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**MICROALGAL BIOMASS PRODUCTION OF “SCENEDESMUS MIX”  
THROUGH HETEROTROPHIC CULTIVATION**

**Anne Caroline Defranceschi Oliveira**

annecaroline@yahoo.com.br

**Eduardo Sassi Caroci**

eduardosassi@gmail.com

**Flávio Júnior Santiago**

flaviojrsantiago@gmail.com

**Iago Gomes Costa**

iago\_gomes\_costa@gmail.com

**Murilo Gasparin Rampi**

murilorampi@gmail.com

**Diego de Lima Souza**

diegofine@gmail.com

**José Viriato Coelho Vargas**

vargasjvcv3@gmail.com

**Vanessa Kava**

vanessagenetica@gmail.com

**André Bellin Mariano**

andrebmariano@gmail.com

Federal University of Paraná (UFPR) – Curitiba – PR – Brazil  
Post-Graduate Program in Engineering and Materials Science (PIPE)  
Post-Graduate Program in Mechanical Engineering (PG-Mec)  
Self-Sustainable Energy Research and Development Center (NPDEAS)

**Abstract.** *Microalgae are microscopic photosynthetic organisms with generally simple nutritional needs. In recent times, the production of microalgae has gained a new focus, production aimed at energy development due to some characteristics present in its biomass. The microalgal biomass can contain from 1 to 70% of lipids, which aroused the interest of research focused on the production of biodiesel. Microalgae are micro-organisms capable of presenting three distinct metabolisms against the source of carbon available for growth: the autotrophic metabolism, where in the presence of light and certain mineral salts the microalgae is capable of fixing carbon dioxide; heterotrophic metabolism, where the microalgae need an organic carbon source present in the culture medium in the absence of light, and mixotrophic metabolism, where the two previous metabolic forms are developed together. This study aimed to optimize biomass production through heterotrophic cultivation of a mix of microalgae from the genus *Scenedesmus*, using an experimental design technique. Variables such as the composition of the culture medium (glucose, sodium nitrate, and sodium phosphate dibasic) were chosen and evaluated in three levels to observe their effect on biomass production. Results showed that glucose had the largest effect on biomass production, resulting in an increase of  $11.215 \text{ gL}^{-1}$  of biomass when the level proposed in the design was used. The variable sodium phosphate had an effect of  $1.215 \text{ gL}^{-1}$ , but it was not statistically significant. The variable sodium nitrate had a negative effect when set at the high level, resulting in a decrease of  $-1.765 \text{ gL}^{-1}$ . However, determining the optimal value of this variable could lead to better results. In conclusion, the use of heterotrophic cultivation to produce biomass from the *Scenedesmus* mix may be an appropriate practice for producing microalgal biomass, resulting in yields greater than those obtained through optimal cultivation of other parameters not determined in this experiment.*

**Keywords:** *Heterotrophic, Biomass, Microalgae.*

## 1. INTRODUCTION

Biofuel is one the sectors that applies biotechnology innovations the most. It has been gaining a greater space since it is based on renewable sources while other fuels such as petrol have a future perspective of shortage and higher impact on pollution.

Renewable energy is no longer an option, but a necessity and the development of new methods for biofuels production has become essential. Energy management strategies must be set to allow the introduction of biofuels without compromising other sectors of the market.

Biodiesel consists in alkyl esters of vegetable oils or animal fat esterified with short chain alcohols. The Brazilian transportation system is mostly road based and diesel is the main fuel that provides energy to the fleet. In Brazil, Biodiesel has been added to diesel in greater proportions, but to keep increasing this biofuel portion it is necessary new research to guarantee a satisfying quality and quantity to attend the country demand.

The main lipid source for biodiesel production in Brazil is soy, since it represents an enormous portion of Brazilian agroindustry. The second source is tallow that is considered a byproduct by the livestock industry and therefore is easily obtained.

The application of new raw materials may be a good solution, and one of the sources of oil that promises to be very promising is microalgae. Microalgae are microscopic photosynthetic beings with relatively simple nutritional needs and have an excellent potential for the generation of biofuels, since they have advantages such as compaction of the space necessary for their cultivation, besides being even possible to cultivate it employing agro-industrial byproducts as substrate. It has been an idea increasingly considered, as it has advantages such as: the reduction of the area needed to obtain biomass, and there is no dispute over the agricultural areas destined for food production.

Catalyzers are required for biodiesel production, and they can be acid or alkali chemical compounds, heterogeneous catalyzers or even enzymes.

Enzymatic catalyze is largely studied due to its advantages: selectivity, no secondary reaction, higher product purity and an easier separation of the catalyzer from the product and remaining glycerin.

Although all the advantages, the enzyme employment still have serious limitations. The main of them is the large time required by the reaction that, despite the lower temperature, increases the energy spent on it. However, the application of biocatalysts has been well studied due to the presentation of interesting characteristics in the obtained product. The high purity of the product obtained is the main advantage of this method, but the yields obtained by this type of catalysis still cannot overcome those obtained by chemical catalysis, which makes its study and optimization so relevant.

The high cost of the enzymes is another limiting factor, but some strategies can be adopted to lower the costs and reduce the steps of its obtainment. The traditional fermentations used to produce them employ expensive nutritional media, but with alternative methods it is possible to substitute these media by residues as substrate for solid-state fermentation (SSF). Beyond serving as substrate, the residue can act as an inert support for the enzyme, allowing it to be reused several times and thereby reducing the costs.

This work aim was to produce biomass from microalgae through heterotrophic culture to extract its oil in order to use it to produce biodiesel from enzymatic reaction.

## 2. MATERIAL AND METHODS

### 2.1 Heterotrophic cultivation of microalgae *Scenedesmus obliquus*

A 2 m<sup>3</sup> inoculum of *Scenedesmus obliquus* was produced in a rectangular tank (0.6 m high x 2.10 m long x 1.6 m wide) using the Chu culture medium (CHU, 1942), with PH 7.0 under constant aeration. This material was used to inoculate a compact 12 m<sup>3</sup> photobioreactor (10 m<sup>2</sup> area, 8 m high, 5 m long and 2 m wide), which has 3.5 km of transparent PVC tube in its structure. The photobioreactor is in the outer area of the laboratory and exposed to the external environment. The photobioreactor used only sunlight as a light source, the only source of carbon was the CO<sub>2</sub> contained in the compressed air injected into the system through a 10 m high and 1.1 m diameter gasser-degasser column. One week after inoculation, a volume of approximately 2 L was withdrawn and used as inoculum for the heterotrophic growth experiments.

### 2.2 Optimization of microalgae production by heterotrophic cultivation

To optimize the biomass production of *Scenedesmus obliquus*, the experimental planning technique was applied. Three variables concerning the composition of the culture medium were selected: glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>, carbon source), sodium nitrate (NaNO<sub>3</sub>, nitrogen source) and dibasic sodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>). The concentrations at which these compounds were varied can be seen in Table 1.

Table 1. Levels proposed for the experimental planning variables for biomass production of *Scenedesmus obliquus*.

| Variable         | -1   | 0    | +1   |
|------------------|------|------|------|
| Glucose          | 30   | 50   | 70   |
| Sodium nitrate   | 8    | 12   | 16   |
| Sodium phosphate | 0.05 | 0.10 | 0.15 |

\* All variables are given in g.L<sup>-1</sup>.

Cultures were carried out in 250 mL Erlenmeyer flasks containing 200 mL of Walne's medium (Andersen, 2005) with pH adjusted to 7.4 in addition to the compounds mentioned above. Each vial received as inoculum  $2 \times 10^8$  *Scenedesmus obliquus* cells obtained as described in the previous item, with the cultures incubated in Shaker at 30 °C and 180 rpm for 11 days. The final biomass content in each culture was observed by gravimetric method. The levels proposed for this factorial planning were based on previously performed.

For this experiment, the value of  $p < 0.05$  was assumed, which indicates the probability that the result obtained is generated by chance or by the variable, in this case, the confidence level of 95% is assumed as significant. The experimental design and analyzes of the effects generated by the variables as well as their  $p$  values were calculated using the software Statistica 7.0.

### 3. RESULTS AND DISCUSSION

#### 3.1 Heterotrophic cultivation of microalgae *Scenedesmus obliquus*

The experimental design was applied with the purpose of obtaining the best culture medium to produce microalgal biomass in a small number of experiments. The results obtained in each assay can be seen in Table 2.

Table 2. Matrix of the experimental planning and results obtained for the biomass production of *Scenedesmus obliquus*.

| Experiment | Glucose | Sodium nitrate (NaNO <sub>3</sub> ) | Sodium phosphate (Na <sub>2</sub> HPO <sub>4</sub> ) | Biomass (g.L <sup>-1</sup> ) |
|------------|---------|-------------------------------------|--|------------------------------|
| 1          | -1      | -1                                  | 0  | 9.43                         |
| 2          | 1       | -1                                  | 0  | 22.96                        |
| 3          | -1      | 1                                   | 0  | 8.63                         |
| 4          | 1       | 1                                   | 0  | 18.87                        |
| 5          | -1      | 0                                   | -1   | 10.87                        |
| 6          | 1       | 0                                   | -1   | 21.50                        |
| 7          | -1      | 0                                   | 1  | 8.87                         |
| 8          | 1       | 0                                   | 1  | 19.33                        |
| 9          | 0       | -1                                  | -1   | 15.07                        |
| 10         | 0       | 1                                   | -1   | 14.73                        |
| 11         | 0       | -1                                  | 1  | 20.33                        |
| 12         | 0       | 1                                   | 1  | 18.50                        |
| 13         | 0       | 0                                   | 0  | 18.93                        |
| 14         | 0       | 0                                   | 0  | 20.90                        |
| 15         | 0       | 0                                   | 0  | 19.53                        |

In all proposed treatments, the secretion of lipases can be observed. The estimation of the effects and values of  $p$  for each variable can be observed in Table 3.

Table 3. Estimation of the effect of the variables on the biomass production of *Scenedesmus obliquus*.

| Variable         | Effect <sup>a</sup> | $p$ Value <sup>b</sup> |
|------------------|---------------------|------------------------|
| Glucose          | 11.215              | 0.000104*              |
| Sodium nitrate   | -1.765              | 0.3736                 |
| Sodium phosphate | 1.15                | 0.5363                 |

<sup>a</sup> Effect caused by the independent variable in biomass production (response variable) when the concentration of the variable is raised from the minimum level (-1) to the maximum level (+1).

<sup>b</sup> 95% confidence level.

Of the three variables tested, only for the variable glucose was observed statistical significance at a confidence level of  $p < 0.05$ , showing an effect of 11.215, that is, when the concentration of this variable was raised from the minimum level to the maximum level there was a gain of 11.215 gL<sup>-1</sup> of biomass.

The other two variables presented lower significance, but the sodium nitrate variable presented a negative effect (-1.765 gL<sup>-1</sup>), which resulted in the determination of its ideal concentrations for the best biomass production, which can be observed in the surface graphs in Figure 1.

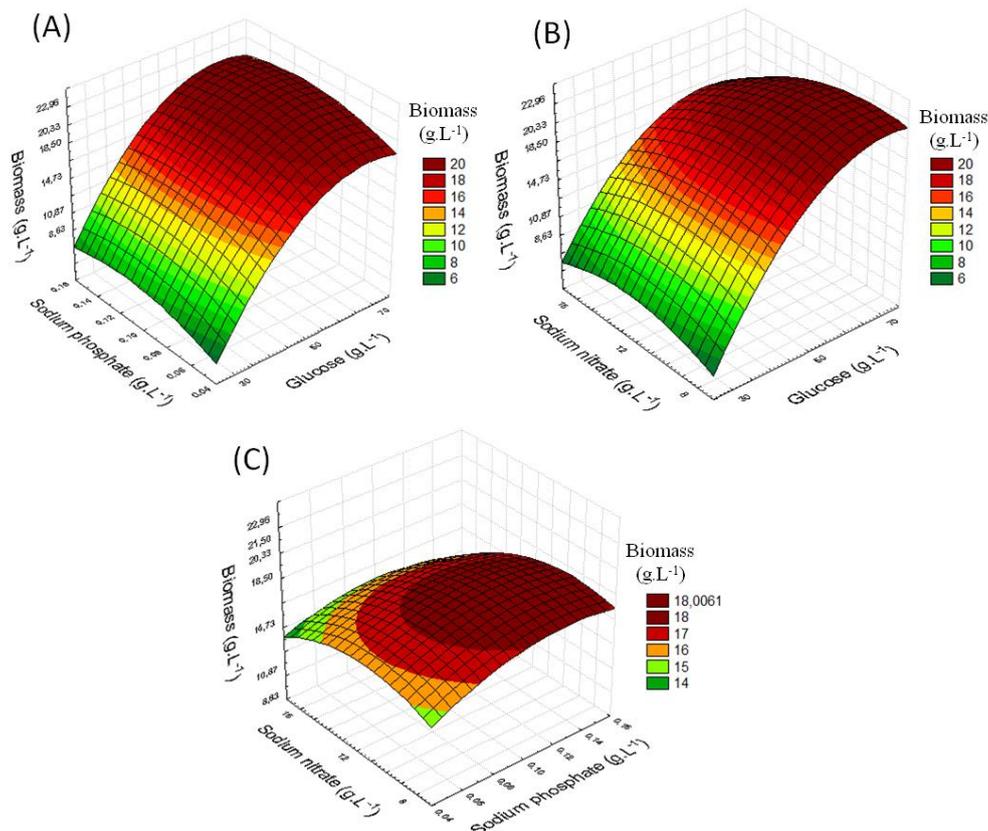


Figure 1. Response surface graphs showing the interaction between (A) glucose and sodium phosphate concentrations; (B) glucose and sodium nitrate and (C) sodium phosphate and sodium nitrate in the production of biomass.

The heterotrophic cultivation has presented expressive results when compared to the autotrophic culture in the production of microalgae biomass. Azma *et al.* (2011) used experimental planning to optimize the biomass production of the *Tetraselmis suecica* microalgae, obtaining about 28 g.L<sup>-1</sup>, a result like that obtained in this experiment. This result is quite promising when compared to the autotrophic culture, where the biomass concentration is around 1 g.L<sup>-1</sup>, a fact that justifies the use of an organic carbon source such as glucose.

#### 4. CONCLUSION

Microalgae oil-based biodiesel and enzymatic catalyze are techniques applicable in lab-scale only, it is necessary the development of larger scales.

The direct application of the fermented solid containing the enzymes in the esterification reaction represents a big advance because it reduces costs and time since it requires fewer steps in the enzyme obtainment.

More studies are necessary aiming the costs reduction and the exploitation of residues for enzymes and microalgae obtainment; this is a promising path for the sustainable development in biofuel production.

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#### 6. REFERENCES

- Aragão, V.C., Anschau, A., Porciuncula, B.D.A, Thiesen, C., Kalil, S.J., Burkert, C.A.V., Burkert, J.F.M., 2009. Síntese enzimática do butirato de isoamila empregando lipases microbianas comerciais. *Quim. Nova.* 32, 2268-2272.  
Bajaj, A., Lohan, P., Jha, P.N., Mehrotra, R., 2010. Biodiesel production through lipase catalyzed transesterification: Na overview. *J. Mol. Catal. B: Enzym.* 62, 9-14.

- Baron, A.M., Sarquis, M.I.M., Baigori, M., Mitchell, D.A., Krieger, N., 2005. A comparative study of the synthesis of *n*-butyl-oleate using a crude lipolytic extract of *Penicillium coryophilum* in water-restricted environments. *J. Mol. Catal. B: Enzym.* 34, 25-32.
- Barrios-González, J., 2012. Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. *Process Biochem.* 47, 175–185.
- Bussamara, R., Fuentesfria, A.M., Oliveira, E.S., Broetto, L., Simcikova, M., Valente, P., Schrank, A., Vainstein, M.H., 2010. Isolation of a lipase-secreting yeast for enzyme production in a pilot-plant scale batch fermentation. *Bioresour. Technol.* 101, 268–275.
- Contesini, F.J., Lopes, D.B., Macedo, G.A., Nascimento, M.G., Carvalho, P.O., 2010. *Aspergillus* sp. lipases: potencial biocatalyst for industrial use. *J. Mol. Catal. B: Enzym.* 67, 163-171.
- Dalla-Vecchia, R., Nascimento, M.G., Soldi, V., 2004. Aplicações sintéticas de lipases imobilizadas em polímeros. *Quim. Nova.* 27, 623-630.
- Damaso, M.C.T., Passionoto, M.A., Freitas, S.C., Freire, D.M.G., Lago, R.C.A., Couri, S., 2008. Utilization of agroindustrial residues for lipase production by solid-state fermentation. *Braz. J. Microbiol.* 39, 676–681.
- Fernandes, M.L.M. Produção de lipases por fermentação no estado sólido e sua utilização em biocatálise. Brazil: Federal University of Paraná; Ph.D. thesis; 2006. p.130.
- Fernandes, M.L.M., Saad, E.B., Meira, J.A., Ramos, L.P., Mitchell, D.A., Krieger N., 2007. Esterification and transesterification reactions catalysed by addition of fermented solids to organic reaction media. *J. Mol. Catal. B: Enzym.* 44, 8-13.
- Ghori, M.I., Iqbal, M.J., Hamed, A., 2011. Characterization of a novel lipase from *Bacillus* sp. isolated from tannery wastes. *Braz. J. Microbiol.* 42, 22-29.
- Gog, A., Roman, M., Tos, M., Paizs, C., Irimie, F.D., 2012. Biodiesel production using enzymatic transesterification e Current state and perspectives. *Renew. Energ.* 39, 10–16.
- Hernandez-Rodriguez, B., Córdova, J., Barzana, E., Favela-Torres, E., 2009. Effects of organic solvents on activity and stability of lipases produced by thermotolerant fungi in solid-state fermentation. *J. Mol. Catal. B: Enzym.* 61, 136-142.
- Hilal, N., Kochkoda, V., Nigmatullin, R., Goncharuk, V., Al-Khati, L., 2006. Lipase-immobilized biocatalytic membranes for enzymatic esterification: Comparison of various approaches to membrane preparation. *J. Membrane Sci.* 268, 198–207.
- Iftikhar, T., Niaz, M., Zia, M.A., Haq, I., 2010. Production of extracellular lipases by *Rhizopus oligosporus* in a stirred fermentor. *Braz. J. Microbiol.* 41, 1124–1132.
- Laane, C., Boeren, S., Vos, K., Veeger, C., 1987. Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* 30, 81-87.
- Li, J., Li, L., Tong, J., Wang, Y., Chen, S., 2012. Research development on lipase-catalyzed biodiesel. *Energ. Procedia.* 16, 1014– 1021.
- Li, N., Zong, M.H., 2010. Lipases from the genus *Penicillium*: production, purification, characterization, and applications. *J. Mol. Catal. B: Enzym.* 66, 43-54.
- Lowry, R.R., Tinsley, J.I., 1976. Rapid colorimetric determination of free fatty acids. *J. Am. Oil Chem. Soc.* 53, 470-472.
- Oliveira, A.C.D., Watanabe, F.M.F., Vargas, J.V.C., Rodrigues, M.L.F., Mariano, A.B., 2012. Production of methyl oleate with a lipase from an endophytic yeast isolated from castor leaves. *Biocatal. Agric. Biotechnol.* 1, 295– 300.
- Paula, A.V., Moreira, A.B.R., Braga, L.P., Castro, H.F., 2008. Comparação do desempenho de lipase de *Candida rugosa* imobilizada em suporte híbrido de polissiloxano-polivinil álcool empregando diferentes metodologias. *Quim. Nova.* 31, 35-40.
- Salihu, A., Alam M.Z., Karim, M.I.A., Salleh, H.M., 2011. Optimization of lipase production by *Candida cylindracea* in palm oil mill effluent based medium using statistical experimental design. *J. Mol. Catal. B: Enzym.* 69, 66-73.
- Salihu, A., Alam, M.Z., AbdulKarim, M.I., Salleh, H.M., 2012. Lipase production: An insight in the utilization of renewable agricultural residues. *Resour. Conserv. Recy.* 58, 36–44.
- Salum, T.F.C., Villeneuve, P., Barea, B., Yamamoto, C.I., Côcco, L.C., Mitchell, D.A., Krieger, N., 2010. Synthesis of biodiesel in column fixed-bed bioreactor using the fermented solid produced by *Burkholderia cepacia* LTEB11. *Process Biochem.* 45, 1348–1354.
- Sharma, R., Chisti, Y., Banerjee, U.C., 2001. Production, purification, characterization, and applications of lipases. *Biotechnol Adv.* 19, 627-662.
- Tan, T., Lu, J., Nie, K., Deng, L., Wang, F., 2010. Biodiesel production with immobilized lipases: A review. *Biotechnol. Adv.* 28, 628-634.
- Tran, D.T., Yeh, K.L., Chen, C.L., Chang, J.S., 2012. Enzymatic transesterification of microalgal oil from *Chlorella vulgaris* ESP-31 for biodiesel synthesis using immobilized *Burkholderia* lipase. *Bioresour. Technol.* 108, 119-127.
- Xie, W., Wang, J., 2012. Immobilized lipase on magnetic chitosan microspheres for transesterification of soybean oil. *Biomass Bioenerg.* 36, 373-380.

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