Metschnikowia pulcherrima BIOMASS PRODUCTION IN SYNTHETIC MEDIA AND ITS CAPACITY TO CONTROL *Penicillium digitatum*

Leda M.F. Gottschalk[1]*, Erika F. de Souza[1], Selma D.C. Terzi[1], Juliana P. Rodrigues[2], Aline S.C. Teles[1], Simone D.D.O. Costa[1], Andressa M. de Souza[1], Edmar D.M. Penha[1], Janine P. Lima[1], Otniel Freitas-Silva[1]

[1]*Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brazil*
[2] *Programa de pós-graduação em Alimentos e Nutrição, UNIRIO, Rio de Janeiro, Brazil*
** Corresponding author's email address: leda.fortes@embrapa.br*

ABSTRACT

The influence of different synthetic media were evaluated on the growth of *M. pulcherrima*, aiming at future scaling of biomass production in a lower cost medium prepared from agricultural waste. Among the media evaluated (YMA, SDA, YPD and YPD with glycerol), the best result was obtained with YMA medium with maximum growth of 8 log CFU/mL in 24 hours, with total reducing sugar consumed. The *M. pulcherrima* biomass produced was concentrated by centrifugation and after that resuspended on phosphate buffer. This solution with 7 log CFU/mL was used for determining *in vitro* antimicrobial activity against *Penicillium digitatum*. The yeast *M. pulcherrima* was very efficient in controlling the growth of *P. digitatum*, pathogen that cause green mold in postharvest citrus.

Keywords: *Metschnikowia pulcherrima, biopesticide,* post-harvest biocontrol, *Penicillium digitatum*.

1 INTRODUCTION

Metschnikowia pulcherrima is a specie of yeast widely found in fruits, nectars, flowers and pollen and present in natural fermentations[1]. Despite its importance often associated with winemaking processes in the non-*Saccharomyces* yeast category, *M. pulcherrima* is considered a producer of lipids that can be a promising alternative to vegetable or fossil oils for application in food and fuels[2].

Furthermore, *M. pulcherrima* and their biomolecules produced have shown efficiency in controlling phytopathogenic fungi and are, therefore, a potential candidate for the development of biopesticides for fruit applications.[3,4] The use of yeast as a post-harvest biocontrol agent has several positive aspects, including the ability to colonize dry surfaces for long periods, simple nutritional requirements, rapid growth, and antagonistic potential against pathogens.[5] A significant portion of production is lost due to post-harvest diseases, with the fungus *Penicillium digitatum*, which causes green mold, being the main cause of this losses[6], since nowadays Brazil is the worldwide largest producer and exporter of orange juice it will be a great strategy to use *M. pulcherrima* in controlling this important post harvested fungi.

In this work, the influence of the Carbon/Nitrogen ratio used in the formulation of synthetic media was evaluated on the growth of *M. pulcherrima*, aiming at future scaling of biomass production in a lower cost medium prepared from agricultural waste. The *M. pulcherrima* biomass produced was evaluate against the fungus *Penicillium digitatum*.

2 MATERIAL & METHODS

Microorganism, media and fermentation conditions

M. pulcherrima DSM 70336, acquired from the Tropical Cultures Collection of the André Tosello Foundation, were inoculated in different media: Medium YMA (Yeast Mold Agar); Medium SDA (Sabouraud Dextrose Agar); Medium YPD (Yeast extract Peptone Dextrose), recommended in general for yeast cultivation; and Medium YPD glycerol (Bedir & Kuleasan (2021), like YPD (Yeast extract Potato Dextrose) medium, but with 5% glycerol added. The assays were conducted in 250 mL Erlenmeyer flasks with 50 mL of medium each with pH adjustment, as shown in Table 1.

Table 1: Formulations used to study the influence of the C/N ratio and media supplementation with yeast and malt extract on the growth of *M. pulcherrima*.

	Medium YMA	Medium SDA	Medium YPD	Medium YPD glycerol
Yeast extract	3 g/L	-	10 g/L	10 g/L
Malt extract	3 g/L	-	-	-
Peptone	5 g/L	10 g/L	20 g/L	20 g/L
Dextrose	10 g/L	40 g/L	20 g/L	20 g/L
Glycerol	-	-	-	50 g/L
pH	6,2	5,6	6,5	5,0
C/N ratio	**10,3**	**24,5**	**9,1**	**16,9**

The flasks were incubated at 30°C and shaken at 150 rpm for 72 h. Yeast growth was monitored by optical density (OD) measurements at 600 nm and by counting viable cells in PDA culture medium. Afterwards, suspensions were spread plating on YPD agar as described previously and yeast colonies were enumerated by colony counting. Then, counts were plotted as log CFU/mL and the mean and standard deviation of samples were calculated. Reducing sugar concentration was determined by DNS colorimetric method and pH was measured by a pH meter. Glycerol concentration was not measured during the process. After this, the kinetics of growth on selected media was evaluated in the same conditions for 48 h. The best medium was used to produce the yeast for the biocontrol tests. Cells produced were recovered by centrifugation 4500 rpm/20 min at 4°C and after that resuspended on phosphate buffer 0,05M pH 6,5. This solution was used for determining in vitro antimicrobial activity.

Method for determining in vitro antimicrobial activity

The agar-well diffusion method was used to perform the in vitro evaluation of the antimicrobial activity of yeasts against orange phytopathogens.[7] A suspension of 100 µL of freshly prepared phytopathogen (*P. digitatum*) with a final concentration of 7 log CFU/mL were inoculated onto the PDA plates, with the aid of a sterile Drigalski loop. Then, wells measuring 10 mm in diameter were cut using a sterile awl. A 250 µL aliquot of the *M. pulcherrima* culture was transferred to the wells. The plates were incubated at 25°C for 3 days. PDA plates inoculated with phytopathogens and sterile water were used to fill the wells as a control. The diameters of the pathogen growth inhibition zones were measured by ImageJ software, excluding the well width. Results were expressed as the average of three independent replications for each combination of *M. pulcherrima* strain phytopathogens with a standard deviation value.

3 RESULTS & DISCUSSION

The results obtained in media YMA, YPD and YPD with glycerol reached around 8 log CFU/mL. All three media were supplemented with yeast extract, malt extract or peptone (Table 1). However, it was not possible to notice a significant effect by replacing part of the yeast extract with malt extract or peptone, besides YMA and YPD have similar C/N ratio. It should also be noted that at the end of the 72 h process, the microorganisms, as shown in Table 2 consumed practically all the reducing sugar and pH increased probably due to cell lysis. When cells lyse, the intracellular ammonia is released into the fermentation broth. Ammonia is a weak base, and its presence can lead to an increase in pH by accepting protons in the medium. However, culture medium SDA, while in medium SDA reached only 6,35 log CFU/mL. not supplemented with yeast extract, still presented residual sugar equivalent to 60 % of sugar initially present in the formulation. In medium SDA, the final pH decrease showed a different behavior than the others, allowing the yeasts not to feel stress due to lack of nutrients. However, the superior C/N ratio may cause an inhibition on yeast growth.

Table 2: Results on pH, reducing sugars concentration and growth of *M. pulcherrima* in different media.

	Medium YMA	Medium SDA	Medium YPD	Medium YPD glycerol
pH initial	6,20	5,60	6,50	5,00
pH final	6,79	4,48	7,02	6,26
[reducing sugar] initial (g/L)	10	40	20	20
[reducing sugar] final (g/L)	0,22	24,29	0,88	0,73
Log CFU/mL	8,01	6,35	7,85	8,16

The kinetics of growth on media YMA, YPD and YPD with glycerol was evaluated in the same conditions for 48 h. The results are shown in Figure 1A, 1B and 1C and in all media evaluated, the growth was higher than 8 log CFU/mL. These results corroborate with those found by Carbon et al. (2023)[8] who also obtained a maximum growth of 8 log CFU/mL using glucose as a carbon source. It should also be noted that at 24 h process it was consumed practically all the reducing sugar. Considering pH it firstly decreases until 24 h and then increased due the lack of carbon source. Yeast growth monitored by optical density measurements at 600 nm showed similar results. For these reason YMA media was chosen for the *M. pulcherrima* biomass production because it had less requirements and a lower cost.

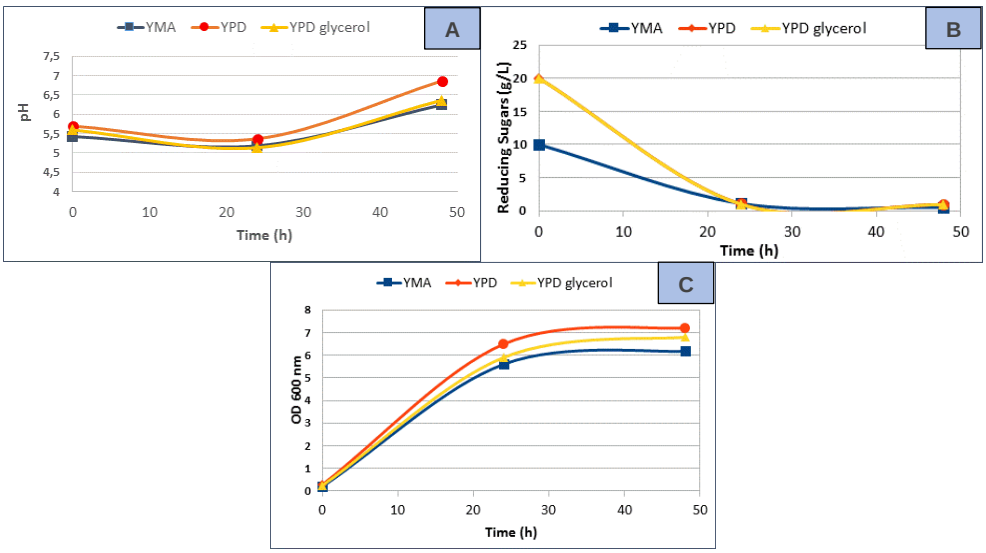

Figure 1: Influence of different media on pH (A), reducing sugars concentration (B) and *M pulcherrima* growth (OD 600 nm) (C).

The solution of *M. pulcherrima* with 7 log CFU/mL was used for determining in vitro antimicrobial activity against *P. digitatum* using the agar-well diffusion method to perform the in vitro evaluation of the antimicrobial activity. The plates were incubated with phytopathogens and sterile water were used to fill the wells as a control. Results were expressed as the average of three independent replications for each combination of *M. pulcherrima* strain phytopathogens with a standard deviation value (Figure 2). The yeast *M. pulcherrima* was efficient in controlling the growth of *P. digitatum* in the concentration of 7 log CFU/mL. These results are in accordance with the literature where two strains of *M. pulcherrima* also reduced the incidence of disease caused by *P. digitatum* and *P. expansum* and the diameter of the lesion in relation to the control.[9] The results obtained indicate that natural yeast-based biocontrol agents can be used to replace pesticides. Yeast-based bioproducts and their modes of action require comprehensive research to develop new formulations against post-harvest spoilage.

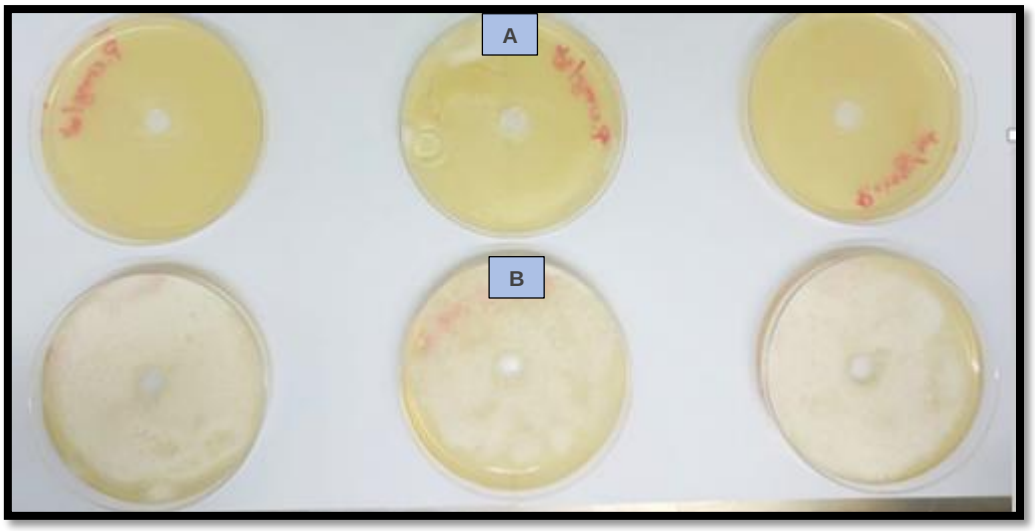

Figure 2: In vitro antimicrobial activity of *M. pulcherrima* against *Penicillium digitatum* (A) and control (B).

4 CONCLUSION

From the experiments carried out and the results obtained with different synthetic media, it was possible to conclude that the effect of organic nitrogen source like yeast and malt extract and peptone does not affect the final yeast biomass production. The C/N ratio indicated to influence and higher C/N ratio may cause an inhibition on yeast growth. The synthetic YMA medium was chosen for the *M. pulcherrima* biomass production (8 log CFU/mL) because it had less requirements and a lower cost. However, new studies will be necessary to optimize biomass production using agricultural waste to obtain an economic media. The yeast *M. pulcherrima* proved to be efficient in controlling the growth of *P. digitatum*, a post-harvest fungus responsible for losses in citrus production chain, especially in oranges.

REFERENCES

1 PALLMANN, C.L., BROWN, J.A., OLINEKA, T.L., et al. 2001. Am. J. Enol. Vitic, 52, 198 – 203.
2 ABELN, F., HICKS, R.H., AUTA, H., MORENO-BELTRÁN, M., LONGANESI, L., DANIEL A. HENK, D.A., CHUCK, C.J. 2020. Biotech. Biofuels, 13, 127.
3 CÔRTES, M.V.C.B.; OLIVEIRA, M.I. DE S.; MATEUS, et al. 2021. Biological Control, 152, 104460.
4 THAKUR, N., KAUR, S., TOMAR, P., THAKUR, S., YADAV, A.N. 2020. New Futur. Dev. Microb. Biotechnol. Bioeng. 243–282.
5 DUKARE, A.S., PAUL, S., NAMBI, V.E., GUPTA, R.K., SINGH, R., SHARMA, K., VISHWAKARMA, R.K. 2019. Crit Rev Food Sci Nutr, 59(9):1498-1513.
6 LIU, Y., YAO, S., DENG, L., MING, J., ZENG, K. 2019. Postharvest Biol. Technol., 152, 100-110.
7 STEGLIŃSKA, A.; KOŁTUNIAK, A.; BERŁOWSKA, J.; CZYŻOWSKA, A.; SZULC, J.; CIECIURA-WŁOCH, W.; OKRASA, M.; KRĘGIEL, D.; GUTAROWSKA, B. 2022. Agronomy, 12, 2546.
8 CARBON, H.N., APLIN, J.J., JIANG, G.Z., GIBNEY, P.A., EDWARDS, C.G. 2023. Food Microbiol. 114, 104308.
9 OZTEKIN, S., KARBANCIOGLU-GULER, F. 2021. Postharvest Biol. Technol.181, 111634.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Brazilian Agricultural Research Corporation (EMBRAPA) and Research Support Foundation of State of Rio de Janeiro (FAPERJ), under process E-26/2011.372/2021, for the financial support to the research.