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Fibrinolytic Enzyme from Green Microalgae: A New Potential Drug for Thrombolytic Therapy?

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Abstract: Thrombosis is characterized by the pathological formation of fibrin clots within a blood vessel, leading to the obstruction of blood flow. Fibrinolytic enzymes from microorganisms have been shown to be more efficient and safer in dissolving clots. Then, this study aimed to evaluate the cell growth and fibrinolytic enzyme production of *Tetradesmus obliquus* under different cultivation conditions. *T. obliquus* grew under autotrophic and mixotrophic conditions using different concentrations of corn steep liquor ($0.25 \leq CSL \leq 4.00\%$). The cells were concentrated and lysed via two different methods (sonication or homogenization) to trigger the release of the enzyme. It was precipitated via acetone or ammonium sulfate additions and purified using ion exchange chromatography. The highest biomass productivity (P_x = 130 ± 12.8 mg·L^{-1}day^{-1}), specific growth rate (μ_{max} = 0.17 ± 0.00 day^{-1}), and fibrinolytic activity (391 ± 40.0 U·mg^{-1}) was achieved on a mixotrophic cultivation at a 0.25% CSL concentration. The results showed that the homogenizing method had better performance in the release of enzyme, and the precipitation with acetone obtained the highest fibrinolytic activity (567 ± 49.3 U·mg^{-1}). The purified enzyme showed a specific activity of 1221 ± 31 U·mg^{-1} and a molecular mass of 97 kDa. So, the fibrinolytic enzyme from *T. obliquus* had higher activity when compared to the other fibrinolytic enzymes, being a potential source for the development of therapeutic agents in thrombosis treatment. Additional studies are needed to investigate the biochemical properties and biological profile of this enzyme.

Keywords: thrombosis; kinetic parameters; cell growth; protein purification; biomass production; Chlorophyceae; agro-industrial sub-product; extraction methods; molecular mass; chromatography

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1. Introduction

Cardiovascular diseases (CVDs) are the main cause of death worldwide and are responsible for about 32% of all global deaths [1]. Thrombosis is a severe CVD complication characterized by the pathological formation of fibrin clots that obstruct blood flow, leading to intense clinical manifestations such as acute ischemic stroke, myocardial infarction, and venous thromboembolism [2,3].

Currently, thrombosis treatment is based on antiplatelet or anticoagulant agents, which may lead to severe bleeding problems, such as hemorrhage [4,5]. Alternatively, fibrinolytic agents such as the tissue plasminogen activator (t-PA), urokinase (u-PA), and streptokinase have been widely used for thrombosis therapy. However, these drugs have some limitations including a short half-life, low specificity to fibrin, high cost, and excessive bleeding [6]. Thus, the search for more effective and safe fibrinolytic enzymes has become the key to thrombosis treatment.

In this sense, fibrinolytic enzymes from photosynthetic microorganisms such as *Chlorella vulgaris*, *Arthrospira platensis,* and *Dunaliella tertiolecta* have shown promising antithrombotic effects [7–9]. However, up to now, there is no report on the fibrinolytic potential of the *Tetradesmus* genus, although some bioactive compounds such as lectin, linoleic acid, and flavonoids with anticancer and antimicrobial activities have already been reported in the literature [10–12].

The production of microalgae has tripled in the last 5 years [13]. The interest in world production is due to some characteristics such as high photosynthetic efficiency, fast growth rate, resistance to various contaminants, and the capacity to grow on non-arable lands and be cultured using different growth conditions (autotrophic, heterotrophic, and mixotrophic growth modes) [14,15]. Specifically, previous studies have shown that mixotrophic conditions using different organic carbon substrates improve enzyme production and the *T. obliquus* biomass yields [16,17].

Organic wastes and by-products are frequently used as substrates for mixotrophic growth and are advantageous for sustainable resource recycling and the cost reduction in microalgal production [18]. Corn steep liquor (CSL) is a by-product from the corn wet-milling industries and has high amounts of carbohydrates, amino acids, vitamins, organic acids, and minerals, being a nitrogen-rich source used for the microalgal cultivation [19]. By the way, this by-product has been successful in the production of fibrinolytic enzymes from *C. vulgaris*, *A. platensis*, and *D. tertiolecta* [7–9]. So, this study aims to evaluate and compare the biomass and fibrinolytic enzyme productions from *T. obliquus* cultivated under autotrophic and mixotrophic (using CSL) growth conditions.

2. Materials and Methods

2.1. Culture Media and Growth Conditions

T. obliquus (SISGEN A5F5402) was isolated from Açude of Apipucos (Recife, Pernambuco, Brazil, coordinates 8°1′13.08″ S; 34°55′56.51″ W) and cultivated under autotrophic condition in 1000 mL Erlenmeyer flasks containing 400 mL of the BG-11 medium [20] with an initial concentration of 50 mg·L^{-1}, temperature of 30 ± 1 °C, continuous light intensity of 40 µmol photons m^{-2}·s^{-1}, and under constant aeration [21]. The mixotrophic condition was defined by the addition of different concentrations of corn steep liquor (0.25, 0.50, 0.75, 1.00, 2.00, and 4.00% (*v*/*v*)) into the BG-11 medium. The corn steep liquor (CSL) (Corn Products Brazil, Cabo de Santo Agostinho, PE, Brazil) was previously treated via autoclaving and precipitation according to Liggett and Koffler [22].

Cell growth was measured daily until the end of the exponential growth phase. The cell biomass was harvested via centrifugation (5000 rpm for 5 min), washed three times with distilled water, freeze-dried, and stored at 4 °C.

The *biomass concentration* was determined by measuring the optical density (*OD*) at λ665 nm via a UV/Visible spectrophotometer using an appropriate calibration curve correlating OD_{665} to the biomass concentration (Equation (1), R^2 = 0.99).

$$Biomass\ concentration = 0.0041 OD_{665} + 0.0486 \quad (1)$$

2.2. Kinetic Parameters

The biomass productivity (P_x) at the end of cultivation was calculated via Equation (2):

$$P_x = \frac{(X_t - X_0)}{t_c} \quad (2)$$

where X_t is the final cell concentration (mg·L^{-1}), X_0 is the initial cell concentration (mg·L^{-1}), and t_c time is the culture's final cell concentration (days).

The maximum specific growth rate (μ_{max}), expressed in day^{-1}, was calculated via the following equation:

$$\mu_{max} = \frac{1}{\Delta t} \ln \frac{X_j}{x_{j-1}} \quad (3)$$

where X_j and X_{j-1} are the cell concentrations at the end and the beginning of each time interval (Δt = 1 day).

2.3. Fibrinolytic Enzyme Extraction

The cell biomass (100 mg·mL^{-1}) was resuspended in 0.02 M Tris-HCl buffer (pH 7.4) and submitted to two different extraction methods: (1) homogenization by constant stirring for 30 min in ice bath [23]; and (2) sonication using a sonicator (Bandelin Sonoplus HD 2070, Microtip MS 72, Berlin, Germany) with 20 pulses for 1 min with intervals of 1 min between each pulse in ice bath [10]. Both the homogenized and sonicated biomasses were centrifuged at 15,000 rpm for 10 min at 4 °C and the cell extract was used for further analysis.

2.4. Precipitation Methods

Cell extract was precipitated using two different solvents: (1) acetone (80%); and (2) ammonium sulfate at 0–40% and 40–70% (*w*/*v*). The protein precipitated by using ammonium sulfate was dialyzed against 0.02 M Tris-HCl buffer for 6 h at 25 °C.

2.5. Protein Purification

The redissolved protein was loaded onto ion-exchange chromatography using DEAE Sephadex column (1.6 × 50 cm) pre-equilibrated with 0.02 M Tris-HCl buffer and eluted with the same buffer at a flow rate of 0.5 mL·min^{-1}, and fractions of 0.5 mL were collected. The absorbance was measured at λ280 nm.

2.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% polyacrylamide gel as described by Laemmli [24]. The molecular mass was calibrated using a Molecular Mass Marker Kit (14,000–200,000 Da, Sigma-Aldrich, St. Louis, MO, USA). Protein bands were detected by staining with silver.

2.7. Protein Concentration Analysis

Protein concentration was obtained using the BCA Protein Assay Reagent Kit (BCA™ Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). Bovine serum albumin was used as the standard.

2.8. Protease Activity Assay

Protease activity was assayed using azocasein as a substrate. The reaction mixture contained 30 µL of 0.08 mM azocasein, 140 µL of 0.02 M Tris-HCl, and 30 µL of the *T. obliquus* cell extracts. After 15 min, the reaction was stopped by the trichloroacetic acid (TCA) addition and the absorbance of liquid fraction was measured at λ450 nm using a microplate reader. One unit of azocasein activity was defined as the amount of enzyme required to increase the absorbance by 0.001 per minute, and the protease activity was expressed as activity units [25].

2.9. Determination of Fibrinolytic Enzyme

2.9.1. Fibrinolytic Plate Assay

The fibrinolytic activity was determined on a fibrin plate [26] with adaptations. Typically, the fibrin plate was prepared by mixing 0.45% bovine fibrinogen and 0.02 M Tris-HCl buffer with 2% agarose dissolved in 0.02 M Tris-HCl buffer and 200 µL of $CaCl_2$. The prepared solution was poured into a Petri plate (90 × 15 mm) containing 200 µL of a thrombin suspension. The fibrinolytic activity of the cell extracts was obtained by creating wells of 5 mm, which were impregnated with 20 µL of the *T. obliquus* extracts and incubated at 37 °C for 20 h. The zone of clearance was defined as the fibrinolytic activity of the cell extracts.

2.9.2. Fibrinolytic Assay Using Spectrophotometry

The fibrinolytic activity was evaluated according to Wang [27] using the spectrophotometric method. Fibrinogen (0.72%) and 0.02 M Tris-HCl buffer was placed in a test tube and incubated at 37 °C for 5 min. After the addition of the thrombin (20 U·mL^{-1}) solution, the resulting mixture was incubated at 37 °C for 10 min, the enzyme solution was added, and incubation continued at 37 °C. At 60 min, the reaction was stopped by adding 0.2 M TCA. Finally, the solution was centrifugated (8000 rpm for 10 min) and the supernatant was measured at λ275 nm. One unit (U) of fibrinolytic activity was defined as the amount of enzyme required to increase 0.01 units of absorbance per minute.

2.10. Statistical Analysis

All the experiments were performed in duplicates and the data are presented as the mean ± standard deviation (SD). The statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey's test as post hoc. p values < 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Cell Growth Profile and Kinetic Parameters of T. obliquus Cultivation under Different Growth Conditions

Cell growth profiles of *T. obliquus* in autotrophic and mixotrophic growth conditions using different CSL concentrations are shown in Figure 1. A short lag phase of two days was observed in autotrophic growth (Figure 1) due to the previous adaptation of *T. obliquus* cells in a culture medium constituted by only inorganic nitrogen sources such as $NaNO_3$ and $(NH_4)_5[Fe(C_6H_4O_7)_2]$. In this condition, the exponential phase was of 16 days reaching the highest maximum biomass concentration (X_m) values (1970 ± 231 mg·L^{-1}). In the mixotrophic cultivation using 0.25% CSL, the exponential growth phase began after 8 days of cultivation and obtained a X_m value of 1625 ± 207 mg·L^{-1} (Figure 1), and when the CLS was increased to 0.50%, slow cell growth was observed during 15 days, reaching the lowest X_m of 936 ± 82.8 mg·L^{-1}. In the CSL concentration higher than 0.50%, no *T. obliquus* cell growth was observed. Similar results were observed in the mixotrophic cultivation of *A. platensis* and *D. tertiolecta* using CSL concentrations above 0.6% and 1.0%, which inhibited cell growth [8,9].

CSL concentration also influenced the cell growth kinetic parameters. As shown in Table 1, the biomass productivity (P_x = 130 ± 12.8 mg·L^{-1}day^{-1}) and specific growth rate (μ_{max} = 0.17 ± 0.00 day^{-1}) in the mixotrophic cultivation using 0.25% CSL was higher than those cultivated with 0.50% CSL (P_x = 93.4 ± 10.9 mg·L^{-1}day^{-1}; μ_{max} = 0.12 ± 0.00 day^{-1}). These results showed that higher CSL concentrations (>0.50%) in the mixotrophic cultivation of *T. obliquus* decrease the P_x and μ_{max} values probably due to stress provoked by the excess of nitrogen [28,29]. CSL is rich in protein content (420 mg·g^{-1}) and the main amino acids available are arginine (44.30 mg·g^{-1}), alanine (35.70 mg·g^{-1}), and glutamic acid (42.00 mg·g^{-1}), showing that CSL is a potential organic N-source [30,31]. By the way, CSL has been considered as a low-cost material for the microbial production of enzymes [18,32,33], and its effects on the fibrinolytic enzyme production of *T. obliquus* has not yet been studied. The highest biomass productivity (130 ± 12.8 mg·L^{-1}day^{-1}) was obtained in the cultivation using 0.25% CSL, which was selected for further steps.

3.2. Protease and Fibrinolytic Productions

The cell growth condition has an important influence on the production of microbial enzymes. As shown in Table 1, *T. obliquus* produced a high amount of protease enzyme when cultivated under mixotrophic conditions using 0.25% CSL (84.7 U·mg^{-1}), followed by the autotrophic (12.5 U·mg^{-1}) and mixotrophic 0.50% CSL (5.85 U·mg^{-1}) conditions. Moreover, the protease activity of *T. obliquus* cultivated on 0.25% CSL is higher than those produced by different marine algae, such as *Ulva lactuca* (6.55–7.33 U·mg^{-1}), *Ulva*

fasciata (8.00 U·mg^{-1}), *Enteromorpha* sp. (6.74–9.60 U·mg^{-1}), and *Chaetomorpha antenna* (9.40 U·mg^{-1}) [34].

Table 1. Cultivation parameters and enzymatic activities of *T. obliquus* cultivated in different growth conditions.

Growth Conditions	X_m (mg·L^{-1})	P_x (mg·L^{-1}·day^{-1})	μ_{max} (day^{-1})	Methods	Total Protein (mg·mL^{-1})	Protease Activity (U·mg^{-1})	Fibrinolytic Activity (U·mg^{-1})
Autotrophic	1970 ± 231 ^{a}	112 ± 13.5 ^{a}	0.19 ± 0.03 ^{a}	Homogenization	0.93 ± 0.00 ^{a}	12.5 ± 1.35 ^{a}	430 ± 40.2 ^{a}
				Sonication	2.99 ± 0.50 ^{b}	4.50 ± 0.40 ^{a}	149 ± 3.8 ^{b}
Mixotrophic (CSL 0.25%)	1625 ± 207 ^{a}	130 ± 12.8 ^{a}	0.17 ± 0.00 ^{a}	Homogenization	0.86 ± 0.00 ^{a}	12.5 ± 2.94 ^{a}	391 ± 40.0 ^{a}
				Sonication	3.32 ± 0.22 ^{b}	84.7 ± 3.51 ^{b}	243 ± 11.5 ^{c}
Mixotrophic (CSL 0.50%)	936 ± 82.8 ^{b}	93.4 ± 10.9 ^{a}	0.12 ± 0.00 ^{a}	Homogenization	2.90 ± 0.09 ^{b}	4.64 ± 3.06 ^{a}	130 ± 1.0 ^{b}
				Sonication	2.76 ± 0.14 ^{b}	5.85 ± 3.68 ^{a}	135 ± 7.0 ^{b}

Maximum cell concentration (X_m), biomass productivity (P_x), specific growth rate (μ_{max}). Data expressed as means ± standard deviations of duplicate experiments. ^{a,b,c} Different superscript letters indicate statistically significant differences ($p < 0.05$).

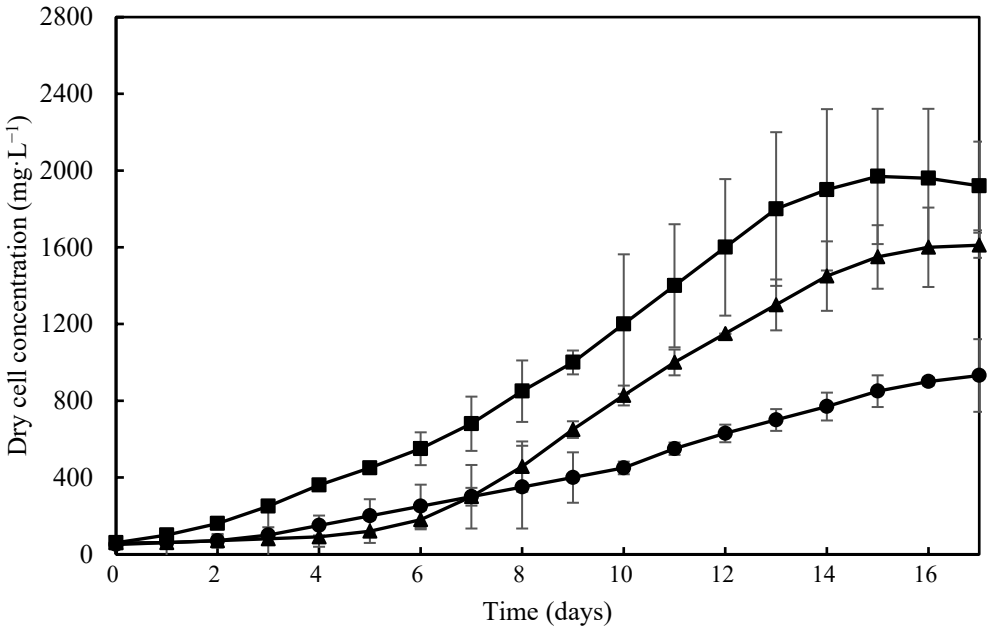

Figure 1. Growth profiles of *T. obliquus* cultivated autotrophically and mixotrophically under different concentrations of corn steep liquor: Autotrophic (■), mixotrophic 0.25% (▲), and mixotrophic 0.50% (●).

No significant difference in fibrinolytic activities was observed between autotrophic (430 ± 40.2 U·mg^{-1}) and mixotrophic using the 0.25% CSL (391 ± 40.0 U·mg^{-1}) cultivations, which were higher than those using 0.50% CSL (135 ± 7.0 U·mg^{-1}). The results showed that high CSL concentrations inhibit protease and fibrinolytic enzyme productions. Fibrinolytic enzyme production from the autotrophic and 0.25% CSL mixotrophic were higher than those obtained from the other photosynthetic microorganisms, such as *A. platensis* (268 ± 10.7 U·mg^{-1}) and *C. vulgaris* (302 ± 37.5 U·mg^{-1}) [7,8]. It is well known that the biochemical composition of the microalgae biomass, e.g., enzyme production, depends on the culture conditions such as the medium composition [35]. Then, the highest enzyme activities were obtained using 0.25% CSL, which also enhanced the enzyme production by *A. platensis* [8]. On the other hand, the cultivation with a higher CSL concentration (≥0.50%) decreases enzyme production, since a high concentration of some nutrients, such as nitrogen, might affect the biomass [36].

3.3. Effect of Extraction Methods on the Enzymatic Activities

The extraction methods influence enzyme activity. The extraction of enzymes was evaluated using the homogenization and sonication methods. Homogenization was the most efficient method to extract protease (12.5 ± 1.35 U·mg^{-1}) and fibrinolytic enzymes (430 ± 40.2 U·mg^{-1}) from the autotrophic cultivation, while the sonication method decreased the protease and fibrinolytic activities to 4.50 ± 0.40 and 149 ± 3.8 U·mg^{-1}, respectively (Table 1). Similar results were observed in the *T. obliquus* extracts from the mixotrophic cultures using 0.25% CSL, which also showed higher protease (12.5 ± 2.94 U·mg^{-1}) and fibrinolytic (391 ± 40.0 U·mg^{-1}) activities using the homogenization method when compared to the sonication methods. These data were similar to those obtained by Silva et al. [9] which showed that the fibrinolytic activity from *D. tertiolecta* was 819 and 422 U·mg^{-1} using homogenization and sonication, respectively. This can be explained by the possible enzyme denaturation caused by the prolonged sonication time, high temperature, or elevated frequency, as reported by Sukor et al. [37] and Ranjha et al. [38]. Then, these results showed that homogenization is more effective in the extraction of the protease and fibrinolytic enzymes from *T. obliquus*.

Therefore, according to Table 1, the mixotrophic cultivation using 0.25% CSL was selected for further steps due to its higher biomass productivity and significantly high enzyme production. Moreover, the extract from the 0.25% CSL cultivation obtained via the homogenization method showed higher fibrinolytic activity compared to the sonication method; thus, this extract was selected for the purification steps.

3.4. Effect of Precipitation Methods on the Enzymatic Activities

The *T. obliquus* extract-rich protein was precipitated using acetone or ammonium sulfate in two fractions of saturation (0–40% and 40–70%). Both 0–40% and 40–70% ammonium sulfate fractions showed similar protease activity (655 ± 69.3 and 623 ± 192 U·mg^{-1}, respectively), which was higher compared to the acetone precipitation (206 ± 17.5 U·mg^{-1}). Then, the ammonium sulfate fractions are more advantageous for the protease activity applications. However, although the 0–40% ammonium sulfate fraction exhibited the highest fibrinolytic activity (625 ± 20.2 U·mg^{-1}), the acetone fraction showed better performance due to its potential fibrinolytic activity (567 ± 49.3 U·mg^{-1}) and the highest enzyme yield of 80.3%. These results are similar to those reported by Barros et al. [8] and Silva et al. [9] for the fibrinolytic enzymes from *A. platensis* and *D. tertiolecta*, which showed fibrinolytic activity of 256 U·mg^{-1} (yield = 53.8%) and 435 U·mg^{-1} (yield = 37%), respectively.

Taking into account that the fibrinolytic activity measures the enzyme capacity of degrading fibrin specifically, and the acetone fraction showed the highest recovery yield, this fraction was considered more advantageous to be studied for thrombosis therapy purposes. Acetone is listed among the Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) since toxicological and medical studies show no adverse effects on human health [39]. Additionally, the use of acetone for precipitation includes some advantages such as simple-step extraction, less cost, and less time consumed [40,41]. Therefore, acetone was selected as the most advantageous precipitating agent to obtain the fibrinolytic enzyme from *T. obliquus*.

3.5. Fibrinolytic Activity in Fibrin Plate

Figure 2 shows a qualitative assessment of the fibrinolytic activity from *T. obliquus* by the fibrin plate method. The cell extract from *T. obliquus* cultivated in 0.25% CSL showed a high clear zone (82 mm^2) when compared to the cell extract obtained from the cell extracts cultivated autotrophically (69 mm^2) or mixotrophically with 0.50% CSL (69 mm^2) (Figure 2A), confirming that the 0.25% CSL cultivation has higher fibrinolytic production by this qualitative analysis. These values are higher than those of the fibrinolytic enzymes from *Bionectria* sp. strains, which ranged from 21.9 to 66.7 mm^2 [42].

The fibrinolytic activities of protein precipitated by different precipitating agents are shown in Figure 2B. Both the 0–40% ammonium sulfate and acetone fractions exhibited a clear zone of fibrin degradation around the well after 48 h, as shown in Figure 2B (letters g and h) and Table 2. On the other hand, the 40–70% ammonium sulfate fraction did not show a clear zone area of hydrolysis (Figure 2B; letter e).

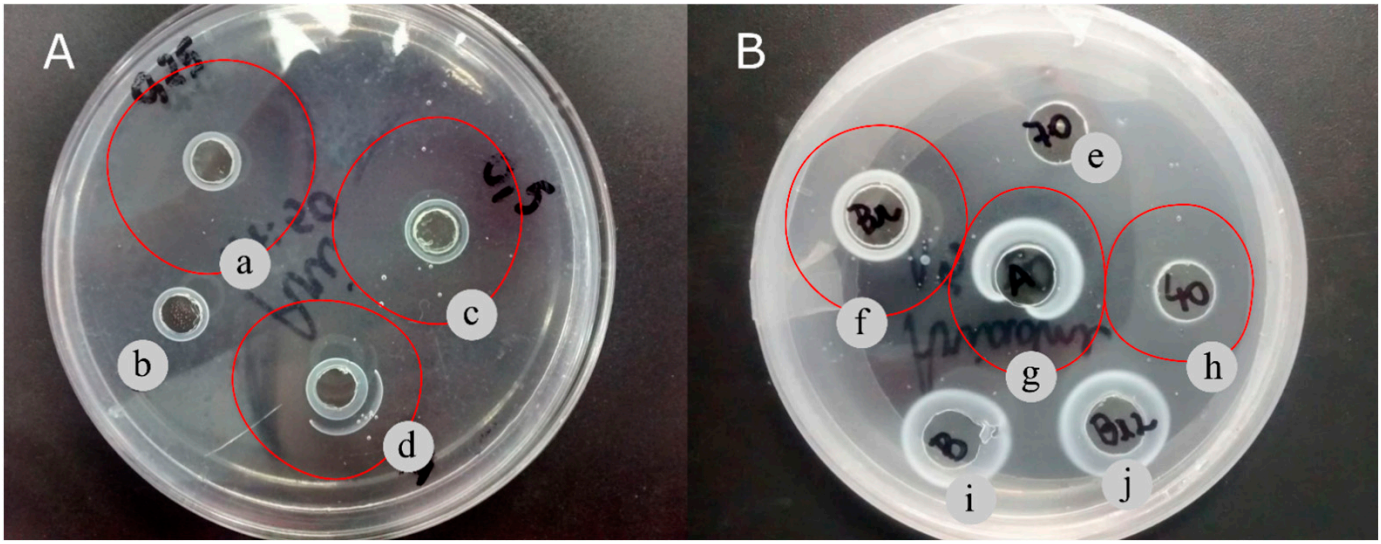

Figure 2. Fibrinolytic activity of *T. obliquus* by fibrin plate method. (**A**) Fibrinolytic activity of homogenized extracts obtained by 0.25% CSL mixotrophic cultivation (a), Tris-HCl buffer control (b), 0.5% CSL mixotrophic cultivation (c), and autotrophic cultivation (d). (**B**) Fibrinolytic activity of 40–70% ammonium sulfate precipitate (e), cell extract (f), acetone precipitate (g), 0–40% ammonium sulfate (h), Tris-HCl buffer control (i), and cell extract extracted twice (j) after 48 h.

Table 2. Comparison of different precipitating agents for precipitation of the homogenized cell extract from *T. obliquus* cultivated in 0.25% CSL.

Precipitating Agents	Volume (mL)	Total Protein (mg)	Total Protease Activity (U)	Specific Protease Activity (U·mg^{-1})	P.F	Yield (%)	Total Fibrinolytic Activity (U)	Specific Fibrinolytic Activity (U·mg^{-1})	P.F	Yield (%)
Cell extract	50	23.3 ± 1.2 ^{a}	4740 ± 1039 ^{a}	203 ± 56.3 ^{a}		100	11,520 ± 1420 ^{a}	494 ± 86.1 ^{a}		100
Ammonium sulfate (0–40%)	40	4.80 ± 0.00 ^{b}	3144 ± 332 ^{b}	655 ± 69.3 ^{b}	3.22	66.3	3000 ± 97 ^{b}	625 ± 20.2 ^{a,b}	1.26	26.0
Ammonium sulfate (40–70%)	40	5.20 ± 1.38 ^{b}	3240 ± 72 ^{b}	623 ± 192 ^{b}	3.06	68.3	2448 ± 0 ^{b}	470 ± 157 ^{b}	0.95	21.7
Acetone	40	16.4 ± 0.9 ^{c}	3384 ± 125 ^{c}	206 ± 17.5 ^{a}	1.01	71.3	9312 ± 396 ^{c}	567 ± 49.3 ^{a,b}	1.14	80.3

Data expressed as means ± standard deviations of duplicate experiments. ^{a,b,c} Different superscript letters indicate statistically significant differences ($p < 0.05$).

3.6. Enzyme Purification

The fibrinolytic enzyme from *T. obliquus* was purified using a combination of acetone precipitation and the DEAE-Sephadex ion exchange column. The chromatogram shown in Figure 3 exhibits a single peak (fractions 4–12). These protein fractions were pooled and concentrated, showing a fibrinolytic activity of 1221 ± 31 U·mg^{-1}. After purification via DEAE-Sephadex, the fibrinolytic enzyme was 2.46-fold purified with a yield of 8.90%, which is relative to that of the cell extract (Table 3). In general, the activity of the purified fibrinolytic enzyme from *T. obliquus* was significantly higher than those obtained by Silva et al. [9] for the fibrinolytic enzyme from *D. tertiolecta*, which showed fibrinolytic activity of 670 U·mg^{-1}. Similarly, the fibrinolytic activity of the fibrinolytic enzyme from *T. obliquus* is also higher compared to diverse macroalgal species, such as *Costaria costata*

(915 U·mg^{-1}), *Codium divaricatum* (6.3 U·mg^{-1}), *Codium fragile* (61.5 U·mg^{-1}), and *Ulva pertusa* (295 U·mg^{-1}), and also various bacterial species such as *Bacillus flexus* (315 U·mg^{-1}), *Bacillus velezensis* BS2 (131 U·mg^{-1}), *Bacillus subtilis* HQS-3 (30.0 U·mg^{-1}), and *Bacillus subtilis* ICTF-1 (280 U·mg^{-1}) [23,43–46].

Table 3. Steps of purification of fibrinolytic enzyme from *T. obliquus*.

Purification Step	Volume (mL)	Total Protein (mg)	Total Protease Activity (U)	Specific Protease Activity (U·mg^{-1})	P.F	Yield (%)	Total Fibrinolytic Activity (U)	Specific Fibrinolytic Activity (U·mg^{-1})	P.F	Yield (%)
Cell extract	50	23.3 ± 1.2 ^{a}	4740 ± 1039 ^{a}	203 ± 56.3 ^{a}		100	11,520 ± 1420 ^{a}	494 ± 86.1 ^{a}		100
Acetone precipitation	40	16.4 ± 0.9 ^{b}	3384 ± 125 ^{a}	206 ± 17.5 ^{a}	1.01	71.3	9312 ± 396 ^{b}	567 ± 49.3 ^{b}	1.14	80.8
DEAE-Sephadex	4.5	0.84 ± 0.02 ^{c}	297 ± 4.2 ^{b}	353 ± 12.4 ^{b}	1.73	6.26	1026 ± 0 ^{c}	1221 ± 31 ^{c}	2.47	8.90

Data expressed as means ± standard deviations of duplicate experiments. ^{a,b,c} Different superscript letters indicate statistically significant differences ($p < 0.05$).

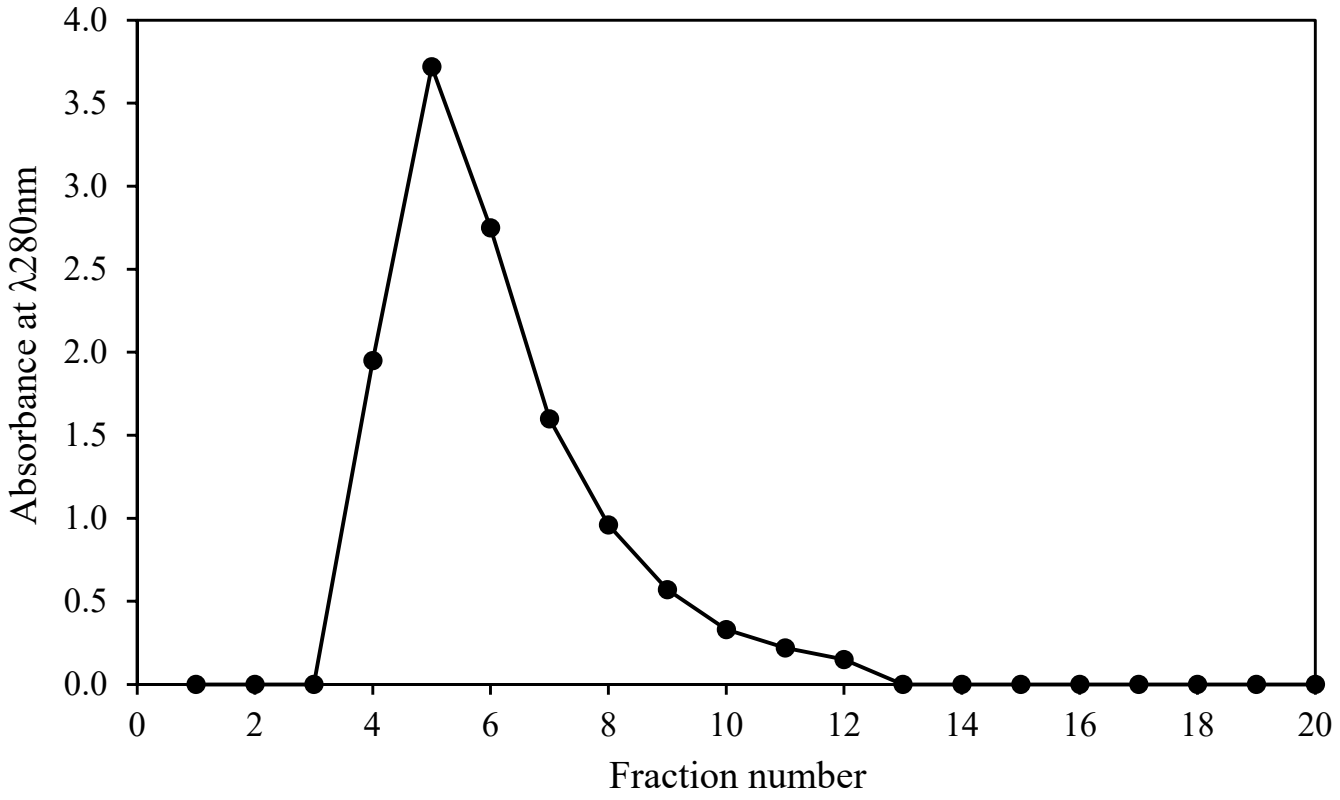

Figure 3. Chromatogram of fibrinolytic enzyme from *T. obliquus* on the DEAE-Sephadex column.

Briefly, the extracts obtained by the homogenization method were purified using acetone precipitation and the DEAE-Sephadex chromatography, showing potential fibrinolytic activity of 1221 ± 31 U·mg^{-1} (Table 3). The same sample was also submitted to the SDS-PAGE analysis.

SDS-PAGE showed one protein band with a molecular mass of probably 97 kDa (Figure 4). This is higher than that exhibited by the other *T. obliquus* proteins reported by Silva et al. [21] and Heide et al. [47] that have a molecular mass of 78 and 12 kDa, respectively. Additionally, the molecular mass of the fibrinolytic enzymes obtained from the other microalgae species, including *A. platensis* (72 kDa), *C. vulgaris* (45 kDa), and *D. tertiolecta* (10 kDa), is also lower than the fibrinolytic enzyme from *T. obliquus* [7–9]. These results show that this is a different protein from those reported previously.

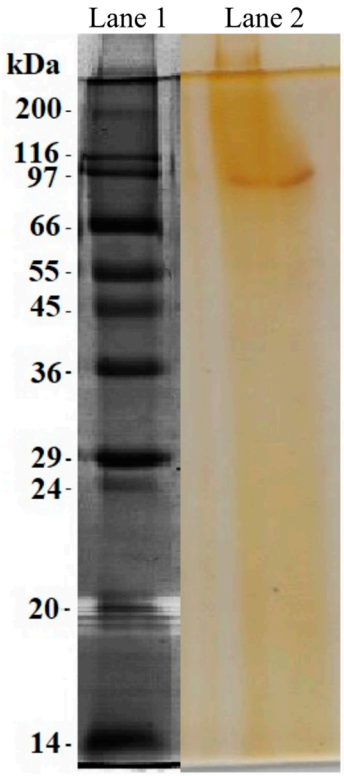

Figure 4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of fibrinolytic enzyme purified by DEAE-Sephadex column. Lane 1, Molecular mass marker; Lane 2, purified enzyme.

4. Conclusions

In the present study, it was possible to extract and purify an enzyme from *T. obliquus* microalgae with a specific activity of 1221 $\pm$ 31 U·mg^{-1}. The mixotrophic cultivation using an inexpensive and advantageous agro-industrial by-product (0.25% CSL) showed higher growth kinetic parameters and fibrinolytic production. Additionally, cell extraction via homogenization had the highest fibrinolytic activity, while the protein precipitation with acetone exhibited the highest recovery yield. In general, these methods are considered simple, efficient, less costly, less time-consuming, and are recognized as safe for human health, which can facilitate this enzyme production as well as its purification. Future research may continue to investigate the enzyme biochemical and biological profile as well as its mechanism of action for thrombolytic activity. Finally, these results can conclude that the fibrinolytic enzyme from *T. obliquus* has wide potential for industrial application besides its promising effects as an alternative to thrombolytic therapy.

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