

ORIGINAL ARTICLE

5'-Ribonucleotides production using 5'-phosphodiesterase from spent malt roots

Produção de 5'-ribonucleotídeos utilizando 5'-fosfodiesterase da radícula de malte cervejeiro

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Cite as: Alves, E. M., Souza, J. F., Macieja, S., & Oliva Neto, P. (2021). 5'-Ribonucleotides production using 5'-phosphodiesterase from spent malt roots. *Brazilian Journal of Food Technology*, 24, e2020246. <https://doi.org/10.1590/1981-6723.24620>

Abstract

5'-ribonucleotides are high value-added molecules widely used in the food and pharmaceutical industries because of their bioactive properties. The present work aims to produce a composition of 5'-ribonucleotides using spent brewer's yeast as cheap source of RNA, and barley malt rootlets as cheap source of 5'-phosphodiesterase (5'-PDE). This is a very promising and innovative strategy because both spent yeast and malt rootlets are residues of the brewing process and are closely linked in a cycle that until now is not yet commercially exploited due to lack of studies. Our results showed that extraction of 5'-PDE was mainly influenced by the fineness of the rootlets and amount of extraction solvent (water). The main molecules formed during RNA hydrolysis were 5'-ribonucleotides, which represented 85.86% of the total hydrolyzed molecules. Finally, the results of the proposed approach can generate a new perspective for the brewing industry regarding the management of its wastes, generating from them products of high added value and with a wide range of applications.

Keywords: Brewing industry wastes; Spent brewer's yeast; Barley malt rootlets; RNA hydrolysis; Enzyme kinetics; Bioprocess optimization.

Resumo

5'-ribonucleotídeos são moléculas de alto valor agregado amplamente utilizadas nas indústrias alimentícia e farmacêutica, em razão de suas propriedades bioativas. O presente trabalho visa produzir uma composição de 5'-ribonucleotídeos a partir de levedura residual cervejeira, como fonte de RNA, e radícula de malte cervejeiro, como fonte barata de 5'-fosfodiesterase (5'-PDE). A abordagem proposta mostrou-se promissora e com caráter inovador, pois ambas, levedura e radícula de malte, são resíduos do processo cervejeiro, estando intimamente ligadas em um ciclo que até o momento ainda não foi explorado comercialmente por falta de estudos. Os resultados do presente trabalho mostraram que a extração de 5'-PDE foi influenciada principalmente pelo tamanho das radículas e pela quantidade de solvente de extração (água). As principais moléculas formadas durante a hidrólise de RNA foram 5'-ribonucleotídeos, os quais representaram 85,86% do total de moléculas hidrolisadas. Por fim, os resultados deste



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trabalho podem gerar uma nova perspectiva para a indústria cervejeira no que se refere ao gerenciamento de seus resíduos, a fim de gerar a partir deles produtos de alto valor agregado e com ampla gama de aplicações.

Palavras-chave: Resíduos da indústria cervejeira; Levedura residual cervejeira; Radícula de malte de cevada; Hidrólise de RNA; Cinética enzimática; Otimização de bioprocessos.

1 Introduction

5'-ribonucleotides and nucleosides are high value-added molecules and are widely used in the food and pharmaceutical industries because of their bioactive properties (Jiao et al., 2019). Nucleosides are composed of a nitrogenous base (purine or pyrimidine) and a pentose (ribose for RNA or 2'-deoxyribose for DNA). When one or more phosphate groups are present, the compound is known as nucleotide and is the most important class of nucleic acid derivatives (Trelles et al., 2019). Phosphodiester bonds connect the 5' carbon of one nucleotide to the 3' carbon of another nucleotide forming polymers known as polynucleotides or nucleic acids. The selective chemical or enzymatic hydrolysis of ribonucleic acids produces a composition of nucleosides and nucleotides.

The process to be chosen for RNA hydrolysis deserves due attention because it directly affects the characteristics of the molecules produced. Chemical RNA hydrolysis can be carried out under alkaline conditions but is considered limited because RNA is degraded to lower molecular weight compounds without any specificity (Smernik et al., 2015). In this process, ribonucleotides with 2'- and 3'-phosphate groups are obtained mostly. These molecules show no flavoring characteristics and are of little commercial interest. 5'-ribonucleotides, on the other hand, are involved in several basic biological processes and are considered high value-added molecules. The production of 5'-ribonucleotides requires the hydrolysis of the crude RNA by means of a very specific enzyme, 5'-phosphodiesterase (5'-PDE). This enzyme hydrolyzes the RNA ester bonds at the 3' position, promoting the release of 5'-ribonucleotides in which the phosphate group is attached to the 5' carbon of the pentose molecule (Luo et al., 2017).

Cohn and Volkin (1953) were the first to demonstrate the presence of 5'-PDE activity in snake venom. Despite its high efficiency, it cannot be used in the agri-food industry for obvious reasons. Other important sources of 5'-PDE include certain fungi such *Penicillium citrinum* (Chen et al., 2019), certain species of *Halobacterium* (Boonpan et al., 2015) and *Aspergillus sp.* (Luo et al., 2017). However, this type of enzyme production requires industrial fermentation and quite complex purification steps, which ultimately results in an expensive enzyme. 5'-PDE from *Penicillium citrinum*, for example, is available commercially at Amano Enzyme among others, under the name Nuclease RP-1G (or EC3, 1.30.1). Because of its very high cost, this enzyme is mainly used on an industrial scale in an immobilized form. Its use in a process for producing yeast extracts enriched in 5'-ribonucleotides induces very large additional enzyme costs. Animal tissues are other sources of 5'-PDE (Morelli et al., 2011), which can also be obtained from germs of some plants, such as rootlets of oats, barley, wheat, corn, rye, millet, sorghum rice and malt rootlets (Xu et al., 2013).

Compared with many other sources, barley malt rootlets are by-products of the brewing industry and, therefore, cheaper materials for the preparation of 5'-PDE and additionally, there is no doubt about the safety of the enzyme. According to Zou et al. (2008), 5'-PDE can be obtained by simple mixing malt root powder in an aqueous solution to extract the enzyme. Barley rootlets are good source of this enzyme among others including deoxyribonuclease, ribonuclease and adenosine-5'-phosphomono-esterase. As previously mentioned, this enzyme can be obtained from a variety of other sources such as plant (Beluhan et al., 2020), animal (Cesarini et al., 2020) and microbial (Okado et al., 2016). However, considering economic aspects, preparations of 5'-PDE from a cheap source residue such malt roots is very promising. Besides, it can serve as good raw material for large-scale production of 5'-PDE.

Based on the explanations above, the present work aims to study the optimal conditions for 5'-PDE extraction from spent malt rootlets as well as its application for hydrolyzing RNA from spent brewer's yeast. It was also objective to determine the enzyme characteristics, including optimal pH and temperature, heat stability and Michaelis' constant (K_m).

2 Material and methods

2.1 Extraction of 5'-PDE

Malt roots were kindly provided by Agrária (Cooperativa Agrária Agroindustrial, Guarapuava, PR, Brazil). Approximately 1 kg of malt roots were sieved and fractioned according to their granulometry (Tamis mesh 16-100, Bertel, Caieras, Brazil). The influence of granulometry and water proportion for the enzyme extraction was evaluated. Malt rootlets (1 g) of the different granulometry were soaked with 6 ml distilled water at 20 °C for 20 min to extract crude 5'-PDE. For the water-rootlet proportion assay, 1g of malt rootlet were soaked with water in the following proportions (w/w): 4:1, 8:1, 12:1, 16:1 and 20:1. After extraction, the samples were filtrated with gauze and centrifuged for 10 minutes at 956 x G (Heraeus Megafuge 16R, Thermo Scientific, Osterode am Harz, Germany). The supernatant was collected and used for 5'-PDE activity assays. Finally, the total protein content of the 5'-PDE extracts was determined according to the classical Kjeldahl method described by AOAC (Association of Official Agricultural Chemists, 2000), thus evaluating the specific activity of the enzyme.

2.2 5'-PDE activity assay

The 5'-PDE activity was determined as described by Fujimoto et al. (1974). It consists of the measure of the absorbance at 260 nm of nucleotides molecules released after RNA hydrolysis by the enzyme. Test tubes (13x100 mm) containing 0.9 ml of RNA solution 1% (w/v) (Sigma-R6625) in acetate buffer solution 0.125 M pH 5.3 and 3 mM Zn^{2+} ion were incubated in a water bath (MA-093, Marconi, Piracicaba, Brazil) at 69 °C for 10 minutes to equilibrate the temperature. Then, 100 µl of crude enzyme solution was added and the tubes were incubated for 15 minutes at 69 °C with shaking every 5 minutes. After incubation, 2.0 ml of ammonium molybdate solution 0.25% (m/v) and perchloric acid 2.5% (v/v) (kept in a refrigerator) were added to inactivate the reaction and precipitate the unhydrolyzed RNA. The tubes were then incubated in an ice bath for 10 minutes and then centrifuged for 10 minutes at 956 x G (Heraeus Megafuge 16R, Thermo Scientific, Osterode am Harz, Germany). The supernatant was collected and diluted 50-fold with deionized water and the absorbance was measured at 260 nm in a spectrophotometer (UV-M51, Bel Photonics, Piracicaba, Brazil) using quartz cuvettes with 1 cm of optical path. The blank was prepared by substituting 100 µl of enzyme solution with deionized water in the reaction mixture, followed by the same procedures. The activity of the enzyme was calculated according to Equation 1.

$$5'PDE \left(\frac{U}{mL} \right) = \frac{(A_{260nm}^{Test} - A_{260nm}^{Blank}) * 2 * 50}{10.6 * 0.1 * 15} \quad (1)$$

Where: 2 = volume (ml) of stopped reaction; 50 = dilution factor; 10.6 = millimolar extinction coefficient ξ ($\mu\text{mol/ml}$) of hydrolyzed ribonucleic acid at 260 nm; 0.1 = volume (ml) of enzyme used and 15 = time (in minutes) of assay as per the Unit Definition. One unit of 5'-PDE activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute.

2.3 5'-PDE characterization

To determine the optimum reaction temperature, the enzyme activity was performed under the same conditions previously mentioned, changing only the temperature values to 30, 40, 45, 50, 55, 60, 65, 70, 80 and 90 °C.

To determine the optimal pH conditions of the reaction, the following 0.125 mol/L buffers were used: glycine-HCl (for pH values in the range of 2.0 to 3), sodium acetate (for pH values in the range of 4.0 to 5.5) and sodium phosphate (for pH values in the range of 6.0 to 8). The enzyme activity was determined as previously described altering only the buffer solution and the pH during the reaction.

The enzyme Michaelis' constant (K_m) was determined by varying the initial concentration of RNA between 0.1 and 30 mg/ml during the reaction. The kinetic model proposed by Michaelis-Menten with substrate inhibition (Equation 2) was adjusted to the experimental results of enzyme activities during the reaction as a function of substrate concentration by means of nonlinear regression using MATLAB software R2017b (Mathworks®), so that the inhibition constant K_i was also determined.

$$V = \frac{V_{max} * S}{K_m + S + \frac{S^2}{K_i}} \quad (2)$$

The effect of heating on the thermostability of 5'-PDE was investigated at 60, 65 and 70 °C for different periods up to 24 hours.

2.4 Enzymatic RNA hydrolysis

Enzymatic RNA hydrolysis for 5'-ribonucleotides production was performed by incubating RNA from spent brewer's yeast with the crude enzyme extract from malt rootlet. Approximately 10 g of centrifuged spent brewer's yeast, having a moisture content of 75%, were weighed and then added water (25.8 ml) so that the autolysis was performed at a ratio of yeast/water (w/v) of 7.5%, on yeast dry basis. After homogenization, the cell suspension was subjected to autolysis for 60 minutes at temperature 60 °C and then centrifugated for 10 minutes at 956 x G (Heraeus Megafuge 16R, Thermo Scientific, Osterode am Harz, Germany). The RNA hydrolysis assay was performed by mixing the supernatant (7.2 ml) with the crude enzyme (0.8 ml) for up to 24 hours. Samples were collected at intervals for 5'-ribonucleotides and ribonucleosides content determination.

2.5 5'-Ribonucleotides and ribonucleosides determination

High Performance Liquid Chromatography (HPLC) was used to verify and quantify the 5'-ribonucleotides and ribonucleosides formed during RNA hydrolysis. Standard solutions containing 10µg/ml of 5'-GMP (G8377-5G, Sigma-Aldrich Brazil Ltda), 5'-CMP (C1006-5G, Sigma-Aldrich Brazil Ltda), 5'-UMP (U6375-5G, Sigma-Aldrich Brazil Ltda.), 5'-IMP (I4625-5G, Sigma-Aldrich Brazil Ltda), 5'-AMP (01930-5G, Sigma-Aldrich Brazil Ltda), Guanosine (G6752-25G, Sigma-Aldrich Brazil Ltda), Cytidine (C4654-5G, Sigma-Aldrich Brazil Ltda), Uridine (U3750-1G, Sigma-Aldrich Brazil Ltda), Inosine (I4125-5G, Sigma-Aldrich Brazil Ltda) and Adenosine (A9251-5G, Sigma-Aldrich Brazil Ltda) were prepared and used for standard curve preparation. The separation of molecules was achieved on reverse phase column Acclaim C30 (2.1 x 150 mm, 3 µm) at 15 °C using an HPLC system (2475 - Multi λ Fluorecence Detector, Waters®) with UV detector at 260 nm and elution gradient. The mobile phase consisted of 3 components: A) deionized water; B) 100 mM ammonium acetate buffer, pH 5 and C) Methanol. The entire run lasted 28 minutes and the elution gradient events are detailed in Table 1. From this methodology, chromatograms of each compound were obtained to determine the retention time of the molecules.

Table 1. Elution gradient.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Flux (mL/min)
0.0	80	20	0	0.25
3.0	80	20	0	0.40
5.0	70	20	15	0.40
8.5	40	20	40	0.25
15.0	40	20	40	0.25
15.1	80	20	0	0.25
23.0	80	20	0	0.25
23.1	80	20	0	0.40

Solvent A: deionized water; Solvent B: 100 mM ammonium acetate buffer, pH 5; Solvent C: Methanol.

3 Results and discussion

3.1 Enzyme extraction

Figure 1 shows the visual appearance of the fractions obtained after malt rootlets sieving. The influence of granulometry, water proportion and time for the enzyme extraction are shown in Figure 2.

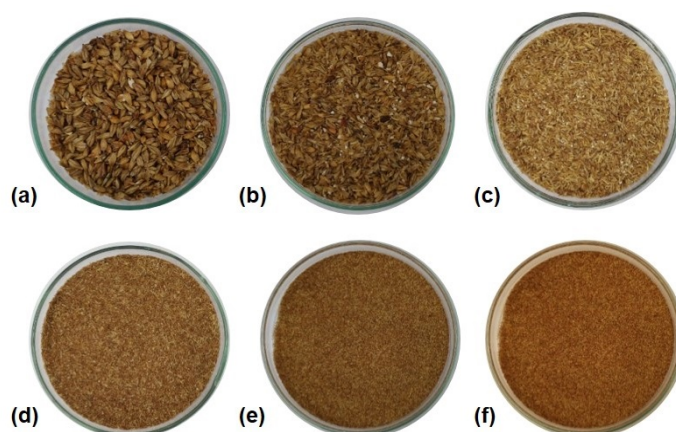


Figure 1. Fractions obtained after granulometric separation of malt rootlets: (a) mesh >16, (b) 16 < mesh < 35, (c) 35 < mesh < 48, (d) 48 < mesh < 60, (e) 60 < mesh < 80 and (f) 80 < mesh < 100.

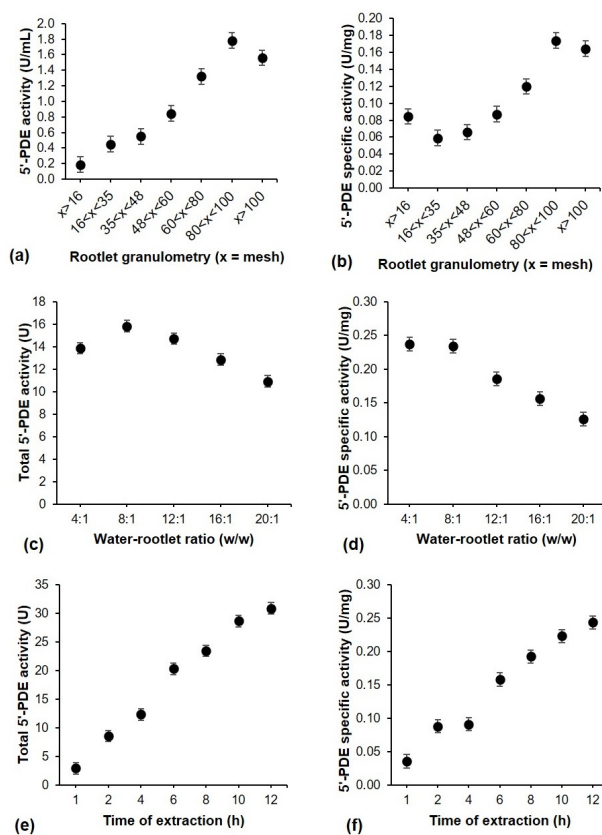


Figure 2. Influence of malt rootlet granulometry (a and b), water-rootlet ratio (c and d) and time (e and f) on the 5'-PDE extraction.

The extraction of 5'-PDE was mainly influenced by the fineness of the rootlets and amount of extraction solvent (water). The smaller the diameter of the rootlet, the greater the yield of the extraction. The best results found for malt rootlet granulometry and water ratio were 80<mesh>100 and 8:1 respectively. Hua & Huang (2010) studied 5'-PDE from barley malt rootlets and their results showed that maximum total enzyme activity was obtained with the fineness of the barley rootlets larger than 120 mesh, pH 7, temperature at 20 °C, water-rootlet ratio of 16:1 and the extraction time of 7 hours. In the present work, with the increase in the extraction time, there was an increase in the extraction of the enzyme in a linear way. In addition, the specific activity also increased up to the maximum time studied (12h) (Figure 2f). Besides, the amount of water of the best water-rootlet ratio result (8:1) was lower in comparison to Hua & Huang (2010) results, which gives a more concentrated crude extract and reduces the cost related to enzyme concentration.

3.2 Enzyme characterization

The results of the influence of pH and temperature on the enzyme activity of 5'-PDE are shown in Figure 3. Figure 3a shows that the enzyme activity is maximal at pH 5. 5'-PDE, showing to be very sensitive to this parameter, thus small variations in the pH can contribute negatively to the enzyme activity being very important and necessary the use of buffer solution for better yield during the hydrolysis of RNA. As noted in Figure 3b, the enzyme activity of 5'-PDE was favored by temperatures in the range of 60 to 70 °C. These results are in accordance with the results of Zou et al. (2008) and Hua & Huang (2010), which also obtained an optimum 5'-PDE activity at temperatures in this range.

Furthermore, the effect of heating on the thermostability of 5'-PDE showed that this enzyme has excellent thermostable properties (Figure 3c). The enzyme activity increased slightly with the increasing of the temperature but overall hydrolysis profile was similar up to 24 hours reaction. As seen in Figure 3d, the enzyme activity is also affected by the concentration of the substrate. 5'-PDE activity reaches a maximum value as the substrate concentration increases up to about 9 mg/ml and after this point, an addition of substrate inhibits the reaction. To mathematically model this behavior, the experimental results were adjusted to the substrate inhibition model according to Equation 2 and the parameters of V_{max} , K_m and K_i were determined by a non-linear regression. A comparison between the experimental and predicted results obtained by the model can be observed in Figure 3d.

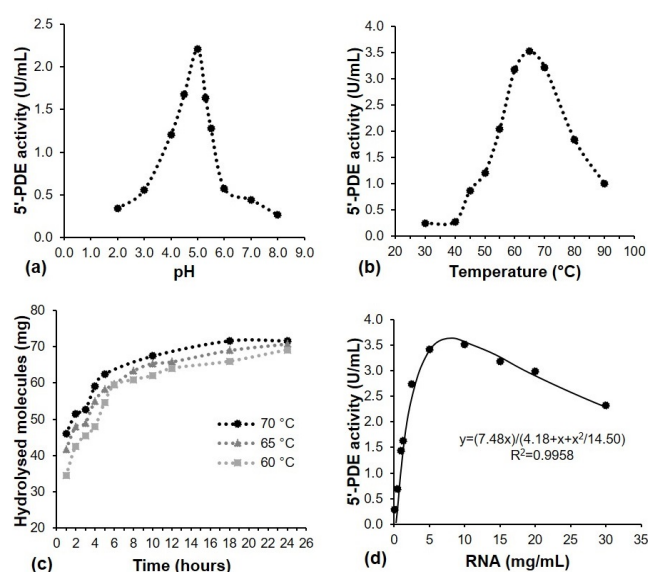


Figure 3. 5'-PDE characterization results: (a) influence of pH and (b) temperature on 5'-PDE activity; (c) effect of heating over the time on the thermostability of 5'-PDE and (d) nonlinear regression for the substrate inhibition model on 5'-PDE activity.

The adjustment of the model (Equation 3) reached a coefficient of determination of 0.9958, indicating that 99.58% of the values of the enzyme activity obtained experimentally can be explained by the variation of RNA concentration.

$$V = \frac{7.48 * S}{4.18 + S + \frac{S^2}{14.50}} \quad (3)$$

Where $V_{max} = 7.48$ U/ml, $K_m = 4.18$ mg RNA/ml and $K_i = 14.50$ mg RNA/ml.

The K_m value obtained is close to those found in the literature for the same enzyme. It is important to note that the choice of the regression method to determine parameters such as V_{max} and K_m is very important because it interferes with the reliability of the results. Several authors have criticized linear regression methods because they have become obsolete over the years. According to Brooks et al. (2012), for example, the Lineweaver-Burke graphs distort the measurements performed at low substrate concentrations and this may give rise to not very accurate estimates of V_{max} and K_m . On the other hand, K_m is a characteristic of the enzyme and its substrate and its value is independent of the amount of enzyme used for its experimental determination, but the same is not true for V_{max} . There is no absolute value of V_{max} and its value depends on the amount of enzyme used. In addition to V_{max} and K_m , K_i are of great importance in the study of enzyme kinetics, since it allows to obtain information about the way in which the activity of the enzyme is affected by the operating conditions, being possible to plan and optimize the parameters of operation in reactors enzymes. 5-PDE is an enzyme very sensitive to RNA inhibition, so it is important to study the ideal concentration of substrate that does not present a risk of inhibition. The K_i value found in this work indicates discourages RNA concentration above 14.50 mg/ml. The K_i value indicates how potent an inhibitor is and can be defined as the concentration of inhibitor in which, under saturating substrate conditions, the reaction rate is reduced to half the maximum reaction rate V_{max} . The lower the K_i value for a given enzyme, the greater the inhibitory capacity of the substrate.

3.3 Enzymatic RNA hydrolysis

Figure 4 shows the UV-260 nm HPLC chromatogram obtained after 12 hours of reaction which allows to clearly observe the efficiency of the enzyme through the formed peaks in comparison with the RNA control, in which enzyme was not added. It was possible to obtain a good separation of the molecules as well as to calibrate a standard curve with correlation between area and the number of ribonucleotides and nucleosides close to 1 (Appendix A and B).

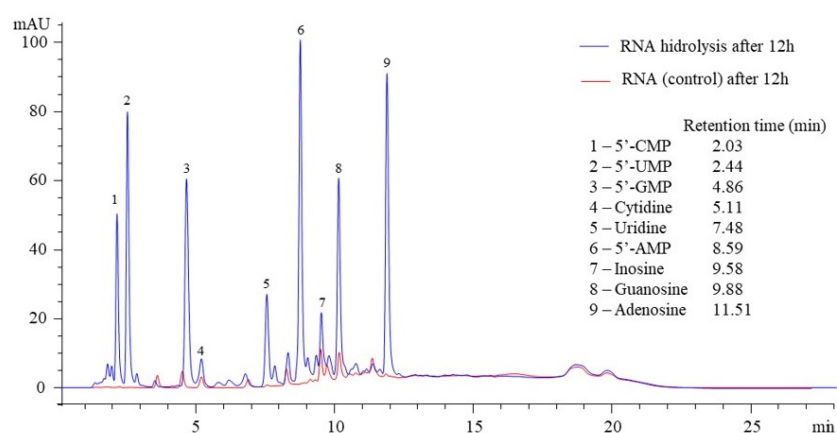


Figure 4. UV-260nm HPLC chromatogram obtained after 12 hours of RNA hydrolysis with 5'-PDE from spent malt rootlets.

Throughout the enzymatic hydrolysis of RNA, we observed that most of the compounds formed were 5'-ribonucleotides (Figure 5). Although obtained in smaller quantities with the proposed approach, nucleosides are important because they are involved in basic biological processes and can be used as precursors to other molecules with therapeutic activity. Some synthetic analogs of natural nucleosides often exhibit biological activities of great pharmaceutical value. Medical applications are related in cancer chemotherapy and AIDS, and as suppressors of the immune response during organ transplantation (Guinan et al., 2020). Some examples of nucleoside analogs with therapeutic application are gemcitabine, clofarabine and cytarabine, effective for chemotherapeutic treatment regimens; D-arabinofuranosylcytidine, effective against myelocytic leukemia; acyclovir, potent anti-herpetic agent, and 3'-azido-3'-deoxythymidine (AZT), an effective anti-HIV compound (Huang et al., 2018; Kumar et al., 2019; Guinan et al., 2020).

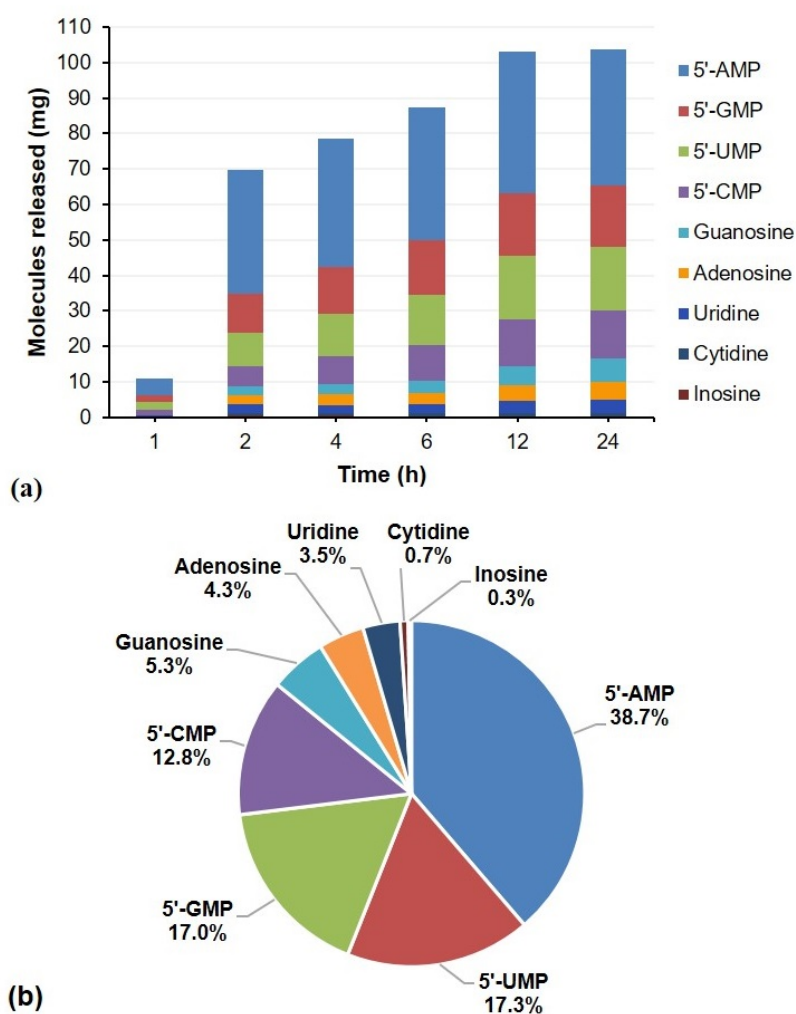


Figure 5. 5'-Ribonucleotides and nucleosides content over the time of RNA hydrolysis (a) and percentage content after 12h (b) using crude extract of 5'-PDE from barley rootlets.

We observed no formation of 5'-IMP and this can be explained by the fact that 5'-IMP is only produced enzymatically by deamination of 5'-AMP using adenylyl deaminase. Under the conditions of the reaction, we observed that the hydrolysis came to an end about 12 hours after the beginning, there was no increase in the number of hydrolyzed molecules after that. The highest yield of molecules formed were 5'-AMP, 5'-GMP and 5'-UMP (Figure 5b). Ribonucleotides represented 85.86% of the total hydrolyzed molecules, which shows the potential of the methodology used to produce these compounds on a large scale. Nucleotides in

general, as well as nucleosides, participate in several biochemical processes essential for the functioning of the organism (Giuliani et al., 2019). When bound to vitamins or their derivatives, the nucleotides constitute a portion of many coenzymes (FAD, NAD and CoA). As major donors and acceptors of phosphoryl groups in metabolism, tri- and diphosphate nucleosides, such as ATP and ADP, play the major role in energy transduction that accompanies metabolic interconversions and oxidative phosphorylation (Rossi et al., 2007). Cyclic nucleotides cAMP and cGMP act as second messengers in hormone-regulated events, and both GTP and GDP play key roles in the cascade of events that characterize signal transduction pathways. Nucleotides, linked to sugars or lipids, are essential biosynthesis intermediates. Sugar derivatives, UDP-glucose and UDP-galactose participate in the interconversions of sugars, as well as in the biosynthesis of starch and glycogen. Similarly, nucleotide-lipid derivatives, such as CDP-acylglycerol, are intermediates in lipid biosynthesis (Rossi et al., 2007).

The range of application of nucleic acids derivatives is wide, which justifies the search for more effective ways of producing them. The molecules produced using the methodology described in the present work can be used in food applications. Nucleotides, when supplemented in the feed, have several beneficial therapeutic effects, and are well evaluated as immunostimulatory agents, promoting protection against bacterial infection and enhancing the immunological function of the organism (Guo et al., 2019). Studies to evaluate the effect of nucleotides in the immune system show that children vaccinated with antigens of protein T had the immune system increased (Lerner & Raanan, 2000). In another study, there was an increase in immunoglobulin in children who received nucleotides in the diet (Martínez-Augustin et al., 1997). Dietary nucleotides increase intestinal iron absorption, affect lipoproteins, and the polyunsaturated long chain fatty acid metabolism has a topical effect on the intestinal mucosa, liver and reduce the incidence of diarrhea (Cosgrove, 1998; Schlimme et al., 2000). Besides that, ribonucleotides such 5'-GMP and 5'-IMP are used as flavor enhancers in the food industry, in products such as corn chips, broth tablets, powdered soups, dehydrated seasoning mixes, among many others (Rocha et al., 2020).

4 Conclusion

Yeast biomass is undoubtedly an underutilized waste from brewing that can be considered an economical source to produce 5'-ribonucleotides and other compounds on a large scale in order to meet the growing demand for new inputs for food and pharmaceutical industries, as well as for the development of biorefineries, using simple and efficient strategies that guarantee economic viability. Our experimental results show that the extraction of 5'-phosphodiesterase from malt roots is simple and economical and can be adapted to industrial production of 5'-phosphodiesterase. The ribonucleotides and nucleosides produced have a wide range of application and can be used as raw materials for biochemical drugs, food additives, and health products. Finally, the approach here proposed can open a new perspective for the brewing industry regarding the management of its wastes, giving a better destination to spent brewer's yeast and spent malt rootlets.

Acknowledgements

The authors gratefully acknowledge São Paulo Research Foundation (FAPESP) for Ph.D. Scholarship (grant 2016/08886-6).

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5'-Ribonucleotides production using 5'-phosphodiesterase from spent malt roots

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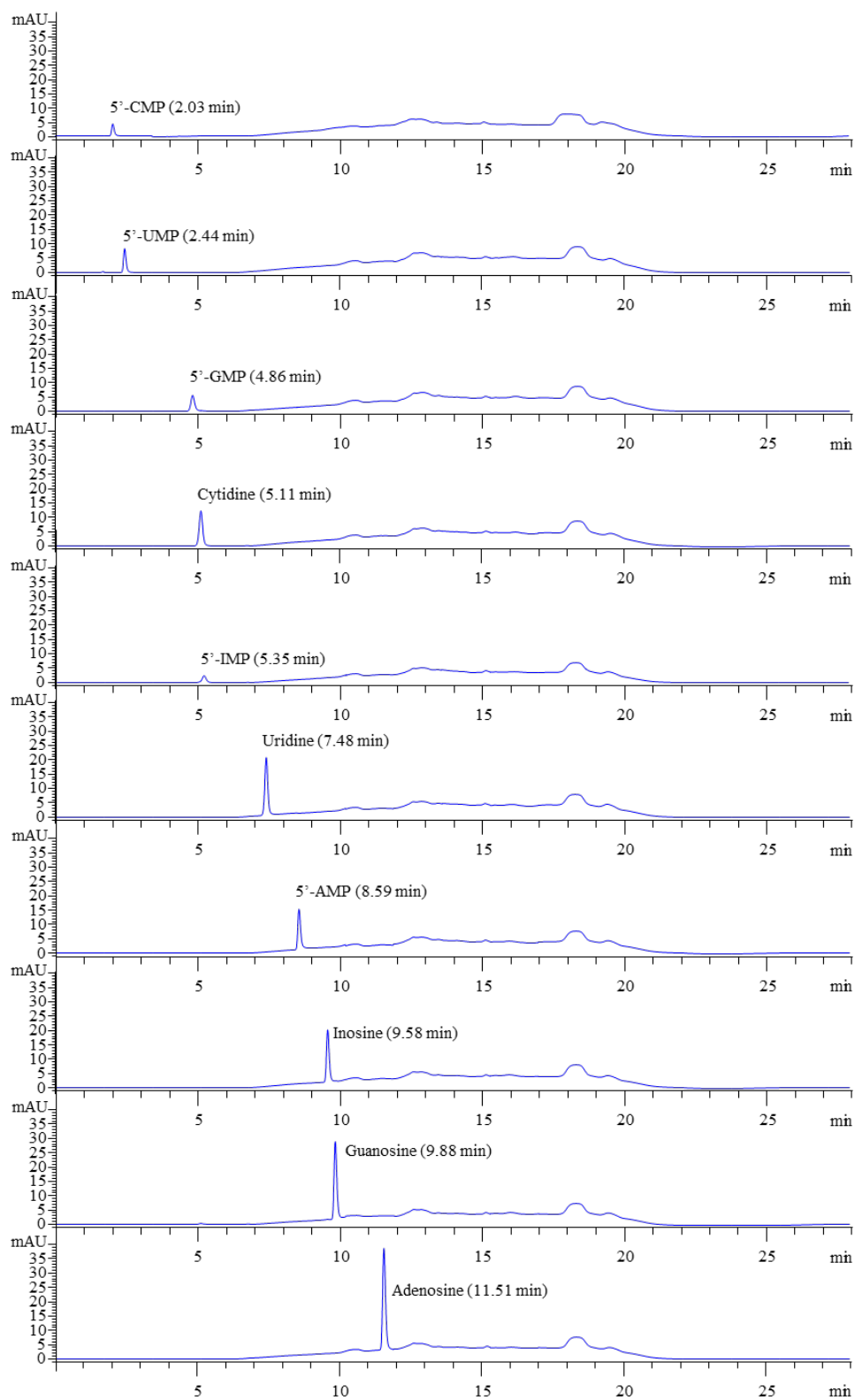
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Funding: São Paulo Research Foundation (FAPESP), grant 2016/08886-6.

Received: Oct. 19, 2020; Accepted: Jan. 05, 2021

Appendix A. 5'-Ribonucleotides production using 5'-phosphodiesterase from spent malt roots UV-260 nm chromatogram obtained for each standard 5'-ribonucleotide and nucleoside.



Appendix B. Standard curves for HPLC quantification of 5'-ribonucleotides and ribonucleosides.

