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Novel extremophilic proteases from *Pseudomonas aeruginosa* M211 and their application in the hydrolysis of dried distiller's grain with solubles (DDGS)

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ABSTRACT

Proteases are the most important group of industrial enzymes and they can be used in several fields including biorefineries for the valorization of industrial by-products. In this study, we purified and characterized novel extremophilic proteases produced by a *Pseudomonas aeruginosa* strain isolated from *Mauritia flexuosa* palm swamps soil samples in Peruvian Amazon. In addition, we tested their ability to hydrolyze Distillers Dried Grains with Solubles (DDGS) protein. Three alkaline and thermophilic serine proteases named EI, EII and EIII with molecular weight of 35, 40 and 55 kDa, respectively; were purified. EI and EIII were strongly inhibited by EDTA and Pefabloc being classified as serine-metalloproteases, while EII was completely inhibited only by Pefabloc being classified as a serine protease. In

addition, EI and EII exhibited highest enzymatic activity at pH 8, while EIII at pH 11 maintaining almost 100% of it at pH 12. All the enzymes demonstrated optimum activity at 60°C. Enzymatic activity of EI was strongly stimulated in presence of Mn^{2+} (6.9-fold), EII was stimulated by Mn^{2+} (3.7-fold), while EIII was slightly stimulated by Zn^{2+}, Ca^{2+} and Mg^{2+}. DDGS protein hydrolysis using purified *Pseudomonas aeruginosa* M211 proteases demonstrated that, based on glycine released, EIII presented the highest proteolytic activity towards DDGS. This enzyme enabled the release 63% of the total glycine content in wheat DDGS protein, 2.2-fold higher that when using the commercial Pronase®. Overall, our results indicate that this novel extremopreoteases have a great potential to be applied in DDGS hydrolysis.

Keywords: extremophilic proteases, *Pseudomonas*, purification, DDGS, glycine

INTRODUCTION

Proteases are one of the most important groups of industrial enzymes and account for approximately 60% of the total enzyme sales in the world [1, 2]. These enzymes find multiple applications in detergent, leather, food, chemical and pharmaceutical industries. In the last years, proteases have also found important biotechnological applications in peptide synthesis in non-aqueous environments, bioremediation processes and management of industrial waste. Although a large number of bacterial species are known to produce proteases, only a few are recognized as commercial producers [3-5]. For the purpose of fulfilling a wide variety of industrial needs; protease-producing bacteria are constantly being sought from different extreme environments such as glaciers, deep sea, saline habitats, mangroves, solvent-contaminated habitats and swamps [6-11]. Swamps are forested freshwater wetlands on inundated soils that constitute unique natural environments. The swamps soils are rich in nutrients due to the leaf litter fall, and their high carbon content supports the growth and

survival of a variety of microorganisms. The microbial diversity of swamp soils depends on several factors such as temperature, pH, oxygen and nutrient content. Different genera of bacteria have been identified in swamp soils including *Streptomyces*, *Micrococcus*, *Bacillus, Staphylococcus, Streptococcus* and *Pseudomonas* [12, 13]. *Mauritia flexuosa* palm swamps called "aguajales" in Peruvian Amazon represent a subtropical natural environment with a rich biological diversity. The average annual temperature is 26°C, fluctuating between 10°C and about 40°C. The high amount of nutrients in these swamps induces growth of plants and algae which increases CO_2 consumption producing alkaline conditions. Moreover, microbial decomposition of nutrients by aerobic microorganisms enhances O_2 consumption generating a hypoxic environment and stressful conditions; while microbial decomposition of the leaf litter fall by anaerobic microorganism generates methane production [14]. This ecosystem is rich in nutrients including proteins because it is the habitat of *Rhynchophorus palmarum*, an invertebrate that is found in decaying trunks of *Mauritia flexuosa* palm characterized by its high protein content [15]. Therefore, the study of microbial diversity from these types of environments will contribute to the isolation and identification of potential bacteria producing proteases with high specificity and activity to be applied in various fields [16]. The *Pseudomonas* genus is a versatile group of bacteria which are found in a very large number of natural environments including soils, fresh and marine waters. Such extensive distribution results from the capacity of the *Pseudomonas* species to adapt to different conditions and to degrade a wide range of substrates including proteins. In swamps soils, these bacteria have been found to be responsible for the decomposition of leaf litter [8, 12, 17]. In addition, *Pseudomonas* has been reported as one of the most important bacteria producing extracellular proteases with properties for industrial and research applications due to their high stability, organic solvent tolerance and thermo-alkaline activity [4, 18-21].

DDGS (Distillers Dried Grains with Solubles) is a by-product derived from distilleries and bioethanol production [22]. Wheat DDGS is composed mainly of polysaccharides (cellulose and hemicellulose) and protein. Protein content varies according to the wheat variety initially used and the production process, but on average is 38% (w/w) [23]. Due to their high content of sugars most of the research has been focused on valorization of this fraction [24]. In contrast, little attention has been paid to the protein fraction, except for its nutritional value in the animal feed market [22]. With the increase of bioethanol production from wheat, significant amounts of wheat DDGS will become available and it is expected to saturate the animal feed market resulting in a lower value for this by-product. For these reasons, recent studies are focusing on wheat DDGS valorization [25]. Wheat DDGS protein presents an amino acid composition rich in glutamine, glycine, proline and tyrosine which can be useful in biocatalytic synthesis to produce bio-based chemicals; and its enzymatic hydrolysis using proteases would be an important step, after DDGS fractionation, to make these amino acids accessible for bioconversion and therefore for their valorization. An initial attempt to produce glutamic acid from wheat protein using commercial enzymes and acid hydrolysis has been reported as a representation of wheat DDGS valorization [26-29]. The aim of this work was to purify and characterize extracellular proteases produced by a *Pseudomonas aeruginosa* M211 strain isolated from palm swamps soil samples collected in Peru. In addition, the purified and characterized proteases were tested for their ability to hydrolyze wheat DDGS protein.

MATERIALS AND METHODS

Materials

Casein from bovine milk (technical grade), trifluoroacetic acid (TFA) (analytical standard), Folin & Ciocalteu's phenol reagent, ammonium sulphate (≥ 99%), InstantBlue™, Protease Type XIV (Pronase®) from *Streptomyces griseus*, amino acid standard (analytical standard)

and other chemicals were purchased from Sigma-Aldrich (Gillingham, UK) otherwise stated. PageRuler™ Plus Prestained Protein Ladder (10 to 250 kDa) and Novex NuPAGE SDS-PAGE Gel System were purchased from Thermo Fisher Scientific (Loughborough, UK). HiScreen™ Capto™ Q column was purchased from GE Healthcare (Little Chalfont, UK). Quick Start™ Bradford Protein Assay was purchased from Bio-Rad (Hertfordshire, UK).

Protease activity assay

Protease activity was measured following the method described by Anson with some modifications using casein as substrate [30, 31]. The enzymatic reaction consisted of 0.5 mL of 0.65% (w/v) casein in 50 mM Tris–HCl (pH 9) preincubated at 40°C for 5 min and the reaction was started by the addition of 0.5 mL of enzyme solution. After 10 min of incubation, the reaction was stopped by adding 0.5 mL of TFA 10% (v/v). The mixture was kept at 40°C for 30 min and then centrifuged at 10000 ×g for 5 min at 4°C. The supernatant (0.3 mL) was mixed with 0.75 mL of 0.5 M Na_2CO_3 and 0.15 mL of 0.5 M Folin & Ciocalteu's phenol reagent, then was incubated at 40° C for 30 min and then the mixture was centrifuged at 5000 ×g for 5 min. The concentration of digested casein in the supernatant was determined at 660 nm (Aquarius UV/Vis spectrophotometer, Cecil Instruments, UK). The calibration curve was made using L-tyrosine as a standard following the same procedure described above. One unit (U) of protease activity was defined as the amount of enzyme that releases 1 µmol of tyrosine equivalent per min at pH 9 and 40°C. The specific activity is expressed in the units of enzyme activity per milligram of protein. Protease activity assay was carried out at 40°C due to temperatures of 37°C, 40°C and 42°C were tested; and crude extract presented its highest activity at 40°C.

Protein quantification

The protein concentration was determined by Bradford method [32] using Quick Start™ Bradford Protein Assay. Bradford assay was performed following the microassay protocol with 300 µL final volume using a microplate reader (FLUOstar Optima, BMG LabTech, Germany) and bovine serum albumin was used as a standard protein.

Amino acids quantification by Ion Chromatography (IC)

Amino acid concentration of the full acid and enzymatic hydrolysis were determined by Ion Chromatography (ICS 5000+ Dionex, equipped with an electrochemical detector and fitted with a 2 x 250 mm analytical AminoPac PA-10 column). The amino acids were eluted with deionized water (eluent A), 250 mM NaOH (eluent B) and 1 M sodium acetate (eluent C) at a flow rate of 0.25 mL/min following method described by ThermoFisher. The calibration curve was made using a standard of amino acids.

Bacterial strain selection

This *Pseudomonas aeruginosa* strain M211 was isolated from *Mauritia flexuosa* palm swamps soil samples collected in Madre de Dios, Peru. For the isolation, the soil samples were resuspended in 0.9% NaCl and serial dilutions were made in sterile phosphate buffer saline (PBS) up to 10^{-12}. The soil samples dilutions (100µL) were spread on agar plates with Tryptone Soya Agar (TSA) and kept for incubation at 37°C for 24 h. Colonies were isolated based on their morphological characteristics. Screening of proteases producing bacteria was carried out on nutrient agar plates supplemented either with gelatin, casein or skim milk (1% w/v). Proteolytic activity was visualized as clear zones around the colonies due to substrate hydrolysis, after 24 h of incubation at 37°C. The strain showing maximum hydrolysis zone diameter was selected as a potential producer of proteolytic enzymes and then identified by 16S rRNA gene sequencing. This selected strain was stored at -80°C as glycerol stocks and maintained at 4°C on nutrient agar slant tubes for further studies.

Production of Pseudomonas aeruginosa M211 proteases

Production of *Pseudomonas aeruginosa* M211 proteases was carried out in a batch culture. The inoculum was prepared by transferring loopful of the strain from nutrient agar slants in TSB supplemented with yeast extract 0.2%. Then, proteases production was carried out in a 5 L laboratory scale bioreactor (Applikon, The Netherlands), the culture contained (g/L): glycerol, 7.5; glucose, 1.25 ;maltose, 7.5; yeast extract, 7.5; peptone, 7.5; $CaCl_2$, 0.5; KCl,0.5; NaCl, 2.5; $MgSO_4.7H_2O$, 0.5 and K_2HPO_4, 0.5 (pH 9) [33] and was inoculated with 10% of inoculum. The fermentation was performed with a working volume of 3L at 40°C and a stirrer speed of 500 ×g, air flow rate of 1 vvm and the dissolved oxygen tension was 40% air saturation. The pH was maintained at 9 by the automatic addition of 1 M NaOH. The dissolved oxygen tension was 40% air saturation. After 24 h, the culture was centrifuged at 10000 ×g for 10 min. The cell-free supernatant was used as crude extract and it was lyophilized for further studies.

Stability of proteases from crude extract of Pseudomonas aeruginosa M211 cultures

The lyophilized powder from crude extract was reconstituted in buffers with pH values ranging from 4 to 10 with or without addition of 1 M NaCl. All buffers used were prepared at 50 mM and were made of: sodium acetate (pH 4 and 5), potassium phosphate (pH 6 and 7) or Tris–HCl/Tris-Base (pH 8 to 10). The stability of proteases was studied by incubating the crude extract with the respective buffer at room temperature. Enzymatic activity was measured under standard assay conditions for up to 24 h of incubation. Residual activity was calculated with reference to the activity of the crude extract at the start of incubation.

Anion exchange chromatography (AEC)

A strong anion-exchange HiScreen™ Capto™ Q column, 7.7 x 100 mm connected to ÄKTA™ pure chromatography system was used (both from GE Healthcare). The buffers used

consisted of 50 mM Tris-HCl (buffer A) or 50 mM Tris-HCl and 2 M NaCl (buffer B), both at pH 9. Prior loading, the lyophilized powder from crude extract was reconstituted in buffer A to a final protein concentration of 50 mg/L and filtered through 0.22 µm pore size PES Millex® filter. 100 mL of the feed were loaded onto a column previously equilibrated with buffer A, washed with the same buffer A and eluted with two linear gradients of buffer B: 0-0.2 M NaCl in 78 min and 0.2-0.6 M NaCl in 30 min. Finally, the remaining material was stripped with buffer B. All steps were performed at the flowrate of 2 mL/min. Fractions of 15 mL were collected and both absorbance at 280 nm and conductivity signals were monitored throughout the run. Protein quantification and protease activity were determined in each fraction. The fractions presenting protease activity were subjected to SDS-PAGE analysis.

Ammonium sulphate precipitation

The protease active fractions collected in AEC that showed a single band and the same molecular weight in SDS-PAGE were pooled together. Solid ammonium sulphate to 70% saturation was added slowly to the active fractions and stirred for 3 h at room temperature. The precipitate was harvested by centrifugation at 5000 ×g for 30 min, and then dissolved in a volume (1/10) of 50 mM Tris-HCl pH 9 and assayed for protein and protease activity. These purified preparations were used for the characterization studies.

Determination of molecular weight

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [34] using Novex NuPAGE SDS-PAGE Gel System. Instant Blue™ was used to visualize protein bands and molecular mass of the denatured proteases were estimated by using a standard molecular weight marker (PageRuler™ Plus Prestained, Thermo Fisher, UK).

Effect of inhibitors on protease activity

The effect of inhibitors on proteases activity was tested incubating 50 mU of purified enzymes solutions in buffer 50 mM Tris–HCl pH 9 with either 10 mM ethylendiaminetetraacetic acid disodium salt (EDTA), 100 µM E-64, 10 µM Pepstatin A or 10 mM Pefabloc at room temperature for 60 min. Then proteolytic activity was measured under standard assay conditions. Residual activity was calculated in percentage referred to the activity of the enzymes incubated in the absence of inhibitors.

Determination of optimum pH and temperature protease activity

The optimum pH of purified enzymes was determined at 40°C using 0.65% (w/v) casein in the following 50 mM buffers: sodium potassium phosphate (pH 7), Tris–HCl (pH 8 to 10) and sodium bicarbonate (pH 11 and 12). Furthermore, the optimum temperature was determined by assaying the purified enzymes at temperatures from 40 to 80°C in 10°C increments using 0.65% (w/v) casein in 50 mM Tris-HCl pH 9. Protease activity was measured under standard assay procedure described previously.

Effect of metal ions on protease activity

The purified enzymes solutions were incubated with 10 mM of $CaCl_2$, $MgCl_2$, $MnCl_2$ and Zn_2SO_4 at room temperature for 60 min. Relative protease activity was measured at optimum pH and temperature conditions, following the standard assay procedure and expressed as the percentage of activity compared with a control without metal ions. Metal ions that increased enzymatic activity at 10 mM were also tested at concentration ranging from 1 mM to 40 mM.

Wheat DDGS protein extraction

Extraction of the protein from DDGS was carried out with 100 mM NaOH and 45% (v/w) ethanol with 10% (w/v) solid loading (dry matter) at 30°C and 250 ×g for 2 h. The mixture

was recovered by centrifugation (10000 ×g at 4°C for 45 min), and the supernatant containing DDGS protein was kept at 4°C until its utilization.

Full acid hydrolysis of wheat DDGS protein

For this reaction, 0.5 mL of wheat DDGS protein (9.25 mg) was transferred to an analysis tube and 3.7 mL of 6 M HCl was added. The reaction mixture was heated at 110°C for 24 h [29]. Then the mixture was centrifuged at 12000 ×g for 10 min and dilutions of the supernatant were used for ion chromatography analysis.

Enzymatic hydrolysis of wheat DDGS protein

Wheat DDGS protein was hydrolyzed with *Pseudomonas aeruginosa* M211 purified enzymes (EI, EII and EIII), *Pseudomonas aeruginosa* M211 crude extract and a commercial enzyme (Protease Type XIV, Sigma-Aldrich), under the conditions showed in Table 1, based on their optimum pH and temperature activities. Previously, wheat DDGS protein was dissolved in the respective buffer and preincubated for 5 min. The reaction was initiated by the addition of the enzyme to give a final enzyme: substrate ratio of 1:100 (w/w). The reaction was carried out in a final volume of 1 mL and in duplicate. After 24 h of incubation, the reaction was stopped by heating it up at 100°C for 10 min, then it was centrifuged at 12000 ×g for 10 min and the supernatants were used for amino acids quantification by ion chromatography.

RESULTS AND DISCUSSION

Bacterial strain selection

A total of 16 different strains were isolated from swamps soil samples of *Mauritia flexuosa,* a typical palm called “aguaje” that grows in the Peruvian Amazon region of Madre de Dios. Its extracellular proteolytic activity was tested using nutrient agar plates either with 1% casein, skim milk or gelatin. Three strains were identified as protease producers due to been able to

hydrolyze at least one of the three used substrates with a zone of hydrolysis higher than 10 mm. For this study, the strain with the highest zone of hydrolysis and with the capability to hydrolyze the three used substrates was selected. The diameter of the zone of hydrolysis around the colonies of this strain was 22.2, 22.3 and 23 mm with casein, skim milk and gelatin; respectively, concluding that the produced proteases have broad substrate specificity. The 16S rRNA sequence of this isolate (GenBank accession number MH130225) was compared using BLAST program, and was confirmed as *Pseudomonas aeruginosa* as it revealed 100% homology with over 100 *Pseudomonas aeruginosa* strains (GenBank accession numbers CP026680, LT969520, CP025229, CP025055, among others). Figure 1 shows the phylogenetic relationship between the *Pseudomonas aeruginosa* strain isolated here and other *Pseudomonas* species. Because our strain is 100% identical over the whole of the 16S sequence with known strains of *Pseudomonas aeruginosa* we are naming this strain as *Pseudomonas aeruginosa* M211. The M211 stands for the code and the region in the Peruvian Amazon where it was isolated (Madre de Dios).

Purification of Pseudomonas aeruginosa M211 proteases

Isolation, purification and characterization of proteases from different *Pseudomonas* strains have been previously reported. Besides, some previous works have demonstrated that *Pseudomonas* species have the capacity to produce more than one type of extracellular proteases with different properties and catalytic activities [18, 35]. For that reason, there is a high probability that the *Pseudomonas* strain described in this study could produce more than one protease which would explain its broad substrate specificity. However, there is scant literature available on *Pseudomonas* multiple proteases purification and characterization. Only Rahman *et al.* [18] have described purification of three proteases from *Pseudomonas aeruginosa* strain K. Because studies of purification of multiple proteases by a *Pseudomonas* strain are unusual, we attempted to purify and characterized the enzymes.

For proteases purification, AEC is one of the most common methods and it implies utilization of buffers at high pH values and high ionic strength (NaCl linear gradients from 0 up to 1 M) [6, 18, 19, 36]. Therefore, prior to proteases purification, it was important to determine their stability under different pH and ionic strength conditions in order to minimize losses of enzymatic activity or avoid protein aggregation during AEC process.

The stability study at pH between 4 and 10 using the crude extract (data not shown) demonstrated that *Pseudomonas aeruginosa* M211 proteases possess elevated stability. It was observed that between pH 5 and 8 these proteases kept almost 100% of their activity up to 24 h of incubation, while at more extreme pH values (4, 9 and 10) the crude extract maintained more than 70% of its proteolytic activity. On the other hand, in presence of 1 M NaCl (high ionic strength) and pH values ranging from 4 to 10 (data not shown), it was noticed that the loss of activity was more predominant, especially for more extreme pH values (4, 9 and 10) where initial proteolytic activity decreased by more than 40% after 3 h of incubation, concluding that stability of proteases of crude extract from *Pseudomonas aeruginosa* M211 was mainly affected by high ionic strength. Additionally, absence of precipitation (due to protein aggregation) was observed at the end of the stability experiments. Based on these results, AEC was used as a first step for *Pseudomonas aeruginosa* M211 extracellular proteases purification.

In the anion exchange chromatogram (Figure 2), five peaks with proteolytic activity were eluted: three major (P1, P2 and P3) and two minor peaks (P4 and P5). The peaks P1 (fractions 11-13), P2 (fraction 14), P3 (fraction 15) and P4 (fractions 17, 18) were eluted during the first gradient (0 - 0.2 M NaCl), while peak P5 (fractions 22, 23) was eluted during the second gradient (0.2 - 0.6 M NaCl). No proteolytic activity was detected in neither flow-through (fractions 1-10) nor strip (fractions 24-26). The most fractions that contained *Pseudomonas aeruginosa* M211 proteases were eluted at pH 9 and low ionic strength (up to 0.2 M NaCl).

These conditions are expected to offer minimal losses of activity according to results from stability studies.

Figure 3 shows the SDS-PAGE analysis of the collected fractions and the crude extract. It was observed that peaks P1, P2 and P3 exhibited a single band with approximately the same molecular weight (35 kDa), suggesting that these three fractions would correspond to a unique enzyme (EI). Elution of several peaks in AEC with the same molecular weight in SDS-PAGE is probably due to the presence of enzyme isoforms. This type of chromatography has been reported as a method that enables to separate proteases isoforms [37-39]. Additionally, peaks P4 and P5 were identified as other two different enzymes named as EII and EIII and with molecular weight of 40 kDa and 55 kDa, respectively. In conclusion, three different extracellular proteases with different molecular masses have been identified in this work, which is in good agreement with previous works on *Pseudomonas* species proteases purification [18, 19, 35, 36, 40-49].

Finally, EI (fractions from 11 to 15), EII (fractions 17 and 18) and EIII (fractions 22 and 23) were recovered by precipitation with ammonium sulphate at 70% saturation. Subsequent to AEC, it was observed that the three enzymes from *Pseudomonas aeruginosa* M211 crude extract were separated with a total enzyme recovery of around 70%. After ammonium sulphate precipitation the total purification factor was 2.8; whereas EI exhibited the highest specific activity, recovery and purification factor; 210.2 U per mg of protein, 80.1% and 5.7, respectively. Purification steps of *Pseudomonas aeruginosa* M211 proteases are summarized in Table 2. Proteases from other strains of *Pseudomonas aeruginosa* have been purified by AEC as a single purification step or by combinations of ammonium sulphate precipitation and chromatographic procedures. Gaur *et al*. obtained 38% recovery and 11.9-fold purification [36] of *Pseudomonas aeruginosa* PseA protease by AEC. By employing ammonium sulphate

precipitation and AEC, 34.7% recovery and 5.7-fold purification was also reported for *Pseudomonas aeruginosa* proteases [40].

Effect of inhibitors on proteases activity

Proteases inhibition test permits to obtain information about the enzyme classification based on its active site and its cofactor requirements [50]. In this work, 4 different types of proteases inhibitors were utilized: EDTA, a metalloproteases inhibitor; E-64, a cysteine proteases inhibitor; Pepstatin A, an aspartyl proteases inhibitor and Pefabloc, a serine proteases inhibitor.

Proteases inhibition experiments (Table 3) showed that EI and EIII were completely inhibited by EDTA, which acts chelating metal ions at the active site of the enzymes which probably plays a role as enzyme cofactors [51]. Besides, EI and EIII lost about 80% and 40% of their activities, respectively, in presence of Pefabloc. On the other hand, EII was completely inhibited only by Pefabloc. Pefabloc reacts with serine residue present at the active site of proteases forming a stable acylated enzyme resulting in a loss of proteolytic activity. These results suggested that EI and EIII belong to serine-metalloproteases family, while EII belongs to serine proteases family. It was reported previously that the majority of proteases from *Pseudomonas* species isolated from different environments have been classified either as metalloproteases [6, 18, 19, 36, 41, 43, 45-48] or serine proteases [42, 49, 52]. However, to the best of our knowledge, it is the first time that extracellular serine-metalloproteases from *Pseudomonas aeruginosa* have been reported.

Determination of optimum pH and temperature protease activity

Pseudomonas aeruginosa M211 purified proteases were found to be active in a range of pH between 7 and 12 (Figure 4 A). The optimum pH found for EI and EII was 8, although 95% and 80% of their activity; respectively was kept at pH 9. Optimum pH for EIII was 11 and

interestingly 98% of its activity was maintained at pH 12 related to its optimum pH. Furthermore, these proteases showed activity at temperatures ranging from 40 to 80°C and their optimum activity for the 3 proteases was exhibited at 60°C. Also, it was observed that EI and EII retained almost 70% of their activity at 70°C; in contrast EIII presented a sharp decrease in its activity at this temperature (Figure 4 B). Based on these results, these *Pseudomonas aeruginosa* M211 proteases could be classified as alkaline and thermophilic proteases [11, 53]. Although some *Pseudomonas* species proteases have been reported to have optimum pH and temperature 8 and 60°C , respectively [6, 36, 42, 46]; optimum activity values ranging from pH 7 to 10 and temperature from 37°C to 70°C have also been reported for *Pseudomonas* species proteases [18,43 ,45, 49]. Thus, the above findings were consistent with data published for other researchers. However, optimum pH of EIII was considerable higher than those reported for proteases from other *Pseudomonas* species previously studied.

Alkali-thermophilic enzymes constitute the group of enzymes with the broadest commercial application in bio-industry [11, 20, 54]. This type of enzymes could be applied in biorefinery bioconversion processes where conditions such as elevated pH and temperatures values are required for feedstock pretreatment. In addition, high temperatures in biocatalytic reactions allow to increase possibilities for easy mixing, better substrate solubility, high mass transfer rate, lower viscosity, enhanced tolerance to organic solvents and reduced risk of microbial contamination [53, 55].

Effect of metal ions on protease activity

The effect of various metal ions on proteases activity was tested at 10 mM concentration and the results are presented in Table 4. EI activity was strongly stimulated in presence of Mn^{2+} (6.9-fold) and a slight activation effect was observed in presence of Mg^{2+}. Also, EII activity was significantly increased by Mn^{2+} (3.7-fold). These results indicate that Mn^{2+} and Mg^{2+} play

an important role in maintaining the active conformation of EI and EII [56]. In contrast, both enzymes were completely inhibited by Zn^{2+}. As the EI and EII characterized in this study, it was found in previous studies that the activity of proteases produced by *Pseudomonas* species have been also stimulated in presence of Mn^{2+} [45, 57], or completely inhibited by Zn^{2+} [6]. Nevertheless, effect of Mn^{2+} on EI and EII found in this study was significantly higher compared to the data reported in earlier references. EIII activity was enhanced in presence of Zn^{2+}, Ca^{2+} and Mg^{2+} (1.1, 1.5 and 1.7-fold, respectively). These findings are in contrast respect to previous works where these ions do not play a stimulating role on *Pseudomonas* species proteases activity [19, 35, 42].

Metal ions that stimulated EI, EII and EIII proteolytic activity at 10 mM were assessed at 1, 5, 20 and 40 mM in order to evaluate the effect of different metal ions concentration in enzymes activity. In general, 10 mM was found the optimum ion concentration to improve protease activity (Figure 5) except for Mg^{2+} in EI activity, which optimum concentration to increase this enzyme activity was 5 mM (Figure 5 A). Similar results were reported by Rahman *et al.* [18] who found that 10 mM concentration of metal ions such as Mn^{2+}, Mg^{2+}, Ca^{2+} and Zn^{2+} produced a higher increase on activity of proteases from *Pseudomonas aeruginosa* strain K. Furthermore, it was observed that EI proteolytic activity was increased by all tested Mn^{2+} and Mg^{2+} concentrations (Figure 5 A). Nevertheless, a slight reduction of EII proteolytic activity was noticed in presence of 5 mM Mn^{2+} (Figure 5 B). Likewise, EIII proteolytic activity presented a reduction by 5 mM Mg^{2+}, Ca^{2+} and Zn^{2+}, and almost a complete loss of activity was observed in presence of 20 mM Zn^{2+} (Figure 5 C). These findings demonstrate that metal ions and their concentration play a relevant role on enzymes conformation and proteolytic activity.

The main properties of the three enzymes from *Pseudomonas aeruginosa* M211 are summarized in Table 5. Here, a comparison with previously reported proteases from the

different species of *Pseudomonas* is also shown. Interestingly, most studies have reported that *Pseudomonas* genus produces only one extracellular protease; however, in this work we have demonstrated that *Pseudomonas aeruginosa* M211 is able to produce up to 3 extreme proteases simultaneously and all of them with different properties. Another relevant find was optimum pH of EIII (pH 11), maintaining almost 100% of its activity at pH 12, demonstrating that it is extremely alkaline compared to others *Pseudomonas* proteases. In addition, *Pseudomonas* proteases have been mainly classified as metalloproteases, while enzymes EI and EIII of this research were classified as serine-metalloproteases and EII as a serine protease. It is remarkable because alkaline serine proteases represent the most important group of commercial enzymes being applied in different industries, and most of them are produced by *Bacillus* genus [58-59].

Enzymatic hydrolysis of wheat DDGS protein

Wheat DDGS protein is mainly composed by glutamine (35%) and glycine (20%). As a result of its full hydrolysis using high acid concentrations (6 M HCl), glutamine is degraded therefore could not be detected. Glycine content obtained from full acid hydrolysis was 827.6 µM and it was used as a reference for the calculation of glycine released by enzymatic hydrolysis using the proteases EI, EII, EIII, crude extract and a commercially available Protease Type XIV (Pronase®) at the conditions described in table 1. The concentrations of glycine obtained using the *Pseudomonas aeruginosa* M211 proteases purified in this study EI, EII and EIII were 28.2, 223.2 and 521.7 µM, respectively. Additionally, with the crude extract the glycine concentration obtained was 152 µM, while using the Protease Type XIV it was 241.2 µM.

These findings showed that EIII, a high-alkaline protease, presented the highest catalytic activity towards wheat DDGS protein based on glycine releasing. This protease released 63%

of the total glycine content in wheat DDGS protein. Additionally, enzymatic hydrolysis with EII allowed to obtain 27% of the total glycine content similar to the percentage obtained using the commercial enzyme Protease Type XIV (29%). Moreover, it was observed that 93.4% (773.1 µM) of the total glycine was released by enzymatic hydrolysis using EI, EII, and EIII separately. Nevertheless, using the crude extract, which contains the three identified proteases only 18.4% (152 µM) of monomeric glycine, was obtained. This could be because in the crude extract EII and EIII present a significantly lower specific activity than EI, which has this protease in a higher concentration.

Of the two principal amino acids in wheat DDGS protein, glutamine results in the formation of glutamic acid and both are mainly produced by microbial fermentation. The enzymatic hydrolysis of waste DDGS could be an alternative source of glutamate. Glycine is currently produced by chemical synthesis and protease hydrolysis of wheat DDGS could represent a potential source material for obtaining glycine. Glycine plays a relevant role in several industries including pharmaceutical and cosmetic; and can be used as an intermediary in the synthesis of other chemical products and to produce bio-based chemicals. This amino acid can be transformed to oxalic acid used as bleaching agent in the textile and pulp industries and in wastewater treatment [60-62].

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NOMENCLATURE

AEC	Anion exchange chromatography

DDGS	Distillers Dried Grains with Solubles
E-64	Trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane
EDTA	Ethylendiaminetetraacetic acid disodium salt
EI	Enzyme I
EII	Enzyme II
EIII	Enzyme III
IC	Ion Chromatography
NAD	No activity detected
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid

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Figure 2. Anion exchange purification of *Pseudomonas aeruginosa* M211 proteases using HiScreen™ Capto™ Q and ÄKTA™ pure. Mobile phase: 50 mM Tris-HCl pH 9 (buffer A), 50 mM Tris-HCl pH 9 with 2 M NaCl (buffer B). Gradient from 0 to 0.2 M NaCl in 78 min and 0.2 to 0.6 in 30 min. Flow rate 2 mL/min. Pink vertical line: start of loading, brown line: conductivity signal in mS/cm, green line: buffer B in %, blue line: absorbance at 280 nm. Proteolytic activity: P1 (Peak 1, fractions 11-13); P2 (Peak 2, fraction 14); P3 (Peak 3, fraction 15); P4 (Peak 4, fractions 17 and 18) and P5 (Peak 5, fractions 22 and 23).

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as the percentage of activity compared with a control without metal ions. Error bar represents the mean of duplicate ± SD.

Table 1. Experimental conditions for wheat DDGS protein hydrolysis with different proteases.

Enzyme	Specific activity[1] (U/mg)	Hydrolysis condition		Added enzyme (mg)	Added DDGS protein (mg)
		pH	Temperature (°C)		
EI	210.2	8.0	60	0.04	4
EII	11.2	8.0	60	0.02	2
EIII	6.5	11.0	60	0.09	9
Crude extract	36.7	9.0	40	0.03	3
Pronase®	4.8	7.5	37	0.03	3

[1]U=Anson Unit

Table 2. Purification of *Pseudomonas aeruginosa* M211 proteases.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification Factor
Crude extract	170.1	4.6	36.7	100.0	1.0
Capto Q chromatography					
$P1^{EI}$,$P2^{EI}$,$P3^{EI}$	107.6	1.3	80.4	63.2	2.2
$P4^{EII}$	1.3	0.3	4.3	0.8	0.1
$P5^{EIII}$	9.4	1.5	6.4	5.5	0.2
Total	118.3	3.1	38.2	69.5	1.0
$(NH_4)_2SO_4$ precipitation					
$P1^{EI}$,$P2^{EI}$,$P3^{EI}$	136.2	0.6	210.2	80.1	5.7
$P4^{EII}$	1.6	0.1	11.2	0.9	0.3
$P5^{EIII}$	4.2	0.7	6.5	2.5	0.2
Total	142.0	1.4	101.4	83.5	2.8

^{EI} Enzyme I: P1 = Peak 1 (fractions 11-13), P2= Peak 2 (fraction 14) and P3= Peak 3 (fraction 15).

^{EII} Enzyme II: P4 = Peak 4 (fractions 17, 18).

^{EIII} Enzyme III: P5 = Peak 5 (fractions 22, 23).

Table 3. Effect of inhibitors on *Pseudomonas aeruginosa* M211 proteases activity.

Inhibitor	Concentration	Residual activity[1] (%)		
		EI	**EII**	**EIII**
Control	-	100.0	100.0	100.0
EDTA 2Na	10 mM	NAD	89.0 ± 10.9	NAD
E-64	100 µM	89.9 ± 0.9	93.4 ± 6.6	98.4 ± 1.6
Pepstatin A	10 µM	100.0 ± 0.0	85.0 ± 9.5	97.3 ± 2.7
Pefabloc	10 mM	18.8 ± 3.9	NAD	57.9 ± 0.9

[1] Residual activity was calculated in percentage referred to the activity of the enzymes incubated in the absence of inhibitors. EDTA 2Na= ethylendiaminetetraacetic acid disodium salt, E-64=*trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane, NAD = no activity detected. Values of residual activity were expressed as mean of duplicate ± SD.

Table 4. Effect of metal ions on *Pseudomonas aeruginosa* M211 proteases activity.

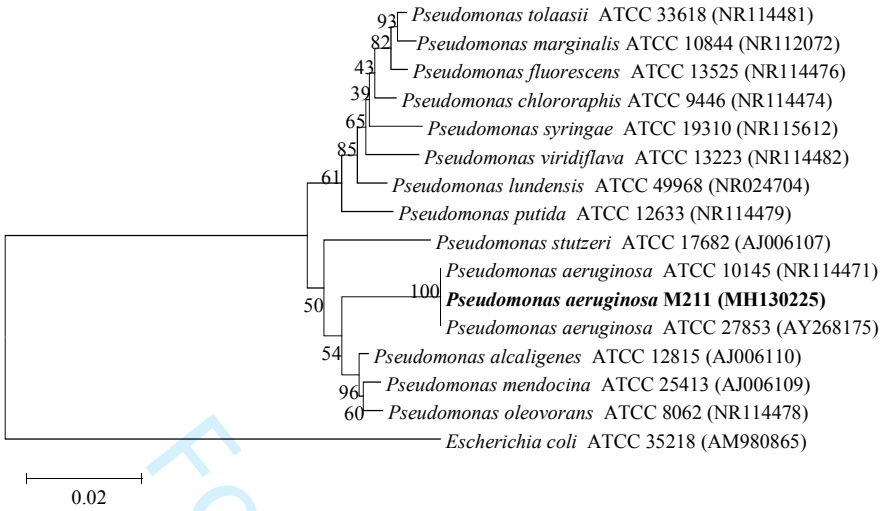
Ion (10mM)	Relative activity[1] (%)		
	Enzyme I	**Enzyme II**	**Enzyme III**
Control	100.0	100 .0	100.0
Ca2+	50.3 ± 5.3	49.7 ± 0.4	147.2 ± 0.1
Mg2+	128.4 ± 12.4	93.1 ± 1.7	173.4 ± 1.7
Mn2+	686.9 ± 43.4	368.0 ± 26.6	58.4 ± 3.9
Zn2+	NAD	NAD	114.8 ± 1.5

[1] Relative activity was expressed as the percentage of activity compared with a control without metal ions. Activity was measured at optimum pH and temperature conditions: EI and EII: pH 8, EIII: pH 11; and EI, EII and EIII: 60°C. NAD = no activity detected. Values of relative activity were expressed as mean of duplicate ± SD.

Table 5. Biochemical characteristics of extracellular proteases from different species of *Pseudomonas* genus.

Bacteria	Molecular weight (kDa)	Optimum pH	Optimum Temperature (°C)	Classification	Ref.
Pseudomonas aeruginosa MN1	32	8	60	Metalloprotease	6
Pseudomonas sp. CL 1457	35	8	60	Serine protease	42
Pseudomonas sp.	25	10	40	Serine protease	49
Pseudomonas aeruginosa	18	9	60	Metalloprotease	46
Pseudomonas aeruginosa PseA	35	8	60	Metalloprotease	19
Pseudomonas aeruginosa K	51	10	70	Metalloprotease	18
Pseudomonas aeruginosa ME-4	33	8	50	Metalloprotease	41
Pseudomonas aeruginosa MTCC 7926	35	9	55	Metalloprotease	47
Pseudomonas aeruginosa PseA	56	8	60	Metalloprotease	36
Pseudomonas aeruginosa	60	9	50	-	40
Pseudomonas fluorescens 07A	50	7.5	37	Metalloprotease	45
Pseudomonas putida A2	38	7	40	Serine protease	52
Pseudomonas fluorescens TBS09	50	7	60	Metalloprotease	48
Pseudomonas aeruginosa M211 (EI)	35	8	60	Serine-metalloprotease	a
Pseudomonas aeruginosa M211 (EII)	40	8	60	Serine protease	a
Pseudomonas aeruginosa M211 (EIII)	55	11	60	Serine-metalloprotease	a

a: this study



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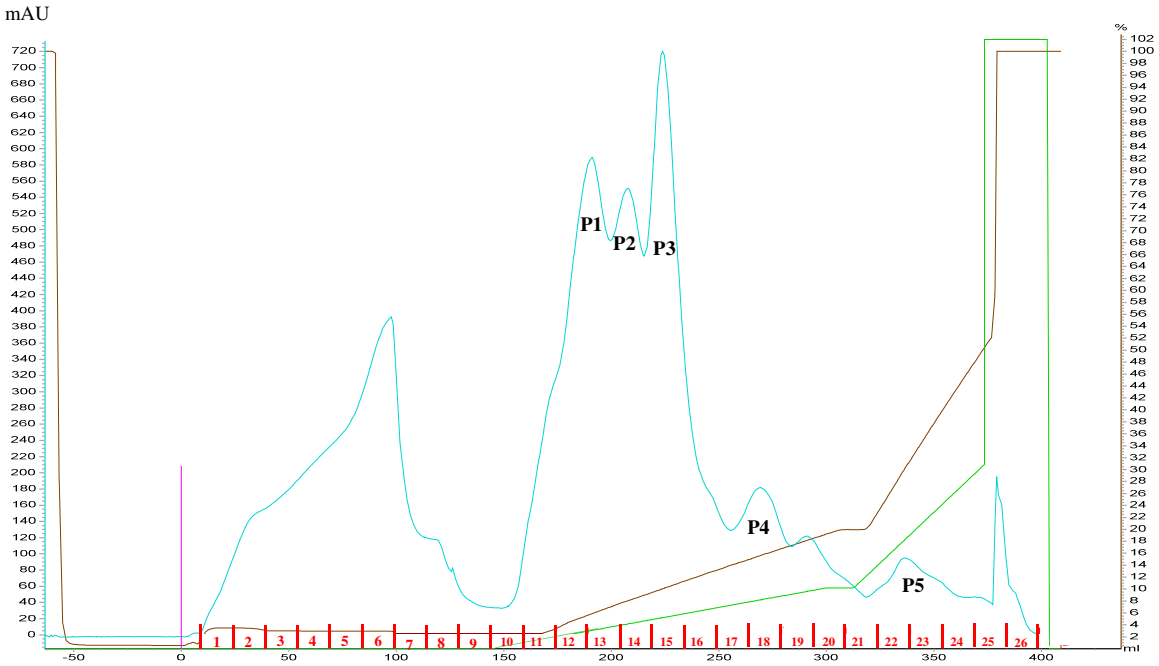

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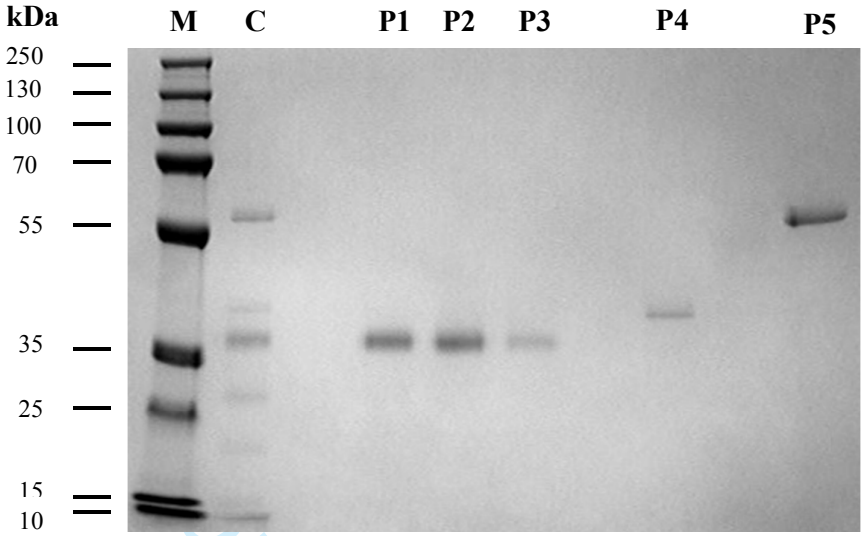

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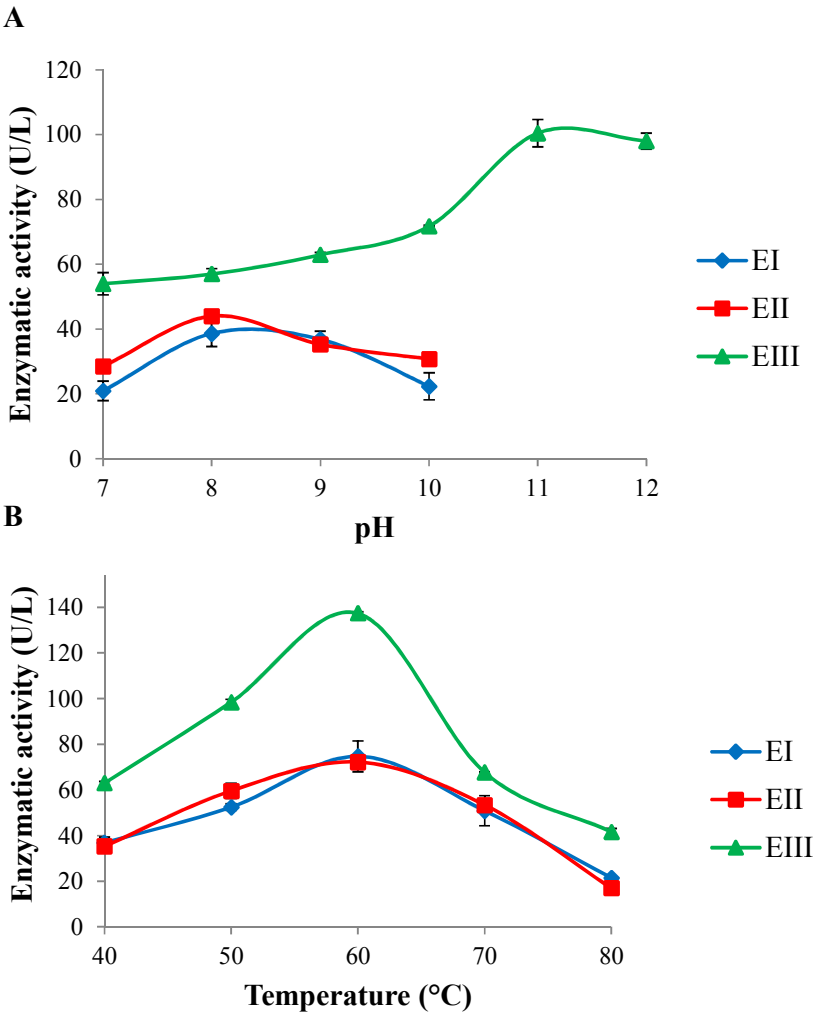

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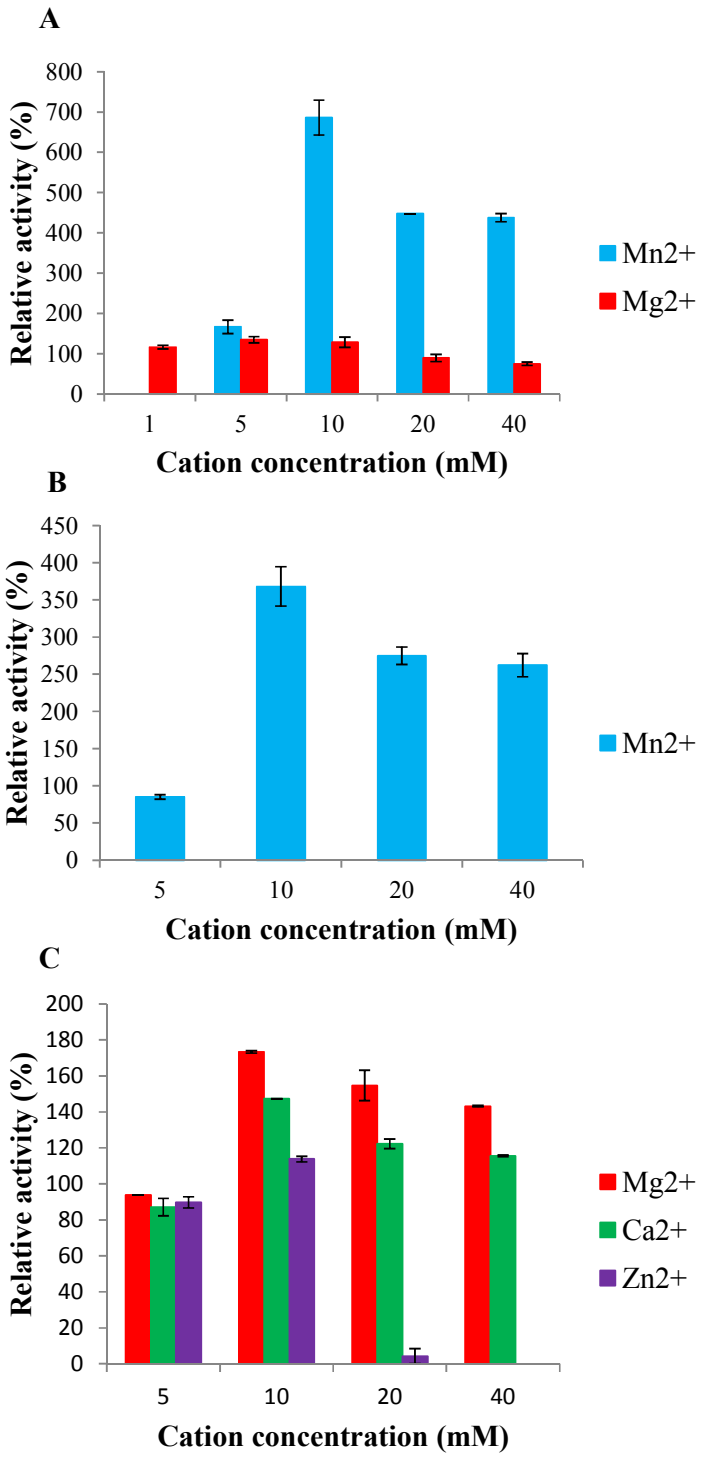

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