**UNIVERSIDADE DE BRASÍLIA**

Faculdade de ciências da saúde

Curso de farmácia

MARCOS AUGUSTO DE ASSIS BRASILEIRO

# PRODUÇÃO DE PROTEASES POR FUNGOS ENDOFÍTICOS DE *Crinum americanum* L. COM POTENCIAL APLICAÇÃO EM BIOCOSMÉTICOS

# "PROTEASE PRODUCTION BY ENDOPHYTIC FUNGI FROM *Crinum americanum* L. WITH POTENTIAL APPLICATION IN BIOCOSMETICS"

ARTIGO

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Trabalho de Conclusão de Curso apresentado ao curso de farmácia como parte dos requisitos necessários à obtenção do título de bacharel em Farmácia.

Orientadora: Paula Monteiro de Souza

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Trabalho de Conclusão de Curso apresentado ao curso de farmácia, na forma de artigo, como parte dos requisitos necessários à obtenção do título de bacharel em Farmácia.

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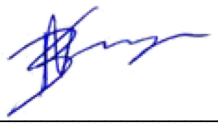

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Prof. Dra. Paula Monteiro de Souza

Orientadora

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Kellen Cruvinel Rodrigues Andrade

Avaliadora

**SUMÁRIO**

Dedico este trabalho aos meus pais, Marcos Brasileiro e Márcia Augusto de Assis Brasileiro, que sempre me inspiraram, apoiaram e me deram o suporte necessário para a conclusão da graduação. Dedico também a todos os meus professores da graduação, que foram de fundamental importância na construção da minha vida profissional, em especial minha orientadora, Paula Monteiro de Souza. À Deus, por me dar forças e ser tão presente e essencial em minha vida.

# PROTEASE PRODUCTION BY ENDOPHYTIC FUNGI FROM *Crinum americanum* L. WITH POTENTIAL APPLICATION IN BIOCOSMETICS

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## RESUMO

Como na escala industrial a produção de cosméticos afeta o meio ambiente, algumas empresas começaram a desenvolver uma linha de produção sustentável, atendendo ao crescente desejo da população em consumir produtos de origem orgânica. Assim, este estudo teve como objetivo avaliar a capacidade de fungos endofíticos isolados de *C. americanum* L. em produzir proteases com atividade queratinolítica e colagenolítica e sua potencial aplicabilidade industrial em biocosméticos. Oito extratos brutos de diferentes espécies de fungos foram testados em três ensaios enzimáticos. O primeiro, usando caseína, foi feito para escolher os melhores fungos produtores de protease que foram então submetidos a ensaios de queratinase e colagenase. A atividade caseinolítica foi interessante em três dos oito extratos brutos, FCEL 2, FCEL 4 e FCEL 7. Esses extratos apresentaram, respectivamente, 42,24 U/mL, 71,70 U/mL e 28,68 U/mL de atividade colagenolítica. No entanto, apenas o FCEL 2 e o FCEL 4 apresentaram atividade queratinolítica que foi de 1,28 U/mL e 2,51 U/mL, respectivamente. Poucos artigos relacionados à produção de queratinases por fungos endofíticos foram observados na literatura e valores relevantes de atividade de colagenase foram obtidos neste estudo, o que comprova a importância de novas investigações sobre esse assunto. As atividades queratinolíticas e colagenolíticas foram observadas com sucesso, enquanto as hidrólises de colágeno apresentaram melhores resultados.



**PALAVRAS-CHAVE:** *Crinum americanum*, fungo endofítico, protease, queratinase, colagenase, biocosméticos.

## ABSTRACT

As in the industrial scale the production of cosmetics has an impact on the environment, some companies have begun to develop a sustainable production line fulfilling the growing desire of the population to consume products of organic origin. Thus, this study aimed to evaluate the ability of endophytic fungi isolated from *C. americanum* L. to produce proteases with keratinolytic and collagenolytic activity and their potential industrial applicability in biocosmetics. Eight crude extracts from different fungal species were tested in three enzymatic assays. The first, using casein, was made to choose the best protease-producing fungi that were then subjected to keratinase and collagenase assays. Caseinolytic activity was interesting in three of the eight crude extracts, namely FCEL 2, FCEL 4 and FCEL 7. These extracts showed, respectively, 42.24 U/mL, 71.70 U/mL and 28.68 U/mL of collagenolytic activity. However, only FCEL 2 and FCEL 4 showed keratinolytic activity which was 1.28 U/mL and 2.51 U/mL, respectively. Few articles related to the production of keratinases by endophytic fungi were noticed in the literature and relevant values of collagenase activity were obtained in this study, which prove the importance of further investigation on this subject. Keratinolytic and collagenolytic activities were observed whereas the collagen hydrolyses showed better results.



**KEYWORDS:** *Crinum americanum*, endophytic fungi, protease, keratinase, collagenase, biocosmetics.

## 1. INTRODUCTION

The genus *Crinum* belongs to the *Amaryllidaceae* family and contains about 110 accepted species with over 270 synonyms. Species of the genus *Crinum* have large, extravagant flowers on leafless stems, are distributed in humid places such as forests, riverbanks, seasonal pools or saline and can be found in the tropics of Africa, Asia, America, Southern Africa, Madagascar and Mascarene and in the Pacific Islands. *Crinum* species extracts have been used in traditional medicine to treat a variety of ailments including fever, pain control, swelling, wounds, cancer and malaria (Presley *et al*., 2016).

Given the relevance of traditional use and investigation of endophytic microorganisms, the study of these species becomes interesting for the scientific community. Plants are usually colonized by endophytic microorganisms for at least part of their life cycle, without causing any apparent symptoms of disease. Fungi are one of the endophytic microorganisms that can be found in a plant. Plant-associated microorganisms are believed to produce metabolites similar to host plant species, so that the therapeutic activity of a particular plant may be due to an endophytic microorganism. Thus, these endophytes are recognized as important secondary metabolite sources, which benefit host species and endophytes as well, including plant growth hormones and proteases (Ayob & Simarani, 2016).

Proteases are enzymes that catalyze hydrolysis reactions with the function of cleaving proteins into smaller peptides (Muri, 2014). These enzymes can be obtained from animals, plants and microorganisms such as fungi and bacteria. Fungi are of great economic interest because they express these enzymes extracellularly and adapt to environmental conditions more easily (Orlandelli *et al*., 2012).

The functional proteins are of great interest to industry from various sectors such as textile, food, pharmaceutical and cosmetic. In the textile industry, proteases are applied to

decrease fabric felting, polish, improve gloss and dyeing stage (Orlandelli *et al*., 2012). In food, proteases are used in the production of bread, wine, beer and dairy products. In addition, these enzymes can improve digestibility and sensory quality of foods, as well as providing health benefits by reducing allergenic compounds (Tavano, 2013).

In the pharmaceutical industry, proteases are widely used for diagnostic and therapeutic purposes, in which bacterial and fungal proteases have also contributed to the development of effective therapeutic agents such as anticancer, anticoagulant, antimicrobial, anti-inflammatory. A relevant therapeutic applicability is the use of *Aspergillus oryzae* protease as a digestive aid to cure lytic enzyme deficiency (Singh *et al*., 2016).

A promising area for protease application is the cosmetic industry, as these products are present daily in people's lives and also by the social appeal represented by the improvement of self-esteem, social inclusion, personal hygiene and consequently disease prevention (Bezerra *et al*., 2018). Thus, the applicability of these functional proteins in the cosmetic industry is through keratinases and collagenases in order to remove scars, regenerate epithelia and accelerate healing processes. Furthermore are explored in the degradation of keratinized skin and preparation of the vaccine for dermatophytosis therapy (Singh *et al*., 2016).

Collagenases have applicability in cases of burns and dermal complications, are also used for dermatological purposes, in biocosmetic formulations, for treatments such as biological peeling, stretch mark removal, hair removal and in the control of oil and seborrhea (Wanderley *et al*., 2017).

However, in the industrial scale, the production of cosmetics has an impact on the environment. Because of this, some companies have begun to develop a sustainable

production line aligning the thinking of "green" chemistry and the growing desire of the population to consume products of organic origin (Bezerra *et al*., 2018).

Biotechnological studies aimed at reducing synthetic products in cosmetic formulations led to the discovery of new active ingredients from plant species and microorganisms, consolidating a new class of products called biocosmetics (Bezerra *et al*., 2018). These products have advantages such as lower toxicity, greater biodegradability, and greater effectiveness in different environmental conditions, such as pH and temperature when compared to products of mostly synthetic composition (Rienzo *et al*., 2015).

Thus, this paper aimed to evaluate the ability of endophytic fungi isolated from *Crinum americanum* L. to produce proteases with keratinolytic and collagenolytic activity and their potential industrial applicability in biocosmetics.

## 2. MATERIAL AND METHODS

### 2.1. Isolation of endophytic fungal species from leaves of *Crinum americanum* L.

After being harvested, the leaves were sanitized with running water and liquid detergent so as not to cause tissue breakage. Then the leaves were subsequently immersed in four containers. The first three containing 2% (v/v) sodium hypochlorite for 60, 90 and 180 seconds respectively, and the fourth containing 70% (v/v) alcohol for 60 seconds. Then, the leaves were immersed for 30 seconds with mechanical agitation in a sequence of three containers containing sterile distilled water for complete removal of hypochlorite and alcohol residues. Then the wet leaves were dried with the aid of previously sterilized filter paper. Finally, the leaves were cut and the fragments were placed in two Petri plates containing Sabouraud Dextrose Agar (SDA) Acumedia® (Petrini *et al*., 1993).

The plates with the leaf fragments and SDA culture medium were incubated at 28 °C for a period of 3 to 5 days and fungal growth was monitored daily. In order to obtain isolated fungal growth, 5 mm discs of mycelium were inoculated into plates containing 20 mL of SDA medium whenever a new fungal colony appeared. After this isolation phase of each visually different fungal colony that grew on the plates, 5 mm discs of mycelium from each fungus were stored in a 10% glycerol solution in distilled water at -80 °C with code assigned to each of them. After this process, a total of eight strains of filamentous fungi were isolated from leaf of *C. americanum* L. and the codes assigned were FCEL 1, FCEL 2, FCEL 3, FCEL 4, FCEL 5, FCEL 6, FCEL 7 and FCEL 8.

**2.2. Cultivation condition**

Previously isolated endophytic fungi were stored in storage solution at -80 °C for preservation. The reactivation was performed using Petri plates containing SDA medium to obtain growth of each fungal species. These plates were incubated in an oven at 28 °C for 7 days until growth. After that, cultivation in liquid medium was done to stimulate protease production by endophytic fungus. For each fungus isolated, a 5 mm diameter mycelium disc was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of a culture medium (2% (w/v) peptone, 1% (w/v) yeast extract, 0.2% (w/v) $KH_2PO_4$, 0.8% (w/v) $NaH_2PO_4$ and 0.25% (w/v) $MgSO_4$). All cultures were incubated in shaker at 28 °C and 120 rpm for 7 days. After incubation the cultures were filtered using filter paper and a Büchner funnel coupled to a Kitasato flask with the aid of a vacuum pump. This process was performed in a biosafety cabinet, the biomass was discarded and the filtrate containing the proteases was called crude extract (CE).

**2.3. Enzymatic assay for caseinolytic activity**

To evaluate the proteolytic activity, the method according to Charney & Tomarelli (1947) with modifications was performed. This assay was performed as a screening for

enzymatic assays of keratinolytic and collagenolytic activity. The substrate used was azocasein, a casein-derived red-orange compound, where a sulfonyl amide group was added. Hydrolysis of this substrate by a protease results in the formation of an orange component soluble in trichloroacetic acid (TCA), which after centrifugation was analyzed by a spectrophotometer. The reaction began by incubating at 55 °C for 40 minutes an aliquot of 500 µL of sample (CE) and 500 µL of azocasein (Sigma Aldrich, St Louis, MO, USA) 0.5% (w/v) diluted in 0.1 M acetate phosphate buffer pH 5.0. Then, the reaction was stopped by adding 500 µL of trichloroacetic acid (TCA) (Sigma Aldrich, St Louis, MO, USA) 10% (w/v) which provided the precipitation of unhydrolyzed casein. After the end of the reaction, the sample was centrifuged at 3780 g for 10 minutes and 500 µL of supernatant was transferred to a cuvette where 500µL of 5.0 N KOH was added for spectrophotometer reading. KOH in reaction with azocasein induces the increase in orange that is characteristic of AZO groups at alkaline pH. The reaction was blanked to zero the spectrophotometer by adding 500 µL 0.1 M acetate phosphate buffer pH 5.0 and 500 µL of azocasein. The assay was performed in triplicate for each sample and the color intensity reading was performed at 430 nm in spectrophotometer.

One unit of activity was defined as the amount of enzyme that produced a 0.01 absorbance per minute reaction among the reaction blank and the sample under the test conditions.

**2.4. Enzymatic assay for keratinolytic activity**

An enzymatic assay was performed, with the best crude extracts from the previous assay, in order to verify the occurrence of keratinolytic activity. The assay was conducted according to Sousa *et al*. (2015) with modifications. First, 20 mg of azoqueratin (Sigma Aldrich, MO, USA) was placed in a 5 mL centrifuge tube; then 3.8 mL of 100 mM tris-HCl buffer, pH 7.8 and 0.2 mL of sample (CE) were added. Samples were analyzed in

triplicate. In addition, two blanks were made; one sample blank containing 4.0 mL of 100 mM tris-HCl buffer pH 7.8 and 0.2 mL of sample, and also a substrate blank containing 4.0 mL of 100 mM tris-HCl buffer pH 7.8 and 20 mg of azoqueratin. The reaction occurred by incubating the tubes at 37 °C and 200 rpm in a shaker for 1 hour. After time, the tubes were placed at 4 °C for 10 minutes. Finally, the samples were centrifuged for 8 minutes at 4 °C and 10,000 g. The supernatant was read at 280 nm in spectrophotometer. A 0.1 increase in absorbance indicated 1 unit of enzymatic activity.

**2.5. Enzymatic assay for collagenolytic activity**

To quantify collagenolytic activity an enzymatic assay was performed according to Rosso *et al*. (2012) with modifications. In this method, Azocoll (Azo dye impregnated collagen - Sigma Aldrich) was used as substrate. Initially, Azocoll was suspended with 0.05 M Tris-HCl buffer pH 7.2 containing 1 mM $CaCl_2$ to a final concentration of 5 mg/mL. Then, the reaction was initiated by placing, in a 5 mL centrifuge tube, 300 µL of sample (CE), 300 µL of 0.05 M Tris-HCl buffer pH 7.2 containing 1 mM $CaCl_2$ and 540 µL of Azocoll suspension. In addition, a sample blank containing 840 µL of buffer and 300 µL of sample and a substrate blank containing 600 µL of buffer and 540 µL of Azocoll suspension was made. All tubes were incubated at 37 °C for 3 hours at 330 rpm and then centrifuged at 10,000 g for 8 minutes resulting in the end of reaction. Finally, an aliquot of the supernatant was placed in a cuvette for absorbance reading at 520 nm using a spectrophotometer. The amount of enzyme per mL that produced an increase in absorbance of 0.1 after 3 hours at 520 nm was defined as one unit of activity (UI).

**2.6. Total protein determination**

Total protein concentration was determined using the BCA Protein Assay Pierce™ Kit. 25 µL of the sample and 200 µL (50: 1 Kit solution) were subjected to the reaction for 30 minutes at 37 °C. After the reaction time the reading was taken at 570 nm in a

microplate reader. A calibration curve was made using albumin at concentrations of 2 to 2000 µg/mL.

## 3. RESULTS AND DISCUSSION

### 3.1. Enzymatic assay for caseinolytic activity

Caseinolytic activity was interesting in three of the eight endophytic fungal crude extracts (Figure 1), namely FCEL 2, FCEL 4 and FCEL 7 with activity of 92.3 U/mL, 121 U/mL and 73.3 U/mL, respectively. These three extracts showed specific activity above 40 U/g and were chosen for the following tests.

A similar study could be observed. Rajesh & Rai (2013), in a screening of hydrolytic enzymes from endophytic fungi of *Ventilago madraspatana*, isolated and identified 15 fungi and obtained protease activity values between 0.587 U/mL and 9.769 U/mL, results lower than those obtained in this paper.

Studies that performed the optimization of the cultivation process using specific methodology were observed. Wahab & Ahmed (2018) used a Plackett–Burman experimental design for screening of the medium components and fermentation conditions for a strain of *Aspergillus niger* and obtained similar results compared to this paper, with the minimum proteolytic activity of 38 U/mL and the maximum of 130 U/mL. Other study with *Aspergillus* genus fungi isolated from the soil of Caatinga (Brazil) showed higher values of protease activity, 5212.5 U (Silva *et al*., 2018).

Therefore, the caseinolytic activity values obtained in this study justify further studies for screening of enzymes for industrial application, including collagenases and keratinases.

### 3.2. Enzymatic assay for keratinolytic activity

Two of the three crude extracts showed keratinolytic activity, namely FCEL 2 and FCEL 4 with activity of 1.28 U/mL and 2.51 U/mL, respectively, as shown in Figure 2.

Sousa *et al* (2015), analyzing soil isolated fungi, obtained similar results to those identified in this study, in which *Aspergillus acuelatus* and *Aspergillus clavatus* presented activity of 1.5 U/mL and 2.2 U/mL respectively. And the maximum activity obtained was 7.35 U/mL from *Aspergillus sulphureus*.

In another study about biodegradation of keratin on feather residue by a soil keratinolytic fungus of the genus *Chrysosporium*, Bohacz (2016) obtained keratinolytic activity of 1.26 U/mL after 7 days of cultivation, a result inferior to that obtained in this article. However, after 42 days of cultivation this activity increased to 5.66 U/mL.

In other papers, higher keratinolytic activities were observed, such as 3873.4 U/mL from *Trichoderma atroviride* (Cao *et al*., 2008) and 27 U/mL from *Microsporum gypseum* (Singh, 2010). However, the cited articles used culture media enriched with chicken feathers or buffalo skin to stimulate the production of keratinases and the process optimization was also observed, varying parameters such as pH and cultivation time in order to obtain better results.

Thus, it is suggested that the use of keratin in the culture medium is important to obtain keratinases produced by filamentous fungi. The use of this protein in natural or synthetic form could increase the keratinolytic activity values. Therefore, in a possible future study, for the investigation of keratinases produced by endophytic fungi from *C. americanum* L., it is necessary to analyze these parameters to optimize the cultivation condition of fungal species.

After survey of articles, it was observed that few studies evaluated the production of keratinases by endophytic fungi, most of the papers reported the production of keratinases by filamentous fungi isolated from the soil. This emphasizes the relevance of this article and the importance of the investigation on this subject.

### 3.3. Enzymatic assay for collagenolytic activity

The three endophytic fungal crude extracts showed collagenolytic activity in the Azocoll assay, being 42.24 U/mL (FCEL 2), 71.70 U/mL (FCEL 4) and 28.68 U/mL (FCEL 7), as can be observed in Figure 3.

Among the papers with non-pathogenic fungal species, the main collagenolytic activity values observed were from *Rhizoctonia solani* with a production of 212.3 U/mL (Hamdy, 2008) and *Penicillium aurantiogriseum* with 231 U/mL (Lima *et al*., 2011).

Some studies used specific substrates in order to get better results, for example in a study with collagenase produced from *Aspergillus sp*. (UCP 1276) using chicken feather industrial residue, Ferreira *et al.* (2016), obtained elevated activity of 1345 U. In another study with fungi of genus *Aspergillus*, Silva *et al*. (2018), obtained 3621 U of collagenolytic activity from *Aspergillus tamari*, showing the potential industrial applicability of this fungi genus.

As this study consists of the specific investigation of two types of proteases, no optimization methodology or a specific culture medium containing collagen or keratin was used during the submerged fermentation process. Thus, this may justify the results slightly below those observed in other studies, which generally investigate these proteases separately. However, relevant values of collagenolytic activity were obtained in this study, which may suggest the potential of the isolated species of *C. americanum* L. to hydrolyze collagen. These results represent the relevance of this study and enable the development of other studies varying parameters such as temperature, pH, carbon and nitrogen in order to achieve optimal conditions of cell growth and biopharmaceutical production.

## 4. CONCLUSION

Even not using collagen or keratin enriched culture medium, crude extracts containing proteases showed interesting activity compared to other articles, which shows the promising capacity of these fungal species to produce collagenases and keratinases.

In summary, keratinolytic and collagenolytic activity from proteases of the strains of endophytic fungi isolated from *Crinum americanum* L. were noticed, whereas the collagen hydrolyses showed better results. Thus, further tests become interesting to evaluate the applicability potential of these proteases in the cosmetics industry. The next steps would be the identification of isolated species, process optimization, purification of keratinases and collagenases, and development and compatibility tests for cosmetic formulations.

## CONFLICT OF INTEREST

There is no conflict of interest in this study.


## ACKNOWLEDGMENT

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**Figure captions**

**Figure 1.** Caseinolytic activity and specific activity values of the eight endophytic fungal crude extracts from *Crinum americanum* L.

**Figure 2.** Keratinolytic activity of crude extracts of endophytic fungi isolated from *Crinum americanum* L.

**Figure 3.** Collagenolytic activity of crude extracts of fungi isolated from *Crinum americanum* L.

**Figure 1**

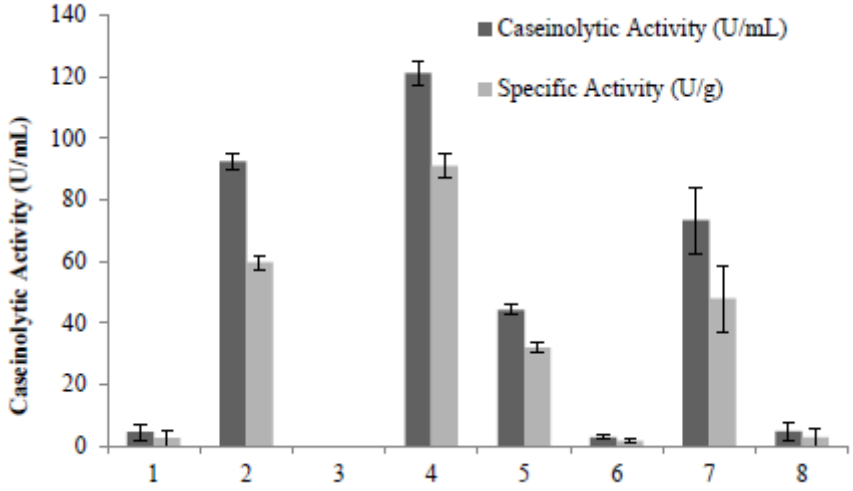

Caseinolytic Activity (U/mL)
Specific Activity (U/g)
Caseinolytic Activity (U/mL)
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8

**Figure 2**

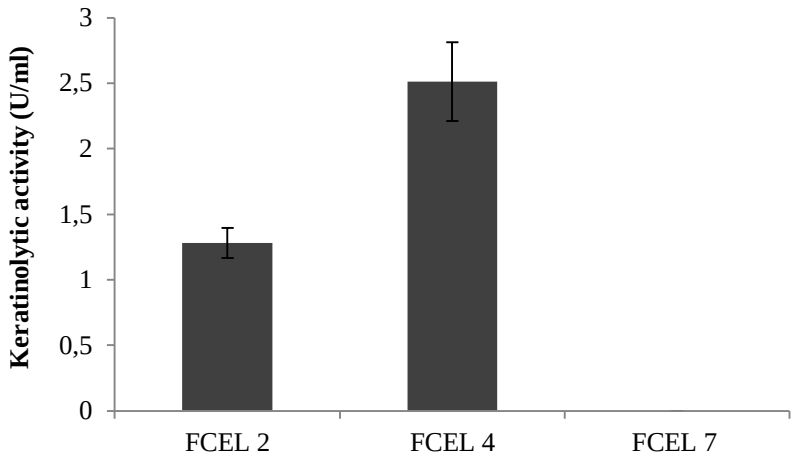
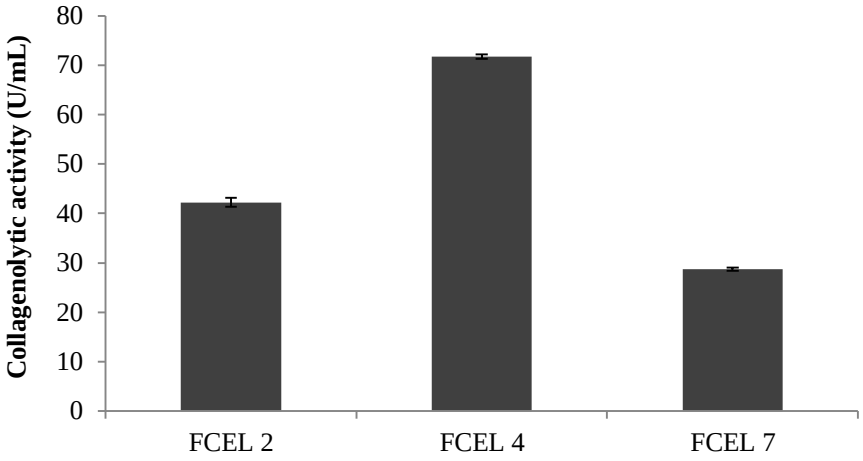


**Figure 3**


Collagenolytic activity (U/mL)
80
70
60
50
40
30
20
10
0
FCEL 2
FCEL 4
FCEL 7