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Antifungal compounds extracted from rice bran fermentation applied to bakery product conservation

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ABSTRACT. The objective of this study was to apply a natural extract derived from the biomass of fermented rice bran as conservative, aiming to increase the shelf life of pizza doughs. The substrate, rice bran, was placed in tray bioreactors and inoculated with *Rhizopus oryzae*, whose initial concentration was 4 x 10^6 spores g^{-1} of bran fungus. The phenolic extracts, in average concentration of 2.47 mg g^{-1}, were applied on the pizzas doughts stored at room temperature. These pizza doughs treated increased by more than ten days life compared with those that were applied conventional conservatives. Thus, this extract phenolic derivative of rice bran showed to be promise as conservative, with results that indicate less fungal contamination as glucosamine, score of molds and yeasts, and invertase enzyme activity in 17.66 μg g^{-1}, 1.3 x 10^1 CFU g^{-1} and 0.04 mg min. proteins^{-1}, respectively.

Keywords: natural conservatives, phenolic compounds, pizzas, propionic acid, *Rhizopus oryzae*.

Compostos antifúngicos extraídos da fermentação de farelo de arroz e aplicados na conservação de produto de panificação

RESUMO. O objetivo deste estudo foi aplicar um extrato natural como conservador, proveniente da biomassa de farelo de arroz fermentado, com o objetivo de aumentar a vida de prateleira de massas de pizza. O substrato, farelo de arroz, foi colocado em biorreatores de bandeja e inoculados com *Rhizopus oryzae*, cuja concentração inicial era 4 x 10^6 esporos g^{-1} de farelo de fungo. Os extratos fenólicos, na concentração média de 2,47 mg g^{-1}, foram aplicados sobre as massas de pizzas armazenadas à temperatura ambiente. Estas massas de pizza tiveram a vida de prateleira aumentada em mais de 10 dias em comparação com aquelas que foram aplicados conservadores convencionais. Assim, este extrato fenólico do farelo de arroz mostrou ser promissor como conservador, com resultados que indicam menor contaminação fúngica como glicosamina, contagem de bolores e leveduras, e atividade da enzima invertase em 17,66 µg g^{-1}, 1,3 x 10^1 CFU g^{-1} e 0,04 mg min. proteínas^{-1}, respectivamente.

Palavras-chave: conservador natural, compostos fenólicos, pizzas, ácido propiônico, *Rhizopus oryzae*.

Introduction

Some food, for their frequent consumption and processing conditions, represent greater toxicological risk, conveying xenobiotics from different sources. Among these are the pizzas, which are widely consumed, not only by the characteristics of practicality and low cost, but also by the pleasant taste and high nutritional value. This product undergoes varied handling during processing, which contributes to its contamination, especially microbial, which in addition to degrading the product may cause damage to health (Botre, Soares, Espitia, Sousa, & Renhe, 2010).

According to Bezerra, Souza, Pereira and Sichieri (2013), there was an increased consumption of pizzas, outside the home, by 42.6%, and this was due to its convenience of preparation and domestic trade, especially those marketed as semi-prepared (semi-cooked pasta). Considering that the presence of conservative is essential in the semi-prepared form, there are two problems: the risk addition levels above the recommended and the inefficiency of conservative function (sometimes).

This concern in production healthy and safe food has been guiding the search for natural conservatives, in a native form or extracted from their sources, replacing chemical conservatives, whose risks are being seen frequently in literature (Guo, Zhang, Wang, Liu, & Xin, 2015; Remington, Taylor, Marx, Petersen, & Baumert, 2013; Schilter et al., 2014). The demand for natural compounds with antifungal activity, in order to inhibit food spoilage by microrganisms, which are more likely to

develop in rich matrices of carbohydrates and in median water activity, may be a solution to limit the use of chemicals antifungal (Botre et al., 2010; Feddern, Furlong, & Soares, 2007).

Among the compounds that are assigned antifungal properties are the phenolic acids, a class of chemical derivatives of phenylalanine and tyrosine that may appear in free form or bound to cellulose and to protein in plant tissues (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012). The inhibitory capacity of fungal species, including toxigenic, was demonstrated in phenolic extract obtained from *Spirulina platensis* (Souza, Prietto, Ribeiro, Souza, & Badiale-Furlong, 2012). The microrganism during fermentation produces enzymes that degrade cellulose and lignin increasing by 40% the content of free phenolic compounds in relation to the original substrate (Schmidt & Badiale-Furlong, 2012; Oliveira et al., 2010; Schmidt, Gonçalves, Prietto, Hackbart & Furlong, 2014).

From these considerations, the aim of this study was to extract antifungal compounds produced during fermentation of rice bran and apply them in a bakery product, in order to replace the conventional conservative.

Material and methods

Feedstock

The Rice bran was provided by a vegetable oil industry, located in the southern state of Rio Grande do Sul - Brazil, to be applied as a substrate for solid state fermentation.

Solid state fermentation

Phenolic compounds were obtained by solid state fermentation of *Rhizopus oryzae* CCT 7560 (Colony Bank Tropical Foundation André Tosello, Campinas, São Paulo State, Brazil), using as substrate the rice bran with 0.5 mm particle.

The fungus was initially grown in potato dextrose agar (PDA – HIMEDIA, India), for 7 days, at 30°C, from where was obtained a spore suspension with 50 mL aqueous emulsion of Tween 80 - 0.2 % (Synth, Brazil). The spore count was performed in a Neubauer chamber (Loptik Labor, Tiefe Depth, Basel, Switzerland). The substrate, rice bran, was distributed, forming a layer of 2 cm thick in tray bioreactors, which mass was the base for the calculation of the nutrient solution, sterile water and spore solution to be added.

Inside a laminar flow chamber (Labconco, SCRUBBER CLASS, Vermont, EUA), was added 45 mL of nutrient solution (2 g L^{-1} of KH_2PO_4 (Nuclear, Brazil) 1 g L^{-1} $MgSO_4$ (Synth, Brazil) and 1.8 g L^{-1} NH_2CONH_2 (Vetec, Brazil) in 0.4 mol L^{-1} HCl (Synth, Brazil)) in each bioreactor with 100 g of rice bran and, thereupon, added to a suspension of spores of the initial concentration of 4 x 10^6 spores g^{-1}. The moisture was adjusted to about 50% by the addition of sterile water. The reactors were covered with sterile gauze and placed in a germination chamber (Tecnal, TE-403, São Paulo, Brazil) at 30°C, for 96h. The samples of fermented bran were removed at 0, 24, 48, 72 and 96 h (Oliveira et al., 2010). Finally, the biomass was frozen (-4°C) for subsequent extraction of phenolic compounds.

Obtainment, separation and identification of phenolic compounds

Phenolic compounds were extracted of the biomass with methanol (Vetec, Brazil), in a proportion of 1:8 (w/v) under orbital shaking (Tecnal TE-141) at 160 rpm, for 3 hours at 25°C.

The methanolic solutions were dried in a rotaevaporator (Quimis®, Q-344B2) and the residue was dissolved in water and 40% ethanol (Synth, Brazil). The extract was clarified with 5 mL of barium hydroxide 0.1 mol L^{-1} (Synth, Brazil), and 5 mL of zinc sulfate (5%, Synth, Brazil), allowed to stand, centrifuged and filtered to obtain phenolic compounds. The free phenol content was determined by the Folin-Ciocateau (Proton chemical, Brazil), the concentration determined by spectrophotometry (Varian Cary 100) at 750 nm, using a standard curve of ferulic acid (Sigma-Aldrich Japan) (1.7 to 12.2 ng mL^{-1}) (Souza, Recart, Rocha, Cipolatti, & Furlong, 2009).

Phenolic compounds have been isolated and identified in accordance with Schmidt et al. (2014), injecting, from suspensions in water and methanol (1:1), 20 mL aliquots in gas chromatograph (Shimadzu, Tokyo, Japan, CLASS137 M10A) at a flow rate of 0.7 mL $min.^{-1}$ at 35°C. From a C18 column (4.6 x 250 mm, 5 um, Discovery®, USA) was performed the separation of the phenolic compound with a gradient of isocratic solvent consisting of methanol and water acidified (acetic acid 1% v / v), at a ratio of 20:80, over 25 minutes, with UV detection at 280 nm at 320 nm at 25 and 15 minutes. The identification of phenolic compounds were made by comparison of retention times and absorption spectra with different patterns of phenols present in rice bran (caffeic acid, chlorogenic acid, p-coumaric, ferulic gallic acid, p-hydroxybenzoic, protocatechuic, syringic and vanillin obtained from Sigma-Aldrich, USA). The limit of detection (LOD)

was calculated from the background noise signal (solution containing the solvents used in the extraction of phenolic substances) at 3:1. The limit of determination (LOQ) was established as three times the LOD.

Preparation of the pizza

The ingredients of the dough were established based on the weight of flour, wherein the formulation used consisted of 100 g of wheat flour, type 1, fortified with iron and folic acid (100%), crystallized sugar (4%), refined salt (2%), soybean oil (3%), fresh yeast (2%) and filtered drinking water (60 - 70%), at 5°C as Limongi, Simões and Demiate (2012). The ingredients were weighed on a precision balance, added and mixed in trough with hook rod type, for 10 minutes on medium speed. After homogenization of the dry ingredients, water was added to perform, again, the beating of mass (Kitchen Aid mixer at 300 watts), at a high speed for 5 minutes, to obtain a homogeneous smooth dough with complete development of the gluten. This development was observed by enlarging the mass in hands until the formation of a thin layer that did not rupture. After this stage, cuts were made in the dough with a circular shape with a diameter of 10 cm and, then, taken to the semi-cooked, for 15 minutes, at a temperature of 180°C.

Establishing the application conditions of conservative

The inhibitory activity of fungal multiplication was tested, employing in the masses (prepared in alcoholic solutions in 40% v / v), immediately after preparation of pizzas, the conservatives of phenolic extract and calcium propionate (Sigma-Aldrich, Japan), individually. The control solution was prepared under the same conditions of the conservatives. The application was carried out from the immersion of bodies in conservative solutions at concentrations of 2.47 mg g^{-1} such that, when applied to mass, absorbed 0.5 mL g_{dough}^{-1} according to each conservative applied. Immediately after this step, the pizzas were dried in a 150°C preheated oven and stored in plastic containers of polyethylene (Costa, Lucera, Conte, Contò, & Matteo, 2013).

Effect of conservatives on the masses of pizzas

The glucosamine content, the scores of molds and yeasts and the activity of the invertase enzyme were used as indicatives of conservatives in action in the masses pizzas, as well as moisture, pH and acidity were carried out to estimate physicochemical characteristics (Instituto Adolfo Lutz, 1985). These determinations were performed every 5 days for 15 days.

Glucosamine produced by mycota in the pizza doughs was extracted by homogenization in a blender (Waring Commercial, 34BL97, California, EUA), with 6 M HCl (Synth, Brazil) added at the ratio 3:5 (w/v). The mixture was heated at 100°C for 20 minutes, neutralized with 3 mol L^{-1} NaOH (Vetec, Brazil), titrated with 1% $KHSO_4$ (Vetec, Brazil), and the volume completed with distilled water to 25 mL. From this solution, 1 mL was transferred to a test tube and was added 1 mL of a solution of acetyl acetone (Sigma-Aldrich, Japan) leading the mixture to boiling water bath for 20 minutes. After cooling, there was added 6 mL of ethanol and 1 mL of Erlich reagent (2.67 g DAB - p_dimethylaminobenzaldehyde - dissolved in 15 mL of ethanol and 15 mL hydrochloric acid), keeping in an oven at 65°C for 10 minutes. The glucosamine content was determined at 530 nm and the concentration estimated by glucosamine standard curve (0.9 to 17.7 μg mL^{-1}) (Saritha, Arora, & Naim, 2012).

The scores of yeast and molds consisted of weighing Twenty-five grams (25 g) of the material aseptically removed and added to 225 mL of peptone water 0.1% (keeping 1 hour resting) under aseptic conditions before the preparation of the dilutions. An aliquot of 0.1 mL of each dilution was spread on Petri plates previously prepared with 15 mL of acidified potato dextrose agar and incubated for 5 days (incubator Q.317M-52, São Paulo, Brazil) at 25°C, as described by Nelson, Tousson and Marasas (1983).

The invertase activity was measured by reducing sugars of the reaction of 1 mL of extract, from 1 mL of a sucrose solution at 0.5 mg mL^{-1} in acetate buffer pH 4.7, at 37°C, for 10 minutes. Reducing sugars were determined using dinitrosalicylic of 3,5 (3,5 DNS) (Vetec, Brazil) on a spectrophotometer at 540 nm, using standard glucose curve (0 to 1 mg mL^{-1}) (Sabaj, 1979).

Statistical analysis

All determinations were performed in triplicate and the differences between treatments were estimated by ANOVA, with 5% significance level, according to Tukey, by the program Statistica 7.0. (Statsoft, 2008).

Results and discussion

The results for phenolic compounds (PC) produced during cultivation of biomass are shown in Table 1. This realizes variation of said compounds during 96h, after being diluted with different solvents to greater quantification.

Table 1. Phenolic compounds determined in different solvent.

Cultive intervals (h)	PC_{water} (mg $g_{biomass}^{-1}$)	$PC_{ethanol}$ (mg $g_{biomass}^{-1}$)
0	1.42 ± 0.02^{d}	1.68 ± 0.02^{d}
24	2.47 ± 0.01^{a}	2.45 ± 0.04^{a}
48	2.16 ± 0.05^{b}	2.44 ± 0.01^{a}
72	2.23 ± 0.07^{b}	2.33 ± 0.05^{b}
96	1.94 ± 0.03^{c}	2.04 ± 0.03^{c}

Mean + standard deviation. Different letters within the same column indicate significant differences (Tukey, $p < 0.05$).

The water soluble phenolic compounds showed a significant increase ($p < 0.05$) after 24 hours, when reached its highest fungal biomass yield (2.5 mg $g_{biomass}^{-1}$). According to Souza et al. (2009), the phenolic compounds are liberated from the decomposition of lignin present in cell walls of rice bran, in other words, the reduction of their levels occurs by the degradation of phenolic structure when releasing other derivatives compounds as result of the production of fungal biomass (Oliveira et al., 2010).

There was no significant difference between the times of 24 and 48h of culture in the content of phenolic compounds soluble in ethanol and water, being the last (48h) chosen for the solubilization of PC biomass for use in pizzas doughs.

In Table 2 are identified and quantified phenolic compounds found in rice bran and rice bran fermented in 24 hours. It is noteworthy that the latter fermentation time was used as a parameter for the composition of Table 2.

Table 2. Phenolic acid content during fermentation of rice bran in 24 hours (mg / gdry wet).

Phenolic compounds	Rice bran	24 hours fermentation time
Gallic acid	2.6 ± 0.8^{b}	3.6 ± 0.3^{a}
Protocatechuic acid	7.7 ± 1.4^{b}	12.5 ± 1.9^{a}
Chlorogenic acid	20.9 ± 0.7^{a}	4.8 ± 1.2^{b}
p-hydroxybenzoic acid	2.4 ± 0.4^{b}	19.1 ± 1.9^{a}
Caffeic acid	4.8 ± 0.9^{b}	2.4 ± 0.2^{a}
Syringic acid	2.1 ± 0.3^{b}	7.6 ± 1.6^{a}
Vanillin acid	8.6 ± 0.4^{b}	14.1 ± 0.1^{a}
p-coumaric acid	14.9 ± 0.9^{b}	40.5 ± 2.7^{a}
Ferulic acid	33.3 ± 2.3^{a}	10.5 ± 2.8^{b}

Values are expressed as means ± sd. The values in each line with the same superscript letter are not significantly different by Tukey test ($p < 0.05$).

According to Table 2, it can be seen that with the fermentation in 24 hours, compared with the results of rice bran, the phenolic compounds which have increased their contents were: gallic acid, protocatechuic acid, p-hydroxybenzoic acid, syringic acid, vanillin acid and p-coumaric acid. These identified phenolic compounds, according to Alves et al., (2013), are natural compounds with potential to inhibit microbial and fungal growth due the positions (para and meta) of their groups (OH and OCH_3) on the benzene ring.

The phenolic compounds can be produced by the decomposition of the linkages between lignin, hemicellulose and cellulose, or by producing one part of rice bran oil. For the fermentation of rice bran, this increase is caused by the cleavage of compounds complexed with lignin, where the enzyme production by filamentous fungi is necessary to break the lignin increasing the free phenolic content (Schmidt et al., 2014; Gharras, 2009; Somsuvra & Shital, 2010).

Effect of conservatives

Applying by immersion each conservative solution in pizza doughs, were determined physicochemical characteristics, which results are shown in Table 3.

As noted, the humidity remained uniform results, exceptions to the 10 and 15th days for treatments with propionic acid and control. These indicated a higher water activity to the product what, according to the results of pH, consists an excellent environment of multiplying spores. Relative to pH, other results showed no drastic changes caused by degradation of the mass. The acidity is a result of organic acids which could be arising to chemical changes caused by microbial growth. In this case the variations were also small, not exceeding 0.4% (Pinho, Machado, & Furlong, 2001).

Table 3. Physico-chemical characteristics of the masses treated by immersion.

	Intervals (days)	Samples		
		CFT	Propionic	Control
Humidity (%)	1	28.05^{a-B}	29.76^{c-B}	35.03^{a-A}
	5	30.64^{a-B}	35.13^{a-A}	30.33^{b-B}
	10	29.51^{a-B}	31.71^{b-B}	35.89^{a-A}
	15	26.14^{b-B}	35.51^{a-A}	35.52^{a-A}
pH	1	5.5^{a-B}	5.8^{a-B}	5.7^{a-B}
	5	5.5^{a-B}	5.7^{a-B}	5.7^{a-B}
	10	5.9^{b-A}	6.1^{a-A}	6.2^{a-A}
	15	5.0^{b-C}	5.3^{a-C}	5.3^{a-C}
Acidity (% acetic acid)	1	0.26^{a-B}	0.21^{b-B}	0.21^{b-B}
	5	0.26^{a-B}	0.20^{b-B}	0.20^{b-B}
	10	0.27^{a-B}	0.24^{b-A}	0.21^{c-B}
	15	0.33^{a-A}	0.26^{b-A}	0.25^{b-A}

CFT = Total Phenolic Compound. The same letters and lowercase letters in the same line (referring to the conservatives used) and equal and uppercase letters in the same column (for each analysis during the time interval) indicated no significant difference between treatments by Tukey test ($\alpha < 0.05$). The results have a lower coefficient of variation than 20%.

The literature suggests that the levels of pH and acidity for bakery products should be in the range 5.2 to 5.6 and 0.25 to 0.43%, respectively. Thus, it was observed (Table 2) in this experiment that the pH of the pizza showed slightly higher than suggested, tended to decrease over 15 days of storage, due to organic acid production (Quaglia, 1991).

The development of fungal contamination was accompanied by the determination of glucosamine, by the activity of the invertase enzyme and,

microbiologically, by enumeration of colonies of molds and yeasts (Table 4).

Table 4. Assessment of fungal contamination in pizza doughs immersed in conservative solutions.

	Intervals (days)	Samples		
		PC[1]	Propionic	Control
Glucosamine ($\mu g\ g^{-1}$)	1	13.3^{c-A}	12.0^{c-A}	12.6^{b-A}
	5	8.5^{d-B}	8.73^{c-B}	11.3^{b-A}
	10	30.6^{a-C}	35.9^{a-B}	38.3^{a-A}
	15	17.66^{b-B}	23.1^{a-A}	16.3^{b-C}
Scores of yeasts and molds (CFU g^{-1})	1	$ND^{2\ a-D}$	$ND^{2\ a-D}$	$ND^{2\ a-D}$
	5	$< 3^{\ a-C}$	$< 3^{\ a-C}$	$< 5^{\ b-C}$
	10	$1 \times 10^{\ c-B}$	$6.6 \times 10^{2\ a-B}$	$3.4 \times 10^{2\ b-B}$
	15	$1.3 \times 10^{1}_{\ b-A}$	$Inc^{3\ a-A}$	$Inc^{3\ a-A}$
Invertase (mg min. proteins^{-1})	1	0.09^{c-B}	$0.15^{a,b-A}$	0.16^{b-A}
	5	0.06^{a-B}	0.11^{b-A}	0.11^{c-A}
	10	0.11^{c-B}	0.17^{a-A}	0.20^{a-A}
	15	0.04^{b-B}	0.11^{b-A}	0.10^{c-A}

[1]PC = total phenolic compound. [2]ND - Not detectable. [3]Inc - an untold number of colonies. The same letters and lowercase letters in the same line (referring to the conservatives used) and equal and uppercase letters in the same column (for each analysis during the time interval) indicated no significant difference between treatments by Tukey test ($\alpha < 0.05$). The results have a lower coefficient of variation than 20%.

Lower values of these indicators are observed in the samples treated with the phenolic solution. In general, all values indicated increased until day 10, however, the increases were smaller than those found for the conventional conservative or control.

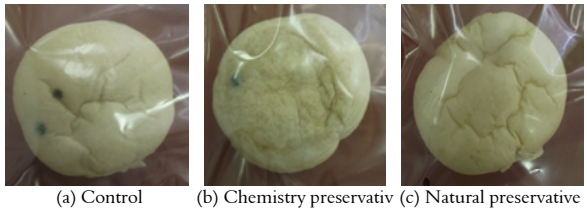
The trend of increasing content of glucosamine during storage is consistent with the proliferation of microrganisms that deteriorate the mass. However, on day 15, the values decreased as compared to initial, suggesting that the fungal biomass, for other changes in the degradation process, found no more conditions for growth in the environment.

The values found for glucosamine and for inhibition of phenolic compounds in fungal growth were similar to Souza et al. (2012) (in the inhibition of fungal with extracts phenolic of *Spirulina platensis*, *in vitro* culture of *Aspergillus flavus*) and to Pagnussatt, Del Ponte, Garda-Buffon and Badiale-Furlong (2014) (greatly reduced radial growth of fungal colonies and average reductions of 40% in the glucosamine levels of the *Fusarium graminearum* in petri dishes throughout the growth period). It is noteworthy that there are no reports in the literature about the inhibition of fungal growth with the direct use of phenolic compounds in a food.

The fungal contamination for enumeration of yeasts and molds, detectable in the 5th day of storage, was defined as countless when showed values higher than 9×10^6 CFU g^{-1}. After the 5th day, the samples treated with natural conservative showed less contamination level in comparison with those which were treated with conventional conservative and control. These results, compared to the activity of the invertase enzyme, confirm that the phenolic compound used as a conservative was effective, and reduction occurred during the first days of invertase activity, which shows the intensity of consumption by microorganisms from the lysing sucrose. However, from the 15th day decreased enzyme activity in all treatments.

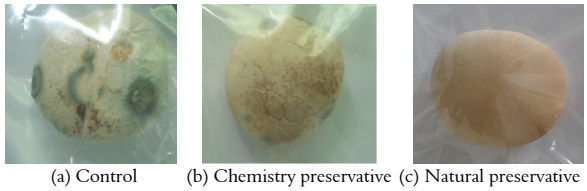
Figures 1c and 2c show the characteristics of the end product, this being one pizza dough immersed in phenolic compound as conservative, packed in polyethylene plastic (0.12 mm) and stored at room temperature for 10 to 20 days, conditions that can be considered suitable for consumption patterns according to Brasil (1997), Ordinance n. 451, which runs until 2011. Stresses that such masses of pizzas were within acceptable standards enumeration of molds and yeasts (less than 5×10^3 CFU g^{-1}).

Figures 1a, 2a and 1b, 2b show pizza doughs treated with control and commercial conservative for 10 and 20 days of storage, respectively. It is evident in the illustration the best look of those treated with natural conservative under the same conditions.



(a) Control (b) Chemistry preservativ (c) Natural preservative

Figure 1. Pizzas treated with different conservatives stored for 10 days.



(a) Control (b) Chemistry preservative (c) Natural preservative

Figure 2. Pizzas treated with different conservatives stored for 20 days.

Conclusion

For these reasons, it is concluded that the phenolic extract object of this study, obtained from the fungal biomass, innovated in applying as natural conservative, proving to be more efficient than the conventional conservative in reducing fungal contamination by an interval of 20 days.

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