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DISRUPTION OF *ASPERGILLUS FLAVUS* CELLS: A BEAD MILL HOMOGENIZATION METHOD

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Summary

The most important mycotoxigenic fungus involved in pre- and post-contamination of crops is *Aspergillus flavus* which causes great health and economic loses worldwide due to production of the most potent natural hepatocarcinogen – aflatoxin B_1. Contamination with this secondary metabolite is getting even worst by global climate changes and other abiotic stressors present in environment. Accordingly, researches with the aim of synthesis or identifying the antiaflatoxigenic and antifungal compounds are of interest. For such efforts realization, use and manipulation with intracellular content of *A. flavus* cells is necessary. The aim of this study was to apply Omni® Bead Ruptor 12 Homogenizer on disintegration of *A. flavus* cells, to find optimal parameters of homogenization and prepare biologically active extracts which can be used for determination of possible strategies for control of contamination with aflatoxins. Results of study showed that bead mill homogenizer Omni® Bead Ruptor 12 Homogenizer can be applied for disintegration of *A. flavus* mycelia and preparation of enzymatically active cell-free extracts. The homogenization mixture in 2 mL homogenization tubes should contain 100 mg of fresh wet mycelia, 1 g of precooled acid washed glass beads of 0.5 mm in diameter and 1 mL of ice-cold buffer. Such mixture should be homogenized at speed of 6 m/s during 120 s, in six cycles of 20 s with cooling of samples in ice-bath between cycles.

Keywords: bead mill, disintegration, *Aspergillus flavus*, catalase, proteins, aflatoxins

Introduction

Saprotrophic mycotoxigenic fungus *Aspergillus flavus* is producer of extremely toxic secondary metabolite - aflatoxin B_1 (AFT B_1), one of the dangerous known natural hepatocarcinogen (IARC, 1993; 2002; 2012), and opportunistic pathogen of crops, animals and humans (Klich, 2007; Šarkanj et al., 2018a). This fungus is continually the subject of scientific research, since its discovery, especially due to health and economic challenges. Moreover, it is affected by global climate changes factors (Battilani, 2016; Helfer, 2014; Magan et al., 2007; Trnka et al., 2014), as temperature, drought stress and CO_2 concentration or new environmental pollutant, abiotic stressors, which causes oxidative status perturbations (Kovač et al., 2017; 2018a; 2018b; 2018c); *A. flavus* is sensitive on such stimuli (Jayashree and Subramanyam 2000; Narasaiah et al. 2006; Reverberi et al. 2010; Miskei et al., 2010; Reverberi et al., 2012; Hong et al. 2013) which results with changes in AFTs production. Moreover, besides aflatoxins (AFTs) production, impact of climate change on the emerging toxins is receiving increasing attention (Battilani, 2016; Abdallah et al., 2017; Kovač et al., 2019).

Above mentioned studies aimed on decrease of pre- and post-harvest contamination with AFTs, imply use and manipulation with intracellular content (Fountain et al., 2018; Šarkanj et al., 2018b; Kovač et al., 2018c), whichrequire application of certain disruption processes. Here stands out disruption of cells with bead mill homogenization, mechanical method of disruption effective for disintegration of filamentous fungi, such as *Aspergillus* spp. fungi. Method is based on intracellular components release due to circulating beads dispersed in homogenization mixture of mycelia and buffer (Doucha and Livansky, 2008). Despite to that, the most of literature data on this topic are about disruption of Gram-negative, Gram-positive bacteria and yeast (Klimek-Ochab et al., 2011). Furthermore, the same group of authors reported that bead milling disruption is not the best option for disruption of *A. fumigatus*. Therefore, the aim of this study is to found optimal parameters for disintegration of *A. flavus* cells by Omni® Bead Ruptor 12 Homogenizer and prepare biologically active extracts which can be used for research of possible AFTs contamination control strategies.

Materials and methods

A. flavus cultivation in yeast extract sucrose (YES) medium

Before *A. flavus* NRRL3251 growth in YES medium with 2% sucrose, stimulation of conidia production was performed by growth on potato dextrose agar during 168 hr, at 29 °C in dark.

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Fungal conidia suspension preparation ($2.5 \cdot 10^4$), inoculation of aflatoxin-inducing YES broth in 250 mL flasks, as well as incubation, were conducted at 29 °C (which favours aflatoxin production) according to Kovač et al. (2017).

For the incubation of the inoculated YES media flasks during 144 hr, the rotary shaker (KS 260 basic, IKA, Germany) settled at 200 rpm was used. After ending of the incubation period, samples of YES broth and *A. flavus* mycelia were collected from the flasks. Separation of mycelia from media was performed by filtration. For comparison of mycelia growth rate and aflatoxins production ability, six random samples were collected. All collected samples of mycelia and media were stored at in 2 mL tubes at −80 °C, for at least 24 hr before experiment. Additionally, mycelia portion was taken prior freezing and lyophilisation, and dried until constant mass (24 hr at 105 °C) in order to determine the dry mycelial weight.

After weighting and freezing of mycelia samples intended for comparison, lyophilisation was performed (Christ, Alpha 1-4 LD, Germany). Drying conditions were as described by Kovač et al. (2018) and as follows: freezing temperature −55 °C; temperature of sublimation −35 to 0 °C; vacuum level 0.22 mbar. The temperature of isothermal desorption varied from 0 to 22 °C under the vacuum of 0.06 mbar. Freeze-drying lasted until the constant mass of mycelia was obtained, which was approximately 5 hr. However, before freezing collected mycelia samples were homogenized by pestle and mortar, separated on portions and transferred into 2 mL tubes. Mycelia stored as this, as well as lyophilised mycelia was used for determination of catalase activity, proteins and aflatoxins content. Also, YES media separated from six samples intended for comparison was subjected to an analysis of aflatoxin content.

A. flavus extract preparation

The extracts of *A. flavus* mycelia were prepared by disintegration of mycelia by homogenization using Omni® Bead Ruptor 12 Homogenizer (Omni International, Kennesaw GA, USA). Disintegration was performed at 2.1, 4 and 6 m/s during 20, 40, 60, 120, 180 and 240 s with sample cooling on ice after every 20 s of disruption. In 2 mL disintegration tubes, 0.1 g of mycelia with 1 g of glass beads (acid washed and precooled; diameter 0.5 mm; Sigma-Aldrich, Germany) and ice-cold extraction buffer (50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA-2Na) was added. Such content of disintegration mixture was previously experimentally determined (Kovač et al., 2017) and is selected according to tubes and device manufacturer technical recommendation. After disintegration process, extracts were clarified by centrifugation (15000 x *g* /4 °C /20 min) at centrifuge Thermo Scientific SL 8R (Thermo ScientificTM, Finland). The prepared extracts were immediately used for analysis with the aim of optimal parameters of described disintegration process determination.

Analysis of catalase activities and protein concentration in extracts

For determination of catalase (CAT; EC 1.11.1.6) activity, spectrophotometric measurements of the decrease in absorbance at 240 nm, due to H_2O_2 decomposition in the presence of CAT, according to Reverberi et al. (2005) were performed.

Concentration of proteins in extracts was determined by Bradford assay according to Bradford (1976). As a standard, bovine serum albumin was used.

Analysis of aflatoxins content in mycelia extracts and YES growth media

Content of aflatoxins (AFTs) in prepared mycelia extracts and separated YES media samples were estimated by *dilute and shoot* LC-MS/MS method described by Kovač et al. (2017). Separation was performed using an Acquity UPLC H-Class system (Waters, MA, USA) on Acquity BEH C18 column (2.1 x 100 mm, 1.7 μm) (Waters, USA), while detection and quantification were performed using a Xevo TQD mass spectrometer (Waters, USA). MassLynx and TargetLynx software (v. 4.1., Waters, USA) was used for data acquiring and processing. Recovery was 92% for all aflatoxins and was assessed by spiking blank YES medium with aflatoxin standard mix (Biopure, Austria) solution at a concentration of 10 ng/mL. Instrumental limits of detection were 0.15 ng/mL, while limits of quantification were 0.5 ng/mL, for all aflatoxins.

Mycelial extracts used for aflatoxin content determination were prepared as described above, but instead of extraction 20% acetonitrile (HPLC grade) solution was used.

Statistical analysis

All data presented here are expressed as the mean value ± SEM. Shapiro-Wilk test was used for normality distribution checking of pooled datasets which were compared by nonparametric statistics methods (Friedman ANOVA and Kendall coefficient of concordance; Kruskal-Wallis ANOVA). The Statistica 13.3 programme package (TIBCO Software Inc, Palo Alto, CA, USA) was used and differences were considered significant when the p value was < 0.05. For the drawing

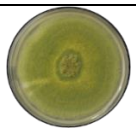
of the Sankey diagrams, Flourish studio was used (Flourish Studio, Kiln Enterprises Ltd, London, UK).

Results and discussion

A. flavus growth and aflatoxin production

At first step of the experiment, *A. flavus* growth rate and aflatoxins production ability was determined. From the all inoculated flasks, six of them were excluded after 144 hr, at the end of the growth period. All actions regarding conidia suspension preparation, inoculation of aflatoxin-inducing YES broth, as well as growth during incubation period, resulted by the pattern typical for tested fungi (Table 1), as previously showed (Kovač et al., 2017; 2018a; 2018b). The results for aflatoxin content are expressed as the sum of mycelial and medial concentrations, as well as the sum of AFT B_1 and B_2, which were detected, where AFT B_1 contributed with more than 99% in the total sum.

Table 1. *Aspergillus flavus* NRRL 3251 growth rate and aflatoxin production ability

 ***A. flavus* NRRL 3251**	**Aflatoxin sum (B_1 and B_2) [ng/mL]**	Mycelia	YES media	Σ
		4.04 ± 0.37	4.39 ± 0.27	8.42 ± 0.64
	Mycelia growth rate [g $_{d.m.w.}$ /50 mL]	0.167 ± 0.04		

Impact of bead mill homogenization speed and time on catalase activity, protein content and aflatoxins concentrations in A. flavus mycelia extracts

The *A. flavus* mycelia extracts prepared under different speed and time of homogenization at bead mill Omni® Bead Ruptor 12 Homogenizer were subjected to determination of the catalase activity, protein and aflatoxin concentrations (Fig. 1-3).

The results of catalase activity in prepared extracts showed proportional dependence to the speed and time of the homogenization (Fig. 1). There was observed homogenization speed-dependent catalase activity, at the lowest rate in the extract prepared at 2.1 m/s and at the highest rate in the extracts prepared at 6 m/s. Statistically significant difference ($p < 0.02$) between this two homogenization speed was determined at all homogenization times, except for 40 s and 180 s of homogenization. Such results obtained for catalase activity imply that optimal homogenization speed for mycelia extract preparation is 6 m/s. When the homogenization time was examined, activity was increased with increase of time of homogenization. However, results showed that maximum activity was achieved at 120 s of homogenization, both at speed of 4 and 6 m/s (Fig. 1). At the applied speed of 6 m/s, almost double catalase activity was determined.

The results of protein content in the prepared extracts of *A. flavus* mycelia showed proportional dependence on the speed and time of the homogenization (Fig. 2), as it was determined in the case of catalase activity (Fig. 1). There is an observed speed-dependent protein release in extracts, at the lowest rate in the extract prepared at 2.1 m/s and at the highest rate in the extracts prepared at 6 m/s. Statistically significant difference between ($p < 0.03$) this two homogenization speed, visible in released protein content, is determined until 120 s of homogenization.

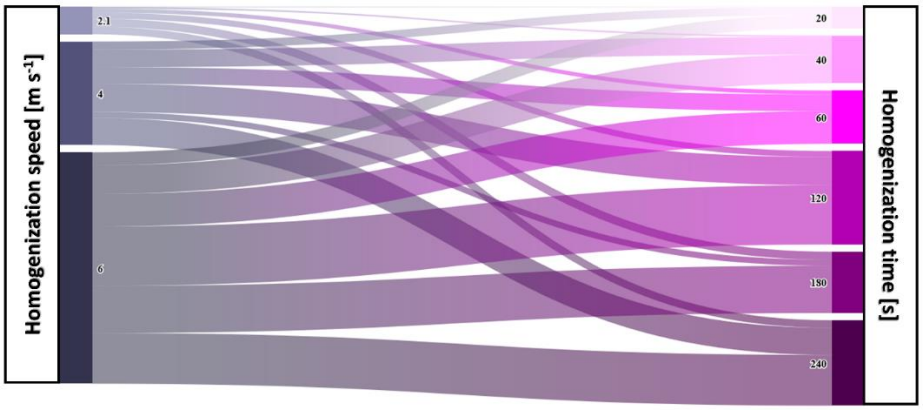


Fig. 1. Catalase activity (U/mL) in *A. flavus* NRRL 3251 extracts prepared by bead mill disintegration at different homogenization speed (● – 2.1, ● – 4 and ● – 6 m/s) and time-points (○ – 20, ○ – 40, ● – 60, ● – 120, ● – 180 and ● – 240 s). The line thickness represents the quantitative value of the catalase activity

Such results of protein content imply that optimal homogenization speed for mycelia extract preparation is 6 m/s. In the case of optimal homogenization time, trend of increase of protein content is observed until 120 s of homogenization, it can be said both for speed of 4 and 6 m/s. However, at the applied speed of 6 m/s, there was higher protein content determined in extracts (Fig. 2).

The release of aflatoxins (sum of AFT B_1 and B_2) from mycelia into extracts during optimization of mycelia homogenization process is showed in Fig. 3. There were no observed statistically significant difference ($p > 0.05$) between applied speed and time of homogenization. Accordingly, all the applied conditions resulted with release of aflatoxin at satisfying level.

A. flavus is the most important mycotoxigenic fungus which is since its discovery subject of numerous scientific researches (Kovač et al., 2018b). The most of them imply use and manipulation with intracellular content (Fountain et al., 2018; Šarkanj et al., 2018b; Kovač et al., 2018c), which require application of certain disruption processes.

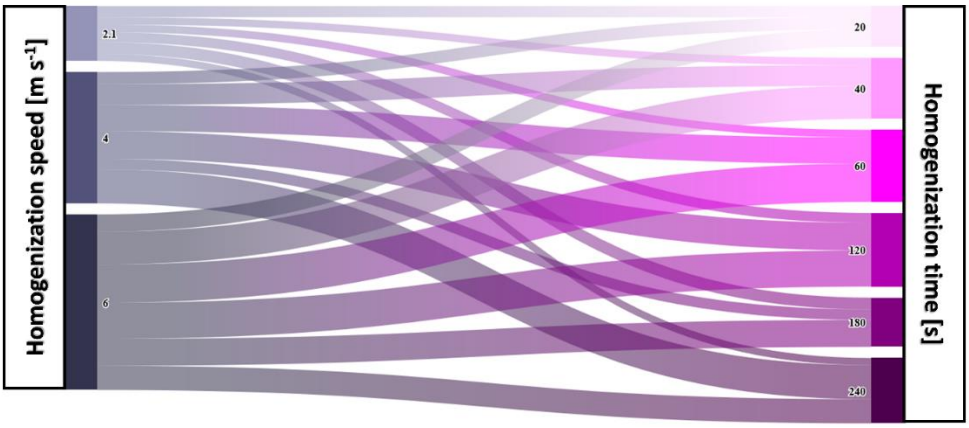


Fig. 2. Protein content (mg/mL) in *A. flavus* NRRL 3251 extracts prepared by bead mill disintegration at different homogenization speed (● – 2.1, ● – 4 and ● – 6 m/s) and time-points (○ – 20, ● – 40, ● – 60, ● – 120, ● – 180 and ● – 240 s). The line thickness represents the quantitative value of the protein content

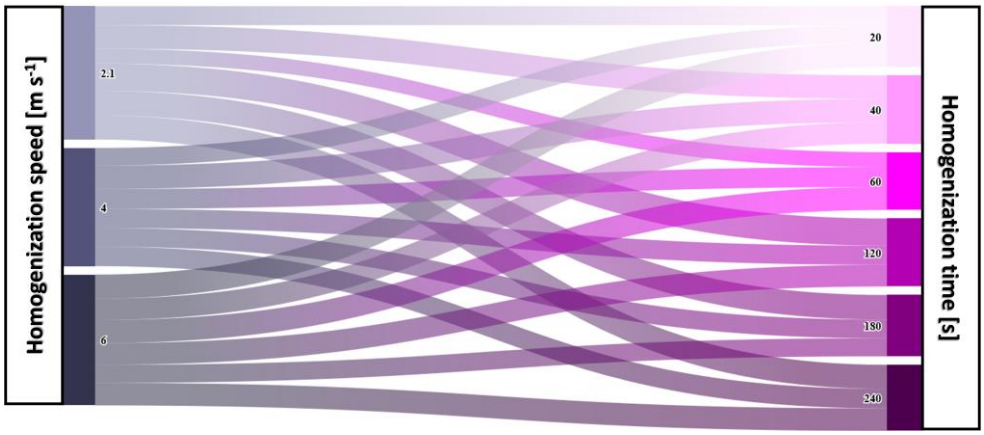


Fig. 3. Sum of aflatoxin B_1 and B_2 (ng/mL) in *A. flavus* NRRL 3251 extracts prepared by bead mill disintegration at different homogenization speed (● – 2.1, ● – 4 and ● – 6 m/s) and time-points (○ – 20, ● – 40, ● – 60, ● – 120, ● – 180 and ● – 240 s). The line thickness represents the quantitative value of the sum of aflatoxin B_1 and B_2

In this case study, bead mill homogenization was applied on cell wall disintegration and activity of catalase, release of proteins and aflatoxins in crude extracts were monitored for optimal homogenization speed and time selection. The main aim of the study was to adjust particular bead mill homogenizer,

Omni® Bead Ruptor 12 Homogenizer, to a particular strain, aflatoxigenic *A. flavus* NRRL 3251. It is known that fungal cell wall is the main factor that regulates retention of intracellular content. This is highly dynamic structure of specific composition and mechanical properties that vary between genera, even between closely related species (Durán and Nombela, 2004; Adams, 2004; Damweld, 2005; Bowman and Free, 2006; Free, 2013). Also, results presented in this study confirms that homogenization method efficiency is dependent on the particular intracellular molecule of interest location behind the fungal cell wall (Fig. 1-3).

Bead mill homogenization process efficiency can be comparable with efficiency of disintegration processes where sonification is applied, at least in the case of *Aspergillus* spp. (Klimek-Ochab et al., 2011). However, at sonification, time of exposure is critical parameter which affects intracellular compounds activity. Furthermore, the most ultrasound energy absorbed by cell suspension is appearing as a heat which also has certain effect on biological material. According to that, and based on previously reported studies (Kovač et. al., 2015; 2016; Šarkanj 2018b), assumption about bead mill homogenization method as a less time and energy consumption and more effective, at the same time, which is confirmed in this study. Despite to that, crude extract overheating still need to be prevented by sample cooling in ice-bath between cycles of homogenization.

Conclusion

To sum up, the bead mill homogenizer Omni® Bead Ruptor 12 Homogenizer can be applied for disintegration of *Aspergillus flavus* mycelia and preparation of enzymatically active cell-free extracts. It can be achieved when 100 mg of mycelia, 1 g of precooled acid washed glass beads of 0.5 mm in diameter and 1 mL of ice-cold buffer is added into homogenization tubes of 2 mL. Such mixture should be homogenized at speed of 6 m/s during 120 s, in six cycles of 20 s with cooling of samples in ice-bath between cycles.

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