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Fermentation of *Trichoderma* for biological control using local inputs in Costa Rica*

Fermentación de *Trichoderma* para control biológico con insumos locales en Costa Rica

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Abstract

Introduction. Supply chain issues have driven up raw material costs and reduced the availability of materials for producing biological control agents. These delays in application could result in increased disease pressure and reduced farm yields. **Objective.** To determine the effect of different amounts of starch and the use of local ingredients for small and commercial-scale fermentation processes for *Trichoderma harzianum*. **Materials and methods.** All trials took place in San José, Costa Rica, between 2016 and 2018. Flask trials were executed to investigate the potential reduction or elimination of starch in commercial fermentation media. Additionally, fermentation vessel trials were conducted to assess the effectiveness of an alternative local medium, encompassing three treatments: 1) Commercial medium as a control, 2) 10% molasses medium, and 3) 10% molasses mediaum with 0.5% yeast extract. Viable spore counts were performed to determine colony forming units (CFU/mL). **Results.** Reducing starch to 10% of the original medium had no impact on CFU/mL. However, the absence of starch led to uneven growth during fermentation, resulting in solid mycelium accumulations. Molasses medium yielded roughly half the CFU/mL compared to the commercial medium, but it still exceeded the 107 CFU/mL threshold commonly used in studies for biological plant pathogen control. Results from a commercial-scale fermenter mirrored those from pilot-scale fermentation. **Conclusion.** While reducing starch content in the commercial medium didn't affect growth, the absence of starch caused solid mycelium accumulations, potentially posing issues in commercial production. Employing locally sourced molasses medium on a commercial scale appears feasible while maintaining a viable spore count meeting the minimum field-use specifications. Overall, these findings support the use of these media for *Trichoderma* production in biological control applications.

Keywords: biological control agents, fermentation, starch, molasses.

Resumen

Introducción. Los problemas en la cadena de suministro han aumentado los costos de las materias primas y reducido la disponibilidad de materiales para la producción de agentes de control biológico. Estos retrasos en la aplicación podrían resultar en una mayor presión de enfermedades y menores rendimientos en las granjas. **Objetivo.** Determinar el efecto de diferentes cantidades de almidón y el uso de ingredientes locales en procesos de fermentación a pequeña y gran escala para *Trichoderma harzianum*. **Materiales y métodos.** Todos los ensayos se llevaron a cabo en San José, Costa Rica, entre 2016 y 2018. Se realizaron ensayos en matraces para investigar la posible reducción o eliminación de almidón en medios de fermentación comerciales. Además, se llevaron a cabo ensayos en recipientes de fermentación para evaluar la efectividad de un medio local alternativo, que incluyó tres tratamientos: 1) medio comercial como control, 2) medio de melaza al 10 % y 3) medio de melaza al 10 % con 0,5 % de extracto de levadura. Se realizaron recuentos de esporas viables para determinar las unidades formadoras de colonias (UFC/mL). **Resultados.** La reducción de almidón al 10 % del medio original no tuvo impacto en las UFC/mL. Sin embargo, la ausencia de almidón resultó en un crecimiento desigual durante la fermentación, lo que provocó acumulaciones sólidas de micelio. El medio de melaza produjo aproximadamente la mitad de las UFC/mL en comparación con el medio comercial, pero aún superó el umbral de 107 UFC/mL comúnmente utilizado en estudios para el control biológico de patógenos de plantas. Los resultados obtenidos en un fermentador a escala comercial fueron similares a los de la fermentación a escala piloto. **Conclusión.** Si bien la reducción del contenido de almidón en el medio comercial no afectó al crecimiento, la ausencia de almidón provocó acumulaciones sólidas de micelio, lo que podría plantear problemas en la producción comercial. El uso de un medio de melaza de origen local a escala comercial parece factible mientras se mantenga un recuento de esporas viables que cumpla con las especificaciones mínimas para su uso en el campo. En general, estos hallazgos respaldan el uso de estos medios para la producción de *Trichoderma* en aplicaciones de control biológico.

Palabras claves: agentes de control biológico, fermentación, almidón, melaza.

Introduction

As plant pathogens continue to develop resistance to traditional chemical pesticides, the application of these pesticides has increased, which results in higher production costs (Baker et al., 2020). Biological control agents (BCAs) refer to organisms used to control plant pathogens and have been developed and studied as an alternative to chemical pesticides (Köhl et al., 2019). *Trichoderma* spp. are often used as BCAs in crop protection systems due to their ability to combat phytopathogens through various mechanisms (Guzmán-Guzmán et al., 2019), such as competition for nutrients, production of lytic enzymes, and induction of resistance in the host plant (Asad, 2022). Products containing *Trichoderma* spp. are commonly formulated using the spores produced from the fermentation process, as they are more stable in adverse environmental conditions compared with the mycelial biomass (Verma et al., 2007).

Due to the impacts of COVID-19 and global supply chain issues, the price of inputs for agriculture has increased, resulting in a rise in food prices (Sridhar et al., 2022). In addition to the problems caused by COVID, the war in Ukraine has exacerbated these issues, leading to higher prices for fertilizers and grains such as wheat and corn (Ben Hassen & El Bilali, 2022; Jagtap et al., 2022). The utilization of local suppliers could help ensure that biologics are available when applications are required, avoiding delays in control programs that can result in increased disease pressure and yield losses (Lamichhane & Reay-Jones, 2021).

Materials to produce Trichoderma spp.-based BCAs are often expensive, leading to research aimed at reducing production costs using alternative ingredients and cutting the costs of these products for farmers (Das & Abdulhameed, 2020). Possibilities for lowering production costs of BCAs include reducing the quantity of raw materials and decreasing the time and labor required. Starch is a common component of fermentation media for filamentous fungi and often requires gelatinization before sterilization of the culture media, increasing the time and labor costs of production (Chávez et al., 2004).

The use of inexpensive and readily available raw material sources for the economical production of BCAs is a critical area of study (Naeimi et al., 2020). It is important to determine the effect on the final product's quality, such as shelf life, spore concentration, and viability when evaluating alternative ingredient sources (Velivelli et al., 2014). Additionally, to improve stability and shelf life, carrier materials are evaluated for compatibility with Trichoderma-based BCAs (Martinez et al., 2023). To achieve effective pathogen control in the field, a viable spore concentration for *Trichoderma* spp.-based products between 106 and 108 colony forming units per mL (CFU/mL) is recommended (Khan et al., 2011). Therefore, a concentration of 107 CFU/ml was selected as the target minimum because this concentration has been shown to be successful in field trials against black Sigatoka (Cavero et al., 2015).

The objective of the study was to determine the effect of different amounts of starch and the use of local ingredients for small and commercial-scale fermentation processes for *T. harzianum*.

Materials and methods

Location and dates of trials

All trials were conducted at Alltech Centroamérica in la Uruca, San José, Costa Rica from 2016 to 2018.

Impact of starch concentration on *Trichoderma* growth

A proprietary strain of Trichoderma harzianum was obtained from Alltech Inc., (Nicholasville, KY, USA). Freeze-dried vials were produced from a stock culture stored at -80 °C. The vials were stored for up to three months at 4 °C before use.

Different starch concentrations of a commercial media were used to evaluate the reduction of starch concentration on the final spore concentration. A vial containing a freeze-dried strain of *T. harzianum* was opened and mixed with 1 mL sterile distilled water. This suspension was then mixed with 5 mL sterile distilled water in a sterile test tube and vortexed. Aliquots of 0.1 mL were placed on potato dextrose agar (PDA) in a Petri dish (100x15 mm) and were placed in an incubator set at 30 °C for 24 hours.

A total volume of 200 mL of commercial media (Table 1) was autoclaved in a 500 mL Erlenmeyer flask for 20 minutes at 121 °C, and the media was then allowed to cool to 24 °C.

A commercial media was prepared as mentioned above, except using only 10 % or 0 % of the original starch content (Table 1). The pH for each culture media was around 6.9. Mycelial plugs of *T. harzianum* were transferred to the flasks, with a sterile loop, from the culture cultivated on PDA in a Petri dish. The flasks were incubated for 72 hours at 200 rpm and 30 °C. Viable spore counts were performed using the standard method outlined by Tournas et al. (2001) to obtain the colony forming units (CFU) per mL. The procedure was completed in triplicate for each media.

Table 1. Media for the cultivation of *T. harzianum* with different starch contents in 2016 in Costa Rica.

Tabla 1. Medios para el cultivo de *T. harzianum* con diferentes contenidos de almidón en 2016 en Costa Rica.

Ingredient	Commercial (g/L water)	Commercial with reduced starch (g/L water)	Starch-free commercial media (g/L water)
KCl	0.5	0.5	0.5
Yeast extract	18	18	18
Dextrose	5	5	5
KH_2PO_4	1	1	1
Starch	60	6	0
$MgSO_4$	1.5	1.5	1.5

Molasses as an alternative ingredient source

Locally sourced molasses was evaluated as an alternative fermentation medium to a commercial medium that required imported materials. A control medium was prepared using the reduced starch commercial medium (Table 2). A volume of 200 mL was prepared for the control medium, a medium with 10% (w/v) molasses, and a medium with 10 % (w/v) molasses (pH 5.5) and 0.5 % (w/v) yeast extract (pH 5.7) (Table 2). This medium was autoclaved in a 500 mL Erlenmeyer flask for 20 minutes at 121 °C and then allowed to cool to 24 °C. The molasses was sourced from Cooperativa de Productores de Leche (Alajuela, Costa Rica).

Table 2. Media for the cultivation of *T. harzianum* using local ingredients from 2017 to 2018 in Costa Rica.

Tabla 2. Medios para el cultivo de *T. harzianum* con uso de insumos locales desde 2017 al 2018 en Costa Rica.

Ingredient	Commercial with reduced starch (g/L water)	Molasses media (g/L water)	Molasses + yeast extract (g/L water)
KCl	0.5	0	0
Yeast extract	18	0	5
Dextrose	5	0	0
KH_2PO_4	1	0	0
Starch	6	0	0
$MgSO_4$	1.5	0	0
Molasses	0	100	100

Mycelial plugs of *T. harzianum* were transferred to the flasks, using a sterile loop, from a colony on PDA following the method described in the previous section. The flasks were incubated for 72 hours at 200 rpm and 30 °C. Viable spore counts were conducted using the method outlined by Tournas et al. (2001) to obtain the CFU/mL. The procedure was carried out in triplicate for each medium.

Scale up to a small fermentation vessel

The procedure described in the previous section was repeated for *T. harzianum* propagation in flasks, with the exception that the incubation time was reduced to 24 hours to ensure transfer during the growth phase. A total volume of 5 L was used for the fermentation.

To gelatinize the starch in the commercial medium before sterilization, all ingredients (except for the starch, as listed in Table 2) were added to a 15 L capacity fermenter and heated to **8 5°C**. The culture medium was then drained into an 18.75 L bucket, and the starch was mixed into the medium. After the starch had gelatinized and the solution became transparent, the medium was transferred back to the fermenter, along with 5 mL of antifoam. It was sterilized at 121 °C for 30 minutes with steam and then cooled to 30 °C.

The inoculum was transferred to the fermenter using a peristaltic pump and sterilized tubing, and then it was incubated for 72 hours at 30 °C and 250 rpm with a constant aeration rate of 20 L/min. Samples of approximately 25 mL were collected at 24, 48, and 72 hours to observe growth and sporulation under a microscope. The spore suspension was filtered to remove mycelium using a sterilized mesh sieve (U.S.A. Standard Sieve #100, 0.149 mm), and the filtrate was collected. Viable spore counts were conducted using the method outlined by Tournas et al. (2001) to determine the CFU/mL. The procedure was carried out in triplicate for both the control medium and molasses medium.

Scale up to a commercial scale fermenter

The procedure described in the previous section was repeated, with the fermentation time adjusted to 24 hours in the 15 L vessel to ensure transfer during the growth phase. A volume of 195 liters of molasses media with 195 mL of antifoam was added to a 250 L capacity fermenter and sterilized at 121 °C for 30 minutes, then cooled to 30 °C. After 24 hours, the media was transferred to the large fermenter and incubated for 48 hours at 30 °C and 250 rpm with constant aeration at a rate of 20 L/min.

The inoculum was transferred to the 250-liter capacity fermenter using a peristaltic pump and sterilized tubing, and then it was incubated for 48 hours at 30 °C and 250 rpm with constant aeration at a rate of 20 L/min. The media was sampled and examined under a microscope to confirm that there was >99 % sporulation after 48 hours. The spore suspension was filtered to remove the mycelium using a sterilized #100 mesh sieve, and the filtrate was collected. Viable spore counts were conducted using the method outlined by Tournas et al. (2001) to determine the CFU/mL. The procedure was performed in triplicate.

Statistical analysis of the results

One-way ANOVA with Tukey's test ($P<0.05$) was employed to assess differences between CFU/mL averages using Minitab 18 software (Minitab, State College, PA, USA). The homogeneity of variances was analyzed using Levene's test ($P<0.05$) prior to ANOVA. The normality of data was examined by assessing residuals and Q-Q plots from the ANOVA. To ensure a final product quality comparable to other Trichoderma-based BCAs, the target minimum was set at 107 CFU/mL, indicating that the final product would meet the necessary quality standards for use on farms.

Results

For all ANOVA tests, there was no evidence of heterogeneity of variance based on Levene's test, and there was also no evidence of non-normality based on Q-Q plots.

Impact of starch concentration on *Trichoderma* growth

There was no significant difference (P>0.05) in the CFU/mL among the different starch concentrations in the commercial media (Table 3). However, solid accumulations of mycelium formed when no starch was used, indicating potential problems during the trials in fermentation vessels (Table 3). To ensure homogeneous growth and avoid issues with solid accumulations in fermentation vessels, the commercial media without starch was not used in future trials.

Table 3. Growth of *T. harzianum* (CFU/mL) in media with different concentrations of starch in Costa Rica in 2017.

Tabla 3. Crecimiento de *T. harzianum* (UFC/mL) en medios con diferente contenido de almidón en Costa Rica en 2017.

Media	CFU/mL	Observation
Commercial	$6.0x10^7 \pm 7.6x10^6$	Homogeneous growth
Commercial with reduced starch	$7.2x10^7 \pm 4.4x10^6$	Homogeneous growth
Starch-free commercial	$6.0x10^7 \pm 2.9x10^6$	Solid accumulations of mycelium

Molasses as an alternative ingredient source

The utilization of molasses media, with or without yeast extract, resulted in lower CFU/mL values compared to the commercial media (Table 4), and the difference between the averages was significant (P<0.05). The addition of 0.5% yeast extract to the media did not result in any significant difference (P>0.05) in the CFU/mL. Growth was homogeneous for all media used, and no solid accumulations were observed during the fermentation process.

Table 4. Growth of *T. harzianum* (CFU/mL) in molasses or starch media in Costa Rica in 2018.

Tabla 4. Crecimiento de *T. harzianum* (UFC/mL) en medio con melaza o almidón en Costa Rica en 2018.

Media	CFU/mL
Commercial with reduced starch	$2.0x10^8 \pm 3.3x10^7$ [a]
Molasses media	$6.7x10^7 \pm 1.7x10^7$ [b]
Molasses media + 0.5% yeast extract	$3.8x10^7 \pm 1.4x10^7$ [b]

Treatments with means followed by the same letter indicate non-significant differences based on the Tukey test (P>0.05)./ Medias de los tratamientos con la misma letra indica una diferencia no-significativa según la prueba Tukey (P>0,05).

Scale up to a small fermentation vessel

After fermentation in the small vessel, the viable spore concentrations were $1.7x10^8 \pm 1.5x10^7$ CFU/mL with the commercial media with reduced starch, and $1.2x10^8 \pm 1.5x10^7$ CFU/mL with the molasses media. Although a lower concentration was observed with the alternative media source, there was no significant difference ($P>0.05$) between the averages. The average concentrations for both media were still above the target of 10^7 CFU/mL.

Scale up to a commercial scale fermenter

There was no significant difference ($P>0.05$) between the viable spore counts obtained from the commercial-scale fermentation compared to the results utilizing the smaller vessel, with an average of $1.4x10^8 \pm 1.7x10^7$ CFU/mL. The average CFU/mL value is above the target of 10^7 after fermentation in the commercial-scale vessel. The results indicate that scaling up to a commercial fermentation vessel with the molasses media does not affect the final viable spore concentration.

Discussion

Due to the high starch content in the commercial media (60 g/L starch), additional time and labor were required to gelatinize the media before sterilization, leading to increased production costs. The absence of starch in the commercial media resulted in the formation of solid accumulations of mycelia, causing issues during commercial-scale fermentation and downstream processes like filtration. Reducing the starch content to 10 % of the original concentration did not impact spore counts. Given that starch gelatinization required more labor and utility costs, reducing the time needed for this step would ultimately lower production costs (Chávez et al., 2004).

Although nitrogen sources are often added to molasses-based media to enhance growth (Lyubenova et al., 2023), there was no difference in growth with the addition of yeast extract compared to molasses media without this nitrogen source. Since there was no observed growth benefit, including yeast extract as a nitrogen source would be unnecessary and would only increase raw material costs. Nitrogen supplementation is not always required (Tamizharasi et al., 2005); however, other sources like ammonium or sodium nitrate should also be evaluated to determine if nitrogen is a limiting factor (Nathan et al., 2014).

In this study, it was possible to cultivate *T. harzianum* at a commercial scale using molasses media sourced locally for fermentation in San José, Costa Rica, resulting in spore counts falling within the range of commercially available BCAs (Khan et al., 2011). The spore counts were lower with the molasses media compared to the original commercial media, leading to higher fixed costs per CFU/mL, such as labor and utilities. However, the use of molasses significantly reduced media costs to only about 3 % compared to the commercial media. Therefore, fermenting *T. harzianum* remains a viable option as the production costs per CFU/mL remain similar.

For future studies involving the use of alternative raw materials, it's essential to consider the production cost of biological control agents and compare these costs with fungicides used in disease management programs. Cultivating organisms in media with different ingredients may lead to changes in the concentrations of secondary metabolites produced by the microorganism (He et al., 2021). Consequently, field trials are necessary to evaluate the efficacy of using alternative culture media for BCA production.

Conclusions

The reduction of starch in the commercial culture media for the fermentation of a proprietary *Trichoderma harzianum* strain did not impact the viable spore counts. However, the absence of starch in the media led to the formation of solid mycelium accumulations. These solid pellets in the media could potentially cause issues during commercial production and downstream processing. This highlights the importance of including starch to achieve a homogeneous fermentation with the commercial media.

The growth of *T. harzianum* cultivated in a molasses-based medium at both small and commercial scales resulted in uniform growth but produced approximately half the viable spore counts compared to the commercial media. However, the spore counts with both the commercial media with reduced starch content and molasses from a local source exceeded the target of 10^7 colony forming units per mL. Overall, this suggests that these media options could be effectively used for producing Trichoderma for biological control applications.

Interests conflict

Author Patrick Becker, an employee of Alltech, conducted graduate research in Costa Rica and in Kentucky, USA, in collaboration with the University of Costa Rica. This study was funded by Alltech Inc., Nicholasville, KY, USA. Alltech reviewed the manuscript but did not influence the study design, data collection, analysis, or interpretation of the data.

The authors Paul Esker and Gerardina Umaña declare that they have no conflicts of interest.

References

Asad, S. A. (2022). Mechanisms of action and biocontrol potential of *Trichoderma* against fungal plant diseases - A review. *Ecological Complexity, 49*, Article 100978. https://doi.org/10.1016/j.ecocom.2021.100978

Baker, B. P., Green, T. A., & Loker, A. J. (2020). Biological control and integrated pest management in organic and conventional systems. *Biological Control, 140*, Article 104095. https://doi.org/10.1016/j.biocontrol.2019.104095

Ben Hassen, T., & El Bilali, H. (2022). Impacts of the Russia-Ukraine War on Global Food Security: Towards More Sustainable and Resilient Food Systems? *Foods, 11*, Article 2301. https://doi.org/10.3390/foods11152301

Cavero, P. A. S., Hanada, R. E., Gasparotto, L., Coelho Neto, R. A., & Souza, J. T. (2015). Biological control of banana black Sigatoka disease with *Trichoderma*. *Ciência Rural, 45*, 951–957. https://doi.org/10.1590/0103-8478cr20140436

Chávez, R. A. P., Tavares, L. C., Carvalho, J. C. M., Converti, A., & Sato, S. (2004). Influence of the nitrogen source on the production of a-amylase and glucoamylase by a new *Trichoderma* sp. from soluble starch. *Chemical and Biochemical Engineering Quarterly, 18*(4), 403–407

Das, M., & Abdulhameed, S. (2020). Agro-processing residues for the production of fungal bio-control agents. *Valorisation of Agro-industrial Residues–Volume II: Non-Biological Approaches*, 107-126

Guzmán-Guzmán, P., Porras-Troncoso, M. D., Olmedo-Monfil, V., & Herrera-Estrella, A. (2019). *Trichoderma* Species: Versatile Plant Symbionts. *Phytopathology, 109*, 6–16. https://doi.org/10.1094/PHYTO-07-18-0218-RVW

He, D. C., He, M. H., Amalin, D. M., Liu, W., Alvindia, D. G., & Zhan, J. (2021). Biological Control of Plant Diseases: An

Evolutionary and Eco-Economic Consideration. *Pathogens, 10*. https://doi.org/10.3390/pathogens10101311

Jagtap, S., Trollman, H., Trollman, F., Garcia-Garcia, G., Parra-López, C., Duong, L., Martindale, W., Munekata, P. E. S., Lorenzo, J. M., Hdaifeh, A., Hassoun, A., Salonitis, K., & Afy-Shararah, M. (2022). The Russia-Ukraine Conflict: Its Implications for the Global Food Supply Chains. *Foods, 11*, Article 2098. https://doi.org/10.3390/foods11142098

Khan, S., Bagwan, N. B., Iqbal, M. A., & Tamboli, R.R. (2011). Mass multiplication and shelf life of liquid fermented final product of *Trichoderma viride* in different formulations. *Advances in Bioresearch, 2*(1), 178-182

Köhl, J., Kolnaar, R., & Ravensberg, W.J. (2019). Mode of action of microbial biological control agents against plant diseases: Relevance beyond efficacy. *Frontiers in Plant Science, 10*. https://doi.org/10.3389/fpls.2019.00845

Lamichhane, J. R., & Reay-Jones, F.PF. (2021). Editorial: Impacts of COVID-19 on global plant health and crop protection and the resulting effect on global food security and safety. *Crop Protection, 139*, Article 105383. https://doi.org/10.1016/j.cropro.2020.105383

Lyubenova, A., Rusanova, M., Nikolova, M., & Slavov, S. B. (2023). Plant extracts and *Trichoderma* spp: possibilities for implementation in agriculture as biopesticides, *Biotechnology & Biotechnological Equipment, 37*(1), 159-166, http://10.1080/13102818.2023.2166869

Martinez, Y., Ribera, J., Schwarze, F. W., & De France, K. (2023). Biotechnological development of Trichoderma-based formulations for biological control. *Applied Microbiology and Biotechnology 107*, 5595–5612. https://doi.org/10.1007/s00253-023-12687-x

Naeimi, S., Khosravi, V., Varga, A., Vágvölgyi, C., & Kredics, L. (2020). Screening of organic substrates for solid-state fermentation, viability and bioefficacy of *Trichoderma harzianum* AS12-2, a biocontrol strain against Rice Sheath Blight Disease. *Agronomy, 10*, Article 1258. https://doi.org/10.3390/agronomy10091258

Nathan, V. K., Esther Rani, M., Rathinasamy, G., Dhiraviam, K. N., & Jayavel, S. (2014). Process optimization and production kinetics for cellulase production by *Trichoderma viride* VKF3. *SpringerPlus **3***, Article 92. https://doi.org/10.1186/2193-1801-3-92

Sridhar, A., Balakrishnan, A., Jacob, M.M., Sillanpää, M., & Dayanandan, N. (2022). Global impact of COVID-19 on agriculture: role of sustainable agriculture and digital farming. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-022-19358-w

Tamizharasi, V., Srikanth, J., & Santhalakshmi, G. (2005). Molasses-based medium requires no nitrogen supplement for culturing three entomopathogenie fungi. *Journal of Biological Control,* 135–140. https://doi.org/10.18311/jbc/2005/3544

Tournas, V., Stack, M., Mislivec, P., Koch, H., & Bandler, R. (2001). *Chapter 18: yeasts, molds and mycotoxins, in: Bacteriological Analytical Manual (BAM)*, Food and Drug Administration, Gaithersburg, MD, USA. https://www.fda.gov/food/laboratory-methods-food/bam-chapter-18-yeasts-molds-and-mycotoxins

Velivelli, S. L. S., De Vos, P., Kromann, P., Declerck, S., & Prestwich, B.D. (2014). Biological control agents: from field to market, problems, and challenges. *Trends in Biotechnology, 32*, 493–496. https://doi.org/10.1016/j.tibtech.2014.07.002

Verma, M., Brar, S. K., Tyagi, R. D., Surampalli, R. N., & Valero, J. R. (2007). Antagonistic fungi, Trichoderma spp.: panoply of biological control. *Biochemical Engineering Journal*, *37*(1), 1-20. https://doi.org/10.1007/s00253-023-12687-x