

Determination of Optimal Condition to Obtain the Bromelain from Pineapple Plants Produced by Micropropagation

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ABSTRACT

This study aimed to obtain the condition of maximum bromelain activity in different parts of pineapple plants produced *in vitro*, by micropropagation. The stems and leaves of Pérola and Imperial cultivar plants were evaluated after three and eight months of *in vitro* cultivation in Murashige and Skoog medium without growth phytohormone, macerated in potassium phosphate buffer at different pH values (5.7, 6.7 and 7.7). Total protein and proteolytic activity were determined in the samples after three- and eight-month cultivation periods. For both the cultivars, the best results were obtained at pH 5.7 in extraction media. Pérola cultivar, showed higher bromelain activity in the leaves cultivated *in vitro* for three months (0.0194 U/mL) while in the Imperial cultivar, it was higher in the stem after eight months (0.0179 U/mL). Imperial cultivar showed higher bromelain activity than the Pérola's.

Key words: Enzymatic activity, bromelain, Pérola pineapple, Imperial pineapple, production *in vitro*

INTRODUCTION

Bromelain is a protease of vegetal origin obtained from several species of the *Bromeliaceae* family. *Ananas comosus* L Merrill is the main source of bromelain, a product of great commercial value that is found in its fruits, stem, leaves and roots. It is used in food and pharmaceutical industries. Its efficacy has been reported to heal several health problems such as angina, indigestion and respiratory problems (Ferreira et al. 2011; Hale et al. 2005). Commercially bromelain is obtained from the pineapple stem. The fruit is also a good

option for its source as it has higher proteolytic activity than in the stem. A good quantity of fruit residue is generated by the pineapple processing factories, which can be used as raw material (Ferreira et al. 2011; Fileti et al. 2009 and 2010; Lopes et al. 2012; Parks 2001).

In vitro cultivation is an important procedure in the multiplication of different species. This technique can be applied on the industrial scale. The micropropagation, besides offering a greater amount of seedlings in a short period of time, enables total control of environmental conditions during the propagation and preservation of matrix

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plants without risk of infection (Pasqual et al. 1998, 2001; Moreira et al. 2003; Barboza et al. 2006). This technique can be an option for large - scale production of vegetal material from pineapple plant aiming to commercially exploit the bromelain enzyme.

Considering the increasing development of biotechnology, it has become necessary to develop new techniques to separate and purify biomolecules, such as enzymes and proteins of animal, vegetal and microbial origin. These techniques should maintain the chemical and physical properties of molecules and, further, should be highly efficient in the purification. Thus, the study on both purification and alternative extraction methods is of great interest, aiming to obtain more economically feasible processes. Hence, solid-liquid extraction applied to a technological process is extremely important for the development of new protein extraction and purification methods (Ferreira et al. 2011; Fileti et al. 2009 and 2010; Lopes et al. 2009; Silveira et al. 2009). This study aimed to quantify bromelain activity in different parts of pineapple plants produced *in vitro*.

MATERIALS AND METHODS

Micropropagation

Pérola and *Imperial* cultivars were provided by the EMBRAPA Tabuleiros Costeiros (Aracaju, SE, Brazil). The micropropagation was carried out using MS medium (Murashige and Skoog 1962) without growth phytohormone as described by

Corrêa et al. (2009), Quoirin et al. (2008), Radmann et al. (2011) and Scherwinski-Pereira et al. (2009). The MS medium was supplied with 30 g/L of sucrose and 7 g/L of agar-agar. The pH was adjusted at 5.8 and the medium was autoclaved at 120°C for 15 min. Inoculum was under s were done on aseptic conditions and light intensity of 52 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ at $27 \pm 2^\circ\text{C}$ for 16 h. The combination of growth regulators for adaptive phase were 2.5 μmol and 0.625 μmol concentrations of amino benzyl purine (BAP) and acetyl-naftalenic acid (ANA), respectively. These in the multiplication phase were 10 and 25 μmol of concentrations of BAP and ANA, respectively. During the multiplication phase, the plants were grown for three and eight months (Corrêa et al. 2009; Quoirin et al. 2008; Radmann et al. 2011; Scherwinski-Pereira et al. 2009).

Obtaining the Enzyme

The raw extract was obtained from the leaf and stem of pineapples *in vitro* using 1g mass which was macerated in 5 mL of 0.2M phosphate buffer at different pH (5.7, 6.7 and 7.7) and filtered twice in gauze (Almeida et al., 2002; Ferreira et al. 2011; Fileti et al. 2009 and 2010; Lopes 2005; Lopes et al. 2009 and 2012). Total protein was determined according to Bradford's method (Bradford 1976; Ferreira et al. 2007; Padilha et al. 2012; Severo Júnior et al. 2007) and enzymatic activity by the casein digestion method, or Kunitz's method, with modifications (Ferreira et al. 2011; Lopes 2005; Lopes et al. 2009; Silveira et al. 2009). The step to obtain the enzyme is shown in Figure 1.

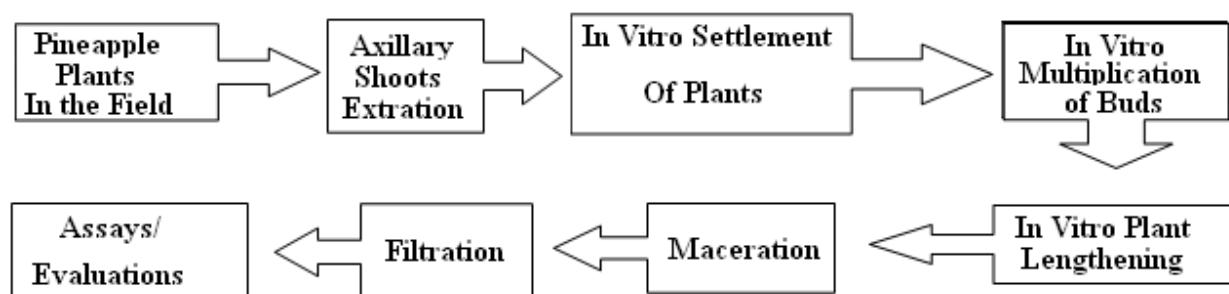


Figure 1 – Flowchart for *in vitro* production of plants and obtainment of bromelain enzyme raw extract.

Preparation of Buffer Solutions

Monobasic potassium phosphate (24.36, 12.72 and 2.2g) and dibasic potassium phosphate (1.18, 6.2

and 10.68g) were dissolved in 1000 mL of distilled water, respectively for pH 5.7, 6.7 and 7.7. Its pH was adjusted with phosphoric acid or potassium

hydroxide (Morita and Assumpção 1995; Ferreira et al. 2011; Ferreira et al. 2007; Severo Júnior et al. 2007; Padilha et al. 2012; Severo Jr et al. 2009).

Preparation of the Samples

The leaves and stems from the *in vitro* *Pérola* and *Imperial* cultivars were macerated separately at different pH values (5.7, 6.7 and 7.7) of phosphate buffer filtered twice in gauze and evaluated (Kiss et al. 1995; Moreira et al. 1999 and 2006; Pasqual et al. 1998 and 2001).

RESULTS AND DISCUSSIONS

Table 1 presents the results of bromelain activities. In *Pérola* cultivar, the activity was lower when a buffer solution with higher pH was used and there was a reduction in the enzymatic activity when the plant cultivation time was longer. There was also a reduction in total protein as the cultivation time was longer, except at pH 7.7

When comparing the cultivation time in a medium with no growth regulator, the concentration

profiles were similar for both the ages and only differed in the basic region, where the leaf's extract whose cultivation time was longer showed an almost constant behavior as the three-month old leaves. pH 5.7 showed the best results for all the cultivar parts and any cultivation period of time, demonstrating to be the optimal pH for this enzyme. Considering the best cultivations conditions for the *Pérola* cultivar, consisting of pH 5.7 and a 3-month cultivation for the leaves, enzymatic activity was 0.0194 U/mL and total protein had a concentration of 10.342 mg/L.

Table 2 shows the results for *Imperial* cultivar. There was a reduction in bromelain enzyme activity when the pH in the extraction solution was increased; indicating that pH 5.7 also should be optimal for this cultivar. However, enzymatic activity increased as the cultivation time increased either in the leaves or in plant stems, an inverse behavior of that presented by *Pérola* cultivar. Total protein showed a reduction when the cultivation time was longer, which was similar to *Pérola*'s cultivar.

Table 1 - Bromelain's activity and protein concentration in the leaves and stems of *Pérola* cultivar regarding distinct cultivation times and pH values for buffer solution.

pH	Activity (U/mL)				Proteins (mg/L)			
	Leaves		Stem		Leaves		Stem	
	3 months	8 months	3 months	8 months	3 months	8 months	3 months	8 months
5.7	0.0194	0.0050	0.0053	0.0097	10.342	10.679	6.3268	4.4458
6.7	0.0073	0.0008	0.0098	0.0040	12.796	11.652	7.5910	5.6837
7.7	0.0012	0.0002	0.0015	0.0010	1.5061	11.497	0.2559	5.4563

Table 2 - Bromelain's enzymatic activity and protein concentration in leaves and stems of *Imperial* cultivar considering different cultivations times and phosphate buffer pH values.

pH	Activity (U/mL)				Proteins (mg/L)			
	Leaves		Stem		Leaves		Stem	
	3 months	8 months	3 months	8 months	3 months	8 months	3 months	8 months
5.7	0.0107	0.0112	0.0148	0.0180	8.759	8.759	4.402	4.087
6.7	0.0083	0.0167	0.0000	0.0076	12.530	8.146	6.270	4.048
7.7	0.0030	0.0114	0.0067	0.0149	12.136	11.248	6.0381	3.995

It was also noticed that the enzymatic activity was higher in the stems than in the leaves and total protein was higher in the latter. A good enzymatic activity for low protein concentrations is considered ideal as a high specific activity is obtained (activity/protein mass). So, the optimal condition to obtain bromelain enzyme from

Imperial cultivar in an *in vitro* production was the one in which the enzyme was extracted from the stem cultivated for eight months in a buffer phosphate at pH 5.7, whose enzymatic activity was 0.0148 U/mL and total protein concentration was 4.087 mg/L.

Results of the Figure 2 corroborated with the analysis of Tables 1 and Table 2. It showed that high activities were found in the leaves for the *Pérola* pineapple variety and steam sample for the *Imperial* pineapple variety and both were high

values for three months cultivation. When comparing the two varieties, the *Imperial* cultivar showed higher production potential for bromelain production than the *Pérola* cultivar.

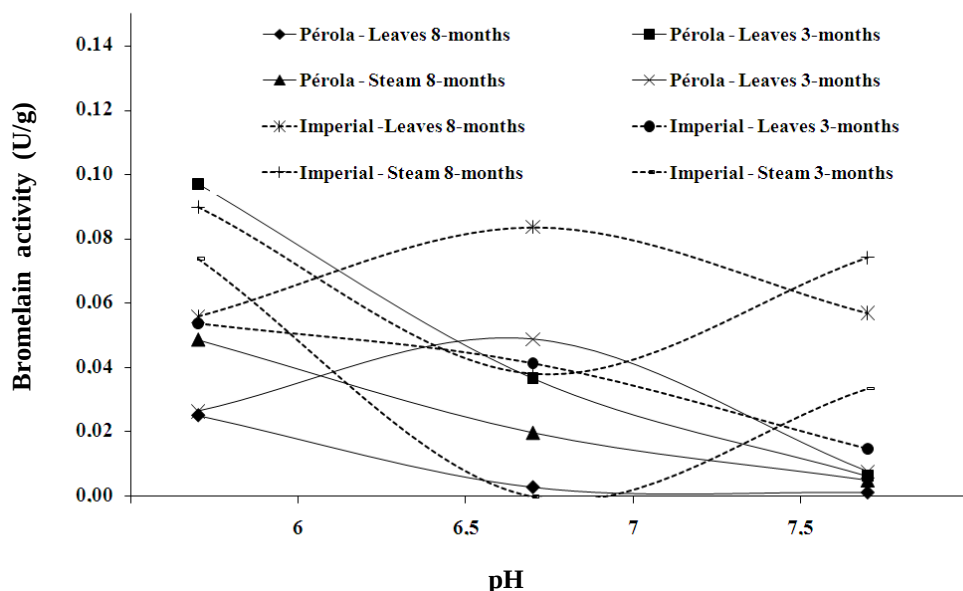


Figure 2 - Enzymatic activities for unity of pineapple plants.

Alcantara et al. (2011) and Pereira et al. (2011) found that longer period affected negatively the *in vitro* explant aspect, leading to a decrease in the survival of the plant during acclimatization for *Eucalyptus grandis*, *Eucalyptus urophylla* and apple plants. However, Radmann et al. (2011) observed that between 20-40 days of growth of 'Flordaguard' rootstock, longer cultivation time was best. Scherwinski-Pereira et al. (2009) showed that effect of the growth time varied the seasons for the potato plants. The crop period was fast in spring-summer (96 days) than autumn-winter (107 days). The micropropagation is used to maximize the plant growth. This technique decreases the production time and increases the productivity and resistance of plants to the diseases, helps in the regeneration, control in propagation genetic improvement, etc. There are other techniques such as genetic transformation that can be incorporated in a genetic breeding program in order to increase yields, shorten production cycles and promote the development of high quality plantations (Alcantara et al. 2011; Corrêa et al. 2009; Pereira et al. 2011;

Radmann et al. 2011; Scherwinski-Pereira et al. 2009).

CONCLUSIONS

Studies aimed to quantify bromelain obtained from the leaves and stems extracts of *in vitro* *Perola* and *Imperial* pineapple cultivars using a simple extraction with phosphate buffer at pH 5.7, 6.7 and 7.7, enabled the following conclusions:

- The proteolytic enzymatic activity in the pineapple plants *in vitro* extracts varied in function of extraction medium pH, variety, part and age of the plant.
- The enzymatic activity increased as the pH of the phosphate buffer pH decreased.
- There was significant variation in protein concentration amount between the pineapple parts evaluated.
- For both the cultivars, the best pH for the extraction was 5.7.

- The best cultivation time for *Perola* cultivar was the three month and for *Imperial* cultivar, it was eight months.
- The best part to obtain bromelain in *Perola* cultivar was its leaf, while in *Imperial* cultivar, it was its stem.
- *Imperial* cultivar had higher bromelain production potential than the *Perola* cultivar.
- The method used in this study was simple and economical, and efficient to evaluate the activity in the enzymatic preparations as well.

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REFERENCES

- Alcantara GB, Bessalho Filho JC, Quoirin M. Organogenesis and transient genetic transformation of the hybrid *Eucalyptus grandis* × *Eucalyptus urophylla*. *Sci Agri*. 2011; 68(2): 246-251.
- Almeida WAB, Santana GS, Rodriguez APM, Costa MAPdeC. Optimization of a protocol for the micropropagation of pineapple. *Revista Brasileira de Frutic*. 2002; 24(2): 296-300.
- Barboza SBSC, Graciano-Ribeiro D, Teixeira JB, Portes TA, Souza LAC. Anatomia foliar de plantas micropropagadas de abacaxi. *Pesq Agropec Bras*. 2006; 41(2): 185-194.
- Bradford MM. A rapid and sensitive method for the quantitative of mg quantities of protein, utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72: 248-254.
- Corrêa CM, Oliveira GN, Astarita LV, Santarém ER. Plant regeneration through somatic embryogenesis of yacón [*Smallanthus sonchifolius* (Poepp. and Endl.) H. Robinson]. *Braz Arch Biol Technol*. 2009; 52(3): 549-554.
- Ferreira GB, Evangelista AF, Severo Junior JB, Souza RRda, Santana JCC, Tambourgi EB, et al. Partitioning optimization of proteins from *Zea mays* malt in ATPS PEG 6000/CaCl₂. *Braz Arch Biol Technol*. 2007; 50(3): 557-564.
- Ferreira JF, Santana JCC, Tambourgi EB. The Effect of pH on Bromelain Partition from *Ananas comosus* by PEG4000/Phosphate ATPS. *Braz Arch Biol Technol*. 2011; 54(1): 125-132.
- Fileti AMF, Fischer GA, Tambourgi EB. Neural Modeling of Bromelain Extraction by Reversed Micelles. *Braz Arch Biol Technol*. 2010; 53(2): 455-463.
- Fileti AMF, Fischer GA, Santana JCC, Tambourgi EB. Batch and Continuous Extraction of Bromelain Enzyme by Reversed Micelles. *Braz Arch Biol Technol*. 2009; 52(5): 1225-1234.
- Hale LP, Greer PK, Trinh CT, James CL. Protein activity and stability of natural bromelain preparations. *Int Immunopharmacol*. 2005; 2: 783-793.
- Kiss E, Kiss J, Gyulai G, Heszky LE. A novel method for rapid micropropagation of pineapple. *Hort Sci*. 1995; 30(1): 127-129.
- Lopes FLG. Recuperação da bromelina a partir da polpa do *Ananas comosus* L. Merrill, utilizando processos de separação por membrana [PhD Thesis]. Campinas, Brazil: University of Campinas; 2005.
- Lopes, FLG, Severo Júnior JB, Souza RRde, Ehrhardt DD, Santana JCC, Tambourgi EB. Concentration by membrane separation processes of a medicinal product obtained from pineapple pulp. *Braz Arch Biol Technol*. 2009; 52(2): 457-464.
- Lopes FLG, Sbruzzi D, Barros KVG, Ferreira JF, Santana JCC, Souza RRde et al. Viability in the production of a drug extracted from *Ananas comosus* by a flat membrane system. *Braz Arch Biol Technol*. 2012; 55(3): 465-470.
- Moreira MA, Pasqual M, Carvalho JG, Fráguas CB. Estiolamento na micropropagação do abacaxizeiro cv. Pérola. *Ciênc Agrotec*. 2003; 27(5): 1002-1006.
- Moreira MA, Sobrinho AA, Pasqual M. Indução ao estiolamento *in vitro* de brotos de abacaxi cv. Pérola. *Rev Univ Alfenas*. 1999; 5: 193-197.
- Morita T, Assumpção RMV. Manual de Soluções, Reagentes e Solventes. Padronização-Preparação-Purificação. 2ª ed. São Paulo: Edgard Blücher Ltda; 1995.
- Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol plant*. 1962; 15: 473-497.
- Padilha GdS, Santana JCC, Monte Alegre R, Tambourgi EB. Extraction of lipase from *Burkholderia cepacia* by PEG/phosphate ATPS and its biochemistry characterization. *Braz Arch Biol Technol*. 2012; 55(1): 7-19.
- Park YK. Produção de enzimas Industriais de Origem vegetal. Biotecnologia Industrial. 1st ed. São Paulo: Edgard Blücher; 2001.
- Pasqual M, Chalfun NNJ, Ramos JD. Aplicações na propagação de plantas. 1ª ed. Lavras: EDUEFLA; 2001.

- Pasqual M, Moreira MA, Sobrinho AA. Biotecnologia aplicada à produção de mudas de abacaxi. *Inf Agrop*. 1998; 19(195): 20-23.
- Pereira JES, Fortes GRL, Silva JB. Baixa temperatura para explantes do porta-enxerto de macieira 'marubakaido' *in vitro* durante a aclimatização. *Sci Agric*. 2011; 58(2): 401-405.
- Quoirin MGG, Biasi LA, Rios JF, Cuquel FL. Micropropagation of *Gypsophila paniculata* through culture of nodal segments. *Scientia Agraria*, 2008; 9(4): 79-83.
- Radmann EB, Bianchi VJ, Fachinello JC, Ferreira LV, Oliveira RP. *In vitro* multiplication of 'Flordaguard' rootstock: cytokinin source and concentration effects, explants orientation and period of permanence in the culture medium. *Braz Arch Biol Technol*. 2011; 54(1): 25-34.
- Scherwinski-Pereira JE, Medeiros CAB, Fortes GRL, Pereira AS. Production of pre-basic potato seed by polyvinyl chloride PVC - articulate gutters hydroponic system. *Braz Arch Biol Technol*. 2009; 52(5): 1107-1114.
- Severo Júnior JB, Oliveira LSS, Sardeiro FS, Souza RRde, Lopes FLG, Santana JCC, Tambourgi EB. Response surface methodology to evaluation the recovery of amylases by hollow fiber membrane. *Braz Arch Biol Technol*. 2007; 50(4): 713-718.
- Severo Jr JB, Souza RRde, Santana JCC, Tambourgi EB. A study of ion-exchange chromatography in an expanded bed for bovine albumin recovery. *Braz Arch Biol Technol*. 2009; 52(2): 427-436.
- Silveira E, Souza-Jr ME, Santana JCC, Chaves AC, Porto ALF, Tambourgi EB. Expanded bed adsorption of bromelain (e.c. 3.4.22.33) from *Ananas comosus* crude extract. *Braz J Chem Eng*. 2009; 26(1): 149-157.

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