XXI SIMPÓSIO NACIONAL DE BIOPROCESSOS
XII SIMPÓSIO DE HIDRÓLISE ENZIMÁTICA DE BIOMASSA

3 a 6 de setembro de 2017
Aracaju, Sergipe, Brasil

OPTIMIZATION OF MICROESCLEROTIA PRODUCTION BY *TRICHODERMA ASPERELLUM*

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ABSTRACT

The Trichoderma genus, despite its widespread use for decades as a biological control in agriculture, has only recently been gaining a share in the biopesticide market, thanks to its versatility in controlling diseases and acting as a plant growth enhancer. Most Trichoderma's commercial products have aerial conidia as active ingredient, but recent studies report the production of another propagule, more robust for formulation and application under field conditions - the microsclerotia (MS). Thus, our objective was to evaluate the nutritional conditions that favor the greater production of MS, using two cultures of T. asperellum with known antagonistic activity. For this, we used the fractional factorial design approach in which we evaluated 5 variables: Carbon source, Carbon concentration, C:N ratio, Strain, Nitrogen source, all influencing the production of MS. The results indicated that all variables were statistically significant to MS production. In the best condition tested, we obtained values higher than 10^4 MS mL^{-1}. In conclusion that the conditions found for the production of MS, using sucrose and lyscell, with carbon concentration 20 g L^{-1} and C:N ratio (10:1) and isolate TR 356, are inexpensive carbon and nitrogen sources may allow us to scale-up this biofungicide.

1. INTRODUCTION

Due to its cosmopolitan occurrence in almost all soils usually associated with organic matter, the genus *Trichoderma* has been used in some cultures since 1960. It is among the most studied biocontrol agent in the world, mainly because it is harmless to non-target organisms and safe to the environment (Harman, 2006). However, the lack of adequate formulations has hampered its broad expansion in modern agriculture. The success in biological control programs depends virtually on three factors: (1) a highly effective biocontrol agent; (2) production of a high level of effective and viable propagules; and (3) delivery systems conducive to the bioprotectant that provide a competitive advantage to the biocontrol agent relative to other microbiota (Harman, 1991). Thus, for the development of an adequate formulation, the choice of a propagule type is very important, since three kinds of propagules can be used in formulations: hyphae, chlamydospores and conidia. Hyphae are more sensitive to dehydration, while conidia and chlamydospores are able to withstand adverse environmental conditions, which explains why almost all products for biological control based on *Trichoderma* sp. contain conidia as the active ingredient (Verma et al, 2007; Cumagun, 2014).

More recently a study reported the production of other kind of propagule – microsclerotia (MS) – by *T. harzianum*. The MS are a resistant structure, very interesting for formulation development, with high persistent in the field and greater stability during storage (Kobori et al., 2015). In this way, our objective was to evaluate the culture conditions that favored the greatest MS production of two cultures of *T. asperellum* with known antagonistic activity.

2. MATERIALS AND METHODS

Aiming to maximize MS production by submerged fermentation, we used the fractional factorial design 2^{5-1} analyzing 5 independent variables that could influence the dependent variable (MS production), totaling 21 assays (Table 1). The tests were performed randomly in sets of 5 assays at a time.

Table 1. Codified levels of the variables studied in the fractional factorial design 2^{5-1}

Variables	-1	+1
Carbon source	Sorghum	Sucrose
Carbon concentration	20 g L^{-1}	50 g L^{-1}
C:N ratio	10:1	30:1
Fungal strain	TR 356	TR 696

Nitrogen source	Yeast extract	Lyscell

2.1. Microorganism maintenance

Embrapa Rice and Beans gently donated cultures of *Trichoderma asperellum*, with known antagonistic activity (Geraldine et al., 2013), which we used throughout this study. The cultures were replicated on potato dextrose agar (PDA) at room temperature for 2 weeks. The sporulated colony was lyophilized and used as a stock culture of *T. asperellum* (lyophilization media containing 10% (w/v) sucrose and 1% (w/v) gelatin). For liquid culture studies, conidia inocula were obtained by inoculating PDA plates with a conidia suspension from lyophilized stock cultures and growing at room temperature for 2-3 weeks. Conidia suspensions were obtained from sporulated agar plates by rinsing plates with 10 mL of a sterile solution containing 0.04 % Tween 80.

2.1. Assessment of liquid culture media

Basal media was prepared according to Jackson et al. (1997), containing in g/L: $KH_2.PO_4$ - 2.0g; $CaCl_2.2H_2O$ - 0.4g; $MgSO_4.7H_2O$ - 0.3g; $FeSO_4.7H_2O$ - 0.05g; $CoCl_2.6H_2O$ - 0.037g; $MnSO_4.H_2O$ - 0.016g; $ZnSO_4.7H_2O$ - 0.014g, pH adjusted to 5.5. Carbon and nitrogen sources were added independently in each treatment, according to factorial design (Table 1). Evaluation of liquid culture media to MS production was performed by submerged fermentation on baffled flask (500 mL) containing 200 mL of culture media. The media were inoculated with a conidial suspension obtained, as previously described, adjusted to deliver a final concentration of 10^5 conidia per mL medium. The flasks were incubated in a rotary shaker incubator at 28° C, 300 rpm agitation for 7 days. Daily 3.0 mL samples were taken from each assay to measure MS concentration and glucosamine. Next, the samples were submitted to a serial dilution and then counts were performed for MS from 100 µL sample spread over a glass slide and examined under a light microscope. After 7 days of incubation, all samples were plated on PDA medium for 12 hours to enumerate CFU mL^{-1}, and also to check for presence of contaminants.

3. RESULTS AND DISCUSSION

Data analysis was conducted through the fractional factorial design 2^{5-1}, using the software Statistic 7.0®. The dependent variable (response) was scored as the MS mL^{-1} at the end of the fermentation period (7 days). In this way, we verified that all the first-order effects of the independent variables were statistically significant, as shown by the Pareto chart (Figure 1) and by the ANOVA table (Table 2).

We observed that in the initial days there is no presence of MS in any of the treatments (Table 3). Rather, MS production was only observed after the 3rd day in some treatments, while in other media formulations MS were not formed during the cultivation period. All kinetic data cannot be shown, but we observed, for all assays, that CFU values were always higher than MS values. This occurred probably because CFU values translate all viable propagules present in the culture broth, which includes hyphae, chlamydospores, conidia and microsclerotia. We did not observe contaminants in any assay.

Others species of the genus *Trichoderma* (*T. harzianum*) were previously reported for their ability to form MS under appropriate nutritional and environmental conditions (Kobori et. al 2015). These authors obtained greater numbers of MS by day 4 in all culture media they tested (2.6 - 4.8 x 10^4 mL^{-1}), with fewer MS observed on day 7, likely due to MS aggregation. They used glucose and acid hydrolyzed casein and a carbon concentration of 36 g L^{-1} with C:N ratio between 10:1 and 50:1. While in the present study, we found better results for medium #3, which produced 2.5 (±0.9) x 10^4 MS mL^{-1} and 3.5 (±1.7) x 10^7 CFU mL^{-1}, using sucrose and lyscell, with carbon concentration 20 g L^{-1} and C:N ratio (10:1) using the isolate TR 356.

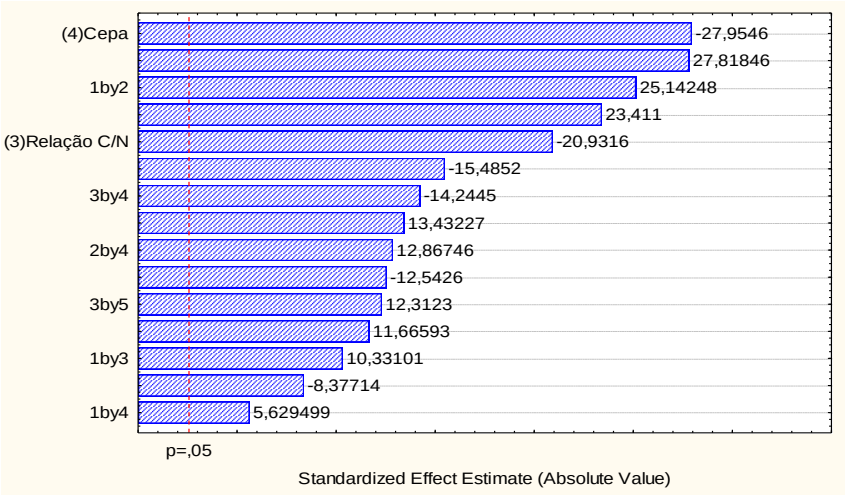

Figure 1. Pareto Chart of Standardized Effects; Variable: MS production, MS Pure Error: 0.01302.

Table 2. ANOVA statistics data of MS production, independent variables and their interactions; r2: 0.99889; 95 % confidence level; MS Pure Error: 0.01302

Factor	SS	Df	MS	F	P
Carbon source	10.07901	1	10.07901	773.8665	0.000001
Carbon concentration	2.04892	1	2.04892	157.3163	0.000057

C:N ratio	5.70631	1	5.70631	438.1303	0.000005
Culture	10.17789	1	10.17789	781.4584	0.000001
Nitrogen source	3.12311	1	3.12311	239.7921	0.000020
1 by 2	8.23318	1	8.23318	632.1441	0.000002
1 by 3	1.39007	1	1.39007	106.7297	0.000146
1 by 4	0.41275	1	0.41275	31.6913	0.002451
1 by 5	7.13825	1	7.13825	548.0750	0.000003
2 by 3	1.77252	1	1.77252	136.0940	0.000081
2 by 4	2.15644	1	2.15644	165.5716	0.000050
2 by 5	0.91399	1	0.91399	70.1765	0.000397
3 by 4	2.64267	1	2.64267	202.9045	0.000031
3 by 5	1.97438	1	1.97438	151.5926	0.000063
4 by 5	2.34991	1	2.34991	180.4259	0.000041
Pure Error	0.06512	5	0.06512		
Total SS	58.42302	20	58.42302		

4. CONCLUSION

We conclude that *Trichoderma asperellum* is a promising producer of MS, likewise other species of *Trichoderma* previously reported. We emphasize that the conditions found for the production of MS, using sucrose and lyscell, with carbon concentration 20 g L^{-1} and C:N ratio (10:1) and isolate TR 356, are inexpensive carbon and nitrogen sources may allow us to scale-up this biofungicide.

5. REFERENCES

Harman, G.E, 2006. Overview of mechanisms and uses of Trichoderma spp. Phytopathology 96, 190-194.

Verma, M., Satinder, K.B., Tyagi, R.D., Surampalli, R.Y., Valéro, J.R., 2007. Review. Antagonistic fungi, Trichoderma spp.: Panoply of biological control. Biochemical Engineering Journal 37, 1-20.

Kobori, N.N., Mascarin, G.M., Jackson, M.A., Schisler, D.A., 2015. Liquid culture production of microsclerotia and submerged conidia by *Trichoderma harzianum* active against damping-off disease caused by *Rhizoctonia solani*. Fungal Biology 119, 179-190.

Cumagun, C.J.R., 2014. Chapter 39 – Advances in Formulation of Trichoderma for Biocontrol. Biotechnology and Biology of *Trichoderma*, 527 – 531.

Geraldine, A.M., Lopes, F.A.C., Carvalho, D.D.C., Barbosa, E.T., Rodrigues, A.R., Brandão, R.S., Ulhoa, C.J., Junior, M.L., 2013. Cell wall-degrading enzymes and parasitism of sclerotia are key factors on field biocontrol of white mold by *Trichoderma* spp. Biological Control 67, 308–316.

Jackson M.A., McGuire M.R., Lacey L.A., Wraight S.P., 1997. Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. Mycological Research 101, 35-41.