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# Amino Acid Supplementation Improves the Production of Extracellular Peptidases by *Aspergillus* Section *Flavi* and their Ionic Immobilization

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## HIGHLIGHTS

- Amino acids modulate peptidases production in submerged bioprocess
- Inhibitors assay indicates serine and metallopeptidases production
- Peptidases were resistant to unfavorable pH and temperature conditions
- Peptidase from *A. section Flavi* can be immobilized in anionic supports

**Abstract:** Bioprocess studies have been highlighted due to the importance of physiological processes and industrial applications of enzymes. The potential of peptidase production from *Aspergillus* section *Flavi* using different amino acids as a supplemental nitrogen source was investigated. A production profile revealed that amino acids had positive effects on peptidase production when compared to the control without amino acids. Optimal production (100 U/mL) was obtained with Arginine amino acid in 96 h of fermentation. Extracellular peptidase from *Aspergillus* section *Flavi* was identified in submerged bioprocesses by in situ activity. Biochemical studies revealed that the maximum activities of the enzyme extract were obtained at pH 6.5 and a temperature of 55°C. The inhibition by EDTA and PMSF suggests the presence of more than one peptidase while the Ni<sup>2+</sup> and Cu<sup>2+</sup> had a negative influence on the enzyme activity. When the crude extract was

reversibly immobilized on ionic supports, DEAE-Agarose and MANAE-Agarose the derivative showed different profiles of thermal and pH stabilities. Hence, this study revealed the basic properties and biochemical characteristics that allowed the production improvement of this class of enzyme. Moreover, with known properties stabilization and immobilization process is required to further explore its biotechnological capacities.

**Keywords:** filamentous fungi; immobilization; MANAE-agarose; submerged bioprocess; protease.

## INTRODUCTION

Enzymes are biocatalyst more efficient than chemical catalyst. Many industrial processes are performed using enzymes, obtained different sources. One of these enzymes used in different fields is peptidases [1]. Peptidases are biocatalysts which are economically important to the world market and have applications in different industrial fields, representing about 60% of the world enzyme market [2,3]. They belong to the class of hydrolases, which catalyze the cleavage of covalent bond using water. Microorganisms are known as a great source of enzymes due to their biochemical versatility, susceptibility to genetic manipulation and their ability to provide a scale-up at lower costs [4,5]. Most of the commercial peptidases have as source different microorganisms. Fungi are shown to be a suitable choice, once peptidases are secreted into the culture medium that favors downstream processes [2].

Fungi are efficient producers of enzymes with biotechnological potential and have advantages, such as production in large amounts, rapid growth and the ability to secrete functional proteins with correct folding and post-translational modifications [6]. The genus *Aspergillus* is a great source for commercial enzymes, and is known for its ability to secrete a high level of enzymes into the environment [7,8]. *Aspergillus* section *Flavi* comprise 22 species known as *Aspergillus flavus*, *A. oryzae*, *A. sojae*, *A. tamarii*, *A. parasiticus* and recently two new species were identified: *A. pseudocaelatus* sp. nov. and *A. pseudonomius* sp. nov.[9].

The industrial production of peptidase is made using a submerged bioprocess and it is one of the most expensive steps in production, therefore studies on the development of culture medium that provide optimization of enzyme production has an important and great biotechnological appeal [9]. Peptidases can be applied in a native or immobilized form. Enzyme immobilization consists of adhesion or confinement of the protein to a defined region maintaining its catalytic activity, aiming at stabilizing and reutilizing the enzyme [10,11].

Therefore, the aim of this study was to define the best composition of culture medium in a submerged bioprocess supplemented with amino acids in order to improve the production of peptidase, and further biochemical characterization of the peptidase(s) in the crude extract and its immobilization in reversible form.

## MATERIAL AND METHODS

### Microorganism and maintenance

*Aspergillus* section *Flavi* was isolated from decomposing passion fruit and identified by the research group run by Prof. Dr. André Rodrigues (Laboratory of Fungal Ecology and Systematics – UNESP). The fungus belongs to a collection of microorganisms in the Enzyme Technology Laboratory, School of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo and was maintained at 4°C on potato-dextrose agar slants (cell bank). For the spore solution, the fungus was cultured on potato-dextrose agar slants at 30°C for 7 days. The spores were resuspended by adding sterile distilled water and quantified in a Neubauer chamber. The inoculum concentration used was  $5 \times 10^5$  spores/mL of culture medium.

### Submerged bioprocess (SmB)

Peptidase production by SmB was performed in erlenmeyer flasks (250 mL), containing 50 mL of liquid medium with the following composition: 0.7%  $\text{KH}_2\text{PO}_4$ , 0.2%  $\text{K}_2\text{HPO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01% yeast extract, 0.01%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , based on Tran and Nagano [12] and 0.1% amino acids (Tryptophan Trp, Arginine Arg, Glutamic acid Glu and Cystein Cys) [13] separately and control without any amino acid supplementation. The medium was adjusted to pH 6.5 and the sterilized flasks were inoculated and incubated in an orbital shaker incubator at 30°C for up to 96 h. The harvested flask was filtered with Whatman paper and centrifuged at 8,000 x g for 20 min at 4°C.

### Determination of proteolytic activity

Proteolytic activity was based on a protocol described by Sarath et al. [14]. The reaction mixture consisted of 1 mL of 1% (w/v) casein (Sigma) prepared in phosphate buffer (50 mM, pH 6.5), 0.1 mL of phosphate buffer (50 mM, pH 6.5) and 0.2 mL of the enzyme solution. The reaction was carried out at 40°C and stopped by adding 0.6 mL of 10% trichloroacetic acid (TCA) after 60 min of reaction. The samples were centrifuged at 10,000 x *g* for 15 min at 25°C and the absorbance of the supernatant was measured by spectrophotometer at 280 nm against their respective blanks. The activity unit (AU) was expressed as the amount of enzyme required to promote the release of 1 µmol tyrosine/min under defined assay conditions [14].

Biochemical studies were performed using 1% azocasein as a substrate [15]. The reaction mixture comprised 0.2 mL of 1% (w/v) azocasein (Sigma), 0.1 mL of buffer suitable for the intended assay and 0.1 mL of the enzyme solution. The reaction was carried out at an adequate temperature and stopped by adding 0.8 mL of 10% trichloroacetic acid (TCA) and the samples were centrifuged at 10,000 x *g* for 15 min at 25°C. The supernatant was mixed with 0.1 M of NaOH solution and the sample absorbance was measured by spectrophotometer at 440 nm against their respective blanks. The activity unit was defined under the assay conditions [16].

### SDS-Polyacrylamide gel electrophoresis and zymogram

The protein profile was verified by 12% polyacrylamide gels SDS-PAGE [17]. Proteins were denaturated by boiling the sample for 10 minutes in the buffer (0.1 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1 M dithiothreitol and 0.001 M bromophenol blue). After the running, the staining was performed using silver nitrate [18].

The *in situ* proteolytic activity, of the sample without heating, was verified by Zymogram in SDS-PAGE copolymerized with 0.1% (w/v) of gelatin and the electrophoresis performed at 4°C. After the running, the gels were washed twice with 2% (v/v) Triton X-100 (30 min at 20°C) and gels were incubated for 30 min at 45°C in sodium phosphate monobasic buffer (50 mM, pH 6.5). The gels were stained using coomassie blue R [19]. The images of SDS-PAGE and Zymogram gels were captured by the Gel Doc EZ system and analyzed by Image Lab™ version 3.0 software.

### Effect of pH, temperature, inhibitors and metal ions on peptidase extract activity

The effect of pH on proteolytic activity was determined at different pH values using buffers at a final concentration of 100 mM. pH 4.5 and 5 (acetate); pH 5.5, 6 and 6.5 (MES); pH 7, 7.5 and 8 (Hepes); pH 8.5 and 9 (Bicine); and pH 9.5, 10 and 10.5 (Caps). The reaction was performed using 1% azocasein as substrate and carried out at 40 °C and the procedures were performed according to the section entitled "Determination of Proteolytic Activity".

The influence of temperature on the peptidase extract activity was investigated at a range of 25°C to 65°C with increments of 5 °C. All experiments were performed using azocasein 1% as substrate and MES buffer (100 mM, pH 6.5) and the procedures were performed according to the section named "Determination of Proteolytic Activity".

The peptidase class was determined by the effect of different inhibitors on the peptidase activity [20]. Peptidase was previously incubated with inhibitors for serine peptidase (phenylmethylsulfonyl fluoride, PMSF), metallopeptidase (ethylenediaminetetraacetic acid, EDTA) and cysteine peptidase (iodoacetic acid, IAA) at a final concentration of 10 mM for 5 min at 55 °C. The experiments were performed using azocasein 1% as substrate and MES buffer (100 mM, pH 6.5) at 55 °C.

The effect of metal ions was determined at a final concentration of 10 mM. Peptidase was previously incubated with different metal ions CoCl<sub>2</sub>, LiCl, MgCl<sub>2</sub>, KCl, ZnSO<sub>4</sub>, NiSO<sub>4</sub>, CuCl<sub>2</sub>, CaCl<sub>2</sub>, MnCl<sub>2</sub>, AlCl<sub>3</sub>, BaCl<sub>2</sub> and NaCl for 10 min at 55°C. The experiments were carried out using azocasein 1% as the substrate and MES buffer (100 mM, pH 6.5) at 55°C.

### Immobilization by ionic adsorption

The enzymes were immobilized on different ionic supports: anionic supports used were MANAE-Agarose and DEAE-Agarose and the cationic support used was Sulphopropyl-sephadex. The crude extract obtained from the submerged bioprocess was dialyzed in buffer Hepes (5 mM; pH 7). Afterwards, this solution was mixed at a 1:1 proportion with buffer Hepes (5 mM; pH 7) and 1.0 g of ionic supports under agitation for 45

and 90 min at room temperature, respectively. The peptidase activity in soluble and immobilized preparations was performed using azocasein 1% as the substrate and MES buffer (100 mM, pH 6.5) at 40°C. The yield of immobilized enzyme was calculated by the difference of activity in suspension solution (support and supernatant) and supernatant. The recovered immobilized enzyme was called the derivative [21].

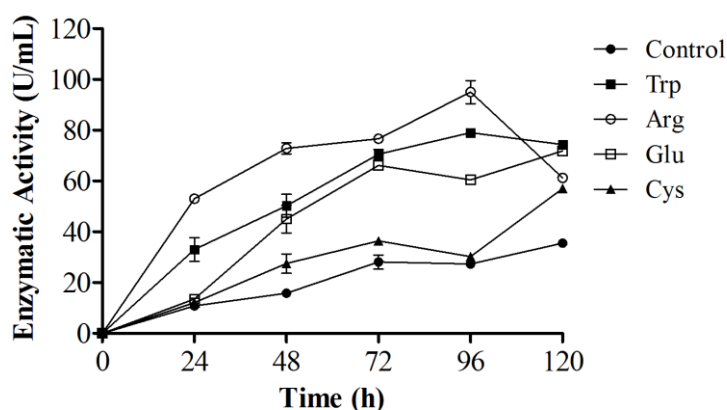
### Thermal and pH stabilities for immobilized peptidase

To study the thermal stability, the immobilized preparation and crude extract were incubated in buffer MES (50 mM; pH 6.5) at different temperatures (30, 40 and 50°C) for 5 and 60 min. The unheated control was considered to be 100% active. To pH stability assay, the immobilized enzyme and the enzyme extract were incubated for 60 min in buffer 50 mM at pH 5, 7 and 9. The residual activities for the thermal and pH stability assays were determined using azocasein as substrate, according to the section entitled "Determination of proteolytic activity", and the control was considered to be 100% of the total activity.

## RESULTS AND DISCUSSION

### Peptidase production in submerged bioprocess

The peptidase production by *A. section Flavi* in a submerged bioprocess was investigated using different amino acids as the supplement (Figure 1).



**Figure 1.** Peptidase production profile in a submerged bioprocess with different amino acids. Peptidase activity in the enzyme extract from *A. section Flavi* cultured. Control (closed circle) - with Trp – Tryptophan (closed square), Arg - Arginine (open circle), Glu – Glutamic acid (open square), Cys - Cysteine (closed triangle).

The profile of peptidase production from *A. section Flavi* in SmB showed, in general, that all amino acids had a positive effect on the peptidase production when compared to the control (without amino acids). The highest peak of peptidase activity (95 U/mL) was obtained in 96 h of the bioprocess in the presence of 0.1% arginine. The total amount of peptidase activity, with tryptophan and glutamic acid supplementation, was about 60 and 79 U/ml, respectively. The peptidase production induced by adding cysteine was higher than the control at 48 (27.5 U/mL) and 72 h (36.4 U/mL) of the bioprocess. We observed the best results with supplementation of arginine in the culture medium.

For *Gluconobacter japonicus* LMG 1373, the growth with asparagine (25 mg/L) was about 70% of the control growth, but arginine, histidine and lysine did not promote growth of this strain. On the other hand, no individual nitrogen sources provided *G. japonicus* CECT 8443 growth and histidine inhibited both strains [22].

The culture medium containing individual amino acid glutamine and histidine at 300 mg/L provided a growth of *G. oxydans* 621H similar to the control medium. On the other hand, histidine at the same concentration was not a viable nitrogen source for *G. oxydans* Po5 strain and the best performances in growth (50% of the control medium) was supported by asparagine, alanine, glutamine and tryptophan. *Acetobacter malorum* CECT 7742 and DSM 14337 strains grew greater than 50% of the control medium with 100 mg/L of the glutamine, glutamic acid and alanine. but the culture in the presence of proline should be mentioned due to the fact that it increased about 1.5 times compared to the control medium [22]. In our study, all amino acids tested showed positive modulation to peptidases synthesis.

The peptidase production by *A. section Flavi* with the selected amino acids had positive effects because its production by filamentous fungi is influenced by culture medium components. Nitrogen sources are vital

for growth and development of the fungi and regarding the fungal nitrogen metabolism, ammonia, glutamine and glutamate are considered primary nitrogen sources [23].

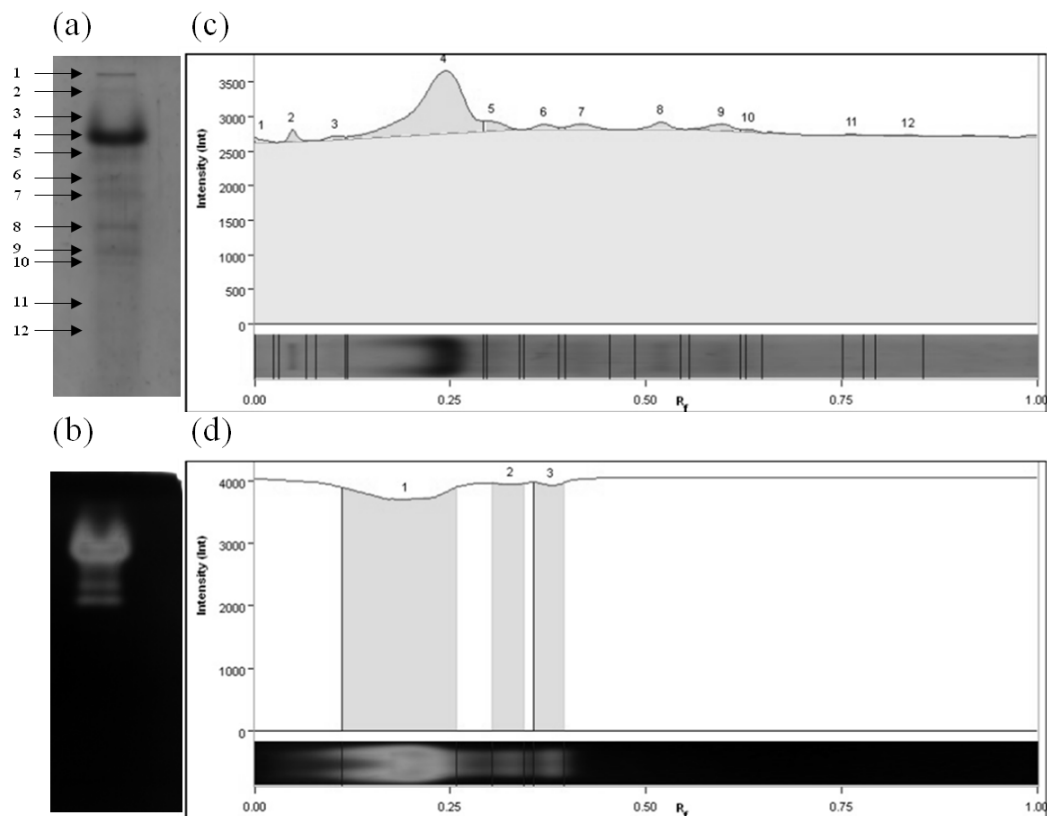
Other nitrogen sources can also be used, and for their acquisition and catabolism the production of catabolic enzymes and permeases are needed [24]. In *Aspergillus nidulans*, global nitrogen metabolism is controlled by the AreA transcription factor which belongs to the GATA zinc finger family [23].

In our study, the positive effects can be justified by the presence of amino acids as an alternative source of nitrogen, and therefore enable the peptidase production by the AreA system. Moreover, arginine, cysteine, glutamic acid and tryptophan are glycoenic amino acids that can be energy sources for enzyme production by gluconeogenesis [25]. The knowledge of the specific microorganism response towards the culture medium composition is vital to define it, and explores the specific conditions and parameters in order to obtain maximum enzyme production [10].

Our results showed that *A. section Flavi* used amino acids to produce peptidases. The differential of this fungus is a production of peptidases in the presence of amino acids. In general, simple molecules such glucose, amino acids are assimilated by fungi and rapidly metabolizable, affecting the peptidases synthesis, [26,27]. Datta et al. [13] showed that *Aeromonas caviae* in the presence of 0.1% tryptophan and cysteine produced 0.112U/mL of peptidases in both conditions, our results were better with production of 60 and 27.5 U/mL, respectively which same amino acids.

### Protein profile and zymogram of crude extract

The extract supplemented with 0.1% of arginine obtained in 96 h of the bioprocess was submitted to SDS-PAGE (Figure 2a) and zymogram with 0.1% of gelatin (Figure 2b) in the native condition to evaluate the protein profile and the activity *in situ*, respectively.

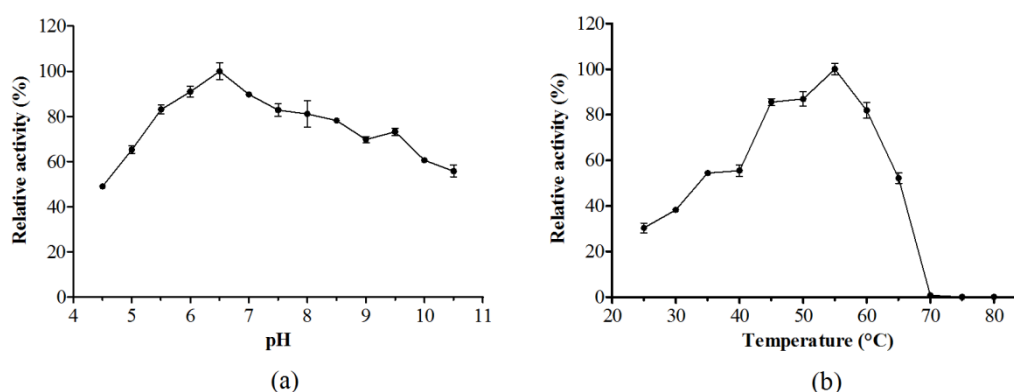


**Figure 2.** (a) Protein profile of the enzyme extract produced by *A. section Flavi* in SDS-PAGE stained with silver nitrate. (b) Proteolytic activity *in situ* in PAGE with 0.1% of gelatin stained with Coomassie R-250. (c) SDS-PAGE virtual analysis of lane and bands. (d) Zymogram virtual analysis of lane and bands. All images were captured in EZ Gel Doc system and were analyzed by Image Lab™ version 3.0 software (Bio-Rad). The arrows (Figure 2a) indicate in total extract profile the protein bands in according identified in figure 2c. The band identified as 1 (Figure 2d) corresponds to bands 4 and 5 indicated by the arrows (Figure 2a), bands 2 and 3 (Figure 2d) correspond to bands 6 and 7, respectively (Figure 2A). The total intensity of the 12 bands corresponds to 100% and the intensities of the bands 4, 5, 6 and 7 correspond to 83.8% of the total intensity. Figure 2c and 2d show the lateral view of the gel from the virtual image and from it the intensity and delimitation information of each band can be extracted.

The number of protein bands in denaturing and zymogram gels and the analysis indicated the presence of 12 (Figure 2c) and 3 (Figure 2d) bands, respectively. The supplementation with arginine improved the secretion of peptidases because, after analyzing the relative quantitative using Image Lab™ version 3.0 software, we determined that the bands with activity (Figure 2d) were correspondents to bands 4, 5, 6 and 7 (Figure 2c) which comprise 83.8% of the secreted total protein. Therefore, in the arginine-induced extract, within the overall secreted protein content, most were peptidases. The protein profile in the SDS-PAGE denaturing gel (Figure 2a) and *in situ* proteolytic activity of the zymogram (Figure 2b) suggest the presence of more than one peptidase in the crude extract with different molecular weight. Moreover, the high secretion of peptidases obtained by arginine supplementation may facilitate the purification processes and is an interesting approach.

### Biochemical properties of crude extract

The extract produced in SmB containing arginine by *A. section Flavi* was subjected to biochemical characterization using azocasein 1% as substrate (Figure 3).



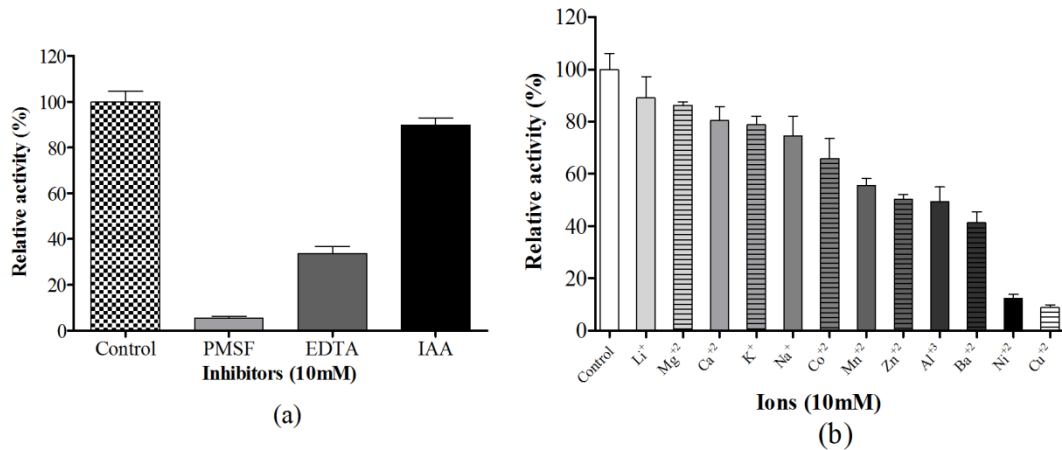
**Figure 3.** (a) Effect of pH and (b) Effect of temperature on peptidase activity from enzyme extract produced by *A. section Flavi* in SmB. The activity was carried out as described in material and methods.

The pH effect on peptidase activity showed maximum activity at pH 6.5 (Figure 3a). Moreover, the activity was maintained above 60% between pH 5 and 10, which indicates more possibilities in different kinds of industrial applications of these fungal peptidases. The maximum activity at pH 6.5 could be influenced by fungus species and culture conditions used to obtain the enzymes. As shown in Figure 3b, the activity was maintained above 80% between 45 and 60°C and the maximum activity was at 55°C.

Both peptidases produced in submerged and solid-state bioprocesses by *Aspergillus fumigatus* [28], and *Eupenicillium javanicum* [29], presented optimum pH at 8.0 and 5.5, respectively. *Penicillium corylophilum* and *Penicillium waksmanii* in a solid-state bioprocess showed the best activities at a pH range of 7.0-8.0 and 7.5, respectively [30]. The profile of temperature effect presented a broad range in which the activity was not affected, but in temperatures above 60 °C the proteolytic activity was drastically reduced. This decrease in the peptidase activity may be related to enzyme denaturation or the effect of interactions with other proteins of the extract.

The optimum temperature is variable to different fungal species. Some authors described that *P. corylophilum* and *P. waksmanii* in a solid-state bioprocess showed optimum temperatures at 60°C and 55°C, respectively [30]. Furthermore, other authors demonstrated the production by *Aspergillus oryzae* of the enzymes with the best proteolytic activities at 40°C [31], and 55°C [32]. Both peptidases produced in submerged and solid-state bioprocesses by *A. fumigatus* [28] and *E. javanicum* [29], presented maximum activities at 50°C and 60°C, respectively.

Analysis of peptidase properties required studies on the profile towards inhibitors and different ions (Figure 4).



**Figure 4.** (a) Effects of inhibitors PMSF (Phenylmethanesulfonyl fluoride), EDTA (Ethylenediamine tetraacetic acid) IAA (Iodoacetic acid) and (b) Effects of ions (Li<sup>+</sup> Lithium chloride, Mg<sup>2+</sup> Magnesium chloride, Ca<sup>2+</sup> Calcium chloride, K<sup>+</sup> potassium chloride, Na<sup>+</sup> sodium chloride, Co<sup>2+</sup> Cobalt chloride, Mn<sup>2+</sup> manganese chloride, Zn<sup>2+</sup> zinc chloride, Al<sup>3+</sup> aluminum chloride, Ba<sup>2+</sup> Barium chloride, Ni<sup>2+</sup> Nickel chloride and Cu<sup>2+</sup> cupric chloride) on peptidase activity from enzyme extract produced by *A. section Flavi* in SmB. The activity was determined in a 100mM MES buffer pH 6.5 at 55°C, using azocasein as substrate. The control assay was performed without inhibitors and relative activity was considered as 100%.

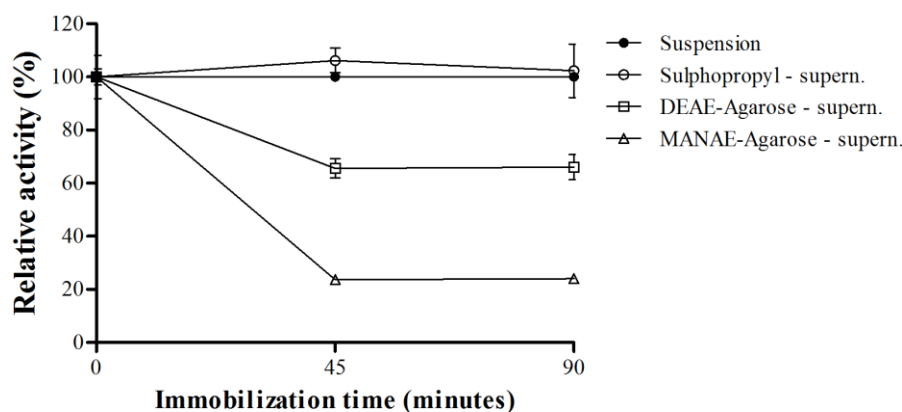
The PMSF and EDTA inhibitors showed a reduction in activity in the proteolytic activity of the peptidase produced by *A. section Flavi*, with a reduction of 94.5% and 66.2%, respectively, when compared to the control without the inhibitor (Figure 4a). The PMSF is a serine peptidase inhibitor whereas EDTA chelates metal ions and can perform as metallo peptidase inhibitor.

The inhibitor assays provide the peptidase classification based on the nature of the catalytic site. In this study, the peptidase activity was reduced by serine and metallo peptidase inhibitors. These results corroborate with *in situ* peptidase activity and emphasize the presence of at least two peptidases that can belong to the classes of serine and metallo peptidases. However, this can also suggest the presence of metal-dependent serine peptidase, likewise the peptidase from *A. fumigatus* [28].

The peptidase activity presented different levels of negative modulation when incubated with mono, di or trivalent ions. Compared to the control, none of the ions presented a positive effect on proteolytic activity, but the negative influence by Ni<sup>2+</sup> and Cu<sup>2+</sup> were of 88 and 91%, respectively. Similarly, Marui et al. [31], showed that the glycine-d-alanine aminopeptidase was inhibited by 1 mM of NiCl<sub>2</sub> and CuCl<sub>2</sub> and the authors indicated that although the chelating reagent EDTA inhibited the enzyme, no ions positively affected the activity.

### Immobilization studies of peptidases

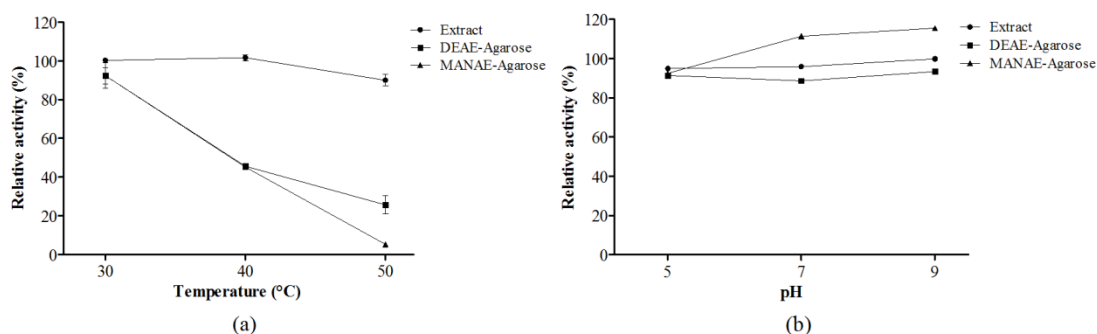
The enzyme extract from the *Aspergillus section Flavi* was immobilized using protocols with ionic supports, and its efficiency is shown in Figure 5.



**Figure 5.** Efficiency of peptidase immobilization on ionic supports. The activity of the supernatants was determined in 100mM MES buffer pH 6.5 at 55°C, using azocasein as substrate after 45 and 90 min of immobilization. The control assay was performed with non-immobilized extract and relative activity was considered as 100%. Anionic supports: MANAE-agarose and DEAE-agarose. Cationic support: Sulphopropyl. Supern.: supernatant.

The anionic support MANAE-agarose showed the best immobilization yield; about 80% of the enzymes were immobilized after 45 min, while on DEAE-agarose the immobilization yield was about 40%. Meanwhile the cationic support Sulphopropyl did not show immobilization of the enzyme. The immobilization on anionic resin rather than on cationic resin indicates that the peptidases from the *Aspergillus* section *Flavi* in pH 7 was negatively charged. This may be an indication that the peptidase does not have a positive charge density on its surface, sufficient to interact with such supports in the immobilization chosen pH. The absence of interaction on cationic support Sulphopropyl corroborates this information.

After immobilization assays, the thermal and pH stability of immobilized enzyme and the control (enzyme crude extract) were evaluated (Figure 6). The thermal stabilities after 60 min showed that at 30°C DEAE-agarose and MANAE-agarose maintained activities above 90%. At 40°C, both derivatives displayed similar profiles with 40% of initial activity whereas at 50°C DEAE-agarose stability was higher than MANAE-agarose (Figure 6a). The pH stabilities showed that the peptidase activity was kept above 90% at pH 5.0, 7.0 and 9.0 for both the crude extract and the ionic supports (Figure 6b). The results indicate that the DEAE-agarose support presented similar profiles to crude extract in different pH conditions; however, MANAE-agarose exhibited the best results at pH 7 and 9.



**Figure 6.** pH and thermal stabilities of enzyme extract, DEAE-agarose and MANAE-agarose immobilized. (a) Thermal stability after 60 min at 30°C, 40°C and 50°C (b) pH stability after 60 min at pH 5,0, 7,0 and 9,0. The assay was performed at 40°C and pH 6.5. The results were presented in relative activities and were compared to activities not exposed to pH and temperatures were considered as 100%.

The immobilization on DEAE-agarose support was lower than on MANAE-agarose. In stabilization assays, the crude extract showed a better performance than ionic supports in a thermal challenge, with a similar performance at different pH ranges. Interestingly, the peptidase activity in MANAE-agarose was higher at pH 7 and 9 than in DEAE-agarose and crude extract, but this fact may be due to biochemical properties of the peptidase and its interaction with the support, once the immobilization process removes the enzyme from the complex environment of the crude extract and places it in a single interaction with the support. The efficiency and stability in the immobilization process may be influenced by interaction between proteins and



the structures of each resin or with other molecules present in the crude extract. Rao et al. [33], also related the interference of conformational changes in the protein after immobilization, to protein structure modifications during the immobilization process and changes in the enzyme microenvironment.

## CONCLUSION

Amino acids are organic nitrogen sources, and in general, organic and complex sources are efficient inducers of enzyme production, but studies indicate that organic and inorganic nitrogen sources exerted physiological effects on microorganism in a specific manner and can influence the proposed biotechnological application. The supplementation with 0.1% of arginine, as organic nitrogen source, provided a high content of secreted peptidases in extracts which have activity in a broad range of pH and temperature. It can also be observed that the crude extract and immobilized form had similar profiles; it is interesting because the immobilized form of the enzyme is more purified than the crude extract and this could facilitate biotechnological applications.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## REFERENCES

1. Choi JM, Han, SS, Kim HS. Industrial applications of enzyme biocatalysis: Current status and future aspects. *Biotechnol Adv.* 2015; 33: 1443–1454.
2. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev.* 1998;62:597-635.
3. Gaur S, Agrahari S, Wadhwa N. Purification of Protease from *Pseudomonas thermaerum* GW1 Isolated from Poultry Waste Site. *Open Microbiol J.* 2010;4:67-74.
4. Kumar CG, Takagi H. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnol Adv.* 1999;17:561-594.
5. Sandhya C, Nampoothiri KM, Pandey A. Microbial Proteases. In: Barredo JL, editor. *Microbial Enzymes and Biotransformations.* Oxford: IRL Press; 2005. p. 165-179.
6. Kosalková K, García-Estrada C, Barreiro C, Flórez MG, Jami MS, Paniagua MA, Martín JF. Casein phosphopeptides drastically increase the secretion of extracellular proteins in *Aspergillus awamori*. Proteomics studies reveal changes in the secretory pathway. *Microb Cell Fact.* 2012;11:5.
7. Oxenbøll K. *Aspergillus* Enzymes and Industrial Uses. In: Powell KA, Renwick A, Peberdy JF, editors. *The Genus Aspergillus: From Taxonomy and Genetics to Industrial Application.* Boston, MA: Springer US; 1994. p. 147–154.
8. te Biesebeke R, Record E, Van Biezen N, Heerikhuisen M, Franken A, Punt PJ, Van Den Hondel CA. Branching mutants of *Aspergillus oryzae* with improved amylase and protease production on solid substrates. *Appl Microbiol Biotechnol.* 2005;69:44-50.
9. Varga J, Frisvad JC, Samson RA. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Stud Mycol.* 2011;69:57-80.
10. Arroyo M. Immobilized Enzymes: theory, methods of study and applications. *Ars Pharm.* 1998;39:23-29.
11. Brena BM, Batista-Vieira F. Immobilization of enzymes: A Literature Survey. In: Guisan JM, editor. *Methods in biotechnology: Immobilization of enzymes and cells.* Totowa, NJ: Human Press; 2006. p. 15-31.
12. Tran LH, Nagano H. Isolation and Characteristics of *Bacillus subtilis* CN2 and its Collagenase Production. *J Food Sci.* 2002;67:1184-1187.
13. Datta S, Menon G, Varughese B. Production, characterization, and immobilization of partially purified surfactant–detergent and alkali-thermostable protease from newly isolated *Aeromonas caviae*. *Prep Biochem Biotechnol.* 2017; 47: 349–356.
14. Sarath G, de la Motte RS, Wagner FW. Protease assay methods. In: Beynon RJ, Bond JS, editors. *Proteolytic enzymes: a practical approach.* Oxford: IRL Press; 1989. p. 25-56.
15. Ducros E, Ferrari M, Pellegrino M, Raspanti C, Bogni C. Effect of aeration and agitation on the protease production by *Staphylococcus aureus* mutant RC128 in a stirred tank bioreactor. *Bioprocess Biosyst Eng.* 2009;32:143-148.
16. Morita Y, Hasan Q, Sakaguchi T, Murakami Y, Yokoyama K, Tamiya E. Properties of a cold-active protease from psychrotrophic *Flavobacterium balustinum* P104. *Appl Microbiol Biotechnol.* 1998;50:669-675.

17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680-685.
18. Blum H, Beier H, Gross H. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*. 1987;8:93-99.
19. Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal Biochem*. 1994; 218:325-329.
20. Dunn BM. Determination of protease mechanism. In: Beynon RJ, Bond JS, editors. *Proteolytic Enzymes: a practical approach*. Oxford: IRL Press; 1989. p. 57-81.
21. Mehdi WA, Mehde AA, Özacar M, Özacar Z. Characterization and immobilization of protease and lipase on chitin-starch material as a novel matrix. *Int. J Biol Macromol*. 2018; 117: 947–958.
22. Sainz F, Mas A, Torija MJ. Effect of ammonium and amino acids on the growth of selected strains of *Gluconobacter* and *Acetobacter*. *Int J Food Microbiol*. 2017;242:45-52.
23. Krappmann S, Braus GH. Nitrogen metabolism of *Aspergillus* and its role in pathogenicity. *Med Mycol*. 2005;43:S31-40.
24. Bugeja HE, Hynes MJ, Andrianopoulos A. AreA controls nitrogen source utilisation during both growth programs of the dimorphic fungus *Penicillium marneffeii*. *Fungal Biol*. 2012;116:145-154.
25. Hynes MJ, Szewczyk E, Murray SL, Suzuki Y, Davis MA, Sealy-Lewis HM. Transcriptional control of gluconeogenesis in *Aspergillus nidulans*. *Genetics* 2007;176:139-150.
26. Rani K, Rana R, Datt S. Review on latest overview of proteases. *IJCLS* 2012; 2:1:12– 18.
27. Souza PM, Bittencourt MLA, Caprara CC, Freitas M, Almeida RPC, Silveira D, Fonseca YMF, Filho EXF, Junior AP, Magalhães PO. A biotechnology perspective of fungal proteases. *Braz J Microbiol*. 2015; 46: 2: 337-346.
28. da Silva RR, de Freitas Cabral TP, Rodrigues A, Cabral H. Production and partial characterization of serine and metallo peptidases secreted by *Aspergillus fumigatus* Fresenius in submerged and solid state fermentation. *Braz J Microbiol*. 2013;44:235-243.
29. Hamin-Neto YAA, Motta CMS, Cabral H. Optimization of metalloprotease production by *Eupenicillium javanicum* in both solid state and submerged bioprocesses. *African J Biochem Res*. 2013;7:146-157.
30. Silva RR, Ângelo T, Cabral H. Comparative evaluation of peptidases produced by *Penicillium corylophilum* and *Penicillium waksmanii* in solid state fermentation using agro-industrial residues. *J Agric Sci Technol B*. 2013;3:230-237.
31. Marui J, Matsushita-Morita M, Tada S, Hattori R, Suzuki S, Amano H, Ishida H, Yamagata Y, Takeuchi M, Kusumoto K. Enzymatic properties of the glycine d-alanine aminopeptidase of *Aspergillus oryzae* and its activity profiles in liquid-cultured mycelia and solid-state rice culture (rice koji). *Appl Microbiol Biotechnol*. 2012;93:655-669.
32. Vishwanatha KS, Appu Rao AG, Singh SA. Production and characterization of a milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341. *Appl Microbiol Biotechnol*. 2010;85:1849-1859.
33. Rao SV, Anderson KW, Bachas LG. Oriented immobilization of proteins. *Microch Acta*. 1998;128:127-143.



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