EVALUATION OF THE ANTIOXIDANT POTENTIAL OF YEAST CELL EXTRACTS

Viviani Tadioto[1,2,3], Angela Alves dos Santos[1], Anderson Giehl[1,3], Stéfany K. Bressan[1], Triciane T. Pereira[1], Miguel de A. de Oliveira[3,4], Aguinaldo R. Pinto[3,4], Gislaine Fongaro[2,3], Izabella Thaís da Silva[2,3] & Sérgio Luiz Alves Jr[1,3]

[1]Laboratory of Yeast Biochemistry (LabBioLev), Federal University of Fronteira Sul, Chapecó, SC, Brazil.
[2]Laboratory of Applied Virology Laboratory (LVA), Department of Biochemistry, Federal University of Santa Catarina, Florianópolis, SC, Brazil.
[3]Postgrauate Program in Biotechnology and Biosciences, Federal University of Santa Catarina, Florianópolis, SC, Brazil.
[4]Laboratory of Applied Immunology (LIA), Federal University of Santa Catarina, Florianópolis, SC, Brazil.
* Corresponding author's email: slalvesjr@uffs.edu.br

ABSTRACT

Yeasts play crucial roles in natural and industrial environments, being capable of generating bioactive metabolites that may benefit human, animal, and plant health. Yeast bioactive compounds influence biological processes and interactions. The ease of use of yeasts in fermentative processes makes them promising for industrial applications, but the lack of knowledge about their bioactive compounds limits their use. Therefore, this work evaluated intracellular extracts produced with five yeasts isolated from different environments of Brazilian biodiversity and a yeast widely used in the Brazilian industry (PE-02). The extracts were obtained with the solvents, hexane, ethyl acetate, and butanol. The aqueous phase was also used as fractionate. The extracts were evaluated *in vitro* for cytotoxicity in two mammalian cell lineages: the macrophage-derived RAW 264.7 and the fibroblast-derived VERO. Furthermore, antioxidant activity was tested in macrophages and their compounds were detected. The cell extracts of three wild yeast strains isolated from caterpillar and bee microbiotas showed promising antioxidant activity. In these extracts, the phenolic compound p-coumaric acid was found at concentrations varying from 0.66 to 0.156 mg/L. Our results thus suggest that yeasts from Brazilian microbiota exhibit significant potential for reducing reactive oxygen species.

Keywords: Intracellular metabolites. Macrophage. Bioactive Compounds. Biodiversity.

1 INTRODUCTION

The rich biodiversity in Brazilian territory represents around 20% of all species in the world, distributed among six terrestrial biomes and three marine ecosystems[1]. In addition to the macroscopic lives that attract our attention, microbial species play a fundamental role in different environments, including plants and animals, with impacts that are often subtle[2,3]. Among the microorganisms that inhabit these varied environments, yeast-like fungi stand out for their wide distribution and presence[4]. This diversity of habitats suggests that these unicellular fungi can produce a wide range of bioactive metabolites, which can be applied in pharmaceutical, food, and other industries[5,6].

Bioactive compounds represent a diverse set of substances capable of interacting with biological processes, bringing benefits to human health, including medicines, nutraceuticals, and personal care products[5,6]. With their effective large-scale fermentative proliferation rates, yeasts are attractive microorganisms for industrial applications, surpassing other microscopic organisms. Its use is well-established in the production of food, beverages, bioethanol and even as probiotics[7,8]. However, the lack of knowledge about their roles as bioactive compounds' producers limits their use[9], although there are reports of antibacterial and anticancer efficacy in yeast-derived compounds[5]. This work aimed to evaluate the antioxidant activity and cytotoxicity of extracts produced from intracellular metabolites of wild and industrial yeasts *in vitro*.

2 MATERIAL & METHODS

Cell extracts were produced from the following yeast strains: CHAP-158 and CHAP-204, both isolated from intestines of *Spodoptera frugiperda* caterpillars[4]; CHAP-208 and CHAP-221, both isolated from *Senna macranthera* flowers; CHAP-243, isolated from Mandaguari bee (*Scaptotrigona postica*); and the industrial yeast *Saccharomyces cerevisiae* PE-2[10]. For the cultivation and prior preservation of yeast cells, they were pre-cultured in YPD liquid medium (1% yeast extract, 2% peptone, and 2% glucose) for 48 hours, with agitation at 200 rpm, at 30°C. The pre-cultivated yeasts were inoculated in Erlenmeyer flasks containing 200 mL of YPDXi culture medium (1% yeast extract, 2% peptone, 2% glucose, and 2% xylose) and incubated until 20–30 of optical density (at 570 nm). Cells were then separated from the culture medium by centrifugation at 10,000 rpm for 5 min at 4°C, followed by weighing and freezing. After that, exhaustive extraction of intracellular compounds was carried out, where the cell biomass was suspended in 10 mL of methanol-water (80:20 v/v) and kept in an ultrasonic bath for 1 hour. In a separation funnel, the cell suspension was mixed with the organic solvents in a 1:1 (v/v) ratio. The organic solvents used to extract compounds were, alternately, hexane, ethyl acetate, and butanol; the raffinate (aqueous phase) was also used as fractionated. The solvents used for the fractionations were vaporized in a rotary evaporator, and then the samples were freeze-dried until the extracts were completely dry.

The extracts used in mammalian cell culture were dissolved in dimethyl sulfoxide (DMSO), and the solutions were diluted in a culture medium before being tested in two mammalian cell lineages: RAW 264.7, derived from murine macrophages, and VERO, derived from African green monkey (*Cercopithecus aethiops*) kidney fibroblast. The culture medium DMEM (Dulbecco's Modified

Eagle Medium) was used for the first lineage, while MEM (Minimal Essential Medium – Eagle) was used for the second. Both media were supplemented with 10% Fetal Bovine Serum (FBS). The cells were incubated in a humidified atmosphere at 37°C, 5% CO_2 in an oven. Cell subcultures were established using the enzyme trypsin in an EDTA solution.

To determine reactive oxygen species (ROS), the antioxidant activity of intracellular extracts was measured using the protocol adapted from Ng and Ooi (2021)[11], which is based on the indirect detection of reactive oxygen species using the 2,7-dichlorofluorescein diacetate probe (H_2DCFDA). For this, cells of the lineage RAW 264.7 were seeded at a concentration of $4{,}10^4$ cells/mL in black 96-well microplates with a transparent bottom coated with collagen matrix. The cells were acclimated at 37°C, 5% CO_2 in oven. After confluence, the cells were treated in the plate with decreasing concentrations of extracts and then incubated again for 24 h. As a positive test control, 100 uM of hydrogen peroxide (100 µM) was used, which was added 1 h before the end of the treatment. In addition, wells were also maintained without any treatment for cell control. Subsequently, the culture medium was removed and replaced with 30 uM of the probe in DMEM previously warmed. After 30 min, the probe was removed and replaced with 100 uL of DMEM. The plate was protected from light incidence, and fluorescence spectroscopy values were acquired using an excitation wavelength of 494 nm and an emission wavelength of 522 nm. Normalization of fluorescence values was performed by determining cell density. Data were subjected to analysis of variance (ANOVA) and the mean comparison test (Tukey test). Each experiment was carried out in triplicate and presented with a 95% Confidence Interval. The softwares used to carry out the analyses were GraphPad Prism 8 and Excel. A high-performance liquid chromatography (HPLC) technique was used to determine possible antioxidant compounds in the extracts. For this, the lyophilized samples were solubilized in methyl alcohol, filtered through 0.45 µm porosity membranes, and transferred to chromatographic vials for injection into a capillary column for phenolic compounds.

Cell viability was analyzed using the sulforhodamine B (SRB) colorimetric assay to evaluate the cytotoxicity of the extracts, as described by Vichai and Kirtikara (2006)[12]. This assay quantified cell death caused by the applied extracts in the lineages RAW 264.7 and VERO in 96-well plates ($4x10^5$ cells per 100 µL for RAW and $2.5x10^5$ cells per 100 µL for Vero, for each cavity). Then, the cells were incubated for 24 hours until cell confluence. To determine the cytotoxicity of the extracts, the concentrations used were gradually decreased. Cells were fixed without removing the supernatant, adding 100 µL of 10% trichloroacetic acid (TCA) to each well and the plate was incubated for 1 h at 4ºC. They were then washed carefully with distilled water three times and kept for 24 hours to dry. Proteins from fixed cells were stained with 100 µL of sulforhodamine B and acetic acid (0.057% w/v) and kept for 30 minutes at room temperature. Excess dye that did not bind to proteins was discarded in three washes with a 1% (v/v) acetic acid solution. Then, the dye was removed with 100 µL of 10 mM Tris Base buffer (pH 10.5) in incubation with light agitation for 10 minutes at room temperature for complete dissolution. Next, the absorbance was measured in a spectrophotometer at 510 nm. The absorbance values obtained in each well containing the extract were converted into a percentage of cell viability in relation to the cell control, where 100% represents the total viability of the cells. Through linear regression analysis, it was possible to calculate the concentration of each sample that reduced cell viability by 50% (CC50). The assays were composed of the average of independent triplicates with a 95% confidence interval. The concentration required to reduce cell viability by 50% (CC_{50}) was calculated by applying non-linear regression analysis, using curves that correlate concentration to effect.

3 RESULTS & DISCUSSION

Among all the extracts tested, four significantly reduced ROS (CHAP-158, CHAP-204, and CHAP-243, in ethyl acetate fractionation, and CHAP-243, in butanol) and demonstrated antioxidant activity in murine macrophages (Figure 1). Compared to macrophage cells exposed to hydrogen peroxide, which has high oxidative activity, cells that were treated with extracts with antioxidant activity reduced cellular oxidation by approximately 0.52, 0.31, 0.0048, and 0.43 log_2 fold, respectively.

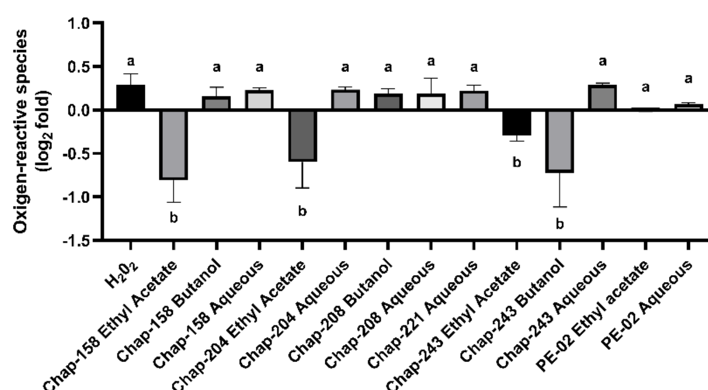

Figure 1 Detection of ROS in the extracts tested in macrophages. Different lowercase letters next to the bars represent significant differences.

The control cell model is abundantly distributed throughout the connective tissue derived from fibroblasts. These cells' main function is to act in the synthesis and secretion of extracellular matrix, thus providing resistance, elasticity, and structure of tissues and organs. In addition, they act in wound healing, in the maintenance of tissues, and in the regulation of inflammation[13]. In order to test not only the antioxidant action of the extracts on macrophages, but also to check whether the extracts affect other cells with important functions in the body, we carried out cytotoxicity tests with fibroblast cells as well. Our results showed that the lineage VERO did not show cytotoxic sensitivity at the concentrations tested (Table 1). In contrast, the RAW cells showed a 50% reduction in cell viability (CC_{50}) at intermediate concentrations of the extracts. The exception was the extract obtained from CHAP-243 fractionated in ethyl acetate, which significantly reduced reactive oxygen species and did not show cytotoxicity at the concentrations tested for either the fibroblast or the macrophage cell model.

Some of the metabolites of the extracts with antioxidant activity were detected. In the extracts from CHAP-204, CHAP-243 (both in ethyl acetate), and CHAP-243 (in butanol), the phenolic compound p-coumaric acid was quantified at 0.156, 0.136, and 0.66 mg/L, respectively, corroborating with Chen and coworkers[14] findings. This compound is described as reducing ROS, which is desirable in treating pathologies such as inflammation, cardiovascular diseases, diabetes, and nervous system diseases[13]. For CHAP-204 extracts, three additional antioxidant compounds — quercetin, myricetin, and kaempferol — were detected, although in negligible concentrations. For CHAP-243 extracts in ethyl acetate and butanol, small amounts of epicatechin, which is attributed with antioxidant action, among other benefits to human health[15], were also detected. Furthermore, the synergy of the compounds present in an extraction fraction must be considered since other compounds present can interfere with the dynamics, even if in small quantities[15].

Table 1 Extract concentration that reduced cell viability (CC_{50}) of lineages VERO and RAW by 50%.

Yeast Cell Extract	VERO	RAW
CHAP-158 Butanol	>200 (ND)	112.1 (101.3–124.9)
CHAP-158 Ethyl Acetate	>200 (ND)	171.9 (127.4–200.0)
CHAP-158 Aqueous	>200 (ND)	113.6 (102.5–126.0)
CHAP-204 Butanol	>500 (ND)	>500 (ND)
CHAP-204 Ethyl Acetate	>200 (ND)	169.7 (160.3–180.3)
CHAP-204 Aqueous	>200 (ND)	>200 (ND)
CHAP-208 Butanol	>200 (ND)	>200 (ND)
CHAP-208 Ethyl Acetate	>500 (ND)	>500 (ND)
CHAP-208 Aqueous	>200 (ND)	27.64 (25.06–49.25)
CHAP-221 Butanol	>500 (ND)	>500 (ND)
CHAP-221 Ethyl Acetate	>500 (ND)	>500 (ND)
CHAP-221 Aqueous	>200 (ND)	>200 (ND)
CHAP-243 Butanol	>200 (ND)	161.6 (145.9–177.3)
CHAP-243 Ethyl Acetate	>500 (ND)	>500 (ND)
CHAP-243 Aqueous	>200 (ND)	>200 (ND)
PE-02 Butanol	>200 (ND)	>200 (ND)
PE-02 Ethyl acetate	130.6 (82.77–200.0)	>200 (ND)
PE-02 Aqueous	195.2 (112.2–200.0)	>200 (ND)
H_2O_2 Control	-	<25 (ND)

4 CONCLUSION

The results suggest that yeast extracts isolated from Brazilian microbiota environments exhibit significant potential for reducing ROS. Furthermore, three strains were shown to produce a variety of phenolic compounds, which could potentially improve human health disorders. Considering the growing demand for alternative sources to produce these compounds, these microorganisms can represent a valuable resource. Therefore, more in-depth studies on the application possibilities of these microorganisms are promising and relevant.

REFERENCES

1 BRASIL. Ministério do Meio Ambiente e Mudança do Clima. 2023. Biodiversidade https://www.gov.br/mma/pt-br/assuntos/biodiversidade-e-ecossistemas#:~:text=O%20Brasil%20ocupa%20quase%20metade,e%20tr%C3%AAs%20grandes%20ecossistemas%20marinhos

2 GIEHL, A., DOS SANTOS, A.A., CADAMURO, R.D., TADIOTO, V., GUTERRES, I.Z., PRÁ ZUCHI, I.D., MINUSSI, G.D.A., FONGARO, G., SILVA, I.T., ALVES, S.L. JR. 2023. Plants. 12 (14). 2688. https://doi.org/10.3390/plants12142688

3 ALBARELLO, M. L. R., GIEHL, A., TADIOTO, V., DOS SANTOS, A. A., MILANI, L. M., BRISTOT, J. C. S., TRAMONTIN, M. A., TREICHEL, H., BERNARDI, O., STAMBUK, B. U., ALVES-JR. 2023. Bioenerg. Res. 16 (4). 2046-2057. https://doi.org/10.1007/s12155-023-10616-4

4 JÚNIOR, S. L. A., TREICHEL, H., BASSO, T. O., STAMBUK, B. U. 2022. Ecology: Yeasts on Their Natural Environment. In Yeasts: From Nature to Bioprocesses; ÁLVAREZ-PÉREZ, S. 2. 1ª ed. Bentham Books: Singapore. 27–57. https://doi.org/10.2174/97898150510631220201

5 SINHA, S., DAS, S., SAHA, B., PAUL, D., BASU, B. 2023. Front. Microbiol. 13. 1088477. https://doi.org/10.3389/fmicb.2022.1088477

6 OLIVEIRA, A. S., FERREIRA, C., PEREIRA, J. O., PINTADO, M. E., CARVALHO, A. P. 2022. International Journal of Biological Macromolecules. 208. 1116-1126. https://doi.org/10.1016/j.ijbiomac.2022.03.094

7 JÚNIOR, S. L. A., TREICHEL, H., BASSO, T. O., STAMBUK, B. U. 2022. In Yeasts: From Nature to Bioprocesses. 2. 1ª ed. Bentham Books: Singapore. https://doi.org/10.2174/97898150510631220201

8 MCFARLAND, L. V. 1994. Jama. 271 (24). 1913-1918. https://doi.org/10.1001/jama.1994.03510480037031

9 DIAS, B., FERNANDES, H., LOPES, M., BELO, I. 2023. Applied Microbiology and Biotechnology. 107 (12). 3925-3937. https://doi.org/10.1007/s00253-023-12565-6

10 TADIOTO, V., DEOTI, J. R., MÜLLER, C., DE SOUZA, B. R., FOGOLARI, O., PURIFICAÇÃO, M., GIEHL, A., DEOTI, L., LUCARONI, A.C., MATSUSHIKA, A., TREICHEL, H., STAMBUK., B.U., ALVES JUNIOR, S. L. 2023. Bioprocess Biosyst Eng. 46 (8), 1133-1145. https://doi.org/10.1007/s00449-022-02812-x

11 NG, N. S., OOI, L. 2021. Bio-protocol. 11 (1) e3877-e3877. https://doi.org/10.21769/BioProtoc.3877

12 VICHAI, V., KIRTIKARA, K. 2006. Nat Protoc. 1 (3). 1112–1116. https://doi.org/10.1038/nprot.2006.179

13 L MESCHER, Anthony. 2013. Junqueira's basic histology: text and atlas. 13ª ed. McGraw Hill Lange.

14 CHEN, F., ZHANG, X., WANG, J., WANG, F., MAO, J. 2024. Current Topics in Medicinal Chemistry. 24 (5). 416-436. https://doi.org/10.2174/0115680266276823231230183519

15 NICHOLS, M., ZHANG, J., POLSTER, B. M., ELUSTONDO, P. A., THIRUMARAN, A., PAVLOV, E. V., ROBERTSON, G. S. 2015. Neuroscience. 308. 75-94. https://doi.org/10.1016/j.neuroscience.2015.09.012

ACKNOWLEDGEMENTS

This work is part of the National Institute of Science and Technology (INCT) "Yeasts: Biodiversity, preservation, and biotechnological innovation". It is supported by grants and fellowships from the Brazilian National Council for Scientific and Technological Development (CNPq, grant numbers 406564/2022-1, 150719/2023-0, and 308830/2023-7), the Research and Innovation Funding Agency of the State of Santa Catarina (FAPESC, grant number 2023TR000234), and the Research Promotion Program from the Federal University of Fronteira Sul (UFFS, grant numbers PES-2022-0221, PES-2023-0349, PES-2023-0350, and PES-2023-0352).