THE OBTAINING OF RESVERATROL FROM AGRICULTURAL SECONDARY FLOW RECOVERY PRODUCTS BY MICROBIAL PECTINOLYTIC EXTRACT TREATMENT

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The present paper deals with the conversion of grape pomace into resveratrol using supernatant of bacterial culture which showed the capacity to synthesize extracellular pectinase. Six microbial strains, noted G, H, T, Mr, My and Mw, were tested in this study and four of them were identified based on the 16S rRNA gene sequences as *Pseudomonas endophytica* (two strains), *Arthrobacter luteolus*, and *Sphingobacterium sp*. After the treatment of grape pomace with bacterial culture, as a general rule, higher amounts of resveratrol, varying from 0.9 to 6.1 mg/L, have been obtained from the supernatant. The highest amount (6.1 mg/L) was recorded for the bacterial strain noted My and identified as *Arthrobacter luteolus*. From the sediment samples, the highest amounts were obtained after two hours of treatment. The highest amount, namely 2.5 mg/L, was observed from the sediment of the G noted strain, identified as *Pseudomonas endophytica*. The accession numbers in DDBJ for the investigated strains are as follow: LC476728, LC476729, LC476730 and LC477070.

Keywords: biodegradation, grape pomace, resveratrol.

INTRODUCTION

Grapes represent one of most important fruit crops and are generally used for wines and other beverage production but also for foods. Following their industrial processing, mainly in the wine industry, the huge quantities of resulting grape pomace could generate serious environmental storage problems (Oliveira & Duarte, 2016; Zacharof, 2017). However, this waste contains several valuable compounds such as polyphenols, an important antioxidant source (Devesa-Rey *et al*., 2011).

A lot of researches were conducted to obtain value-added products from the grape pomace, in order to be used as animal food (Zacharof, 2017), food additives (Kafantaris *et al*., 2018), fertilizers (Ferrer *et al*., 2001), grape oil (Baydar *et al*., 2007) or anthocyanins (Bridle & Timberlake, 1997). Some of the researches had as target

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to obtain only one final product, but recent papers showed that using a cascade approach, the grape pomace could be converted simultaneously in more than one product (Jin *et al*., 2018).

Resveratrol (3,5,4'-trihydroxystilbene) is one of best-known plant metabolites due to its antioxidant properties, also anticancer activity, beneficial effects for human health and other roles for the environment (Colica *et al*., 2018). The interest about resveratrol from grapes was a consequence of some epidemiological studies indicating a relationship between wine consumption and hearth diseases (Colica *et al*., 2018; Das & Das, 2010).

On the other hand, this compound could be used as a unique carbon source for the growth of some bacterial species like *Acinetobacter* sp., which can act in rhizosphere for their degradation (Kurt *et al*., 2018).

However, some recent studies revealed that lactic acid bacteria can use the by-product from wine processing industry to enhance storage stability of certain foods and could help address the environmental impact of the storage of grape pomace and increase the diversity of probiotic food (Dias *et al*., 2018).

A lot of methods for extraction of resveratrol were optimized and the influence of temperature and extraction flow rate are well documented (Guerrero *et al*., 2008; Wu *et al*., 2019), but there is not knowledge about the use of bacterial cultures containing pectinase for this purpose. In this regard, the present paper shows the results obtained by conversion of grape pomace in resveratrol by using bacterial cultures. The bacterial strains have been isolated from the grape pomace and tested for their pectinase activity which appears to be involved in the conversion of waste into resveratrol.

MATERIALS AND METHODS

Bacterial cultures were represented by wild bacterial strains isolated from two samples of grape pomace supplied by the company Salmed Farma, Medias and obtained from the processing of grapes during wine production. The strains isolation was performed in two steps on nutrient agar. Initially, from grape pomace samples there were performed serial dilutions that were inoculated in melted nutrient agar medium and after solidification, the Petri dishes were incubated at 30 °C for 48 hours. Then, some strains were randomly selected and grown on modified nutrient agar with the following composition: a) 5 g/L peptone and 5 g/L carboxy-methyl-cellulose (for testing cellulase activity) and b) 5 g/L peptone and 10 g/L pectin (for testing pectinase activity). The plates were incubated for 72 hours at 30 °C and the enzymatic activities were evaluated by the methods described by Farkas *et al*., 1985 and Rohban *et al*., 2009.

The molecular identification of the selected strains was performed by 16S rDNA amplification and sequencing. To this purpose, the strains were grown on nutrient broth for 72 hours, centrifuged at 9500 rpm for 10 minutes and the

genomic DNA was extracted from the obtained biomass using the Qiagen kit following the manufacturer's protocol. The PCR amplification was performed using the following primers: 27F (AGAGTTTGATCACTGGCTCAG) and 1492R (ACGGCTTACCTTGTTACGACTT) in an Eppendorf Mastercycler pro S with the following programme: 2 min denaturation at 95 °C, followed by 35 cycles of 30s denaturation at 95 °C, 30s annealing of primers at 54 °C, 90s extension at 72 °C, and a final extension step of 5 min at 72 °C. The sequencing was performed by the commercial company CeMIA (Larissa, Greece) and the nucleotide sequences obtained were compared against the NCBI database using BLASTN (https://blast.ncbi.nlm.nih.gov).

Resveratrol obtaining by treatment of grape pomace: the microbial strains having extracellular pectinolytic activity were grown in 20 mL of nutrient broth for 48 hours at 30 °C and then centrifuged at 9500 rpm for 10 minutes. The resulted supernatant was used in the next step. A quantity of 0.05 g of red grape pomace was placed in Eppendorf tubes and mixed with one mL of the above supernatant for two, four and 24 hours. At the end of incubation intervals, the tubes were centrifuged for 10 minutes at 9500 rpm and the resulted supernatants and sediments were used for the determination of the resveratrol amount.

Resveratrol determination was performed by HPLC. For the sediment samples, 0.1 g were mixed with 10 mL methanol HPLC reagent grade and boiled at 55 °C for two hours and then filtered using a 0.45 μm diameter filter. For the supernatant samples, the filtration step was applied. The stationary phase of HPLC was represented by the Phenomenex Nucleosil C18 column, 100Å, 25 cm x 3.2 cm, 5μm and Gemini C18 4X2 cartridge. The mobile phase was represented by a mixture of distilled water and acetonitrile (50 v/v). The parameters of the systems were: 5 μl injection volume, 0.7 mL/min elution debit and 300 nm wavelength. For the calibration curve, resveratrol solutions with concentrations between 0–150 mg/L were used. The amount of resveratrol has been calculated using chromatogram area and the etalon curve.

RESULTS AND DISCUSSIONS

Isolation of bacterial strains and detection of cellulolytic and pectinolytic activities

After the incubation of the samples, the colony-forming units/mL was determined by counting the bacterial colonies. The results presented in Table 1 revealed a relatively low number of microorganisms in sample 1 compared to sample 2. In the last case, the colony forming units could be quantified only after the fourth dilution of the sample. This data could be argued by the preservation status of the samples and by their manipulation during the industrial processing steps.

Table 1

The colony forming units in the investigated grape pomace samples;
TNTC = too numerous to count; numbers = colony forming units/mL

Dilutions / Samples	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
1	85	1	1	0	0
2	TNTC	TNTC	TNTC	520	0

Approximately 10% of the microbial strains were randomly selected and screened for pectinolytic and cellulolytic activities. Six of them showed positive results for pectinase and/or cellulase, but only five bacterial strains (noted G, H, Mr, My and Mw) were selected for further investigation considering that the sixth (noted T) proved to be a fungal strain. Based on Gram staining, all the investigated strains were represented by Gram-negative rods except the My strain that has shown to be Gram-positive rods. Furthermore, the oxidase and catalase tests have demonstrated that all the investigated strains are positive.

Regarding the extracellular enzymatic activities, the data summarized in Table 2 revealed that, for the selected strains, the pectinase activity prevailed and the cellulase activity was only recorded for a low number of strains. Should be noted that most of the strains synthesized only pectinase, the cellulolytic activity being recorded only for the strains which produce pectinase. The diameter of the hydrolysis zone varied from 1.7 cm to 3.5 cm for pectinase and 1.6 cm in case of cellulase (Table 2; Fig. 1).

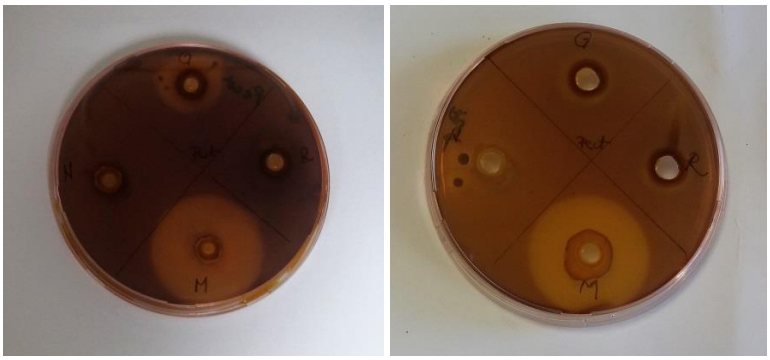
Table 2

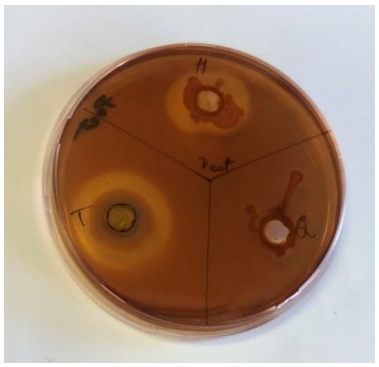
The presence of pectinase and cellulase activities in some selected bacterial strains.
The diameter of hydrolysis zone is in cm and includes the well diameter (0.7 cm)

Strains	Pectinase (diameter of hydrolysis zone)	Cellulase (diameter of hydrolysis zone)
H	2.4	-
T	3	-
G	1.7–2	-
Mw	3.5	1.6
My	weakly positive	-
Mr	weakly positive	-

Regarding the molecular identification of the pectinolytic selected strains, the analysis revealed that the isolates noted Mw, My, Mr and G show strong sequence homology to *Pseudomonas endophytica* (Mw and G), *Arthrobacter luteolus* (My) and *Sphingobacterium* sp. (Mr). The accession numbers in DDBJ are as follow: LC476728, LC476729, LC476730 and LC477070.

(a)





(b)

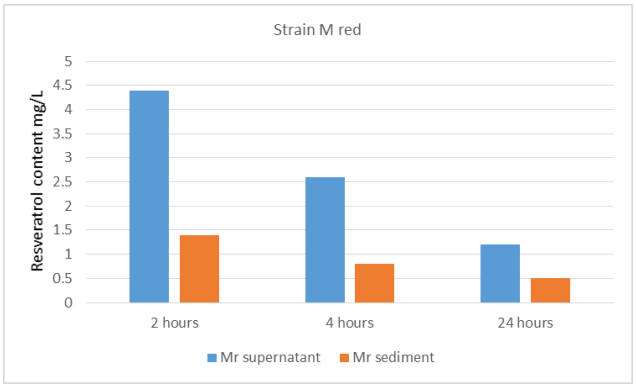
Fig 1. The presence of cellulolytic activity (a) for the strain noted Mw and pectinolytic activity for the strains noted Mw, H, T, G (b).

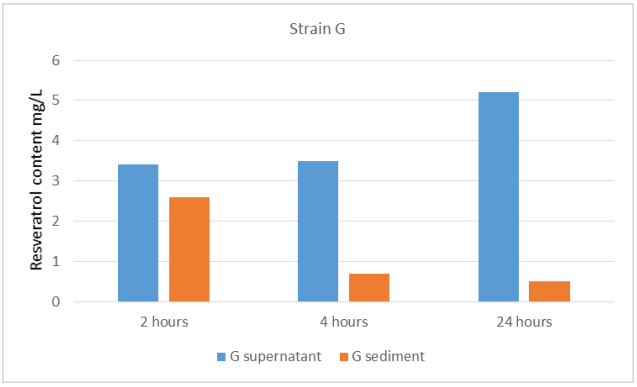
Resveratrol determination

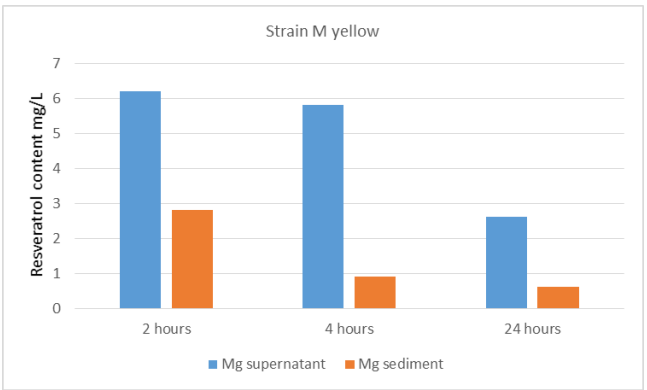
Results presented in Figure 2 show that, as a general rule, the highest amounts of resveratrol, varying from 0.9 until to 6.1 mg/L, have been obtained from the supernatant. Amounts varying from 0.4 until to 2.5 mg/L were obtained from the sediment. Excepting strain G, highest resveratrol amounts from supernatant were obtained after two or four hours of grape pomace treatment. After 24 hours of treatment, the yields significantly decreased. The highest amount, 6.1 mg/L (the

chromatogram in Figure 3), was recorded in the case of strain My, identified as *Arthrobacter luteolus*.

Generally, from the sediment samples, high amounts were obtained after two hours of treatment and the yield decreased afterword, for all tested strains. The highest amount, namely 2.5 mg/L, was obtained from the sediment of strain G, identified as *Pseudomonas endophytica*.







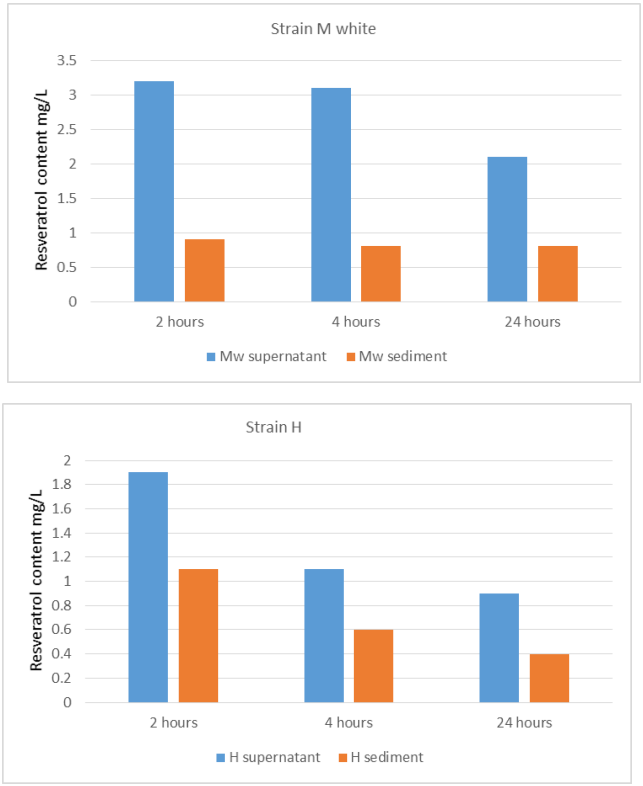
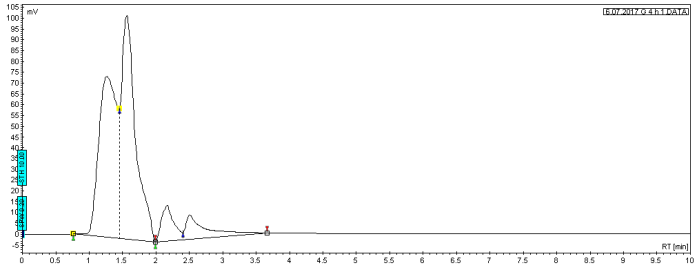
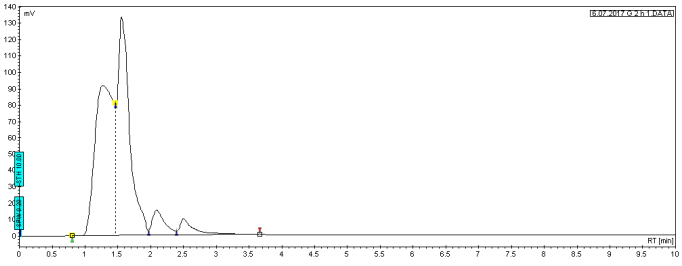


Fig. 2. The amount of resveratrol obtained after bacterial pectinase treatment of grape pomace.



(a)



(b)

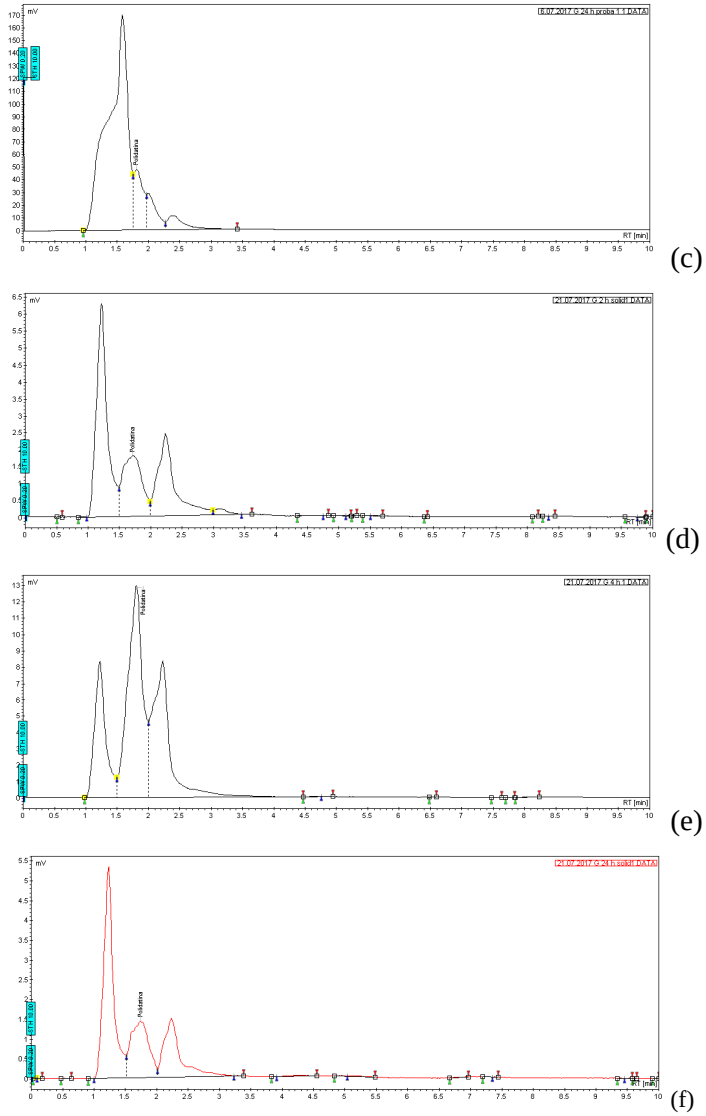


Fig. 3. The HPLC chromatograms for strain G, *Pseudomonas endophytica*: a – c, supernatant after 2, 4 and 24 hours of treatment and d – f sediment after 2, 4 and 24 hours of treatment.

CONCLUSIONS

The results revealed that all pectinolytic tested strains have the capacity to degrade grape pomace with the obtaining of different resveratrol yields for each particular strain. The highest amounts of resveratrol were generally obtained from

the supernatant. The data revealed that, using pectinolytic bacterial strains, the waste resulted from grape processing in various food and beverage industries could be environmentally friendly transformed into value added products.

Future investigations will be conducted for the characterization of the bacterial strains by polyphasic taxonomy approach and also for the description of the optimal parameters of the biotechnological process for conversion of grape pomace into value-added products (including resveratrol) using the bacterial cultures tested in the present study.

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