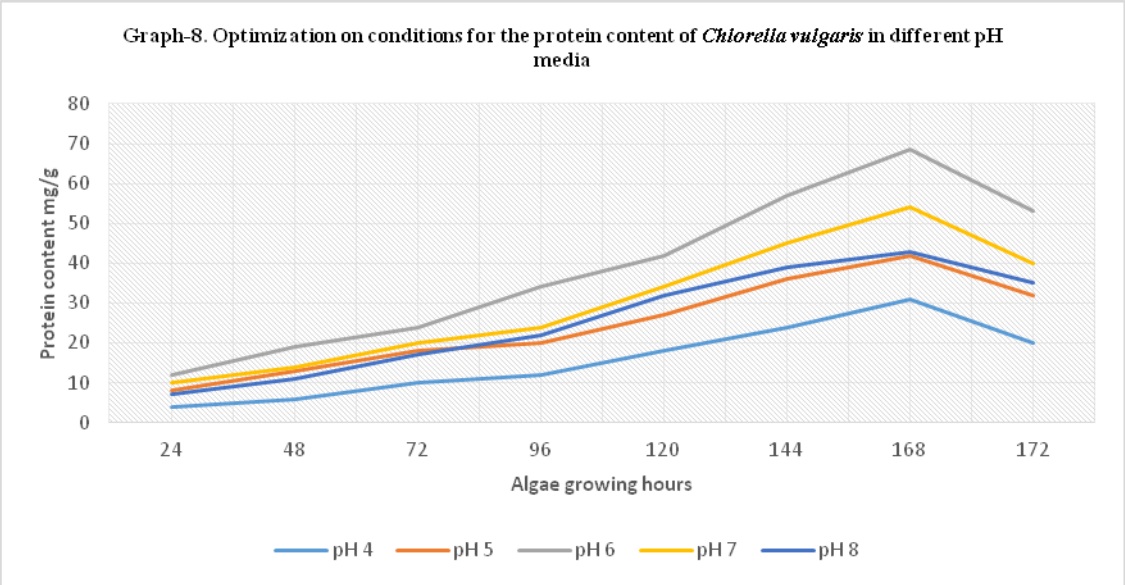
Chlorella vulgaris survived in all the different initial pH chosen in the basal medium. *Chlorella vulgaris* showed a gradual increase in cell number from pH 5.0 to 7.0. Maximum growth bio mass of 6.6 mg/mL was recorded at pH 7.0 on 7th day. The increment in cell number at this condition was more than 5.0% to that of control (pH 6.0) (Graph-7). In addition, the organism at pH 6.0 registered a maximum total protein content of 68.51 mg/mL on 7th day (Graph-8). Ginalska *et al*. (2007) found that maximum lipase production was at pH 6 by a newly isolated strain of *Geotrichum* like R59 [21]. However, Vishnupriya *et al*. (2010) reported that there are no significant changes obtained in the pH range [22].



A- Standard marker; B- Partial purified lipase

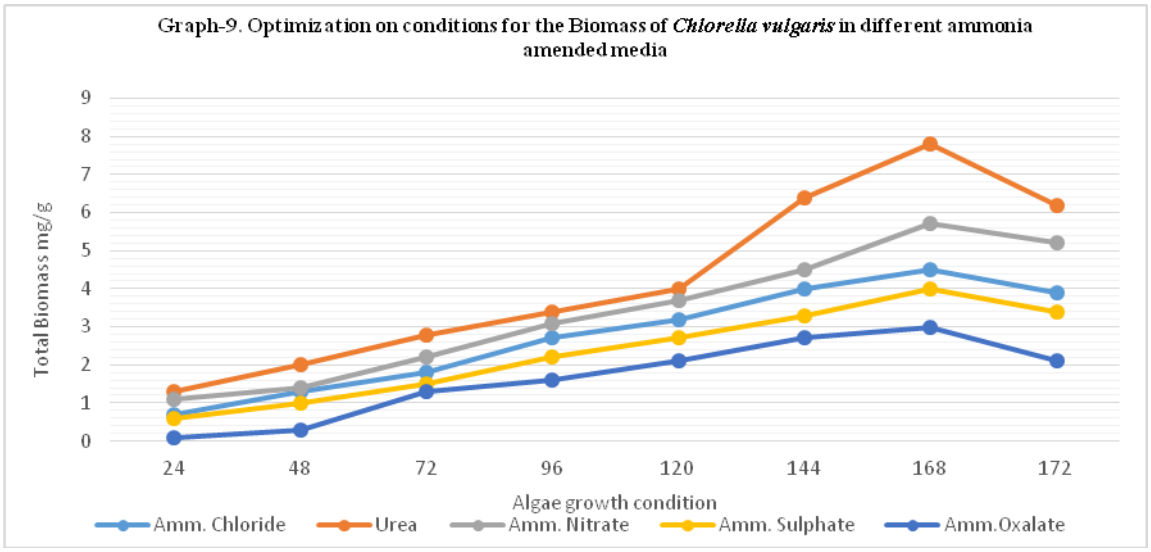
Fig-1: Molecular mass determination of partial purified Lipase by SDS-PAGE

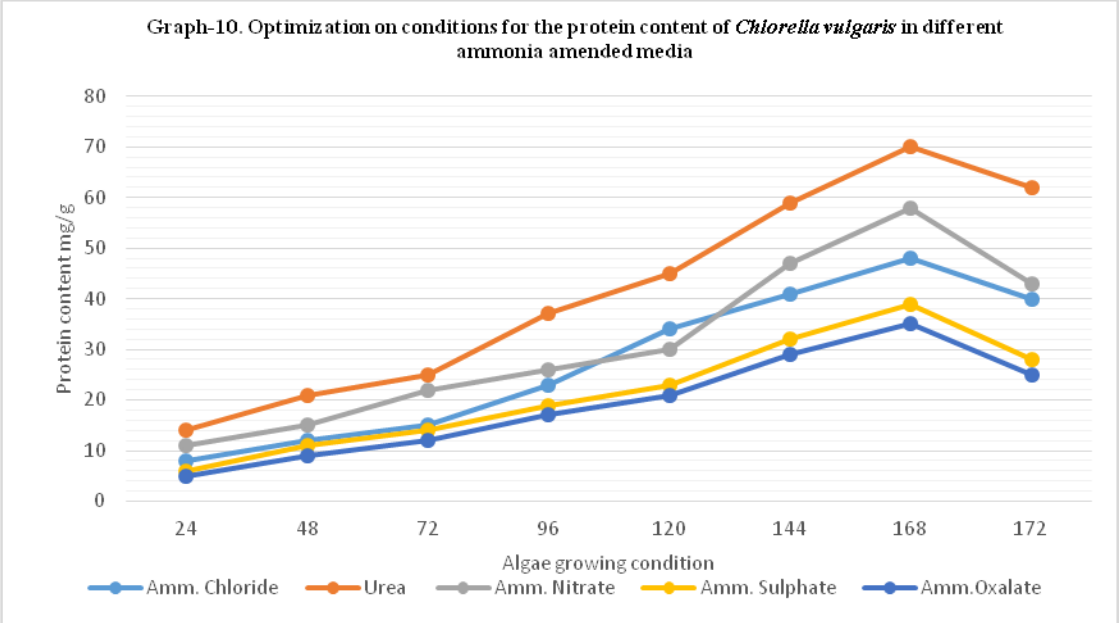




Optimization of conditions for the growth of *Chlorella vulgaris* in different ammonia amended media

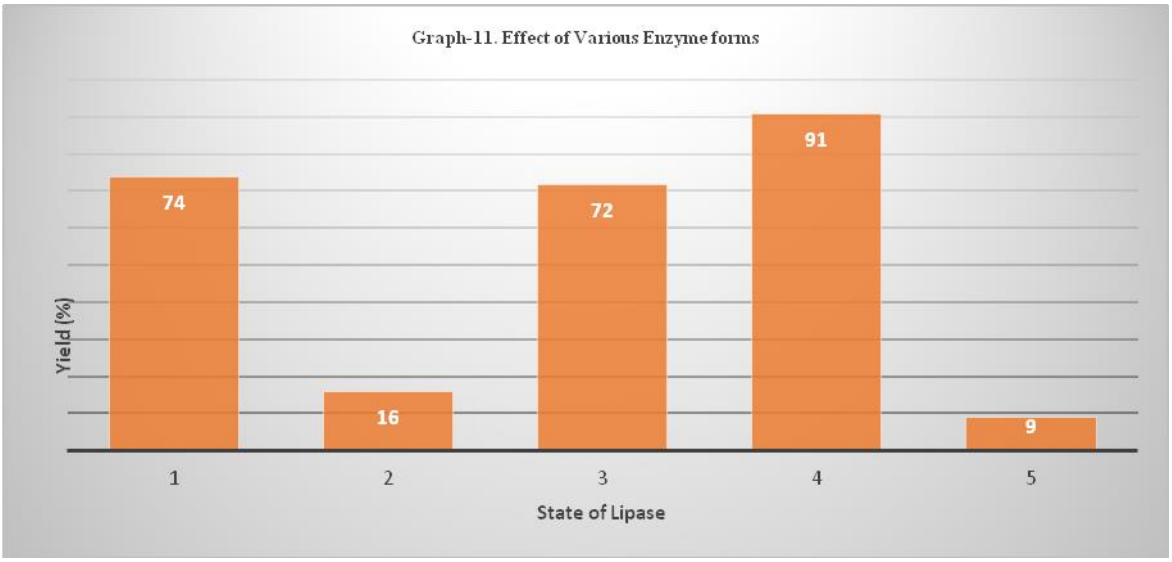
Chlorella vulgaris survived in all the different ammonia amended in the basal medium. Maximum growth bio mass of 7.8 mg/mL was recorded urea on 7th day. The increment in cell number at this condition was more than 6.0% to that of control (Graph-9). Further, the organism urea registered a maximum total protein content of 70 mg/mL on 7th day (Graph-10). The findings of our work are in agreement with those publicized by Zhu *et al.* (2002) whose results depicted that algae grew best in media adjusted with an initial pH of 7.10 and thus algae have shown fastest growth when the pH was near 8.0 and also concluded that in growth medium with a pH of 5.0 and 9.0 algae did not show apparent growth, thus the desired pH for growing algae is 7-8 [23]. Similar results were found in another study where microalgae was able to grow at pH 7.5 and above 8 the cells showed decline in growth rate [24]. Several inorganic forms of nitrogen, including nitrate, nitrite, ammonia ion, as well as urea, have been found to serve as N sources for algae growth [25].





Immobilized state of Lipase enzyme

Enzyme conformations in active state with the aid of hydrogen bonding, hydrophobic bond, van der Waals forces. The lyophilized alginate beads with immobilized enzyme had almost the same activity compared to the free enzyme. Generally, the use of microporous supports might improve the catalytic properties of immobilized lipase compared to those of free lipase because the substrate concentration of the liquid-solid interface is higher than that in solution. In addition, the immobilization could lead to partial coverage of the enzyme active sites, and lost the part of active center. Resulting in gel enzyme activity decreased to a certain degree. Alginate itself also had some catalytic activity to hydrolysis (Graph-11) (Fig-2).



1- Novozymes non immobilised (soluble) 2- sodium alginate gel; 3- calcium alginate gel enzyme, 4- freeze dried calcium alginate gel enzyme, 5- freeze dried calcium alginate gel.

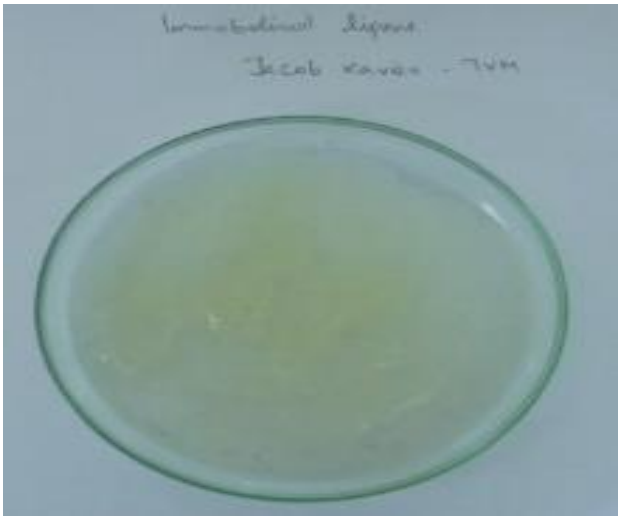
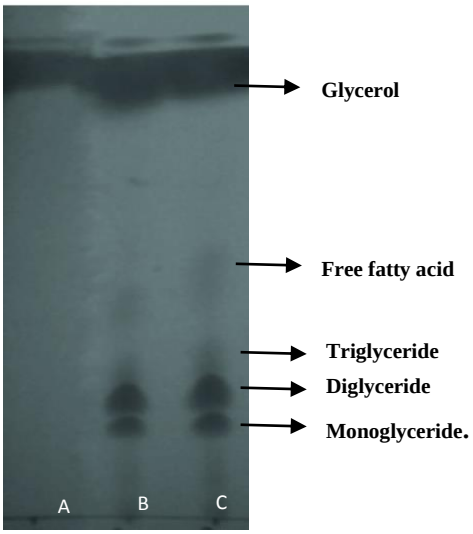


Fig-2. Immobilized Lipase

Effect of Lipase-Catalyzed Transesterification

To convert algal lipids into FAMEs, we first test the effect of acyl acceptors on esterification of fatty acids or on transesterification of TAG. Previous studies suggested methanol is an effective and commonly used acyl acceptor. The conversion efficiency using methanol (83%) suggesting methanol was more effective than these acyl acceptors in lipase based conversion of algal lipids. Methanol was thus used in the following experiments for enzyme conversion. The progress of the reaction was monitored by removing aliquots (20 μL) at various time intervals (12 and 24 hrs). The aliquots were appropriately diluted (with hexane) and analysis by thin layer chromatography (Fig-3).



A. standard (methyl oleate, Sigma Chemicals, 99% Pure),B and C: reaction samples.

Fig-3. Lipases catalyzed biodiesel production, TLC plate.

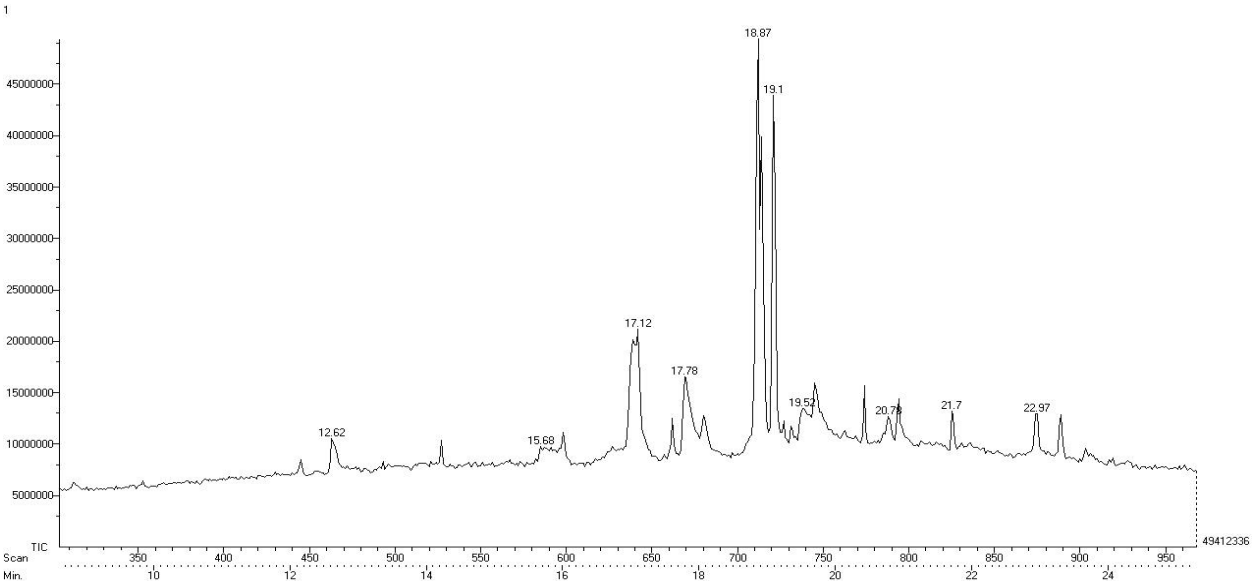


Fig-4. The analytical result of fatty acid methyl esters of Lipase-Catalyzed *Chlorella vulgaris* oil by Gas chromatography

S.No.	RT	Name of the compound	Molecular formula	MW
1	12.62	Palmitic acid anhydride	$C_{16}H_{32}O_2$	256.42
2	15.68	Oleic Acid	$C_{18}H_{34}O_2$	282.46
3	17.12	Pentadecanoic acid, 13-methyl: methyl ester	$C_{17}H_{34}O_2$	270.45
4	17.78	Methyl 10-Oxo hexadecanoate	$C_{17}H_{32}O_3$	284.43
5	18.9	10-Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	296.48
6	19.15	Heptadecanoic acid, 9-methyl, methyl ester	$C_{19}H_{38}O_2$	298.50
7	19.52	Octanal,[2,4-dinitrophenyl] hydrazone	$C_{14}H_{20}N_4O_4$	308.33
8	20.78	Dasycarpidan-1-methanol, acetate (ester)	$C_{20}H_{26}N_2O_2$	326.43

Contents of fatty acid methyl esters of Lipase-Catalyzed *Chlorella vulgaris* oil

The percentage of FAMEs has been found to increase due to the hydrolysis of triglycerides in the presence of moisture and oxidations. Degradation of the CJCO results higher concentration of FAMEs (Fig-4).

CONCLUSION:

As demonstrated at this point, microalgal biodiesel is technically possible. It is the only renewable biodiesel that can potentially completely displace liquid fuels derived from petroleum. Financial side of producing microalgal biodiesel need to improve substantially to make it competitive with diesel, but the level of improvement necessary appears to be within reach. Producing low-cost microalgal biodiesel requires primarily improvements to algal biology through genetic and metabolic engineering. Employ of the biorefinery concept and advances in photobioreactors engineering will further lower the

cost of making. In view of their much greater productivity than raceways and tubular photobioreactors are likely to be used in producing much of the microalgal biomass required for making biodiesel. Photobioreactors give a controlled environment that can be tailored to the specific demands of highly productive microalgae to attain a consistently good annual yield of oil content.

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