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## **PRODUCTION OF AGENTS FOR BIOCONTROL OF APPLE FUSARIUM ROT BY SOILBORNE STREPTOMYCETES**

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## **Abstract**

*Within this paper, the potential of *Streptomyces hygroscopicus* in biocontrol of two isolates of *Fusarium avenaceum* isolated from apple fruits in the storage was tested. Production of *S. hygroscopicus* biocontrol agent was performed in 3 l stirred tank bioreactor at an aeration rate of 1.5 vvm and mixing speed of 150 rpm. Bioprocess was realized at  $26 \pm 1^\circ\text{C}$  for 168 hours. The activity of bioagents production was tested using in vitro diffusion method with wells. Also, residual glycerol, residual nitrogen content, and cell biomass were determined to analyze bioprocess parameters. The results showed that the highest production of bioagents was observed in 96h of cultivation when the largest zone diameters were formed against *F. avenaceum* KA12 and KA13 of  $40.67 \pm 1.15$  and  $43.33 \pm 0.58$  mm, respectively. The activity of the produced bioagents was confirmed in planta research, where the difference between necrosis diameter of treated and control samples were 2.79 (for KA12) and 2.17 (for KA13) times smaller.*

## **Keywords**

Biocontrol, Bioprocess, *Fusarium Avenaceum*, *Streptomyces Hygroscopicus*.

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## **1. Introduction**

The emergence of overpopulation and an increasing number of people around the world has led to the intensification of agricultural activities aimed at direct food production (Önder & Kamil, 2019). The key to a healthy life is reflected in consuming healthy food (Adedokun, Ojewola & Ahamefule, 2018). Apple fruit is one of the most widely used fruits in the world. Adults, children, and babies as well use this important food not only as fruit but also as processed in the form of compotes, jams, baby food, and more. However, during storage, transport, and marketing, apple fruit can be infected with various phytopathogenic fungi. This is expected given that the apple is full of water and sugar, which is a good medium for microorganisms.

Phytopathogenic fungi from genera *Penicillium*, *Monilinia*, *Colletotrichum*, and *Alternaria* are considered the most common causes of apple disease in storage. However, in the last few years fungi of the genus *Fusarium* have been increasingly mentioned as causes of apple diseases in

storages (Wenneker et al., 2016; Juhnevica-Radenkova, Radenkova & Seglina, 2016; Sever, Ivić, Kos, & Miličević, 2012). Certain species of fungi are especially important for humans due to the possibility of producing mycotoxins harmful to human health. The most important fungi, producers of mycotoxins are *Alternaria*, *Fusarium*, and *Penicillium* genera, and their presence on apple fruits must be controlled (Gashgari et al., 2019; Bjelić et al., 2018).

The intensive application of synthetic pesticides in modern agriculture has led to several side effects that are primarily related to environmental pollution. Some of the most significant disadvantages of synthetic fungicides are: contamination of crop products with harmful chemical residues; contamination of soils and groundwater; health risks to those who apply agrichemicals; genetic resistance; most chemical pesticides are nonspecific etc. Because of that, scientists around the world are making efforts to find alternative methods to replace the use of synthetic chemicals, and one alternative way is the use of beneficial microorganisms (Grahovac et al., 2020). Many scientists have confirmed that the genus *Streptomyces* has great potential in the production of various secondary metabolites many of which can be used against fungal pathogens (Mitrović et al., 2021; Mojićević et al., 2017; Singh & Rai, 2012; Sadhasivam, Shanmugam & Yun, 2010). Streptomycetes are generally not considered pathogenic to humans. Many scientists around the world were studying the use of various streptomycetes and their secondary metabolites. In particular, when it comes to their use in biological control, many products such as Mycostop (*Streptomyces griseoviridis* K61) and Actinovate (*Streptomyces lydicus*) have been produced and used (Olanrewaju & Babalola, 2019).

This paper aims to analyze the bioprocess of bioagents production in a laboratory bioreactor using the production microorganism, *S. hygroscopicus*. This is important to determine at which moment of bioprocess the highest production of an antifungal bioagent effective against two *F. avenaceum* isolate occurs. The efficacy of cultivation medium supernatant will be tested *in vitro* and *in planta* every 12 h of the bioprocess.

## **2. Material and Methods**

To fully understand the bioprocess of biocontrol agents production obtained by the cultivation of *S. hygroscopicus* in a laboratory bioreactor, appropriate methods for sample analysis were selected. Sampling was performed at precisely defined intervals, and the obtained samples were further analyzed. The methods were chosen to give the clearest possible results of this research.

## **2.1. Streptomyces and Strain Preparation**

Soilborne *Streptomyces hygroscopicus* was stored in the Faculty of Technology Novi Sad, Serbia in the Microbial Culture Collection (GenBank accession number KT026467).

Medium used for the strain preparation had the following composition: glucose (15 g/l), soybean meal (10 g/l), CaCO<sub>3</sub>, (3 g/l), NaCl, (3 g/l), MgSO<sub>4</sub>, (0.5 g/l), (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, (0.5 g/l), K<sub>2</sub>HPO<sub>4</sub>, (1 g/l). The pH of the medium was adjusted to 7.2 ± 0.1 before autoclaving. The inoculum was prepared on a rotary shaker at 150 rpm, for 72 h.

## **2.2. Fungal Pathogens**

*Fusarium avenaceum* isolates labeled as KA12 and KA13 were used in this study as isolates to be tested. The isolates were obtained and identified as described in the paper by Grahovac et al. (2020).

## **2.3. Residual Nutrients Determination**

For the determination of nitrogen and glycerol content in the sample, the cultivation liquid sampled every 12 h of the bioprocess, was centrifuged. To obtain the supernatant required for further analysis, the liquid was centrifuged for 10 minutes at 10,000 rpm.

The Kjeldahl method was used for the determination of nitrogen content (EPA Manual). The content of residual glycerol was determined by UHPLC as described in the paper of Mitrović et al. (2021).

## **2.4. Biomass Determination**

The sampled cultivation liquid was centrifuged for 10 minutes at 10,000 rpm and the supernatant was discarded. The cell pellet was re-suspended in distilled water and the centrifugation procedure was repeated. The resulting precipitate was dried at 105°C overnight and then measured. The experiment was done in two replicates (Meanwell & Shama, 2008).

## **2.5. Bioreactor Experiment**

Production of agents for biocontrol of two *Fusarium avenaceum* apple phytopathogens was realized in a bioreactor (BiostatAplus, Sartorius AG, Germany). A used bioreactor is adequate for laboratory experiments with a volume of 3 l. During 168 h of bioprocess aerobic conditions were enabled by the application of a 1.5 vvm aeration rate and 150 rpm mixing speed. Other process parameters were controlled automatically.

A 10% of fresh prepared *S. hygroscopicus* culture was inoculated in the bioreactor, containing following media composition: glycerol (20 g/l), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.25 g/l), K<sub>2</sub>HPO<sub>4</sub> (1.5 g/l), CaCO<sub>3</sub> (3 g/l), NaCl (3 g/l) and MgSO<sub>4</sub> (0.5 g/l) at pH 7.0 ± 0.1.

## **2.6. In Vitro and in Planta Testing**

Diffusion method with wells was used to examine the activity of produced *S. hygroscopicus* bioagents against *F. avenaceum* KA12 and KA13 *in vitro* (Tadijan, Grahovac, Dodić, Grahovac & Dodić, 2016).

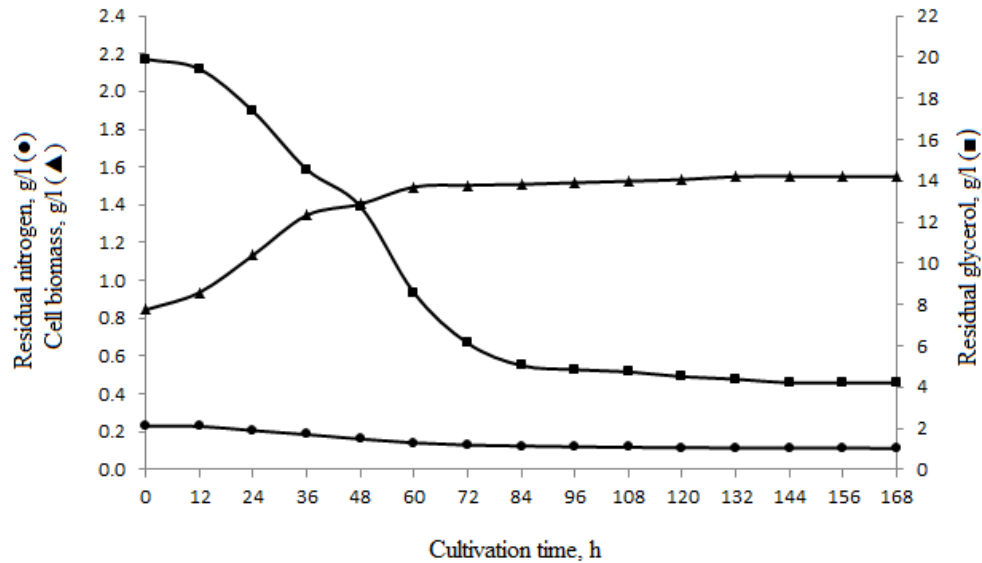
To confirm the effect of the produced bioagents on the stored apple fruits, *in planta* testing was performed. Testing was performed on the Golden Delicious apple fruits according to the method described by Mitrović et al., 2021. Each apple fruit was surface sterilized with 70% ethanol for 2 minutes. After that, the apple fruits were washed with sterile distilled water. In artificially made wounds (4x3 mm), first was added 10 µl of *S. hygroscopicus* supernatant and then mycelial plug (3 mm diameter) of the tested fungal isolate (*F. avenaceum* KA12 and KA13) into each wound. Sterile water was used as a control treatment. The fruits were incubated in a plastic container at a temperature of 21–23°C, 97% relative humidity. The diameter of developed necrotic lesions on the inoculated fruits was measured 10 days after inoculation.

## **3. Results and Discussions**

The results of this work were obtained by monitoring the bioprocess of biocontrol agent production using the microorganism *S. hygroscopicus*. Within the results, the course of this bioprocess was examined, by monitoring the change in the consumption of the most important media nutrients and the amount of generated biomass. Also, the results show the effect of produced bioagents using *in vitro* and *in planta* tests on two phytopathogens of storage apple fruits, *F. avenaceum* KA12 and KA13.

### **3.1. Substrate Consumption and Cell Growth**

Substrate consumption and cell growth were monitored during 168 hours of *S. hygroscopicus* cultivation. This is significant given that the increase in biomass and substrate consumption can indicate at which point the exponential phase ends and the stationary phase of the bioprocess begins.



**Figure 1:** Substrate consumption and cell growth during 168 h of *Streptomyces hygroscopicus* cultivation  
(Source: Data obtained in the experiment)

According to the fact that *S. hygroscopicus* produces secondary metabolites during the stationary phase, from Figure 1, it can be concluded that this phase begins after 72 h of the bioprocess.

Results of glycerol testing during cultivation (Fig. 1) show that the value of this nutrient was significantly consumed by the third day of bioprocess because microbial cells were intensively consumed glycerol for growth and biomass production. A similar flow was shown by the nitrogen utilization curve during the bioprocess (Fig. 1). As with glycerol, consumption of nitrogen decreases significantly until the third day.

Also, in accordance with the consumption of the two most important nutrients, at the same time, there is an increase in the cells biomass of the production microorganism, *S. hygroscopicus* (Fig. 1). Similar nutrient consumption was observed in the work of Tadijan et al. 2016. However, faster consumption of the carbon source in their work is due to the fact that the most suitable carbon source for streptomycetes is glucose (Kanini, Katsifas, Savvides & Karagouni, 2013; Singh, Mazumder & Bora, 2009). Certainly, in addition to glucose, glycerol has been reported by some scientists as the most suitable for the production of antifungal metabolites from the *Streptomyces* genera (Singh & Rai, 2012).

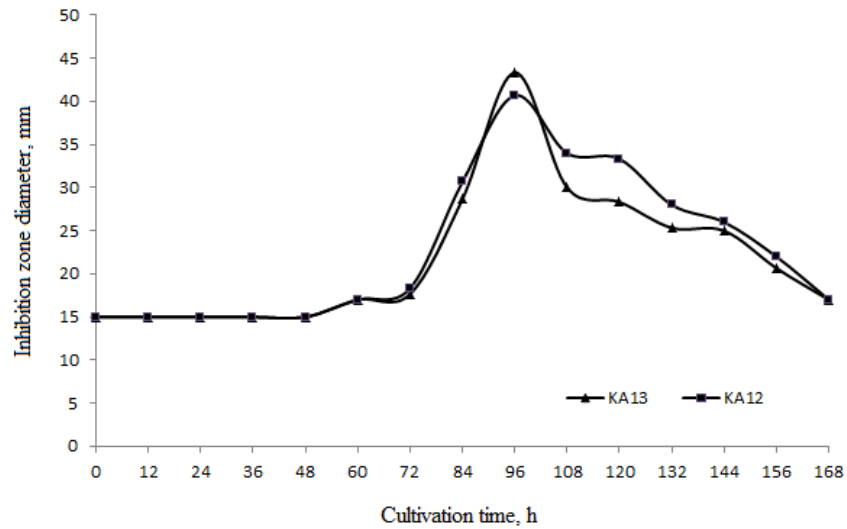
Based on Figure 1 it can be seen that the exponential phase of the bioprocess ends after the third day when the stationary phase, the phase in which *S. hygrosopicus* produce antifungal metabolites, begins.

### **3.2. In Vitro Results**

*In vitro* method represents the first step in the testing of the efficacy of produced bioagents against phytopathogenic isolates. Based on the obtained inhibition zone diameters it can be concluded at which hour of the bioprocess the largest production of bioagents occurs.

Following Figure 1, Figure 2 shows that no antifungal bioagents were produced until the third day of cultivation. After the third day, there was a gradual increase in the zone diameter of the *F. avenaceum* KA12 and KA13 mycelial growth. The largest diameter of inhibition zone formed on isolates *F. avenaceum* KA12 and KA13 was observed on the fourth day of (96 h of cultivation).

By analyzing Figure 2 it can be concluded that the *F. avenaceum* KA12 isolate was more resistant to the produced antifungal bioagents, forming a maximum mean value of mycelial growth inhibition zone diameter of 40.67 mm. At the same time, the maximum mean value of the mycelial growth diameter of the inhibition zone for *F. avenaceum* KA13 isolate at 96 hours of cultivation was 43.33 mm. After 96 h of cultivation, there was a gradual decrease in the efficiency of antifungal bioagents produced by *S. hygrosopicus*. Since these are isolates of the same species, similar results obtained in the experiment were expected. These results are related to the results obtained in the work of Grahovac et al., 2020, which was done on a similar medium composition but using different conditions of mixing and aeration of the cultivation liquid.



**Figure 2:** Mean values of mycelial growth inhibition diameter (mm) for *F. avenaceum* KA12 and KA13 isolates caused by *S. hygroscopicus* supernatant  
 (Source: Data obtained in the experiment)

It is assumed that the favorable conditions in the bioreactor are the result of large inhibition zone diameters formed on isolates *F. avenaceum* KA12 and KA13. This is expected since Yen and Li (2014) proved that an aeration rate of 1.5 vvm was best for rapamycin production by using *S. hygroscopicus*. Similar results were obtained by Sousa, Lopes & Pereira (2002) by examining *S. parvulus* and actinomycin D production.

### 3.3. In Planta Results

To confirm the obtained *in vitro* results, the next step is an *in planta* experiment. Table 1 shows the results of necrosis obtained after 10 days of incubation of apple fruits treated with supernatant *S. hygroscopicus* (obtained in 96 h of cultivation) and necrosis results of control samples.

The results show that there was a statistically very significant difference between the treated samples and the control ( $p < 0,01$ ).

**Table 1.** Mean values of necrosis diameter on treated and control apple fruits after incubation of 10 days

Pathogen	Necrosis diameter $\pm$ Sd (mm)	
	Artificially inoculated apple fruits	Control samples
	Bioprocess 3 l	
<i>F. avenaceum</i> KA12	6.68 $\pm$ 0.58	18.68 $\pm$ 0.58
<i>F. avenaceum</i> KA13	7.68 $\pm$ 1.15	16.68 $\pm$ 1.15



(Source: Data obtained in the experiment)

Results in Table 1 show that the difference between necrosis diameter of treated and control samples were 2.79 (for KA12) and 2.17 (for KA13) times smaller.

#### **4. Conclusion**

The results obtained in this paper show the good potential of *S. hygroscopicus* in biocontrol of stored apples from fungal disease caused by *F. avenaceum* isolates. The biocontrol agents produced by *S. hygroscopicus* in the laboratory bioreactor with a mixing speed of 150 rpm and aeration rate of 1.5 vvm show a significant effect against phytopathogenic isolates *F. avenaceum* KA12 and KA13. *In vitro* experiments and *in planta* experiments realized in this study show the high efficiency of produced bioagents. Based on the obtained results, it can be concluded that in 96 h of cultivation the highest efficiency of *S. hygroscopicus* supernatant on phytopathogenic isolates of *F. avenaceum* KA12 and KA13 was achieved. In practical terms, this means that the bioprocess can be shortened from 7 days to 4 days, making this bioprocess more economical. This research represents the first step towards increasing the volume of this production process. Therefore, as a continuation of the research, a scale-up should be done. This implies the further examination of *S. hygroscopicus* biocontrol agent production on a larger scale under the conditions obtained in this paper. Also, given that the cost of bioagents production is quite high, ways to reduce production costs should be considered. One of the possibilities is certainly the use of adequate waste effluents instead of synthetic substrates, which would significantly affect the cost of production.

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