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# Enhanced Production of Fibrinolytic Protease from Microalgae *Chlorella Vulgaris* using Glycerol and Corn Steep Liquor as Nutrient

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## Abstract

Cardiovascular diseases are the leading causes of death worldwide and may be caused by the accumulation of fibrin in the blood vessels. Microbial fibrinolytic enzymes have attracted much more attention than typical thrombolytic agents because of the expensive prices and the side effects. The objective was evaluated cell concentration ($X_m$), cell Productivity ($P_x$) and Total Fibrinolytic activity ($FT_{act}$) from the *Chlorella vulgaris* microalgae using glycerol ($C_{gly}$) and corn steep liquor ($C_{csl}$) by $2^2$ plus star central composite experimental design combined with response surface methodology. In the optimized condition of the 0.9% of $C_{gly}$ and 1.2% of $C_{csl}$, $X_m$ was 1520 mg $L^{-1}$, 50% higher than in autotrophic conditions. $P_x$ increased with increase of $C_{gly}$ and $C_{csl}$, obtaining the highest value of 232 mg $L^{-1}$ $day^{-1}$. Maximum protease production of 21.7 U $mL^{-1}$ was optimized in the condition of 1.1% $C_{gly}$ and 0.9% $C_{csl}$, while that to fibrinolytic to protease activity ratio ($F_{act}/P_{act}$) of 2.1 was using 2.0% $C_{gly}$ and 0.8% $C_{csl}$. The present study showed that *C. vulgaris* grown in culture medium containing glycerol and corn steep liquor byproducts, as well as to enhance protease and fibrinolytic enzyme production.

## Keywords

*Chlorella vulgaris*, Fibrinolytic enzyme, Corn steep liquor, Production

## Introduction

According to the World Health Organization (2012), in 2030, almost 3.6 million people will die from Cardiovascular Diseases (CVDs), mainly from heart disease and stroke [1]. One of the pathophysiological Conditions which causes myocardial infarction, stroke and other CVDs, is the formation of a fibrin clot by the proteolytic action of thrombin on fibrinogen, and consequent accumulation of fibrin in the blood vessels usually leads to thrombosis [2].

Thrombolytic agents, anticoagulants, antiplatelet and direct thrombolytics are used for the treatment of thrombotic disorders [3]. Based upon their mechanism of activation of the fibrinolytic system, fibrinolytic agents are classified into two types. One is plasminogen activator such as Tissue-type Plasminogen Activator (t-PA) and urokinase [4,5]. The other one is plasmin-like fibrinolytic enzymes which can directly degrade the fibrin in blood clots, thereby dissolving the thrombi rapidly. However, all these thrombolytic agents still suffer significant shortcomings, including requirement of large therapeutic dose, short plasma half-life, limited fibrin specificity, reocclusion, antigenic reactions and bleeding complications [6].

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Microalgae are a special group of microorganisms that have demonstrated to be excellent producers of new bioactive metabolites, such as antibiotics [7], anticancer [8] and anti-diabetic agents [9]. Few studies have been conducted on fibrinolytic enzymes biosynthesized by microalgae and no reports are available concerning statistical medium optimization from microalgae.

Obtaining a suitable medium for maximum production during the enzymatic bioprocess is of great importance since the elements that make up the culture medium significantly affect the yield of the product and the cost of production [10]. Enhanced productivity with economical viable cost is a key requisite for the fulfillment of market demand of fibrinolytic enzyme.

Photosynthetic microorganism can produces various useful metabolites under photoautotrophic and mixotrophic cultivation. Cellular concentrations in photoautotrophic cultures are usually very low, due to light limitation, with the consequent high cost of downstream processing. A feasible alternative for photoautotrophic culture is to use mixotrophic culture in which organic carbon sources and $CO_2$ are simultaneously assimilated in the presence of light and both respiratory and photosynthetic metabolism operates concurrently [11-13].

Organic residues from agriculture and industries, e.g. soybean residue, cane molasses, glycerol and Monosodium Glutamate Waste Liquor (MGWL) had been increasingly exploited in bioprocesses because those were excellent substrates for heterotrophic microorganisms growth by supplying the essential nutrients [14] and reducing production costs. In general, these organic residues have been utilized to bacteria, yeast, fungi and, in limited number, microorganism photosynthetic cultivation.

The present study evaluated the fibrinolytic activity from cell extract of *Chlorella vulgaris* microalgae cultivated in low-cost agro-industrial residues, initiating a new era of bioprospecting of fibrinolytic enzymes from microalgae. This is the first report about of fibrinolytic enzyme from *C. vulgaris* microalgae.

## Materials and Methods

### Microalgae and media

*C. vulgaris* (UTEX 1803) was obtained from the UTEX (University of Texas, Austin). Stock cultures were maintained in liquid Bold's Basal medium [15].

The culture medium was sterilized in autoclave at 121 °C for 20 min. Corn steep liquor (Corn Products Brazil,

**Table 1:** Effects of BBM culture medium supplemented with glycerol or corn steep liquor on protease and fibrinolytic activities.

| Days | Test | Culture medium | Protease activity (U $mL^{-1}$) | Fibrinolytic activity (U $mL^{-1}$) |
|---|---|---|---|---|
| 7 | 1 | BBM | 5.00 ± 2.0[a] | UN |
| 8 | 2 | BBM + Corn Steep Liquor 0.5% | 292 ± 26[b] | 741.5 ± 30.0[a] |
| 10 | 3 | BBM[1] + Corn Steep Liquor 0.5% | 1.48 ± 6.0[c] | 1,099 ± 56.0[b] |
| 12 | 4 | BBM + Glycerol 0.5% | 7.00 ± 2.0[a] | UN |

UN = Unrealized; [1]BBM without $NaNO_3$; [a,b,c] = Values with the same superscript are not significantly different according to the Tukey test ($p > 0.05$).

**Table 2:** Experimental results of *Chlorella vulgaris* cultivation as a function of two independent variables, glycerol ($C_{gly}$) and corn steep liquor ($C_{CSL}$), and their responses variables ($X_m$, $P_x$, $PT_{act}$, $SF_{act}$ and $F_{act}/P_{act}$).

| Days | Test | $x_1$[a] | $x_2$[b] | $C_{gly}$[c] (%) | $C_{csl}$[d] (%) | $X_m$[e] (mg $L^{-1}$) | $P_x$[f] (mg $L^{-1}$ $dia^{-1}$) | $PT_{act}$[g] (U $mL^{-1}$) | $SF_{act}$[h] (U $mg^{-1}$) | $F_{act}$ (U $mg^{-1}$)/$P_{act}$ (U $mg^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 1 | -1 | -1 | 0.5 | 0.5 | 881.2 | 146.0 | 13.00 | 226.6 | 1.314 |
| 6 | 2 | -1 | 1 | 0.5 | 1.5 | 1,325 | 220.0 | 17.40 | 158.6 | 0.703 |
| 9 | 3 | 1 | -1 | 1.5 | 0.5 | 680.7 | 75.00 | 18.09 | 467.1 | 1.992 |
| 5 | 4 | 1 | 1 | 1.5 | 1.5 | 1,165 | 232.0 | 12.15 | 590.2 | 1.416 |
| 8 | 5 | 1.414 | 0 | 1.7 | 1.0 | 1,200 | 150.0 | 17.58 | 662.6 | 1.907 |
| 9 | 6 | -1.414 | 0 | 0.3 | 1.0 | 1,250 | 139.0 | 16.11 | 183.2 | 1.031 |
| 6 | 7 | 0 | 1.414 | 1.0 | 1.7 | 1,260 | 213.0 | 16.96 | 198.8 | 0.904 |
| 5 | 8 | 0 | -1.414 | 1.0 | 0.3 | 650.0 | 130.0 | 19.22 | 313.6 | 1.065 |
| 8 | 9 | 0 | 0 | 1.0 | 1.0 | 1,430 | 179.0 | 20.69 | 153.8 | 1.383 |
| 8 | 10 | 0 | 0 | 1.0 | 1.0 | 1,480 | 185.0 | 21.21 | 261.8 | 1.602 |
| 8 | 11 | 0 | 0 | 1.0 | 1.0 | 1,430 | 179.0 | 24.03 | 242.2 | 1.754 |
| 8 | 12 | 0 | 0 | 1.0 | 1.0 | 1,480 | 185.0 | 20.51 | 143.0 | 1.358 |
| 7 | 13[i] | - | - | - | - | 698.7 | 99.80 | 5.000 | UN | UN |
| - | 14[j] | 1.450 | 1.600 | 0.9 | 1.2 | 1,520 | - | 21.00 | - | 1.517 |
| - | 15[l] | 1.530 | 1.445 | 1.1 | 0.9 | 1,384 | - | 21.70 | - | 1.796 |
| - | 16[m] | 2.000 | 1.400 | 2.0 | 0.8 | 666.1 | - | 12.00 | - | 2.100 |

[a]$x_1$: Variable coded for $C_{gly}$; [b]$x_2$: Variable coded for $C_{csl}$; [c]$C_{gly}$: Glycerol concentration (%); [d]$C_{csl}$: Corn steep liquor concentration (%); [e]$X_m$: Maximum biomass concentration; [f]$P_x$: Cell Productivity; [g]$P_{act}$: Protease activity; [h]$F_{act}$: Fibrinolytic activity; [i]Autotrophic conditions; UN: Unrealized. Values calculated by regression equation of [J]Xm, [l]$PT_{act}$, [m]$F_{act}/P_{act}$.

Cabo-PE, Brazil) was treated according to Liggett and Koffler [16]. Initially, the corn steep liquor was centrifuged at 8000 rpm for 10 minutes to remove the solid particles, and then concentrated NaOH was added to adjust pH 8 and autoclaved at 121 °C. Corn steep liquor was then filtered and the filtrate was used in the cultures. The organic nutrients such as glycerol (Dynamic, PA - Purity = 99.5%) and treated corn steep liquor were separately sterilized in different bottles and aseptically added into the culture medium.

## Culture conditions

*C. vulgaris* was grown autotrophically and mixotrophically on 400 mL of culture media (initial pH = 6.8) in 1000 mL Erlenmeyer flasks at 27 ± 1 °C, under a constant fluorescent light intensity of approximately 74 µmol photons $m^{-2}$ $s^{-1}$ measured by a LI-250 Light Meter with a LI-190 quantum sensor (Li-Cor, Lincoln, NE, USA). Sparging air provided agitation and aeration during cell growth. Initial cell concentration was 50 mg $L^{-1}$. Initially, preliminary tests were carried out with 0.5% glycerol and corn steep liquor (Table 1) to evaluate the influence of these nutrients on production of fibrinolytic enzymes. All experiments were duplicated. Then, experimental design was utilized to optimize the concentration of glycerol ($C_{gly}$) and corn steep liquor ($C_{csl}$) on the response variables (Table 2) Periodic samples were taken from the flasks to determine the response variables.

## Experimental design and results analysis

Response Surface Methodology (RSM) was used to determine the influence of the two independent variables, Glycerol Concentration ($C_{gly}$) and corn steep liquor concentration ($C_{csl}$), on the response variables, namely maximum cell concentration ($X_m$), cell Productivity ($P_x$), Total Protease activity ($PT_{act}$), Specific Protease activity ($SP_{act}$), Total Fibrinolytic activity ($FT_{act}$), Specific Fibrinolytic activity ($SF_{act}$) and Fibrinolytic to Protease activities ratio ($F_{act}/P_{act}$). To this purpose, multivariable regression analyses were done under experimental design called "star planning" proposed by Barros Neto, et al. [17] which consists of two factors in five levels of independent variables. The central point was fourfold repeated to check the reproducibility of results (Table 2). The independent variables $C_{gly}$ and $C_{csl}$ and its corresponding ranges were selected on the basis of the results of Liang, et al. and Mahboob, et al. [11,18] respectively.

A regression method was used to fit the second order polynomial equation (1) to the experimental data and to identify the relevant model terms *p*-values of less than 0.05 were considered to be statistically significant. RSM was applied to the experimental data using STATISTICA software (Version 5.5, 1999 Edition; Statsoft Inc., Tulsa, OK, USA). The same statistical software was used to generate the statistical and response plots.

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_{ii}^2 + \sum b_{ij} x_i x_j \quad (1)$$

Where, Y is the response, $b_0$ is an intercept, $b_i$, $b_{ii}$ and $b_{ij}$ are the coefficients of the linear, quadratic and interaction terms, respectively. $x_i$ and $x_j$ represent the coded independent variables. The fitted polynomial equation is expressed as surface plots in order to visualize the relationship between the response and experimental levels of each factor.

## Preparation of microalgae extracts

Lyophilized microalgae were homogenized in Phosphate Buffer (PB) 0.1 M pH 7.0, at cell concentration of 50 mg $mL^{-1}$, in room temperature for 30 minutes. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 4 °C.

## Analytical methods

**Determination of cell density:** Cell concentration was daily determined by measuring the optical density of samples at 685 nm using a spectrophotometer (Agilent 8453, P. R. China) [19]. The cell density was performed in duplicate. Cell productivity ($P_x$, g $L^{-1}$ $day^{-1}$) was estimated by an equation (Equation 2) where *X* (g $L^{-1}$) was the concentration of biomass at the end of the cultivation, $X_0$ (g/L) was that at the beginning, and *t* was the duration of cultivation.

$$P_x = \frac{(X - X_0)}{t} \quad (2)$$

**Protease activity:** Protease activities assayed by spectrophotometric using azocasein as substrate. One unit (U) of enzyme activity was defined as the amount of enzyme able to hydrolyze azocasein giving an increase of 0.001 units of absorbance per minute, at 450 nm [20]. The activity was performed in duplicate.

**Fibrinolytic activity:** The specific enzyme activity was evaluated using an assay of fibrin degradation [21,22]. In this assay, one unit (Fibrin Degradation Unit, FU) of enzyme activity is defined as a 0.01-per minute increase in absorbance at 275 nm. The activity was performed in duplicate.

**Protein concentration:** Protein concentrations were determined by using BCA or Micro BCA protein assay reagent Kit (BCA™ Protein Assay Kit, Thermo SCIENTIFIC). Bovine serum albumin was used as protein standard. The dosage was performed in triplicate.

# Results and Discussion

## Preliminary test of glycerol and corn steep liquor to protease and fibrinolytic enzymes productions by *C. vulgaris*

Protease and fibrinolytic activities of the cells extracts

were showed in the Table 1. In the tests 1 and 4, without corn steep liquor addition, were observed low protease activity while that those with the corn steep liquor addition was obtained protease activity of 292 U $mL^{-1}$ (Test 2; Table 1) and 1.48 U $mL^{-1}$ (Test 3; Table 1). The removal of inorganic source ($NaNO_3$) of BBM increased proteases production (Table 1) which is not feasible for the purpose of the work. One of characteristics of the ideal thrombolytic agent is fibrin speciefic, which allow direct degradation of fibrin at the clot surface. Furthermore, higher fibrin specificity would limit activation of circulating plasminogen and thus degradation of fibrinogen - attributes that would be expected to reduce the risk of bleeding [23]. The cell extract with corn steep liquor (0.5%) and BBM (with $NaNO_3$) had low protease activity and high fibrinolytic activity (Table 1, Test 2). Therefore, BBM medium with $NaNO_3$ was selected for further optimization study using statistical approaches.

From the results, Plackett-Burman statistical design and Response Surface Methodology (RSM) was performed to optimized glycerol and corn steep liquor concentrations added in standard BBM on dependents variables maximum cell concentration ($X_m$), cell Productivity ($P_x$), Protease Total activity ($PT_{act}$), Specific Protease activity ($SP_{act}$), Total Fibrinolytic activity ($FT_{act}$), Specific Fibrinolytic activity ($SF_{act}$) and ratio to Fibrinolytic to Protease activities ($F_{act}/P_{act}$). High fibrinolytic enzyme concentration can reduce production costs and facilities the steps of downstream, since purification steps will be used in future.

## Effects of glycerol ($C_{gly}$) and corn steep liquor ($C_{csl}$) concentrations on growth profiles of the *C. vulgaris* microalgae

Growth profiles of the *C. vulgaris* microalgae with 1% $C_{gly}$ and different $C_{csl}$ are showed in Figure 1. The in-

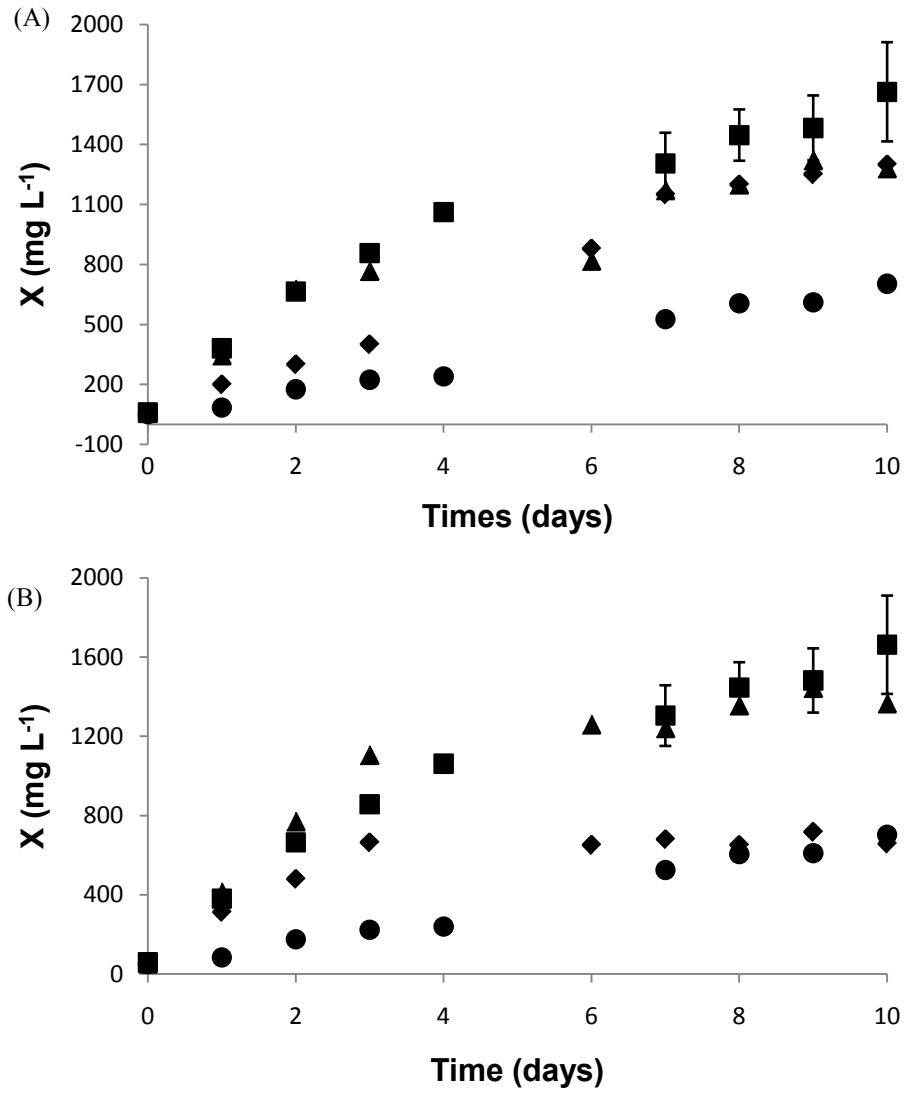


**Figure 1:** A) Growth profile of the *C. vulgaris* microalgae using 1.0% $C_{gly}$ and differents $C_{csl}$: (♦) 0.3%; (■) 1.0%; (▲) 1.7% ; B) Growth profile of the microalgae *C. vulgaris* using 1.0% $C_{csl}$ and differents $C_{gly}$: (♦) 0.3%; (■) 1.0%; (▲) 1.7%; Autotrophic conditions (●).

creasing of $C_{csl}$ results in higher $X_m$ values, showing that *C. vulgaris* can use nutrients in corn steep liquor for its growth. In mixotrophic microalgae culture, the metabolism assimilates $CO_2$ autotrophic and organic carbon sources provided to it. According to the literature, some microalgae show high cell concentration when mixotrophically cultivated which can be justified by the fact that the additional carbon source minimizes the consequences generated by self-shading effect [24,25]. The assimilation of inorganic carbon is hampered due to low light availability caused by high cell density. Thus, corn steep liquor can be used as organic carbon source by microalgal since it is rich in protein and carbohydrates. In addition, exogenous protein supplement from corn steep liquor can induce protease production by microorganism [26]. Hence, protease activity in the culture without corn steep liquor was not detectable in this work. Similar results of protease induction by protein were observed by Dhar and Kaur [27] in *Metarhizium anisopliae* production using casein. Cultures with 1.0% and 1.7% of $C_{csl}$ showed similar cell concentration with a significant increase compared to 0.3% of $C_{csl}$ (Figure 1A). Stationary phase of growing using 0.3% $C_{csl}$ was after 3 days, obtained $X_m$ of 665 mg $L^{-1}$. In cultures with 1% of $C_{csl}$, $X_m$ was 1,305 mg $L^{-1}$ after 7 days while that using 1.7% $C_{csl}$, $X_m$ was 1,097 mg $L^{-1}$ after 6 days. *C. vulgaris* growth using 1% glycerol and 1% $C_{csl}$ obtained highest cell concentration and can be quite viable when applied on larger scale. Mahboob, et al. [18] related that corn steep liquor and urea, which are low cost nitrogen sources, were highly stable for *C. vulgaris* growth**.**

The growth profile of the *C. vulgaris* cultivates in BBM with 1% $C_{csl}$ and 1.7% $C_{gly}$ exhibited a limited effect on the growth of microalgae (Figure 1B). Konh, et al. [28] showed that *Chlorella vulgaris* utilize glycerol as a sole carbon substrate for the production of biomass and biochemical components, such as photosynthetic pigments, lipids, soluble carbohydrates and proteins. The stationary phase of cell growth cultivated in 0.3% $C_{gly}$ was after 6 days, achieving $X_m$ values of 878 mg $L^{-1}$. When *C. vulgaris* was cultivated in BBM with 1% and 1.7% $C_{gly}$, the stationary phase reached after 7 and 8 days, and $X_m$ was of 1,305 and 1,191 mg $L^{-1}$, respectively. Liang, et al. [11] observed an inhibitory effect on *C. vulgaris* growth using 2% glycerol, obtained $X_m$ of 656.0 mg $L^{-1}$ at 6 days of cultivation. Heredia-Arroyo [29] mixotrophic growth of *C. vulgaris* using 80: 20% glucose: glycerol, observed an increase in cell concentration, while increased levels of glycerol, about 15 g $L^{-1}$ was inhibitory. Cheng, et al. [30] optimized the growth of *C. protothecoides* under three variables independents ($NaNO_3$, $MgSO_4.7H_2O$ and proteose concentrations) obtaining the maximum cell concentration of 1,190 mg $L^{-1}$ after 11 days of cultivation, though use of proteose in industrial scale is not feasible. The present work showed that it is possible to use two agro industrial byproducts to obtain high microalgae concentration (1450 mg $L^{-1}$) using 1% $C_{gly}$ and 1% $C_{csl}$ since the costs for the cell production was minimal.

## Protease and fibrinolytic productions

Extract of *C. vulgaris* obtained protease (12.15 U $mL^{-1}$) and fibrinolytic (590.2 U $mg^{-1}$) productions when cultivated in the BBM supplemented with 1.5% $C_{gly}$ and 1.5% $C_{csl}$ (Test 4; Table 2). The best result was using 1.7% $C_{gly}$ and 1% $C_{csl}$ (Test 5, Table 2) which obtained relatively low protease production of 17.58 U $mL^{-1}$ and high fibrinolytic production of 662.6 U $mg^{-1}$, showing that fibrinolytic protease produced has the ability to directly degrade fibrin. Fibrinolytic enzyme from *Codium* macro algae was obtained by Matsubara, et al. [31-33]. The enzyme extract of the *C. intricatum* macroalga showed specific activity of 691 U $mg^{-1}$for CIP-I and 533 U $mg^{-1}$ for CIP-II *C. divaricatum* showed specific activities of 6.3 U $mg^{-1}$ Fibrinolytic activities from *C. vulgaris* obtained in this study were comparatively higher than the earlier report of *Codium* macroalgae [33].

Currently, not there are papers reporting the use of enzymes produced by microalgae in combating cardiovascular disease. On the other hand, fibrinolytic enzymes produced by other organisms, mainly bacteria and fungi, have been widely studied and optimized the conditions of cultivation. Vijayaraghavan and Vincent [34] related that fibrinolytic activity (1,573 U $mL^{-1}$) produced by *Pseudoalteromonas* sp. IND11 in optimized medium using cow dung substrate in solid-state culture three times higher than the unoptimized medium. Silva, et al. [35] showed that several other microorganisms may be producers of fibrinolytic enzymes, among which the *Bacillus* genus. *Bacillus subtilis* 168 (S1) produced 252 U/mg fibrinolytic enzymes [36] whereas *B. licheniformis* KJ-31 produced 242.8 U $mg^{-1}$ [37]. Bacteria of the genus *Streptomyces* are also producers of fibrinolytic enzymes, with activity of 136.2 U $mg^{-1}$ [38] and 19 U $mg^{-1}$ [39]. Moreover, fungi of the genus *Aspergillus* [40] and *Fusarium* [41] may also be promising sources in the production of these enzymes. This present study obtained fibrinolytic activity from *C. vulgaris* microalgae varied between 143.0 to 662.6 U $mL^{-1}$, proving to be a source future in combating cardiovascular disease, specifically in direct degradation of fibrin clot. These fibrinolytic enzymes may have wide application in pharmaceutical industry. Fibrinolytic enzymes from this kind of food grade organisms could effectively prevent and treat cardiovascular diseases [42].

## Optimization of $C_{gly}$ and $C_{csl}$ concentrations on response variables

Response surface methodology is an approach that

combines various statistical and mathematical techniques and is useful for developing, improving and optimizing of process [43]. In the present study, Box-Behnken model for two independent variables $C_{gly}$ (0.5-1.5%) and $C_{csl}$ (0.5-1.5%) with their low, medium and high levels, was used as the experimental design model for optimization of mixotrophic growth of *C. vulgaris*. Cell concentration (mg $L^{-1}$), cell productivity (mg $L^{-1}$ $d^{-1}$) and total protease activity (U $mL^{-1}$), specific protease activity (U $mg^{-1}$), total fibrinolytic activity (U $mL^{-1}$), specific fibrinolytic activity (U $mg^{-1}$) and ratio to fibrinolytic to protease activities (U $mg^{-1}$/U $mg^{-1}$) were taken as response (Table 2). The goodness of fit of the model was evaluated by the coefficient determination ($R^2$) and the Analysis of Variances (ANOVA).

**$X_m$:** As showed in the Table 2, $X_m$ varied between 650 to 1,480 mg $L^{-1}$. Based in these values, the regression analysis was applied to $X_m$ in function of both $C_{gly}$ and $C_{csl}$. It is possible to get a quadratic polynomial equation to express the relationship between $X_m$ and the selected independent variables. To get better fitting of the model, the interaction coefficients between the independent variables were omitted, because they were proved not significant ($p > 0.05$).

$$X_m = -517.28 + 985.22\,C_{gly} + 2612.47\,C_{csl} - 547.03\,C_{gly^2} - 1077.64\,C_{csl^2} \quad (3)$$

Where $X_m$ is the predicted response, i.e. the cell concentration (mg $L^{-1}$), and $C_{gly}$ and $C_{csl}$ are values of glycerol and corn step liquor concentrations, respectively.

The statistical significance of the model equation was evaluated by Analysis of Variance (ANOVA) which showed that adjust coefficient of determination ($R^2$ = 0.95) of the regression was statistically significant ($p < 0.05$). This indicates that the model was adequate for prediction within the range of experimental variables.

Estimated $X_m$ by the model was 1,516 mg $L^{-1}$. Li, et al. [44] using 0.1% $C_{gly}$ in photoheterotrophic cultivation of *C. minutissima* UTEX 2341 obtained $X_m$ = 770 mg $L^{-1}$ in 7 days of culture. Liang, et al. [11] noted that the growth of *C. vulgaris* under autotrophic conditions resulted 722 mg $L^{-1}$ of algal biomass in 6 days. Carbon, nitrogen and phosphorous sources are three main influencing important nutrients microalgae growth [45] and the use of glycerol as a carbon source strongly stimulates its growth, since about 45% of microalgae organism is composed of carbon [46]. The great importance of glycerol on the production of biomass also is emphasized in the literature [47].

The present work presented about twice the $X_m$ obtained by Li, et al. [44] and Liang, et al. [11] demonstrating that the use of glycerol and corn steep liquor, an agro industrial byproducts, improve $X_m$. Likewise, glycerol uptake for some *Chlorella* species has been described in other studies. For example, simultaneous utilization of multiple organic substrates, glycerol, glucose and B. peptone by *Chlorella saccharophila* was reported by Isleten-Hosoglu, et al. [48], where *C. saccharophila* was capable of utilizing glycerol as a carbon source but its cell

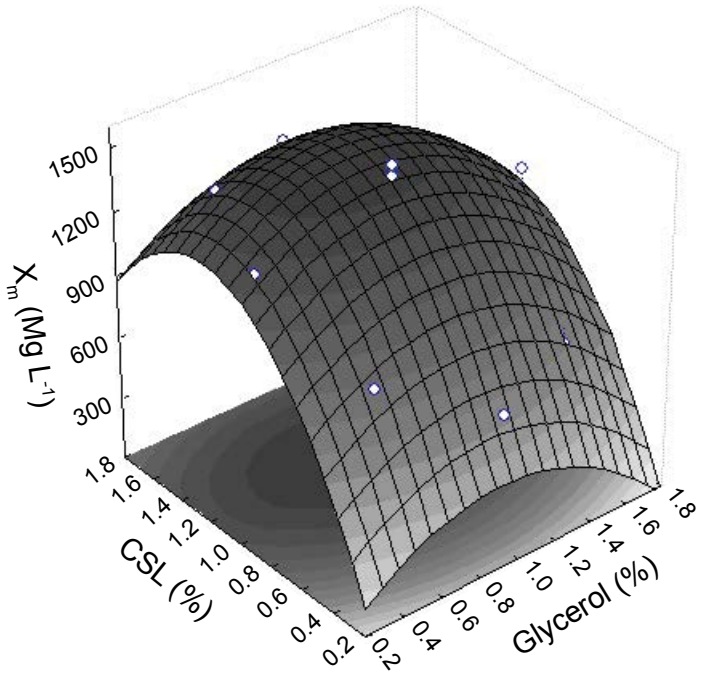


**Figure 2:** Response surfaces showing the mutual effect of glycerol ($C_{gly}$) and corn steep liquor concentrations ($C_{csl}$) in maximum cell concentration.

growth on glucose is much better than that on glycerol.

Three-dimensional response surfaces were based on the model equation to investigate the interaction among the variables and to determine the optimum concentration of each factor on $X_m$ (Figure 2). The optimal conditions to maximize $X_m$ estimated by deriving Equation (2) were $C_{gly}$ = 0.9% e $C_{csl}$ = 1.2%.

**$P_x$:** High $C_{gly}$ and $C_{csl}$ values increase $P_x$, obtaining higher values of 232 mg $L^{-1}$ $day^{-1}$ (Table 2 and Figure 3). This result was obtained since there was a decrease in the cultivation time in the experiment with high $C_{gly}$ and $C_{csl}$. By the way, cell productivity is calculated as function of cell concentration ($X_m$) and time cultivation, short time of cultivation provides higher cell productivity. Li, et al. [44] related that *C. minutissima* UTEX 2341 productivity of 110 mg $L^{-1}$ $day^{-1}$ was obtained by addition of 1 g $L^{-1}$ of glycerol and Cheng, et al. [30] obtained *C. protothecoides* productivity of 108 mg $L^{-1}$ $day^{-1}$ using 0.25 g $L^{-1}$ proteose, a product of hydrolysis of proteins. These values were 50% lower than the present work.

The second order polynomial equations fitted to the experimental data of the CCD for predicting $P_x$, which showed $p < 0.05$ are given in Equation (4).

$$P_x = 108.57 + 42.15\,C_{gly} + 30.37\,C_{csl} - 68.06\,C_{gly^2} - 12.84\,C_{csl^2} + 83.00\,C_{gly}C_{csl} \quad (4)$$

Where $P_x$ is the predicted response, i.e. the cell productivity (mg $L^{-1}$ $day^{-1}$), and $C_{gly}$ and $C_{csl}$ are values of glycerol and corn step liquor concentrations, respectively.

The values $R^2_{Adj}$ = 0.80 (Equation 4) indicate a high degree of agreement between the observed and predicted values for cell productivity, suggesting that the proposed model equations provide satisfactory and accurate results (Figure 4A). ANOVA of the regression model demonstrates that the model is highly significant, as indicated by the calculated $p$-value < 0.05 for cell productivity.

**Fibrinolytic and protease activities:** A second-order polynomial regression model was employed dependent variables Total Protease activity ($PT_{act}$), Specific Protease activity ($SP_{act}$), Total Fibrinolytic activity ($FT_{act}$), Specific Fibrinolytic activity ($SF_{act}$) and Fibrinolytic to Protease activity ratio ($F_{act}/P_{act}$). It was observed lack of fit significant to $SP_{act}$ ($p$ = 0.3899), $FT_{act}$ ($p$ = 0.1665), $SF_{act}$ ($p$ = 0.8182), suggesting that this model did not accurately represent data in the experimental region.

The experimental results of $PT_{act}$ and $F_{act}/P_{act}$ were fitted to a second-order quadratic equation, giving two numerical correlations to estimate the responses $PT_{act}$ and $F_{act}/P_{act}$ according to equation 5 and equation 6.

$$PT_{act} = -9.53 + 34.90\,C_{gly} + 28.15\,C_{csl} - 12.03\,C_{gly^2} - 9.49\,C_{csl^2} + 10.34\,C_{gly}C_{csl} \quad (5)$$

$$F_{act}/P_{act} = -0.24 + 1.31\,C_{gly} + 2.27\,C_{csl} - 0.32C_{gly^2} - 1.31\,C_{csl^2} \quad (6)$$

Where $PT_{act}$ or $F_{act}/P_{act}$ is the predicted response, i.e. the Protease activity (U $mL^{-1}$) or ratio between fibrinolytic and protease activity, and $C_{gly}$ and $C_{csl}$ are values of glycerol and corn step liquor concentrations, respectively.

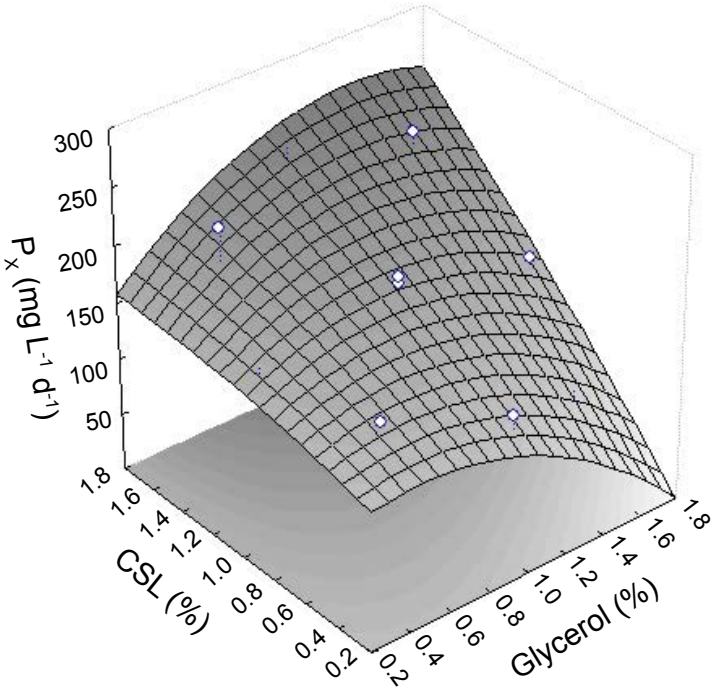


**Figure 3:** Response surfaces showing the mutual effect of glycerol ($C_{gly}$) and corn steep liquor concentrations ($C_{csl}$) in cell productivity ($P_x$).

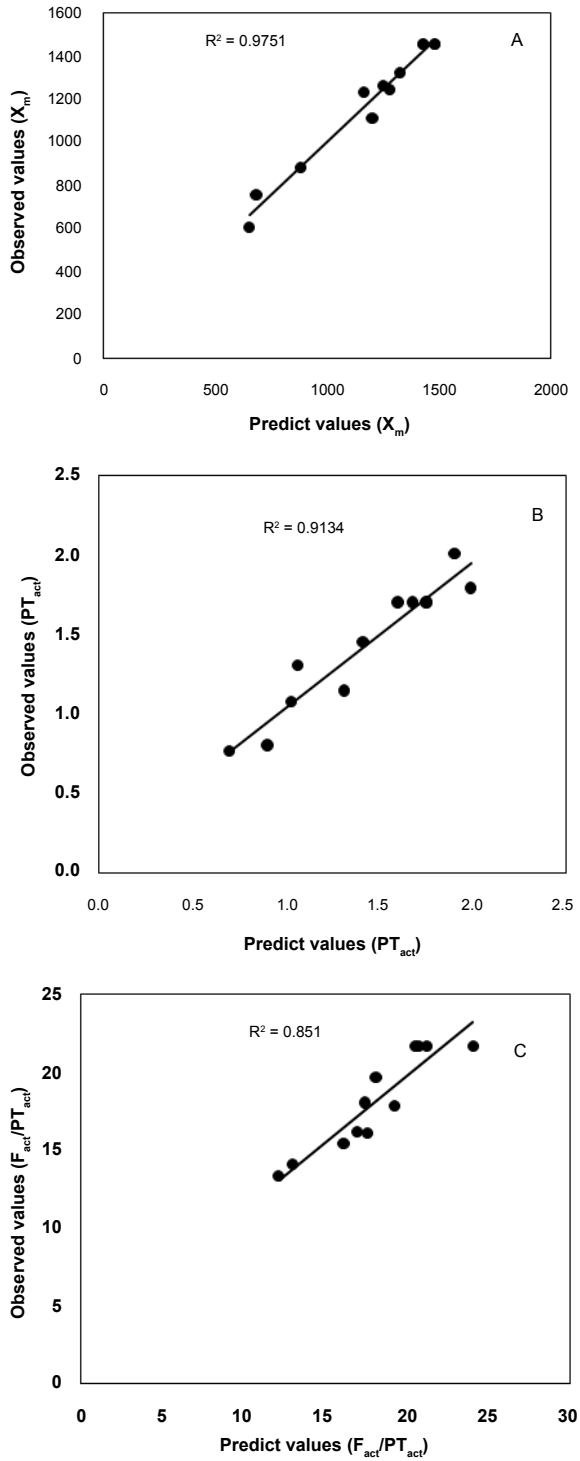


**Figure 4:** Linear relationship between the predicted and experimental values of A) Maximum cell concentration; B) Protease total activity; C) Fibrinolytic and Protease activities ratio ($F_{act}/P_{act}$).

The results of the quadratic model for $PT_{act}$ and $F_{act}/P_{act}$ in the form of Analysis of Variance (ANOVA). The values of $R^2$ found also advocated a high correlation between the observed and predicted values. This means that regression model provides an excellent explanation of the relationship between the independent variables (glycerol and corn steep liquor concentrations) and the responses ($PT_{act}$ and $F_{act}/P_{act}$). This implies that 73% and 87% of the sample variation for $PT_{act}$ and $F_{act}/P_{act}$ activities are explained by the independent variables. The examination of the fit summaries output revealed that the quadratic models are statistically significant for the responses and therefore these equations may be used for further analysis.

Figure 5 illustrate the three-dimensional response surface graphs of the quadratic model for protease total activities. As shown in the figure, it is also very easy and convenient to locate optimum levels of two variables. The best value of $PT_{act}$ (21.7 U $ml^{-1}$) occurred close to the upper point indicating the values optimized of glycerol concentration and corn steep liquor was 1.06% and 0.89%, respectively.

The effect of $C_{csl}$ and $C_{gly}$ concentrations on the $F_{act}/P_{act}$ ratio was investigated by using Response Surface Methodology (RSM) and were constructed for determining the optimum conditions for a required $F_{act}/P_{act}$ values (Figure 6). The developed prediction equation shows that increasing of $C_{gly}$ up to 1.2% increase $F_{act}/P_{act}$ values, while that the increase of $C_{gly}$ improve Fact/Pact values only in the high $C_{csl}$, indicating that the $F_{act}/P_{act}$ values is more dependent of $C_{csl}$ than $C_{gly}$. High proteins and peptides concentrations are presents in corn steep liquor [49] and it can induce the production of proteases by microalgae *C. vulgaris*. Ferrero, et al. [50] related that protease production by *Bacillus licheniformis* MIR 29 was repressed in the glycerol presence although cell growth had been observed.

The optimum operating conditions obtained from the quadratic form of the RSM were 2% of $C_{gly}$ and 0.8% of $C_{csl}$ with 2.1 of predicted $F_{act}/P_{act}$ ratio values.

The values of $PT_{act}$ and $F_{act}/P_{act}$ experimentally obtained and predicted from the related empirical models showed in Figure 4B and Figure 4C indicated that for both independent variables, the calculated values of $PT_{act}$ and $F_{act}/P_{act}$ agreed very well with the predicted values of $PT_{act}$ and $F_{act}/P_{act}$ at all concentration combinations studied.

## Conclusion

The results showed that the use of corn steep liquor to production of fibrinolytic enzymes from the microalgae *Chlorella vulgaris* UTEX 1803 is potentially viable. Statistical analysis proved to be a useful and powerful tool in developing optimum conditions. The statistical analysis based on a Box-Behnken design showed that 0.9% of glycerol and 1.2% of CSL concentrations were the best conditions to biomass production. Protease production was optimized in the condition of 1.1% of glyc-

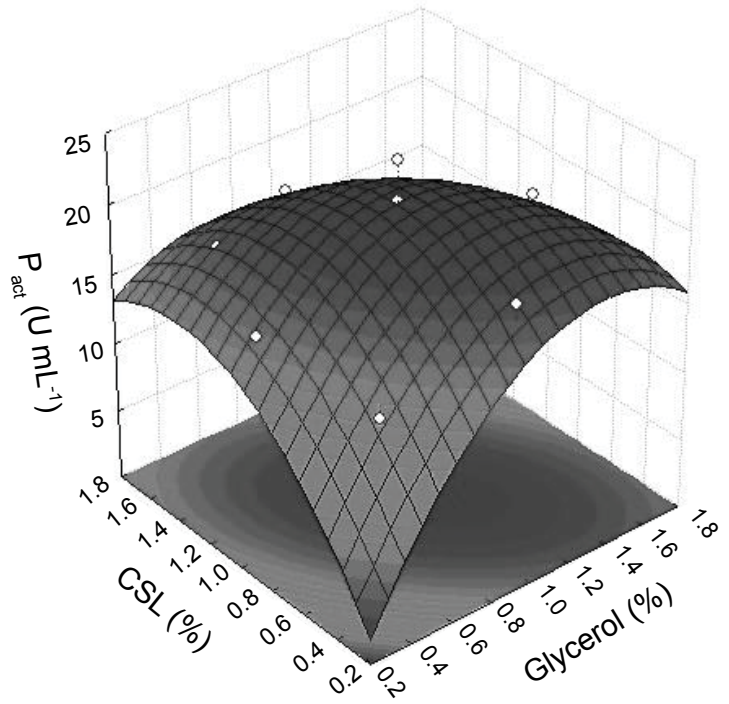


**Figure 5:** Response surfaces showing the mutual effect of glycerol ($C_{gly}$) and corn steep liquor concentrations ($C_{csl}$) in Protease activity ($P_{act}$).

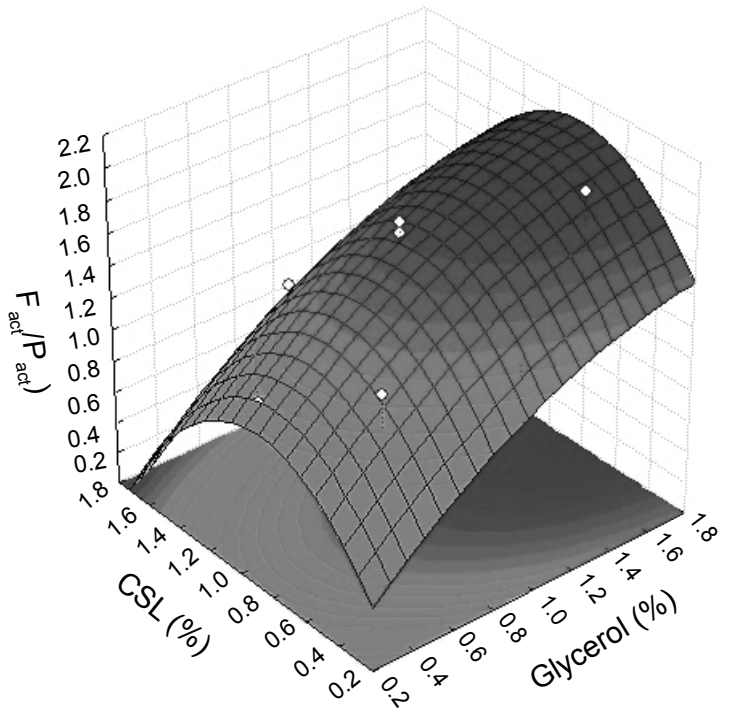


**Figure 6:** Response surfaces showing the mutual effect of $C_{gly}$ and $C_{csl}$ on ratio between Fibrinolytic and Protease activities ($F_{act}/P_{act}$).

erol and 0.9% of corn steep liquor, while that to $F_{act}/P_{act}$ ratio was 2.0% of glycerol and 0.8% of corn steep liquor. The agro-industrial byproducts proved to be good for increasing cell concentration, protease production, obtaining potential for future industrial and biotechnological applications in cardiovascular disease treatment.

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