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MICROBIOLOGY

Cost-effective fibrinolytic enzyme production by microalga *Dunaliella tertiolecta* using medium supplemented with corn steep liquor

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Abstract: A fibrinolytic enzyme from the microalga *Dunaliella tertiolecta* was produced under mixotrophic conditions using different corn steep liquor (CSL) concentrations (0 ≤ CLS ≤ 0.75%), purified using a combination of salting out and ion-exchange chromatography, and then biochemical characterized. Cultivation of this microalga using 0.5% CSL led to the highest maximum cell concentration (1.960±0.010 mg L^{-1}) and cell productivity (0.140g L^{-1} day^{-1}), besides a high fibrinolytic activity of the extract obtained by the homogenization method (102 ±1 U mL^{-1}). The enzyme extracted from the microalgal biomass was 5-fold purified with a 20% yield and was found to have a specific activity of 670 U mg^{-1}. The enzyme, whose molecular weight determined by fibrin zymography was 10 kDa, was shown to be stable at pH 3.0–9.0 and up to 70°C with optimal pH and temperature values of 8.0 and 50°C, respectively. When compared to other fibrinolytic enzymes, this protease stood out for its high fibrinolytic activity, which was enhanced by Fe^{2+}, inhibited by Zn^{2+}, Cu^{2+}, Mg^{2+}, and Ca^{2+}, and strongly inhibited by phenylmethylsulfonyl fluoride, suggesting that it belongs to the serine metalloprotease family. Moreover, thanks to its thermal stability, the enzyme may be easily preserved and activated under high-temperature conditions.

Key words: biochemical characterization, corn steep liquor, extraction, production, protease, purification.

INTRODUCTION

Accumulation of fibrin in the blood vessels can lead to thrombosis, resulting in cardiovascular events such as acute myocardial infarction, ischemic heart disease, high blood pressure, and stroke, which are the main causes of death worldwide (WHO 2021). Fibrinolytic enzymes can hydrolyze insoluble fibrin fiber, lyse the thrombus, and restore blood flow to the area of ischemia (Huang et al. 2013). In addition, fibrinolytic therapy has shown advantages in treating patients with acute respiratory distress syndrome, lung lesions, and hypoxemia caused by COVID-19 infection (Barrett et al. 2020, Wu et al. 2020).

Fibrinolytic enzymes and plasminogen activators, such as tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), and streptokinase, have been purified from several sources and used as thrombolytic agents (Mihara et al. 1991). Human-derived activators are generally safe but expensive, while others exhibit undesired side effects, low specificity for fibrin, short half-life, and relatively high prices (Wang et al. 2006). Therefore, the search for new fibrinolytic proteases is still necessary

(Montriwong et al. 2012, Mander et al. 2011, Silva et al. 2022).

Thrombolytic agents have been obtained from various sources such as fermented food products, food-grade microorganisms, insects, earthworms, snake venom, and mushrooms (Wang et al. 2005, 2006, Ahn et al. 2003, Di-Simone et al. 2005, Deepak et al. 2008). However, microbial fibrinolytic enzymes are considered the main alternative for thrombosis treatment (Lee et al. 2005). So far, fibrinolytic enzymes from marine microorganisms such as microalgae remain largely unexplored, which suggests strong potential for the discovery of new enzymes with unique properties for biotechnological application (Lee et al. 2010).

Marine microorganisms can produce enzymes with advantages over the traditional ones such as non-vulnerability to contaminants when grown under controlled conditions. In addition, halotolerant marine organisms produce metabolites that may be applied as an alternative for therapeutic purposes (Rasmussen & Morrissey 2007, Mahajan et al. 2012). Sabu (2003) reported that the saline nature of seawater is chemically closer to the human blood plasma and this could provide enzymes with little or no toxicity and side effects when used for therapeutic applications in humans. Similarly, Vaitkevicius-Antão et al. (2022) reported that aqueous extracts of *A. platensis* and *D. tertiolecta* showed low toxicity to human peripheral blood mononuclear cells, Velayutham et al. (2022) observed that the *O-acetyltransferase* from *A. platensis* had no cytotoxic effect *in vitro* or against zebrafish larvae, and Saggu et al. (2019) purified a protease from *Microbacterium* strain with no cytotoxicity to epidermoid cells.

Fibrinolytic enzymes from different marine algae belonging to the genus *Codium* spp. (Matsubara et al. 1998, 1999, 2000) and the cyanobacterium *A. platensis* (Barros et al. 2020) were purified and investigated, while Silva et al. (2018) extracted a fibrinolytic protease from the microalga *Chlorella vulgaris*. However, there are few works on fibrinolytic enzymes from other algae. Particularly, *Dunaliella* is a marine microalgal genus poorly exploited biotechnologically, which until now has been used especially to recover carotenoids, glycerol, and proteins. Nevertheless, several biomolecules isolated from *Dunaliella* spp. have shown pharmaceutical activities, including antihypertensive, bronchodilator, antiserotonin, polysynaptic block, analgesic, muscle relaxant, and anti-edema activities (Borowitzka 1995, Tafreshi & Shariati 2009). *Dunaliella* genus has several advantages in the synthesis of new high-added value compounds (Zanette et al. 2019, Chen et al. 2011). Its cultivation in high salinity media provides a remarkable degree of selectivity against most contaminating organisms, reducing the production costs of these new compounds. *Dunaliella tertiolecta* is simple to cultivate, does not clump on the surface, and is highly salt tolerant, which might be useful in large-scale outdoor cultivation (Elenkov et al. 1996). This microalga has several advantages over other microorganisms in industrial enzyme production due to its minimal nutritional requirement (natural or artificial light, CO_2, water, nitrogen source, and some salts), which reduces costs (Brasil et al. 2017). Furthermore, it can also be cultivated under mixotrophic conditions (Chavoshi & Shariati 2019).

Microalgae mixotrophic cultivation has been shown to ensure higher cell density than autotrophic ones (Silva et al. 2018, Barros et al. 2020). However, the cost of the organic carbon sources (usually in the form of glucose or acetate) is high compared to those of all other added nutrients. To obtain a reduction in

production costs, supplementation with organic carbon/nitrogen sources from by-products or industrial waste has been used (Melo et al. 2018, Pereira et al. 2019). Corn steep liquor (CSL), a byproduct of corn processing, is widely used in the cultivation of microorganisms to produce several biotechnological products (Silva et al. 2018, Barros et al. 2020, Ernandes et al. 2013, Tian et al. 2016).

Based on this background, the mixotrophic cultivation of *D. tertiolecta* using CSL has been investigated in this study for fibrinolytic protease production. Even though *D. tertiolecta* has already been studied in mixotrophic cultivation, this is the first report on CSL cultivation and fibrinolytic enzyme production by this microalga. In addition, this enzyme was purified and biochemically characterized for future applications in the pharmaceutical industry.

MATERIALS AND METHODS

Microalga and culture conditions

Dunaliella tertiolecta (UTEX LB999) was obtained from the UTEX (University of Texas, Austin) and cultivated under either autotrophic or mixotrophic conditions. The F/2 medium formulated by Guillard & Ryther (1962) was used as standard medium for autotrophic cultivations, while it was supplemented with 0.25%, 0.50%, 0.75%, or 1.00% (v/v) of previously treated corn steep liquor (CSL) (CornProducts Brazil, Pernambuco, Brazil) for the mixotrophic ones (Liggett & Koffler 1948). The microalga was inoculated with an initial biomass concentration of 50 mg L^{-1} and grown at a temperature of 30 ± 2 °C, with continuous light intensity of 40 ± 4 µmol photons m^{-2} s^{-1}, under constant aeration, until the end of the exponential growth phase.

Cell growth and cell productivity determination

Cell concentration was determined by measuring the optical density (OD) at 680 nm (Chen et al. 2011) with a UV-Vis spectrophotometer (Agilent 8453, Santa Clara, CA, USA) and expressed in grams of dried biomass per liter of medium (g L^{-1}) through a calibration curve relating OD to dry biomass weight. Biomass productivity (P_x; g L^{-1} day^{-1}) was estimated by the equation:

$$P_x = \frac{(Xm - X_0)}{t} \quad \text{(Eq.1)}$$

where X_m (g L^{-1}) and X_0 (g L^{-1}) were the concentrations of biomass at the end and the beginning of cultivation, and t was the duration of cultivation.

Enzyme extraction

The freeze-dried biomass (50 mg mL^{-1}) was resuspended in 100 mM phosphate buffered saline (PBS), pH 7.0, and submitted to two different extraction methods: (1) sonication using a sonicator (Bandelin Sonopuls HD 2070, Berlin, Germany) with 20 pulses of 1 min in an ice bath, and (2) homogenization by constant stirring for 40 minutes in an ice bath (Matsubara et al. 2000). The extracts were centrifuged at 10,000 × *g* for 10 min at 4°C, and the cell-free supernatants, denominated crude extracts, were used for further analyses.

Purification of fibrinolytic enzyme

The crude extracts were precipitated by slow addition of a pre-cooled 80% acetone solution at 4ºC (Manni et al. 2010, Crowell et al. 2013). The precipitated proteins were collected by centrifugation (8,000 × *g* for 30 min at 4 °C), re-dissolved in 0.1 M Tris-HCl buffer, pH 7.0, and submitted to anion exchange chromatography using a DEAE-Sephadex® A-25 (7.5 x 3 cm)

column (Sigma-Aldrich, St. Louis, MO, USA). The sample was eluted with the same buffer using a discontinuous gradient of 0.0, 0.3, and 0.5 M NaCl at a flow rate of 2 mL min^{-1}, and fractions of 8 mL were collected in each tube. The elution profile of proteins was determined by absorbance at 280 nm. The active fractions were pooled, dialyzed, and subjected to gel filtration in a Sephadex G-25® (1.0 cm × 80 cm) column (Sigma-Aldrich) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.0). The protein eluted at a flow rate of 1 mL min^{-1} was determined at 280 nm. The peak was pooled, lyophilized, and resuspended in a small volume to determine total protein concentration and fibrinolytic activity.

High-performance liquid chromatography (HPLC) analysis

For HPLC analysis, an aliquot of the purified enzyme obtained by gel filtration column was diluted in 0.1% trifluoroacetic acid (TFA), injected, and analyzed in a C18 column (Beckman peptides, 150 x 5mm, 5 mm) on a reverse-phase using an Agilent 1200 series HPLC system (Agilent Technologies, Wokingham, UK) equipped with a UV detector. The mobile phase (flow rate of 0.7 mL min^{-1}) consisted of two solvents, namely 0.1% TFA in distilled water (Solvent A) and 90% acetonitrile (solvent B). A linear elution gradient (5 - 100%) of 90% acetonitrile was applied to reach an acetonitrile/TFA ratio of 100/0.1 (v/v) for 68 min. The elution was monitored at 215 nm.

Fibrinolytic activity

The fibrinolytic activity was evaluated using the spectrophotometric method described by Wang et al. (2011). Briefly, fibrin degradation was performed using 0.72% (w/v) fibrinogen from bovine plasma (dissolved in 0.15 M Tris-HCl buffer, pH 7.5) and 20 U mL^{-1} thrombin from bovine plasma (dissolved in 0.2 M sodium phosphate buffer, pH 7.0) to form a fibrin clot. The enzyme was added and incubated for 1 h at 37ºC. After stopping the reaction by the addition of 0.2 M trichloroacetic acid followed by centrifugation (8,000 × *g* for 10 min at 4 °C), the supernatant was collected and measured at 275 nm. One unit (U) of fibrin degradation activity was defined as the amount of enzyme able to cause a 0.01 absorbance increase per minute.

Dosage of total proteins

Total protein concentration was determined by the BCA reagent Kit (BCA™ Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) using bovine serum albumin as protein standard.

Determination of molecular weight by fibrin zymography

Fibrin zymography was performed according to Kim et al. (1988). A separating gel solution (12.0% w/v) was prepared in the presence of 0.12% bovine fibrinogen (w/v) and thrombin (7.5 U mL^{-1}). The enzyme samples were diluted in a zymogram sample buffer consisting of 0.5 M Tris-HCl (pH 6.8), 10% SDS, 20% glycerol, and 0.03% bromophenol blue, and applied to fibrin-copolymerized gel in a cold room (at 4 °C). After electrophoresis, the gel was soaked in 2.5% (v/v) Triton X-100 for 1 hour at room temperature (26 ± 0.5 °C). After washing the gel thrice in distilled water, it was incubated in the reaction buffer (0.1 M glycine, pH 8.4) at 37 ºC for 18 h, stained with Coomassie blue for 1 h, and then detained. The achromatic bands on the gel indicated regions of fibrinolytic enzyme on a blue background. The SDS-PAGE analysis with the 12% (w/v) separating gel solution as described elsewhere was used to determine the molecular weight of the fibrinolytic enzyme using a molecular weight marker (GE Healthcare 17044601, São Paulo, SP, Brazil) as a standard (Kim et al. 1998).

Effects of metal ions and protease inhibitors on fibrinolytic activity

The effects of metal ions and inhibitors on fibrinolytic activity were investigated by pre-incubating the purified enzyme with different salts at 5 mM concentration,namely $CaCl_2$, $CoCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$, or $FeSO_4$, or enzyme inhibitors, namely 1 mM pepstatin A,1 mM iodoacetic acid, 10 mM 2-mercaptoethanol (2-hydroxy1-ethanethiol, C_2H_6SO), 10 mM phenylmethylsulfonyl fluoride (PMSF, $C_7H_7FO_2S$), 10 mM ethylenediaminetetraacetic acid (EDTA), for 60 min at 37º C. Enzyme activity in the absence of metal ions or protease inhibitors was assumed to be 100%.

Effect of temperature on fibrinolytic activity and stability

The effect of temperature on enzyme activity was evaluated from 20°C to 90°C during the reaction in 0.1 M Tris–HCl buffer (pH 8.0) using fibrin as a substrate. The thermal stability of the enzyme was determined by incubating it for 30 min at different temperatures. The enzyme activity under standard assay conditions at pH 8.0 and 37°C was assumed to be 100% (Mahajan et al. 2012, Krishnamurthy et al. 2018, Narasimhan et al. 2018).

Effect of pH on fibrinolytic activity and stability

The optimal pH for the enzyme fibrinolytic activity was determined in a pH range of 3.0 – 11.0 using different 0.2 M buffers, namely sodium citrate (pH 3.0–5.0), sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 8.0–9.0), and carbonate-bicarbonate (pH 10.0–11.0). For this purpose, the enzyme was separately incubated for 60 min at 37°C in each of the different buffers. Enzyme activity under standard assay conditions at pH 8.0 and 37°C was assumed to be 100% (Mahajan et al. 2012, Krishnamurthy et al. 2018).

Statistical analysis

Experiments and assays were performed in triplicate and analyzed by one-way analysis of variance (ANOVA). For all statistical analyses, *p*-values less than 0.05 were considered significant according to the Tukey's test.

RESULTS

In general, the mixotrophic cultivations performed statistically better than the autotrophic ones ($p < 0.05$, Fig. 1), showing mean values of X_m (1.943 ± 0.070 g L^{-1}) and P_X (0.138 g L^{-1} day^{-1}) about 3.5-fold higher (Table I). On the other hand, increasing the supplementation of CSL from 0.25 to 0.75% no increase in either X_m or P_X was observed, while at 1.0% there was even a decrease in both parameters (Table I).

To stepwise increase fibrinolytic activity, CSL-free basal F/2 medium and the same medium supplemented with CSL at different concentrations were tested. As shown in Table I, the fibrinolytic activity was lower in crude extract from *D. tertiolecta* cells cultivated in a CSL-free medium, regardless of the extraction method (Table I). The lowest fibrinolytic enzyme levels were, in fact, detected in the extracts obtained by either homogenization (118 U

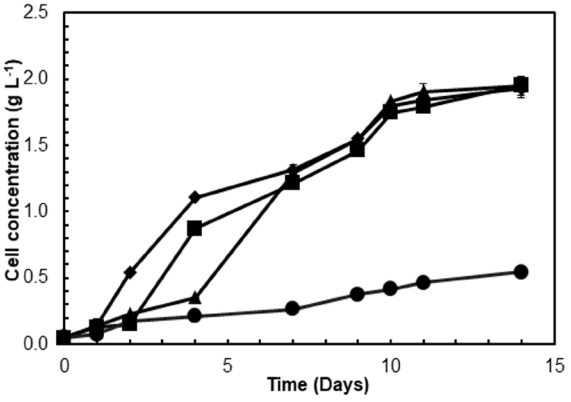

Figure 1. ***Dunaliella tertiolecta*** **growth in F/2 medium supplemented with corn steep liquor (CLS) at different concentrations. (◆) F/2 + 0.25% CLS; (■) F/2 + 0.5% CLS; (▲) F/2 + 0.75% CLS; (●) autotrophic condition.**

mL^{-1}) or sonication (184 U mL^{-1}) of biomass grown autotrophically in basal medium, but the CSL addition up to 0.5% increased them by almost 8.5 and 3.5 times, respectively.

The fibrinolytic enzyme was purified from the cell extracts of *D. tertiolecta* by a three-step procedure consisting of acetone precipitation followed by anion-exchange chromatography on DEAE-Sephadex and exclusion chromatography on Sephadex G-25. The results of each purification step are gathered in Table II. The crude extract containing 153 mg of proteins showed a specific activity of 134 U mg^{-1}. After the first step of acetone precipitation, which resulted in a 3.2-fold increase in the specific activity, the enzyme solution was loaded on a DEAE-Sephadex column, which yielded three fractions (peak 1, 2, and 3) according to the increase in the ionic strength (Fig. 2a). The first peak, eluted without the addition of NaCl, showed high absorbance at 280 nm but no fibrinolytic activity, meaning that the enzyme of interest remained bound to the anion-exchange resin. This attraction was satisfactorily reduced by modifying the ionic strength with the addition of 0.3 and 0.5 M NaCl, which made it possible to obtain peaks 2 and 3, respectively. Although both fractions showed absorbance at 280 nm, peak 2 had less than one-half of the fibrinolytic activity of peak 3 (670 U mg^{-1}). The peak 3 eluted at 0.5 M NaCl was purified 5.0-fold, which corresponds to a 20% recovery yield based on the crude extract (Table II).

The peak 3 collected from the DEAE-Sephadex column was concentrated by freeze-drying and submitted to molecular exclusion chromatography using a Sephadex G-25 column, which resulted in a single elution peak (Fig. 2b) with decreased specific activity (494 U mg^{-1}) and yield (0.8%), but with similar purity (4.7 times). When this peak was loaded on a reverse-phase HPLC system, only one peak was obtained (Fig. 2c), highlighting protein purification.

So, peak 3 from DEAE-Sephadex chromatography containing the enzyme was submitted to fibrin zymography that showed a homogeneous profile and a clear zone in substrate gel indicating a strong activity of the enzyme (Fig. 3), for which it was possible to estimate an apparent molecular weight of only about 10 kDa.

The results listed in Table III show that Zn^{2+} and Co^{2+} exerted only a slight inhibition on the fibrinolytic activity, unlike Cu^{2+}, $Ca^{2+,}$ and Mg^{2+} whose inhibiting effects compared to the control were as high as 54.1, 66.9 and 68.9%, respectively.

Table I. Effects of different concentrations of CLS and extraction method under fibrinolytic activity.

Culture medium	X_m (g L^{-1})[1]	P_x (g L^{-1} day^{-1})[2]	Fibrinolytic activity (U mL^{-1})	
			Homogenization	Sonication
f/2	0.545 ± 0.090^{a}	0.039^{a}	$118 \pm 49^{a,A}$	$184 \pm 20^{a,A}$
f/2 + 0.25% CLS	1.930 ± 0.070^{c}	0.137^{b}	$819 \pm 49^{b,B}$	$422 \pm 20^{b,B}$
f/2 + 0.50% CLS	1.960 ± 0.100^{c}	0.140^{b}	$1025 \pm 1^{d,D}$	$660 \pm 44^{c,D}$
f/2 + 0.75% CLS	1.952 ± 0.300^{c}	0.139^{b}	$625 \pm 15^{c,C}$	$500 \pm 28^{d,C}$
f/2 + 1.00% CLS	*	*	*	*

Values with the same superscript are not significantly different according to the Tukey test ($p < 0.05$). Lowercase letter compares among treatments and uppercase letter compares between extraction methods. [1]X_m: Maximum cell concentration; [2]P_x: Maximum cell productivity: *It was not cell growth.

On the other hand, the enzyme activity was strongly enhanced by Fe^{2+}, indicating that the enzyme is iron-dependent (Table III).

The effects of typical inhibitors of different enzyme families were also investigated (Table III). The fibrinolytic activity was entirely suppressed by PMSF, slightly reduced by 2-mercaptoethanol, and not statistically influenced by EDTA, iodoacetic acid, and Pepstatin A. Instead, the inhibitory effect of 2-mercaptoethanol was very weak.

As shown in Fig. 4a, the purified enzyme was active over a wide range of pH (3.0–9.0) with maximum activity and stability at pH 8.0. Particularly, the enzyme retained more than 60% of its initial activity for 60 min in the pH range 6.0–9.0, but above pH 9.0 it dropped sharply (Fig. 4a).

The enzyme was active between 20 and 70°C with residual activity above 70%. The highest catalytic performance and stability of the purified enzyme were both found at 50°C (Fig. 4b). The purified enzyme was especially stable in the temperature range of 20-50°C, within which it retained more than 80% of its initial activity after incubation for 30 min, thus showing good thermostability (Fig. 4b). On the other hand, it completely lost its activity at temperatures above 70°C (Fig. 4b).

DISCUSSION

The effect of corn steep liquor (CSL) concentration on *Dunaliella tertiolecta* maximum cell density (X_m), cell productivity (P_x), and fibrinolytic enzyme production was investigated under either autotrophic or mixotrophic conditions. *D. tertiolecta* grew more under mixotrophic conditions because CSL contains several organic compounds such as proteins (24%), carbohydrates (5.8%) and others (Joshi et al. 2018), which are capable of promoting microbial growth. These results agree with previous studies where CSL was shown to enhance the growth of the microalgae *Chlorella vulgaris* (Silva et al. 2018, Melo et al. 2018, Wang et al. 2011, Mirzaie et al. 2016) and *Tetraselmis suecia* (Cid et al. 1992) as well as the cyanobacterium *Arthrospira platensis* (Barros et al. 2020).

It is well known that mixotrophic conditions can allow microalgae to exploit both respiratory and photosynthetic metabolisms, increasing biomass productivity compared to photoautotrophic cultivations (Barros et al. 2020, Pereira et al. 2019, Mirzaie et al. 2016, Perez-Garcia et al. 2011).

Table II. Steps of purification of the fibrinolytic enzyme from *D. tertiolecta*.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Fold	Yield (%)
Crude extract	20	153	20736	134	1	100
Acetone	10	18.0	7839	435	3.2	37
DEAE-Sephadex (Peak 3)	40	6.30	4248	670	5.0	20
DEAE-Sephadex (Peak 2)	8.0	1.77	296	296	2.2	2.5
Sephadex G-25	1.5	0.35	178	494	4.7	0.8

Few studies have investigated the mixotrophic cultivation of *D. tertiolecta*, and no studies have done so in a medium supplemented with CSL. Chavoshi & Shariati (2019), using *D. salina* and glucose or acetate as carbon sources, observed that cell concentration was approximately 2.5-fold higher in mixotrophic culture than in the photoautotrophic one. Zanette et al. (2019) reported that *D. tertiolecta* biomass production was 8.57-fold higher in mixotrophic cultivation using lactose as a carbon source when compared to photoautotrophic culture.

As previously mentioned, such an increase in the fibrinolytic enzyme production induced by CSL, which is in agreement with the findings of previous reports (Silva et al. 2017, 2018, Barros et al. 2020, Melo et al. 2018) was likely due to the high contents of proteins, carbohydrates and fats in this byproduct, which can be used under mixotrophic conditions as carbon and nitrogen source (Joshi et al. 2018). On the other hand, the fibrinolytic enzyme production remarkably decreased at CSL concentrations higher than 0.5%, probably due to repression of protease synthesis caused by some amino acids present at high concentrations in the CSL-containing medium (Joshi et al. 2018), such as alanine, arginine, and leucine (Sharma & Singh 2016), and/or inhibition of fibrinolytic activity by salts such as $CaCl_2$ (550 mg L^{-1}) and $MgSO_4$ (583 mg L^{-1}) (Deng et al. 2018). The same negative effect was observed for fibrinolytic enzyme production by *C. vulgaris* and *A. platensis*, in the presence of CSL up to 0.9% and 0.2%, respectively (Barros et al. 2020, Silva et al. 2017).

The method used to disintegrate the cells and allow the extraction ideally should not affect the activity and properties of the enzyme (Taubert et al. 2000); however, this is practically impossible for intracellular enzymes like some fibrinolytic proteases, the release of which requires a lysis step (Kumar & Punekar 1997, Strigáčová et al. 2001). For this purpose, two different extraction techniques,

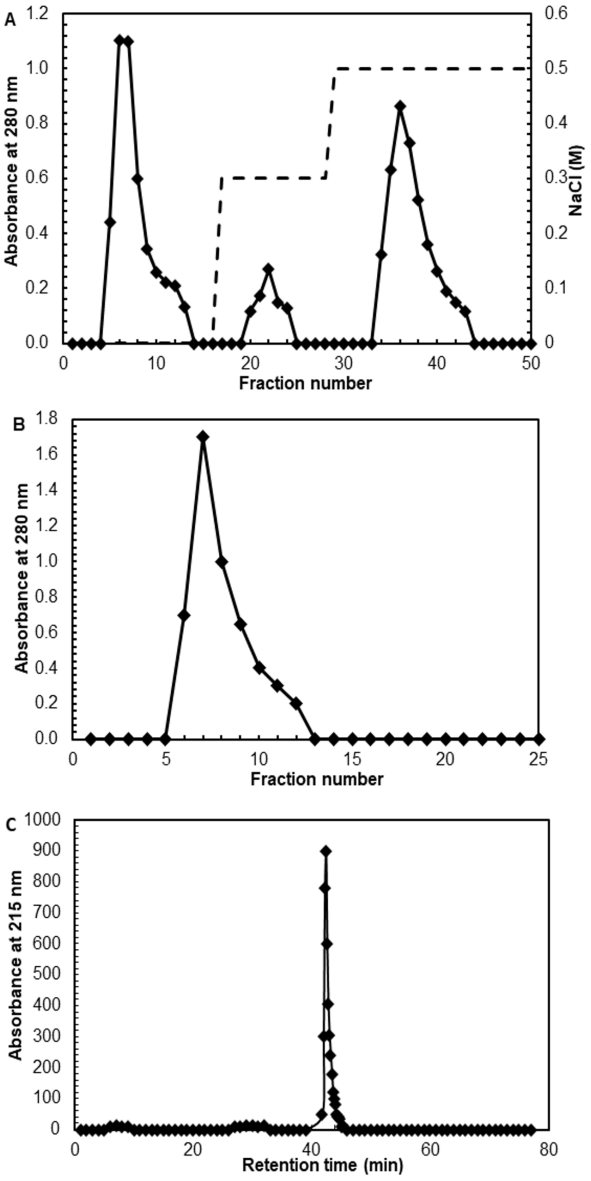

Figure 2. Purification of the fibrinolytic enzyme from *D. tertiolecta*using DEAE-Sephadex chromatography column. (●) ABS at 280 nm; (- - -) discontinuous gradient of NaCl (a); gel filtration on Sephadex G20 column eluted with 10 mM Tris-HCl buffer, showing the fractions (from 6 to 14) with fibrinolytic activity(b); HPLC analysis of the purified enzyme isolated from *Dunaliellatertiolecta*. The purified enzyme was analyzed using a reversed phase C18 column, and the isolated protein appeared as a single peak with a retention time of 43.2 min(c).

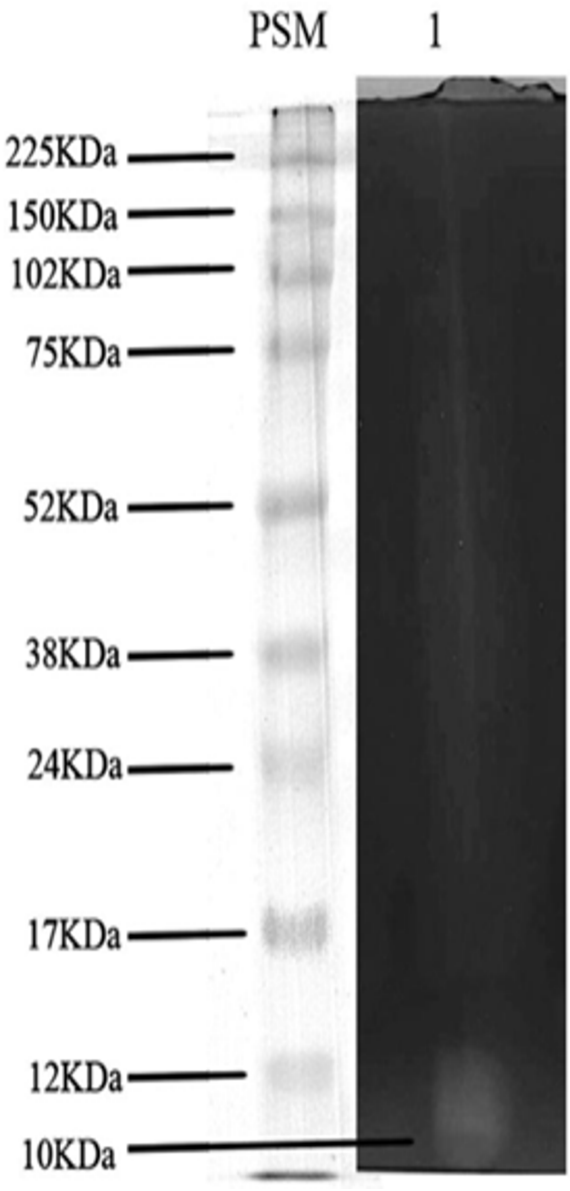

Figure 3. Fibrin zymography analysis. Lane PSM, protein size maker by SDS-PAGE analysis; Lane 1, fibrin zymography of the fibrinolytic enzyme purifiedby ion-exchange chromatography.

namely sonication, and homogenization were tested in this study. Regardless of culture conditions, the homogenization method allowed for higher values of fibrinolytic activity when compared to the sonication one, likely due to partial enzyme denaturation resulting from the shear forces and high temperatures generated during the ultrasonic treatment (Özbek& Ülgen, 2000). The results reported in the literature on microalgae disruption to extract their enzymes are rather scarce. Previous studies reported the use of homogenization to extract fibrinolytic proteases from the marine green alga *Codium divaricatum* (Matsubara et al. 2000) and the cyanobacteria *Anabaena fertilissima* (Sauer et al. 1989) and *A. platensis* (Barros et al. 2020). On the other hand, Zanette et al. (2019) observed higher efficiency of β-galactosidase extraction from *D. salina* by disrupting the cells by sonication rather than by glass bead milling. These findings suggest that the choice of the right cell-disruption process is dependent on cell properties such as the composition of the cell wall as well as the intracellular location of the enzyme (Sauer et al. 1989, Banerjee et al. 2013).

The maximum specific activity obtained in the present study (670U mg^{-1}) was significantly higher than those of fibrinolytic enzymes from the macroalgae *Codium fragile* (61.5 U mg^{-1}) (Choi et al. 2013) and *Codium divaricatum* (6.3 U mg^{-1}) (Matsubara et al. 2000), more than 60% higher than that of the fungus *Neurospora sitohila* (415.6 U mg^{-1}) (Deng et al 2018), all submitted to several chromatographic steps, but about 27% lower than the one of the macroalga *Costaria costata* (915.5 U mg^{-1}), which was purified using three chromatographic steps (Kim et al. 2013). A fibrinolytic enzyme from *C. vulgaris* was one-step purified using anion-exchange chromatography with a yield of 4.0% and a 2-fold overall purification (Silva et al. 2018).

Fibrinolytic enzymes with comparably small molecular weights have been reported for three members of the green algae genus *Codium* (17-48 kDa) (Sabu 2003, Matsubara et al. 1998, 1999) and different fungi and bacteria (12-18 kDa) (Mander et al. 2011, Cha et al. 2010, Xiao-Lan et al. 2005). This result is quite promising taking into account that small size proteins often exhibit low

Table III. Effects of metal ions and protease inhibitors under fibrinolytic activity.

Chemicals	Concentration	Relative activity (%)
Control	**None**	**100.0±7.5**
$CuSO_4$	**5mM**	**45.9±0.1***
$FeSO_4$	**5mM**	**178±9.0***
$ZnSO_4$	**5mM**	**79.1±0.3***
$CaCl_2$	**5mM**	**33.1±1.5***
$MgSO_4$	**5mM**	**31.3±3.0***
$CoCl_2$	**5mM**	**91.2±0.6**
Pepstatin A	**1mM**	**89.8±3.1**
Iodoacetic acid	1mM	92.9±1.6
2-mercaptoethanol	10mM	86.6±1.7*
EDTA	10mM	88.8±3.5
PMSF	10mM	0.00±0.3*

Data represents mean ± SD (n=3); *p<0.05 (significantly different values from controls; one-way ANOVA).

immunogenicity, thereby being relatively safe for human use (Krishnamurthy et al. 2018).

The effect of metal ions on the catalytic activity of fibrinolytic enzymes is important as blood is known to contain several metal ions (Krishnamurthy et al. 2018). Therefore, various metal ions were tested for their influence on the residual activity of the fibrinolytic enzyme after its incubation in the presence of each of them. Fibrinolytic activity was slightly inhibited by Zn^{2+} and Co^{2+} not influenced by Cu^{2+}, $Ca^{2+,}$ and Mg^{2+} and strongly enhanced by Fe^{2+}, indicating that the enzyme is iron-dependent. Similar results were observed for fibrinolytic enzymes from the fungus *Neurospora sitophila* and the cyanobacterium *A. fertilissima*, which were inhibited by Zn^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+} and Mg^{2+} (Deng et al. 2018) and by Cu^{2+} (Banerjee et al. 2013), respectively. Fe^{2+} addition can restore or even enhance the apoenzyme catalytic activity thanks to the flexible metal coordination geometries of iron, as already observed for purified fibrinolytic enzymes from *C. vulgaris* (Silva et al. 2018), *Mucor subtilissimus* UCP 1262 (Nascimento et al. 2015), and *Cordyceps militaris* (Cui et al. 2008) when incubated with Fe^{2+}. An analogous effect on the activity of a fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* has been reported for Mg^{2+}, Mn^{2+} or Zn^{2+} (Krishnamurthy et al. 2018).

The fibrinolytic activity was entirely suppressed by PMSF and slightly reduced by 2-mercaptoethanol. Since PMSF is a well-known inhibitor of serine proteases, which irreversibly sulfonates serine residues inside or near their active sites, its so strong inhibiting effect observed in this study suggests that the *D. tertiolecta* fibrinolytic enzyme belongs to this enzyme family. Fibrinolytic enzymes from different sources such as bacteria (Mahajan et al. 2012, Narasimhan et al. 2018, Taneja et al. 2019), fungi (Deng et al. 2018, Nascimento et al. 2016), macroalgae (Matsubara et al. 2000, Kim et al. 2013), and cyanobacteria (Choi et al. 2013) have been reported as serine protease.

On the other hand, the very weakt inhibitory effect of 2-mercaptoethanol, which is well known to reduce the activity of cysteine proteases by cleaving disulfide bonds or reacting with thiol groups of their active site, is in line with a previous observation that these groups may not participate in the catalytic reaction of fibrinolytic enzymes (Mahajan et al. 2012, Deng et al. 2018, Nascimento et al. 2016, Moon et al. 2014).

The pH of a solution can have several effects on the structure and activity of enzymes because it influences the state of ionization of acidic or basic amino acids, thus altering their tertiary structure, leading to their inactivation and reducing their activity (Vishwasrao & Ananthanarayan 2018). The purified enzyme was active over a wide range of pH with maximum activity and stability at pH 8.0. Such a value is in accordance with optimum alkaline conditions

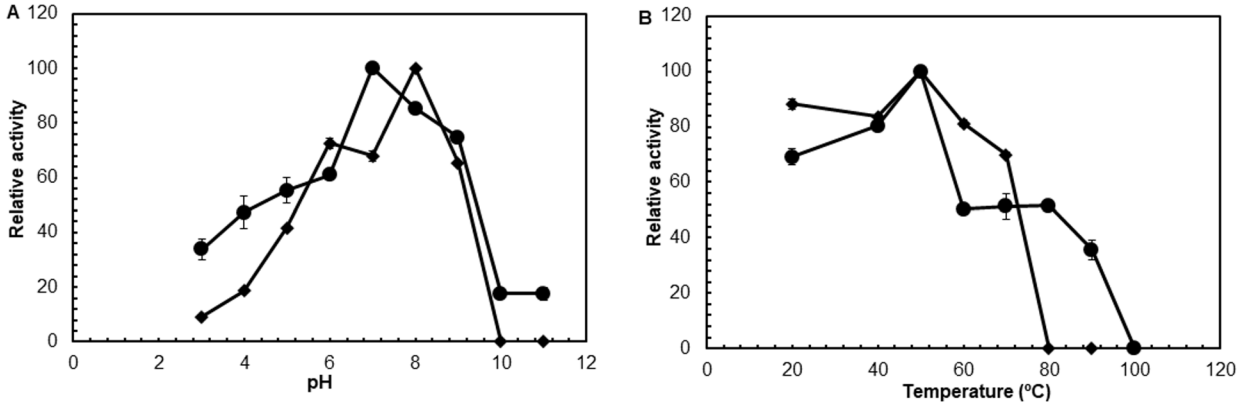

Figure 4. (a) Effect of pH (◆) on activity and (●) stability of the purified fibrinolytic enzyme from *Dunaliella tertiolecta* after 60 min of incubation at the predetermined pH range; (b) Effect of temperature (◆) on activity and (●) stability of the purified fibrinolytic enzyme from *Dunaliella tertiolecta* after 30 min of incubation at the predetermined temperature range. Fibrinolytic activity was normalized to 100% of control.

reported for fibrinolytic enzymes from different organisms such as cyanobacteria (*A. fertilissima*) (Banerjee et al. 2013), macroalgae (*Codium* spp.) (Matsubara 1998, 1999, 2000), fungi (Deng et al. 2018), bacteria (Mahajan et al. 2012), and even annelida (*Whitmaniapigra*) (Chu et al. 2016). Particularly, the enzyme retained more than 60% of its initial activity for 60 min in the pH range 6.0–9.0. Similar long-term stability especially under alkaline conditions (pH 5.0-11.0) has been reported for fibrinolytic enzymes produced by *A. fertilissima* (Banerjee et al. 2013) and *Bacillus subtilis* (Mahajan et al. 2012). The property of an enzyme to function effectively over a wide pH range is particularly important when its therapeutic use is envisaged (Krishnamurthy et al. 2018).

The enzyme was active between 20 and 70°C with residual activity above 70%. The highest catalytic performance and stability of the purified enzyme were both found at 50°C, an optimum value comparable with those of other fibrinolytic enzymes (Mahajan et al. 2012, Krishnamurthy et al. 2018, Deng et al. 2018). As expected, a further increase in temperature led to thermal denaturation that induced a quick reduction of fibrinolytic activity. The purified enzyme showed a good thermostability, losing its activity only at temperatures above 70°C. These results corroborate with earlier reports on fibrinolytic enzymes from *Bacillus cereus* (Narashimhan et al. 2018), *B. subtilis* (Mahajan et al. 2012), and *Serratia marcescens* subsp. *sakuensis* (Krishnamurthy et al. 2018).

CONCLUSIONS

In the current study, it was possible to produce, purify and characterize a fibrinolytic enzyme from the marine microalga *Dunaliella tertiolecta* cultivated mixotrophically using corn steep liquor as a cost-effective ingredient of the production medium. This is the first report about fibrinolytic enzyme production by this microalga and their purification. The broad pH stability and remarkable tolerance up to 70°C of this fibrinolytic enzyme are interesting advantages for its application on an industrial scale. This study introduces a new candidate for cheap production of a fibrinolytic enzyme to be used for medical applications.

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Author contributions

TAFS and PECS defined the design research; TAFS conducted experiments and wrote original draft; TPN and RMPBC contributed with analytical tools and their validation; ALFP acquired resources and funding; RPB managed the project, conceptualized, revised the writing; AC revised the paper and contributed to results interpretation.