Acta Univ. Sapientiae, Alimentaria, **7** (2014) 54–62

Effect of carbon sources on the production of the biofungicide by *Streptomyces hygroscopicus*

I. Tadijan[1]
email: tadi@uns.ac.rs

J. Grahovac[2]
email: johana@uns.ac.rs

J. Dodić[1]
email: klik@uns.ac.rs

M. Grahovac[2]
email: milapedja@gmail.com

S. Maširević[2]
email: stevanm@polj.uns.ac.rs

D. Vučurović[1]
email: dvdamjan@uns.ac.rs

S. Dodić[1]
email: dod@polj.uns.ac.rs

[1]University of Novi Sad, Faculty of Technology,
Bulevar Cara Lazara 1, Novi Sad, Serbia

[2]University of Novi Sad, Faculty of Agriculture,
Trg Dositeja Obradovića 8, Novi Sad, Serbia

Abstract. Fungi from the genera *Alternaria*, *Colletotrichum* and *Fusarium* are listed among the most important storage pathogens of apple fruits. Isolate of *Alternaria sp.* was obtained from apple fruit samples expressing rot symptoms. Biological control of plant pathogens by means of microorganisms is considered as an attractive alternative to chemical-based treatments, with minimal impact on the environment. Actinomycetes are known to have a great potential for control of plant fungal diseases and their antifungal activity greatly depends on the medium

Keywords and phrases: *Streptomyces hygroscopicus*, carbon sources, biofungicides, *Alternaria sp.*

used for their cultivation. The aim of this study was to determine the influence of ten different carbon sources on the production of the biofungicide produced by *Streptomyces hygroscopicus* against *Alternaria sp.* as a test microorganism. *In vitro* antifungal activity of the cultivation liquids on *Alternaria sp.* grown on potato dextrose agar were examined using wells technique. The maximum inhibition zone was reached after 72 hrs of incubation at 28 °C in mediums with fructose and starch (diameter >20 mm). However, the efficacy of other eight carbon sources was significantly higher compared to the control. The obtained results indicate that tested isolate of *Streptomyces hygroscopicus* shows great potential as a tool for the biological control of *Alternaria sp.*

1 Introduction

Post-harvest losses are caused by fungal pathogens due to high amount of nutrients and water in fruits, low pH and loss of intrinsic resistance that protects them while they are attached to the plant (*Nunes*, 2012). Well-known mycotoxin producers are *Aspergillus*, *Fusarium*, *Alternaria* and *Penicillium* species, and they are listed among the most important storage pathogens of apple fruits (*Andersen et al.*, 2006). In apples, some Alternaria spp. causes various types of post-harvest fruit rot. Also, *Alternaria sp.* can grow at low temperatures, which means that the contamination of refrigerated foodstuffs during transport and storage is possible.

Post-harvest pathogens may be the most suitable target organisms for biological control due to two reasons: first of all, they are managed in controlled environment, such as storage, and, secondly, the use of synthetic fungicides after harvest is already prohibited in many European countries (*Adaskaveg & Forster*, 2010). Biological control using microbial antagonists has emerged as one of the most promising alternatives, either alone or as part of integrated pest management to reduce pesticide use. During the past 30 years, several biocontrol agents have been exploited and widely investigated against different post-harvest fungal pathogens (*Saravanakumar et al.*, 2008).

According to *Kunoh* (2002), endophytic *Streptomyces* may play an important role in the development and health of plants because it affects plant growth due to its assimilation of nutrients and production of secondary metabolites (*Dhanasekaran et al.*, 2012). Streptomycetes are known to have a great potential for the control of plant fungal diseases (*Doumbou et al.*, 2001) and their antifungal activity greatly depends on the medium used for their cultivation. With regard to carbon sources, species-specific variation occurs within

Streptomyces for cell growth and production of secondary metabolites. Microorganisms usually break down high molecular weight carbon sources into small molecules, convert these to amino acids, nucleotides, vitamins, carbohydrates and fatty acids, and finally build these basic materials into proteins, coenzymes, nucleic acids, mucopeptides, polysaccharides and lipids used for growth. Glucose, which commonly is an excellent carbon source for cell growth, has been shown to influence the formation of several antimycotics. Other carbohydrates, such as glycerol, maltose, mannose, sucrose and xylose, have also been reported to interfere with the production of secondary metabolites. However, the optimal carbon source and the morphology varies between many antimycotic producing *Streptomyces* (*Jonsbu et al.*, 2002).

In the present study, the ability of *Streptomyces hygroscopicus* to assimilate different carbon sources and produce high-value metabolic compounds with antifungal activity against isolate of *Alternaria spp.* was investigated.

2 Materials and methods

Fungal pathogen

Isolates of *Alternaria sp.* were obtained from apple fruit samples expressing rot symptoms. Apple samples were collected during 2012 from Ultra Low Oxygen storages in Vojvodina Province, Serbia. The pathogen was identified according to pathogenic, morphological and ecological characteristics. The isolates were initially grown on PDA (Potato Dextrose Agar) plates for seven days. After seven days, a small amount of mycelium of each isolate was added to flasks containing 50 ml of potato dextrose broth. The flasks were incubated for 48 hrs on a rotary shaker (150 rpm) at 25 °C. Before use, culture liquid was filtered through the double layer of sterile cheesecloth.

Antifungal component production

Production microorganism *Streptomyces hygroscopicus* was isolated from the natural environment and stored in the Microbial Culture Collection of the Faculty of Technology in Novi Sad. The medium used for the growth of production microorganism had the following composition (g/L): glucose (15.0), soybean flour (10.0), $CaCO_3$, (3.0), NaCl, (3.0), $MgSO_4$, (0.5), $(NH_4)_2HPO_4$, (0.5), K_2HPO_4, (1.0). The pH of the medium was adjusted to 7.2 ± 0.1 prior to autoclaving.

For the preparation of the fermentation mediums, we used the following ten carbon sources: glucose, starch, lactose, mannitol, arabinose, galactose, fructose, maltose, sucrose and glycerol. Other components were the same as in the medium used for growth. The pH of mediums was adjusted to 7.2 ± 0.1 prior to autoclaving. The isolate was grown in a 100 cm^3 shake flask containing 30 cm^3 of the culture medium. The fermentation medium was inoculated with 10% (v/v) of a preculture after 48 hrs of growth and incubated at 26 ± 1 °C for 7 days under conditions of spontaneous aeration. Rotary shaker at 150 rpm was used for the mixing of fluids during the cultivation.

After cultivation, the sample of the cultivation medium was centrifuged at 10,000 g for 10 min and the supernatant of the cultivation medium was used for *in vitro* antagonistic activity assay.

In vitro antagonistic activity assay

In vitro antagonistic activity assay was performed in 85-mm Petri plates using wells technique (*Segy*, 1983). In short, two layers of PDA medium were spread in plates. The first layer consisted of 2% PDA medium. After solidification, a new layer composed of 1.2% PDA and filtered fungal culture liquid (35%) was added. Three wells per plate with a diameter of 10 mm were made and two plates represented one treatment. For each treatment, 100 µl of test liquid was added in each well. The treatments included: supernatant of *Streptomyces hygroscopycus* cultivation medium and sterile distilled water served as negative control treatment. After 72 hrs of incubation at 28 °C, the radius (mm) of mycelial growth inhibition zone around wells was measured.

The values were subjected to further analysis by factorial analysis of variance (factorial ANOVA) and the Duncan's multiple range test, using software Statistica 12 (Statistica, 2012).

3 Results and discussion

It is well known that designing an appropriate fermentation medium is of crucial importance in the production of secondary metabolites. To design effective medium, it is necessary to evaluate the effects of different carbon sources on the production of bioactive metabolites. Carbon source is required for the synthesis of microorganism cells and as an energy source. In the case of secondary metabolites, special attention is paid to the choice of carbon source because of their inhibitory effect on the biosynthesis of secondary metabolites. Also, biomass yield and cell morphology are strongly influenced by carbon

source. Media which contains starch, lactose, glycerol or fructose as a carbon source may result in reduced growth with low biomass yield (*Brzonkalik et al.*, 2011). Carbon sources which are rapidly metabolized, such as glucose, often lead to the maximum growth rate of the biomass, but a reduced production of many secondary metabolites (*Gallo* & *Katz*, 1972). However, some carbon sources have better influence on the biomass growth, while others significantly affect the synthesis of secondary metabolites. There is a diversity in carbon sources that can be metabolized by different *Streptomyces* species.

In order to examine the growth of *Streptomyces hygroscopicus* on different carbon sources, shake flask cultures were carried out. A selection of different carbon sources was tested, which included representatives of monosaccharides, disaccharides, polysaccharides and alcohols. As expected, the evaluated treatments had significant ($p \leq 0.01$) influence on the mycelial growth inhibition radius (mm).

Table 1: Mean values of the inhibition zone diameter [mm] after 72 hours of incubation at 28 °C and significance of differences at 5% level probability

Carbon source	**Inhibition zone (mm)±Sd**
Sucrose	20.6 ± 1.2^{c}
Maltose	23.0 ± 3.6^{c}
Galactose	26.3 ± 1.5^{b}
Glycerol	27.6 ± 2.1^{ab}
Glucose	27.6 ± 0.6^{ab}
Arabinose	28.0 ± 1.7^{ab}
Lactose	28.6 ± 1.2^{ab}
Mannitol	30.0 ± 0.0^{a}
Starch	30.3 ± 2.5^{a}
Fructose	31.0 ± 1.7^{a}

*The mean values with the same lowercase letters in the column "Inhibition zone radius [mm]" are not significantly different at 5% level of probability. (Duncan's multiple range test).

The results shown in *Table 1* indicate that the tested isolate of *Streptomyces hygroscopicus* shows great potential as a tool for the biological control of Alternaria rot on apple, and that the medium containing different carbon sources ensures its high activity (diameter > 20 mm).

The results indicate that there was no statistically significant difference between inhibition zone diameters when seven different carbon sources were applied for medium preparation. The efficacy of the other three carbon sources was also on a significantly higher level compared to the control. In *Figure 1*, the inhibition zones formed around wells with 100 µl of *Streptomyces hygroscopicus* are shown for isolates with fructose (1), starch (2) and control plates (3) after 72 hrs of incubation at 28 °C. The least efficiency to test microorganism showed mediums with sucrose and maltose.

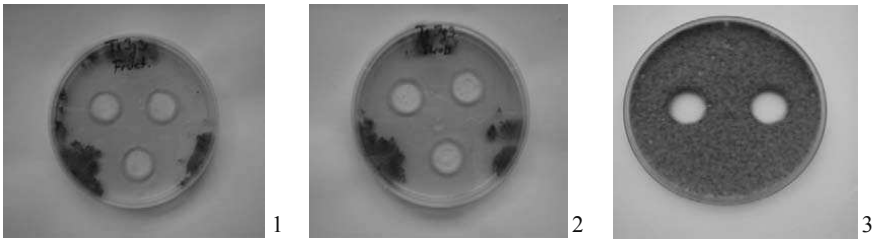

Figure 1: Inhibition zones formed around wells with 100 µl of *Streptomyces hygroscopicus* for isolates with fructose (1), starch (2) and control plates (3) after 72 hrs of incubation at 28 °C.

However, between glycerol, glucose, arabinose, lactose, mannitol, starch and fructose, as the seven best carbon sources in this experiment, there were no statistically significant differences. On the other hand, sucrose, maltose and galactose showed the least efficiency to test microorganism. *Singh et al.* (2009) also showed that the addition of carbon sources, such as maltose, sucrose and galactose, to the medium favoured the growth of *Streptomyces tanashiensis*, but the antibiotic production was less when compared with glucose, for example.

The choice of the carbon source, which will be used for potential industrial production, greatly depends on its availability and price. For example, an increase in biodiesel production results in an increase in the amount of waste glycerol. Waste glycerol constitutes a versatile carbon source with many possible applications in industrial fermentations, so it can be used in industrial microbiology for the production of valuable products, such as biofungicides (*Reungsang et al.*, 2013). That means that the use of glycerol as waste material significantly affects the price of biofungicide production process. *Jonsbu et al.* (2002) showed that glycerol was the carbon source that supported high specific growth rate at the same time as high nystatin production by *Streptomyces noursei*.

However, lactose and fructose showed a great potential as a carbon source for the production of antifungal compounds in this experiment. *Vinogradova et al.* (1985) detected a high level of heliomycin on lactose and *Sanchez & Demain* (2002) have reported positive effects of lactose on the biosynthesis of penicillin and erythromycin (*Gesheva et al.*, 2005). On the other hand, *Jonsbu et al.* (2002) concluded that fructose showed, in comparison to glucose, a trend of a more efficient utilization for the production of nystatin by *Streptomyces noursei*.

Demain & Fang (1995) have investigated that polysaccharides (e.g. starch) and oligosaccharides (e.g. lactose, maltose, sucrose) are often preferable for fermentations yielding secondary metabolites. This was in accordance with our work, where starch and disaccharides have proved to be very good carbon sources for the production of components for biological control of *Alternaria* rot on apple.

The development of efficient fermentation processes for the production of secondary metabolites by *Streptomyces* requires the examination of a diversity of species-specific features, including carbon-source nutrition and morphology (*Jonsbu et al.*, 2002). In conclusion, the findings of the present study showed that naturally occurring actinomycetes have a great potential to assimilate different carbon sources and produce high-value metabolic compounds with antifungal activity against the isolate of *Alternaria spp.* This fact allows that for the production of targeted antifungal components we can use different carbon sources depending on their availability on the market and prices.

Acknowledgement

The study is the result of the investigations conducted within the project "Development and scale-up of a biotechnological process for production of apple storage pathogens antagonists" (114-451-5041/2013) funded by Provincial Secretariat for Science and Technological Development of Autonomous Province of Vojvodina, Republic of Serbia.

References

[1] J. E. Adaskaveg & H. Forster, New developments in postharvest fungicide registrations for edible horticultural crops and use strategies in the United States. In: D. Prusky, L. M. Gullino, (eds.), Postharvest Pathology series: *Plant pathology in the 21st century*, Springer, 2. (2010) 107–111.

[2] B. Andersen, J. Smedsgaard, I. Jorring, P. Skouboe, L. Hagsholm Pedersen, Real-time PCR quantification of the AM-toxin gene and HPLC qualification of toxigenic metabolites from *Alternaria* species from apples. *International Journal of Food Microbiology*, 111. (2006) 105–111.

[3] K. Brzonkalik, T. Herrling, C. Syldatk, A. Neumann, The influence of linebreak different nitrogen and carbon sources on mycotoxin production in *Alternaria alternate*. *International Journal of Food Microbiology*, 147. (2011) 120–126.

[4] A. L. Demain & A. Fang, Emerging concepts of secondary metabolism in actinomycetes. *Actinomycetology*, 9. (1995) 98–117.

[5] D. Dhanasekaran, N. Thajuddin, A. Panneerselvam, Applications of actinobacterial fungicides in agriculture and medicine. *Fungicides for Plant and Animal Diseases*, 2. (2012) 29–54.

[6] C. L. Doumbou, M. K. Hamby, D. L. Crawford, C. Beaulieu, Actinomycetes, promising tools to control plant diseases and promote plant growth. *Phytoprotection*, 82. (3) (2001) 85–102.

[7] M. Gallo & E. Katz, Regulation of secondary metabolite biosynthesis catabolite repression of phenoxazinone synthase and actinomycin formation by glucose, *Journal of Bacteriology*, 109. (1972) 659–667.

[8] V. Gesheva, V. Ivanova, R. Gesheva, Effects of nutrients on the production of AK-111-81 macrolide antibiotic by *Streptomyces hygroscopicus*. *Microbiological Research*, 160. (2005) 243–248.

[9] E. Jonsbu, M. McIntyre, J. Nielsen, The influence of carbon sources and morphology on nystatin production by *Streptomyces noursei*. *Journal of Biotechnology*, 95. (2002) 133–144.

[10] C. Nunes, Biological control of postharvest diseases of fruit. *European Journal of Plant Pathology*, 133. (2012) 181–196.

[11] A. Reungsang, S. Sittijundac, I. Angelidaki, Simultaneous production of hydrogen and ethanol from waste glycerol by *Enterobacter aerogenes* KKU-S1. *International Journal of Hydrogen Energy*, 38. (2013) 1813–1825.

[12] S. Sanchez, A. L. Demain, Metabolic regulation of fermentation processes. *Enz. Microb. Technol.*, 31. (2002) 895–906.

[13] D. Saravanakumar, A. Ciavorella, D. Spadaro, A. Garibaldi, M. Lodovica Gullino, Metschnikowia pulcherrima strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples through iron depletion. *Postharvest Biology and Technology*, 49. (2008) 121–128.

[14] L. S. Singh, S. Mazumder, T. C. Bora, Optimisation of process parameters for growth and bioactive metabolite produced by a salt-tolerant and alkaliphilic actinomycete *Streptomyces tanashiensis* strain A2D. *Journal de Mycologie Médicale*, 19. (2009) 225–233.

[15] Statistica, Data Analysis Software System, Version 1.2. – Tulsa, (2012) USA.

[16] K. A. Vinogradova, N. P. Kirilova, Z. G. Sokolova, P. A. Nikolau, M. V. Shalgina, G. N. Skvortsova, A. N. Polin, Regulation of heliomycin biosynthesis by carbon sources. *Antibiot. Med. Technol.*, 30. (1985) 264–270.