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ASSESSMENT OF THE FERMENTATION OF ROSE OIL DISTILLATION WASTEWATER (RODW) BY *TRICHODERMA ASPERELLUM SL-45* AS ADDITIONAL STEP FOR FUNGAL BIOMASS PRODUCTION, TO THE RODW PHENOLICS EXTRACTION

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Abstract: The industrial production of rose oil from *Rosa damascena* flowers by water steam distillation results in generation of large quantities of phenolics - rich rose oil distillation wastewater (RODW). *Trichoderma asperellum SL-45* is a rhizospheric micromycete strain with high potential for application in soil and potting substrate improvement, as well as xylanases and cellulases production. The present study assesses the RODW fermentation capacity of *T. asperellum SL-45* and the impact of fermentation on RODW phenolics composition. The *in vitro* shake flask culture studies showed high growth rate of *T. asperellum SL-45*, without the need for changes in RODW parameters and composition prior cultivation. Further analysis showed only minor modifications of the RODW phenolics composition following T. asperellum SL-45 fermentation. The results suggest that *T. asperellum SL-45* fermentation of RODW could be efficiently applied as additional step for fungal biomass production prior or following the RODW phenolics extraction and valorization. The possibilities of using RODW fermentation for larger scale *T. asperellum SL-45* biomass production are discussed.

INTRODUCTION

The industrial production of rose essential oil generates annually a great amount of solid and liquid waste products, including around 30 000 tons of rose oil distillation wastewater (RODW), which has been previously shown to be a rich source of sugars and phenolics (Rusanov *et al.,* 2014). Rusanov *et al.* (2014) developed efficient procedure for extraction of the RODW phenolics composition and later studies proved that the obtained extract, rich in quercetin, kaempferol and phenylethyl alcohol glycosides as well as ellagic acid, possesses biological activities with diverse potential applications (Rusanov *et al.,* 2014; Solimine *et al.,* 2016; Wedler *et al.,* 2016). The developed procedure, although extracting the phenolics composition of RODW, still leaves behind large amounts of sugars, which might be utilized by microorganisms allowing the use of RODW as a culture media (Rusanov *et al.,* 2014).

Trichoderma spp. have been known for their wide range of industrial applications including their use in plant organic farming due to the positive effect of several species on plant growth stimulation and protection against plant pathogens (Blaszczyk e*t al.,* 2014; Waghunde *et al.,* 2016). *Trichoderma asperellum SL-45* is a rhizospheric micromycete strain, which has been extensively tested for its ability to produce and excrete xylanases and cellulases with potential application in the paper industry (Badalova, 2017; Evstatieva *et al.,* 2013; Velichkova *et al.,* 2014). Additionally, *T. asperellum SL-45* produces phytohormone-like metabolites, which have been shown to positively affect the growth of several plant species including *Cyclamen persicum*, *Chrysanthemum grandiflorum* and *Euphorbia pulcherrima.* The plant growth stimulation effect of *T. asperellum SL-45* liquid cultures makes them suitable for application as liquid microbial fertilizer (Badalova, 2017). Although *T. asperellum SL-45* possesses a number of desired characteristics with potential industrial applications, all previous studies carried out with isolate in liquid cultures involved the use of optimized liquid media like Mandel's media (Badalova, 2017). Preceding studies have successfully demonstrated the cultivation of *T. asperellum* (previously known as T. *viride*) using a number of different liquid and solid waste products (Ahuja & Bhatt, 2018; Bai *et al.,* 2008; Emerson & Mikunthan, 2015; Govindan *et al.,* 2017). In the current study we investigated the potential of using RODW as a source of cheap liquid media for cultivation of *T. asperellum SL-45* and compared the obtained results to two endophytic fungi representing *Phoma* sp. and *Fusarium* sp. isolated from medicinal plants and which have previously shown to grow well in RODW and ferment RODW sugars and phenolics (Rusanova *et al.* 2019).

MATERIALS AND METHODS

Fungal strains

Trichoderma asperellum SL-45 was obtained from the microbial collection of the Faculty of Biology of Sofia University, Bulgaria. Endophytic fungi *Phoma* sp. E30 isolated from leaves of *Salvia officinalis* and *Fusarium* sp. E119 isolated from *Valeriana officinalis* leaves are maintained in the fungal collection of Agrobioinstitute, Sofia, Bulgaria.

Sampling of rose oil distillation wastewater and preparation of RODW culture media

Rose Oil Distillation Wastewater (RODW) liquid samples were collected during the 2014 rose oil distillation campaign from cooling pool immediately after discharging waste waters from the rose oil distillers. Volumes of five liters were collected in plastic bottles and stored at -20°C till use. Portions of RODW filtered through 2 mm nylon mesh were used for preparation of RODW culture media by autoclaving at 121°C for 20 min, without pH correction and further changes of RODW parameters and composition.

Growth of fungal strains in RODW

All experiments including fungal cultivation in RODW were performed in the following manner: a small piece (approximate area of 25 mm^2) of well-developed mycelium grown on solid PDA medium was scrapped from the medium with a sterile wooden spatula and transferred to 5 mL sterile RODW medium in 30 ml polystyrene tubes and cultivated at 26°C, 190 rpm for 96 h. The obtained cultures were transferred to 100 mL sterile RODW medium in 500 mL flasks and cultivated for 188 h at 26°C, 190 rpm on a ThermoStable™ IS-20R shaking incubator (Daihan). Samples taken on the 24^{th}, 72^{nd}, and every 24h after that until the 188^{th} hour of cultivation were used to follow the dynamics of reducing sugars and phenolics content. Samples for HPTLC and HPLC/UV analyses were taken at the end of cultivation (188th hour).

Microscopic imaging

Microscopic imaging of *T. asperellum SL-45* mycelium was performed on an Axio Imager A1 microscope (Carl Zeiss GmbH) following staining of the fungal mycelium with methylene blue (Sigma). Well-developed *T. asperellum SL-45* mycelium after 140 hours of cultivation in liquid RODW media was taken from the culture with a blue pipette tip and transferred to a microscopic slide. The slide was dried using alcohol burner and the sample was covered with 0.1 % methylene blue in ethanol. After staining for 15 min at room temperature the slide was washed with distilled water, covered with a cover slip and observed under the microscope.

Phenolics extraction

Phenolics were extracted from RODW using 10 mL PD-10 columns (GE Healthcare) filled with 1 g of SepabeadsTM SP-207 resin (Mitsubishi Chemical) purchased from Sigma. Prior extraction the resin was activated by addition of 4 mL methanol, washed with ultrapure water, treated with 8 mL of 1M NaOH and washed three times with ultrapure water. Phenolics extraction was carried out by resin adsorption after addition of ten milliliters of sample to the column. The phenolics-depleted liquid was removed and the resin was washed three times with 10 mL of ultrapure water. The phenolics-enriched extract was obtained after two consecutive elutions with 2 mL methanol. The two eluates were mixed giving a RODW extract sample for further analysis.

Spectrophotometric methods

All samples subjected to analysis of phenolic and sugar content were centrifuged in 1.5 mL centrifuge tubes at 14000 rpm for 20 min at 4°C in Sigma 1-15PK centrifuge. The supernatants were transferred to a new tube and analyzed. Total phenolic content was determined by modified Folin-Ciocalteau method (Singleton *et al.,* 1999) adapted for 96x micro plate reader (Biochrom EZ Read 400). Ten microliters of sample were mixed with 790 µL distilled H_2O and 50 µL Folin-Ciocalteu′s phenol reagent (Sigma) in a 96 Nunc® DeepWellTM plate (Sigma). After brief vortexing, the plate was incubated for 5 min at room temperature, followed by the addition of 150 µL 20% Na_2CO_3. The samples were again vortexed and incubated for 2 h at room temperature. Aliquots of 200 µL of each sample were transferred to a 96x micro plate, and the absorption was measured at 650 nm. The phenolic content was calculated as gallic acid equivalents (GAE) according to a calibration curve. Reducing sugar content was determined by the DNS method (Miller, 1959) adapted for 96x micro plate reader (Biochrom EZ Read 400). One hundred microliters of sample were mixed with 100 µL of DNS reagent in a 96 Nunc® DeepWellTM plate (Sigma). After vortexing, the samples were heated at 94°C for 15 min followed by cooling on ice. One milliliter of ultrapure water was added to the cooled samples. Aliquots of 200 µL of each sample were transferred to a 96x micro plate, and the absorption was measured at 562 nm on a Biochrom EZ Read 400 plate reader. Reducing sugars content was calculated as mg/L D- glucose according to a calibration curve.

HPTLC analysis

Five microliters of RODW extract samples were applied on a glass 20 x 10 cm HPTLC silica gel 60 F254 plate (Merck) using CAMAG Automatic TLC Sampler 4 (ATS 4). The samples were applied at a constant application rate of 150 nL/sec band with a band width of 6 mm. Glass chamber (ADC 2) was saturated for 20 minutes with the mobile phase of EtOAc/formic acid/glacial acetic acid/H_2O (100/11/11/26, v/v/v/v) prior to plate development. Following the saturation step the plate was placed in the mobile phase, and ascending development was performed to a distance of 8 cm.

Detection was at UV 254 and 366 nm before staining, and at 366 nm after staining with natural product reagent (0.5% diphenylboryloxyethylamine in EtOAc; Sigma-Aldrich) followed by PEG solution (5% polyethylene glycol-4000 in dichloromethane; Sigma-Aldrich) in CAMAG TLC scanner 4. WinCATS software was used for data processing.

HPLC-UV analysis

Two milliliters of each RODW extract sample were placed in a 15 mL Falcon tube and evaporated at 40°C for 6 hours using CentriVap Benchtop Vacuum Concentrator (Labconco) for ethanol removal. The remaining water extract was frozen in liquid nitrogen and freeze-dried using Martin Christ Alpha 1-2 LD Plus freeze drier. The dry RODW extracts were dissolved in 10% aqueous DMSO containing 3 mg/mL TBHQ (Sigma) as internal standard to a final concentration of 1 mg/mL phenolics and filtered through 0.45 μm MS® nylon filter (Membrane Solutions). The HPLC analysis of the extracts was carried out on a Hitachi Elite LaChrom HPLC system consisting of a L-2130 pump and L-2420 UV-Vis detector operated by EZ Chrom Elite ver. 3.3.2 SP2 (Agilent) and using H_2O (A) and ACN (B) as mobile phases both containing 1% acetic acid. Twenty microliters of each sample were manually injected and analyzed on an Inertsil® ODS-4 (4.6 x 250 mm, 5 μm, GL Sciences) column at room temperature using the following gradient program: 0 min 10% B, 0-40 min to 46% B, 40-42 min to 100% B, 42-52 min 100% B isocratic, 52-54 to 10%B. UV absorbance was recorded at 254 nm. Compounds were identified based on co-chromatography with standard compounds (SIGMA) as well as compounds purified by preparative HPLC from RODW extract and identified in a previous study (Rusanov *et al.,* 2014).

Statistical analysis

Experimental data mean values, standard deviation as well as all charts were calculated and plotted using Microsoft Excel 2016.

RESULTS AND DISCUSSION

Cultivation of *T. asperellum SL-45* in RODW

We tested the ability of *T. asperellum SL-45* to grow in 100 mL liquid RODW media for a period of 188 hours. In parallel we also grew two isolates of endophytic fungi E119 and E30, belonging to *Fusarium* sp. and *Phoma* sp. respectively as controls. Figure 1 shows a microscopic image of *T. asperellum SL-45* mycelium in RODW media following 140 h of cultivation. The well-developed mycelium and abundance of vegetative spores indicated normal development conditions as similar picture was also observed during growing *T. asperellum SL-45* in optimized Mandel's media (Badalova, 2017).

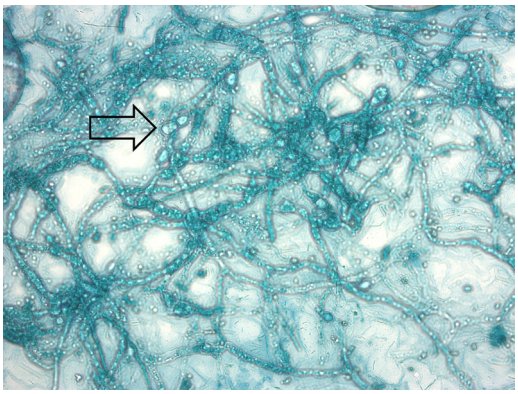

Fig. 1 Asexual sporulation of *T. asperellum SL-45* RODW liquid media. The arrow shows vegetative spores developed in *T. asperellum SL-45* mycelium

Figure 2 shows the dynamics of reducing sugar content in RODW measured every 24 h for a period of 188 h. As can be seen from **Fig. 2**, the two control endophytic isolates E30 and E119 representing *Phoma* sp. and *Fusarium* sp., respectively, showed faster assimilation of the reducing sugar content in RODW and reaching a plateau of 30% from the initial content after 72 h of cultivation. It took much longer for *T. asperellum SL-45* to reach a plateau, which happened after 144 h of cultivation, when it also reached a plateau at around 20% of the initial reducing sugar content.

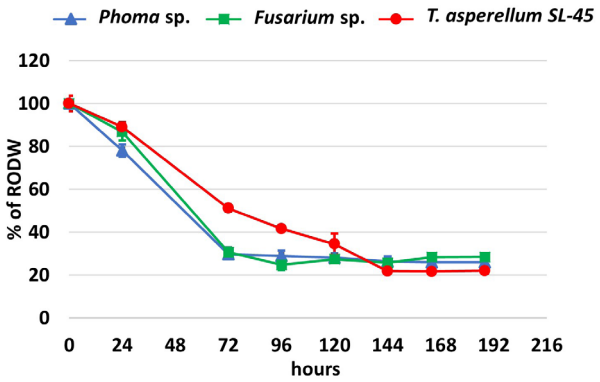

Fig. 2 Dynamics of reducing sugar content in RODW media during cultivation of *T. asperellum SL-45* and two endophytic fungi isolates E30 and E119 representing *Phoma* sp. and *Fusarium* sp., respectively

Figure 3 shows the dynamics of phenolics content during the cultivation period of 188 h. Both *Fusarium* sp. E119 and *Phoma* sp. E30 isolates showed reduction in the content of total phenolics, which was an indication for their possible assimilation and biotransformation in different compound classes. In contrast, *T. asperellum SL-45* showed almost no change in the initial concentration of total phenolics indicating that the cultivation of this strain in RODW was not related to assimilation of the RODW phenolics.

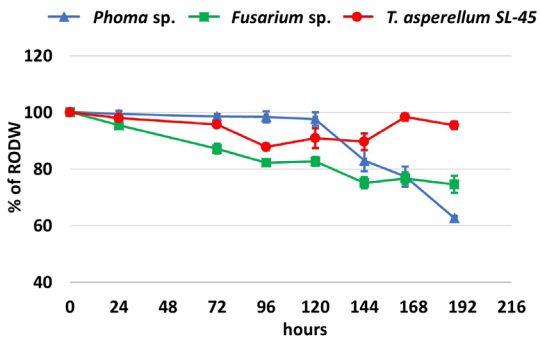

Fig. 3 Dynamics of phenolics content in RODW media during cultivation of *T. asperellum SL-45* and two endophytic fungi isolates E30 and E119 representing *Phoma* sp. and *Fusarium* sp., respectively

Characterization of the phenolics composition after cultivation of *T. asperellum SL-45* in liquid RODW media

Since the biological activity of the extract from RODW is related to its phenolics composition (Solimine *et al.,* 2016; Wedler *et al., 2016*), we analyzed the changes in the RODW phenolics extract composition following 188 h cultivation of *T. asperellum SL-45* and two control endophytic fungi isolates representing *Phoma* sp. and *Fusarium* sp. in RODW liquid media. Figure 4 shows HPTLC analysis of extracts from the culture media after cultivation with the tested microorganisms.

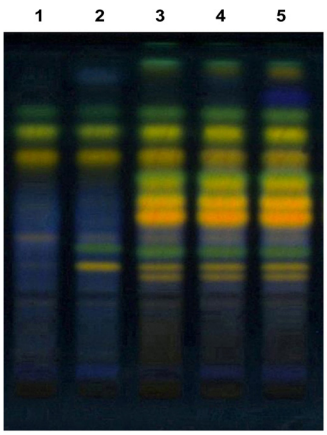

Fig. 4 HPTLC analysis of RODW media extracts obtained after cultivation of different fungi in liquid RODW media.
1-*Phoma* sp. E30, 2-*Fusarium* sp. E119,
3 and 4-*T. asperellum SL-45*,
5 - extract from RODW control media

The two phenolic extracts obtained from RODW media, following cultivation of the two control *Phoma* sp. and *Fusarium* sp. isolates (Fig. 4, lines 1 and 2), showed significant changes in the observed HPTLC bands compared to the parallel control extract without fungi cultivation (Fig. 4, line 5), indicating partial assimilation of the initial RODW phenolics content. At the same time the RODW phenolics extracts following 188 h of T. asperellum SL-45 cultivation (Fig. 4, lines 3 and 4) showed almost the same HPTLC band pattern when compared to the control extract without cultivation (Fig. 4, line 5).

We also performed HPLC/UV analysis of the RODW extracts following 188 h cultivation with *T. asperellum SL-45* and the two control endophytic fungi isolates *Phoma* sp. E30 and *Fusarium* sp. E119.

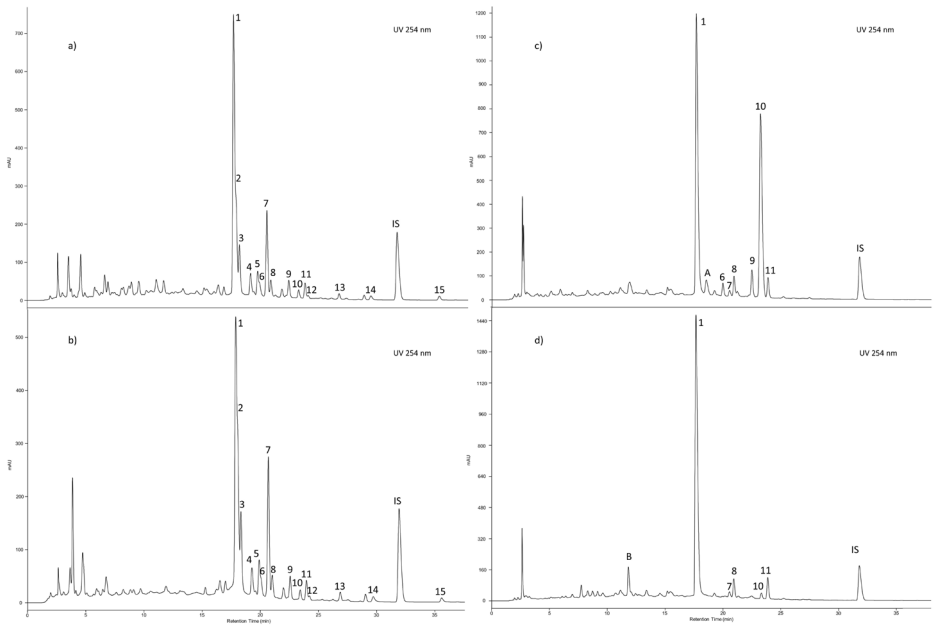

Fig. 5 HPLC/UV chromatograms of extract from RODW media.
a) Fermentation with *T. asperellum SL-45.* b) Control without fermentation, c) Fermentation with *Fusarium* sp. E119, d) Fermentation with *Phoma* sp. E30. 1- Elagic acid, 2- Hyperoside, 3- Isoquercitrin, 4- Kaempferol-3-O-rutinoside, 5- Kaempferol-3-O-galactoside, 6- Quercetin-O-methyl-disaccharide, 7- Astragalin, 8- Quercitrin, 9- Kaempferol-3-O-xyloside, 10- Multiflorin B, 11- Kaempferol-3-O-arabinoside, 12- Kaempferol-3-O- rhamnoside, 13- Multiflorin A, 14- Quercetin, 15- Kaempferol, IS- internal standard, A and B - newly synthesized compounds

As can be seen from Fig. 5, the cultivation of T. asperellum SL-45 in RODW liquid media resulted in almost no changes in the composition of RODW phenolics following 188 h of cultivation (Fig. 5 a) when compared to the RODW without fungal fermentation (Fig. 5 b). At the same time the cultivation of the control Fusarium sp. isolate resulted in significant changes in the RODW phenolics composition including both changes of the relative composition of the initial phenolic compounds present in RODW and synthesis of new compounds as a result of biotransformation (Fig. 5 c, d). The results of RODW fermentation by *T. asperellum SL-45* suggest that it can be efficiently used for fungal biomass production in parallel to the RODW phenolics extraction. The lack of significant changes of RODW phenolics composition suggests that *T. asperellum SL-45* fermentation can be included as intermediate step prior to the final RODW phenolics extraction. On the other hand, the applied RODW phenolics extraction using phenolics adsorption / desorption on microporous resin (Rusanov *et al.,* 2014) results in less than 20% reduction of RODW sugar content (unpublished data). This makes possible the fungal biomass production step via *T. asperellum SL-45* fermentation to be also applied as final step, following RODW phenolics extraction. Our experience on successful scale up of the RODW phenolics extraction to semi-industrial volumes suggests that both scenarios for introduction of *T. asperellum SL-45* fermentation step are feasible and worth testing at semi-industrial scale of RODW volumes in a follow-up study.

CONCLUSIONS

The results from our study showed that *T. asperellum SL-45* can be successfully cultivated in RODW liquid media without the need for correcting the RODW parameters and composition. The *T. asperellum SL-45* fermentation does not significantly change the composition of the biologically active RODW phenolics composition. The results of the study suggested that *T. asperellum SL-45* fermentation of RODW could be efficiently applied in parallel to the RODW phenolics extraction for low cost production of *T. asperellum SL-45* fungal biomass.

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DECLARATION OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHORSHIP STATEMENT

M.R., K.R., S.M., T.Z., Y.E., D.N. and I.A. contributed equally to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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