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Application of the *Trichoderma* to the release of sugars from vegetable waste raw materials

Introduction

Trichoderma sp. are commonly found, asexually reproducing moulds fungi. Well-developed enzymes biosynthesis, and thus an ability to exploit a wide range of nutrient sources and high resistance to many toxic compounds of microbial and chemical origin, contributed to the occurrence of *Trichoderma* in almost all climatic zones [*Kubicek et al., 2001; Pandya et al, 2011*].

The biological adaptive determinants of *Trichoderma* sp. fungi and the ability to produce a number of other metabolites, trigger the potential use of these organisms in a variety of industrial areas, [*Harman et al., 2004*] i.a. as a microbiological cellular automata for the production of heterologous proteins, and potentially in the food industry and agriculture, where they have considerable predispositions, not only as a biocontrol factor, but also as an effective producer of second generation biofuels [*Schuster and Schmoll, 2010*].

The enormous potential of fungi *Trichoderma* is reflected mainly in the industrial production of enzymes and biocontrol, the process of eradication of phytopathogens by safe microorganisms that, unlike chemical plant protection products, are neutral or even beneficial for the environment. Their effective mechanism against pathogens is based on processes such as: competition, mycoparasitism or antibiosis [*Chet et al., 1997*]. The *Trichoderma* fungi compete with pathogenic microorganisms mainly for the living space and available nutrients, and the dominance allows for the rapid colonization of the environment under various conditions, as well as the ability to produce biologically active compounds - siderophores. Mycoparasitism is based on the physical contact with pathogenic moulds and lysis of its cell wall. Pathogen growth restriction by *Trichoderma* sp. also occurs through the production of antibiotics and other secondary metabolites [*Benitez et al, 2004;Wojtkowiak-Gębarowska, 2006*].

The *Trichoderma* sp. moulds exhibiting the ability of intensive production of hydrolysis enzymes like xylanase, celulase and polygalacturonase are potentially applicable in degradation of plant waste materials, mainly lignin-cellulosic compounds, coming in huge quantities from the paper industry, as well as from agriculture and forestry. The resulting products as a mixture of simple and complex sugars may become valuable raw materials base [*Mach et al., 1999*].

The primary quantitative and qualitative methods to analyze the degradation process of macromolecular compounds are high performance liquid chromatography (HPLC) and gas chromatography (GC). The GC method is more sensitive to compounds at low concentrations e.g. sugars, however, the preparation of the sample prior to analysis is much more time-consuming than in the case of HPLC. For this reason, the HPLC method is often used to analyze these compounds [*Agblevor et al., 2006*].

The aim of this study was to analyze the sugars released into the aqueous phase from the plant cell wall building polysaccharide complexes after biodegradation with *Trichoderma* moulds, using high performance liquid chromatography.

Materials and methods

Microorganisms

In the present study the following strains were used: *Trichoderma citrinoviride* C1 and *Trichoderma harzianum* T33 [*Piegza et al., 2014*], deposited in the local culture collection of *the Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences*. Microorganisms were stored on 12 g/L PDB (*Potato Dextrose Broth*) agar slants at 4°C.

Culture

Six different raw waste materials from the food industry were tested: grape cherry seeds (PE), grape pears (G), red cabbage (CZ), grape dogwood (D) grape with seeds (WIN) and beep pulp (W). Culture media were prepared by mixing 30g of each compound in 30 ml water and sugar beep pulp (W) in the amount of 10g with 30 ml of water. One ml of the suspension (10^8 spores/ml) was used to inoculate 100 ml of PDB (Potato Dextrose Broth) 24 g/l medium in 250-ml *Erlenmeyer* flasks. Cultures were conducted for 7 days at the temperature of 25 °C on an orbital shaker *SHELLAB SI4*. Thus prepared inoculum were inoculated the solid-state cultures of which were held for 33 days, at a temperature of 25 ° C, at 160 rpm.

Methods of analysis

Post culture preparations obtained on the day 5, 9, 13, 19, 26 and 33 were analyzed for the presence of carbohydrates such as sucrose, glucose, galactose, cellobiose, rhamnose and GLcNAc (N-acetylglucosoamine) using the HPLC technique on a column of *Aminex HPX 87 h* combined with the UV detector at a wavelength of 254 nm and RI detector at room temperature. The flow rate of the liquid phase, 20 mM H_2SO_4, the column was 0.6 cm^3/min.

Results and discussion

During the 33-day culture, preparation were collected on the 5^{th}, 9^{th}, 13^{th}, 19^{th}, 26^{th} and 33^{th} day, and then the content of carbohydrates such as glucose, galactose, rhamnose, xylose, cellobiose and N-acetylglucosamine was evaluated. According to the literature data, ligninocellulotic substrates like municipal solid waste (MSW) and pulp, as well as paper mill waste (PMW) were applied for the production of glucose. In cultures on the MSW the PMW medium 23.5 g/L and 14.5 g/L of sugar was obtained, respectively [Douglas et al, 1983]. Another often-discussed example is the technique of obtaining simple sugars from the waste materials by chemical methods from materials such as newspapers, newsprint and wheat, which allowed to acquire glucose or xylose. When it comes to the amount of glucose obtained in this process for these substrates, the concentration ranged from 4.01 to 6.86 g/L and xylose 4.30 to 34.68 g/L. Galactose was obtained from newspaper at 2.34 g/L, but not on any other substrate [*Foyle et al., 2004*].

The concentration of sugars found in cultures of *Trichoderma citrinoviride* C1 varied depending on the substrate and the culture period. In the case of glucose the concentration obtained ranged from 0.06 g/L on the 19^{th} day in pear medium to 1.90 g/L on the 13^{th} day in dogwood medium. Only on the 9^{th} day none of the analysed sugars was detected on any substrate. Higher concentrations were observed for rhamnose , that reached the value from 0.02 g/L on the 9^{th} day times in the medium with dogwood and grapes to 8.41 g/L on the 5^{th} day in pear medium.

In the case of other sugars: xylose, xalactose, rhamnose, and N-acetylglucosamine, their presence was detected in culture only in individual cases (Fig. 1). Respectively: xylose on the 33^{th} day in pear medium (2.1 g/L) and in the dogwood medium (1.3 g/L); galactose on the 19^{th} day, in dogwood (0.61 g/L); cellobiose on the 33^{th} day in grape cherry seed medium (0.06 g/L), pear (0.85 g/L), cabbage (0.12 g/L) and on the 13^{th} day in sugar beep pulp medium (0.33 mg/L), GLcNAc on the 5^{th} day with cabbage as a substrate and culture medium (0.003 µg/L).

For the *Trichoderma harzianum* T33 strain the values obtained for glucose were in the range of 0.02 g/L to 3.06 g/L on the 19^{th} day in the culture with the red cabbage.

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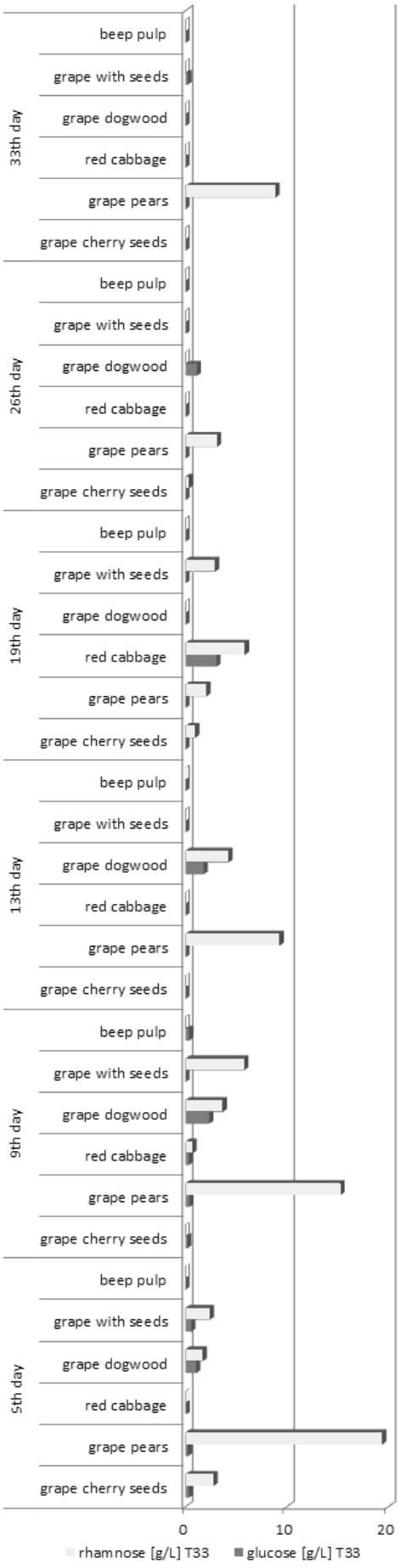

Fig. 1. Selected sugars concentration depending on day and culture of *Trichoderma* C1

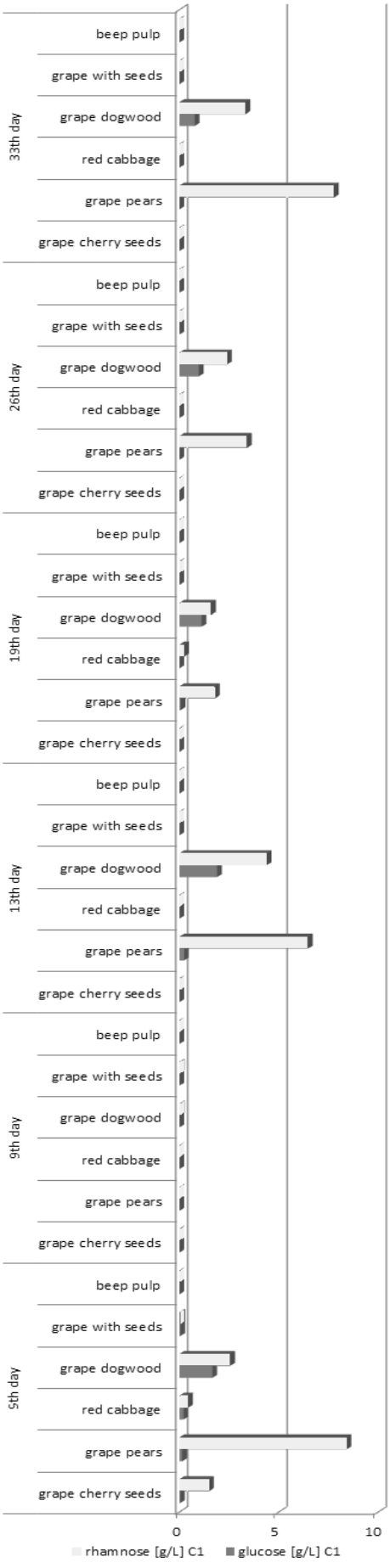

Fig. 2. Selected sugars concentration depending on day and culture of *Trichoderma* T33

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Rhamnose concentrations reached much higher values and ranged from 0.33 g/L on the 26th day in grape cherry seeds medium to 19.41 g/L on the 5th day for pear medium.

As in the case of C1 strain, the presence of xylose, galactose, cellobiose and N-acetylglucosamine was observed only in individual cases (Fig 2). Respectively: xylose on the 26th day in the cabbage medium (0.12 g/L) and on the 33th day with pear (1.5 g/L); galactose on the 9th day, in sugar beep pulp medium (0.13 g/L), on the 19th day in dogwood medium (0.9 g/L) to on the 26th day at the level 1.8 g/L and also in grapes (0.2 g/L); cellobiose on the 33th day in grape cherry seeds medium (0.07 g/L), pear (1.18 g/L); GLcNAc on the 5th day in grapes and the 9th day in cabbage presents (0.02 g/L), in 13 day in grapes (0.11 g/L) and on the 19th day, also in grapes (0.03 g/L).

Statistical analysis

The results were subject to statistical analysis in order to determine the relevance of one of the three factors affecting the process of sugars release, and thus their concentration in outcoming preparations. The first step was to analyze the impact of the medium, day and strain for the degradation of plant cell wall building polysaccharide complexes.

Significant impact of culture time on the release of four of the six analyzed sugars was denoted: galactose (19 day), cellobiose (33 day), rhamnose and glucose (5 day). Also the substrate type used in cultures turned out to be significant for the release of galactose (dogwood) and cellobiose and rhamnose (pear). In the case of glucose the application of the strain T33 was significant.

Tab. 1. Statistical analysis of released sugars as a function of culture period (homogenous groups given)

Released sugar	Day	1	2	3
GLUCOSE	26 th	a		
	33 th	a		
	13th	a	b	
	19th		b	
	9 th		b	
	5 th			c
RAMNOSE	13 th	a		
	33 th	a		
	19 th	a		
	9 th	a	b	
	26 th	a	b	
	5 th		b	
GALACTOSE	5 th	a		
	33 th	a		
	26 th	a		
	9 th	a		
	13 th	a		
	19 th		b	
CELOBIOSE	26 th	a		
	9 th	a		
	5 th	a		
	13 th	a		
	19 th	a		
	33 th		b	

The next stage of statistical analysis was to examine the interaction of substrate-day, day-strain and substrate-strain on the quantity released in the sugars.

In the case of the interaction of the substrate- day for cellobiose and N-acetylglucosamine showed the relationship for pear medium on the 33th day, for galactose on the 19th day, and rhamnose on the 5th day.

Analysis of the interaction of day-strain relationship revealed only in the case of *Trichoderma harzianum* T33 on the 33th day of culturing for cellobiose. Last analyzed interaction of substrate-strain showed an important dependency for cellulose in the case of the T33 strain in the pear medium (Tab. 1).

Conclusions

The conducted analysis showed a high variability in the level of the released sugars from hydrolysis of high-complex compounds, to the aqueous phase. A long-time culture resulted in pronounced fluctuations in the quantity and quality of designated sugars. In most cases, it was definitely a wider profile quantitative detectable sugar in the strain of *Trichoderma harzianum* T33, and to be the best substrate to attain high concentrations of sugars was grape pears and dogwood.

The highest concentration of sugars found on the 5th day C1 strain culture in pear medium, and on the 5th and 9th day for the T33 strain in pear medium.

The above results allowed concluding that it is culture time strictly determines the level and intensity of the release of a specific saccharide.

LITERATURE

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