Research Article

Received: 19 November 2022 Revised: 19 January 2023 Accepted article published: 23 January 2023 Published online in Wiley Online Library: 2 February 2023

(wileyonlinelibrary.com) DOI 10.1002/jctb.7326

Biodegradation of phenolic compounds from grape pomace of *Vitis vinifera* Asyrtiko by *Chlamydomonas reinhardtii*

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Abstract

BACKGROUND: Grape pomace from *Vitis vinifera* Asssyrtiko is an important sub-product of the Greek wine industry. However, its accumulation is a serious problem with a negative environmental impact. Grape pomace is rich in bioactive compounds and its utilization for alternative uses, such as a fertilizer, is an interesting area of research. On the other hand, its high concentration of phenolic compounds inhibits germination processes. Therefore, there is a need to decrease the high phenolic level in grape pomace before it can be used as a fertilizer. The main objective of our study is the fast reduction of polyphenols in grape pomace, and more specifically catechin and epicatechin in *Vitis vinifera* Assyrtiko. For this purpose, *Chlamydomonas reinhardii* was used for polyphenol biodegradation in grape pomace extract. It is shown that the bioremediation proceeds very fast, while the final product has a high potential to be used as a soil conditioner.

RESULTS: The results of this study identify that after 6 days of cultivation, *C. reinhardtii* was able to reduce the total polyphenolic amount by 43%, while catechin and epicatechin were decreased by 100%. In addition, it is shown that the final aqueous product is rich in minerals, such as sodium, phosphorus and potassium.

CONCLUSIONS: Through this work we were able to develop a method that allows for the efficient decrease of polyphenols in grape pomace. *C. reinhardtii* biodegrades a sufficient amount of polyphenols in grape pomace with a high rate, while the product obtained 6 days after cultivation contains high concentrations of minerals and low levels of polyphenols. These results suggest that there is a great potential in using the *Vitis vinifera* Asssyrtiko biodegraded product as a soil conditioner.

Keywords: *Vitis vinifera*; grape pomace; *Chlamydomonas reinhardtii*; biodegradation; soil improver

INTRODUCTION

Agro-industrial waste or byproducts are generated during agricultural production, industrial manufacturing, processing and distribution. Many of such wastes contain significant amounts of essential bioactive compounds and can thus be utilized further. Many developed countries are putting their efforts into turning wastes into useful resources. Grape (*Vitis vinifera*) is one of the most significant fruit crops. In 2018–2019, worldwide grape production was 22.15 million metric tons.[1] Approximately 75% of the produced grapes is intended for wine production, out of which 20–30% represents waste products.[2] Because of the increasing consumption of wine in recent years, the increase of grape pomace production has recently drawn attention. Grape pomace is a biodegradable solid byproduct of the winemaking process, obtained after mechanical pressing or fermentation, encompassing peels (skin), seeds and some parts of the stem.[3] It is estimated that the worldwide production of grape pomace ranges between 10 and 13 million tons annually.[4] Its chemical composition consists approximately of water (55–75%), polysaccharides (30%), proteins (6–15%), lipids, sugars and unsaturated fatty acids.[5] Also, according to the literature, grape pomace is a substrate rich in polyphenols such as phenolic acid, anthocyanidin, proanthocyanidin, catechin and other flavonoids, with catechin and epicatechin being the most abundant.[6-9] The main disadvantage of grape pomace is that a considerable amount of it is disposed in landfill and thus causing negative environmental effects.[10] Grape pomace disposal into soils causes a series of considerable issues for the environment, including phytotoxic and antimicrobial effect, due to the existence of antimicrobial substances, such as tannins and polyphenols as well as low pH.[10-12] Furthermore, the toxicity of phenolic compounds stems from their hydrophobic character as well as their ability to form free radicals.[13] The management and valorization of grape

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pomace have the potential for decreasing the ecological impact by reducing the presence of the high concentrations of phenolic compounds.[14,15] In addition, it is rich in organic matter and inorganic nutrients which can be used to improve soil fertility and plant growth.[11,16]

Many microorganisms, including bacteria and fungi, are capable of biodegrading a wide variety of organic compounds under solid-state fermentation.[17] The catalytic capabilities of these microorganisms rely on their capacity to biodegrade phenolic compounds,[18] by utilizing photosynthesis as a method for simultaneous oxygen production.[19] This biodegradation might be used to treat grape pomace and convert it into a byproduct capable of improving soil conditions. The biodegradation of polyphenols by bacteria and fungi has been extensively studied, but only recently has there been more interest in investigating the capabilities of some algae for phenol biodegradation.[20-22] *Chlamydomonas reinhardtii* is a single-cell green microalga and it is a well-studied model microorganism that was recently shown to be able to biodegrade phenol.[23,24] It was shown that *Chlamydomonas* cells biodegrade phenol since they can use it as carbon source for the maintenance of homeostasis.[23] In addition, high concentration of phenol was found to induce higher levels of biodegradation as a response to stress, while at the same time the presence of an alternative organic carbon source (acetic acid) had a prominent role in the alleviation of stress effects.[24]

The aim of the study reported here was to evaluate the ability of *C. reinhardtii* to biodegrade *Vitis vinifera* Assyrtiko grape pomace via aerobic processes. Initially, the ability of the microorganism *C. reinhardtii* to decrease the phytotoxicity of grape pomace by reducing its polyphenolic compounds was investigated. In particular, the decrease of total polyphenolic compounds as well as the decrease of catechin and epicatechin were studied since these phenols are the most abundant in *Vitis vinifera* Assyrtiko and they are phytotoxic and inhibit seed germination.[9] Minerals such as sodium, potassium and calcium were quantified after the biodegradation of grape pomace.

EXPERIMENTAL

Grape pomace pretreatment

Grape pomace from *Vitis vinifera* Assyrtiko was collected directly from a basket press available at Diamantakis Winery, an enterprise located in the southwest part of Heraklion city, outside Kato Assites village (Crete Island, Greece; latitude 35°12′43 and longitude 24°59′33). The fresh material was collected during the harvest of 2020 (in September) and was stored at −20 °C until use.

Grape pomace extraction

Preliminary experiments indicated that the quality and quantity of extracted polyphenols were the same when organic solvent and water extractions were compared (data not shown). Water extraction is an eco-friendly method that is increasingly used as an alternative to traditional extraction techniques.[25] As a result, the extraction of polyphenolic compounds was carried out using deionized water as a solvent. In particular, 15 g of grape pomace was added into 100 mL of deionized water. The mixture was autoclaved at 120 °C for 20 min.

C. reinhartdtii growth conditions

Mother cultures of *C. reinhartdtii* (CC-125, wild type) were grown photoheterotrophically in Tris-acetate-phosphate (TAP) medium[26] at 25 °C in 1000 mL Erlenmeyer flasks, shaken at a rate of 140 min^{-1} under a light intensity of 70–80 μmol photons m^{-2} s^{-1}.[23] Cell growth was determined, every 24 h, by measuring the packed cell volume (PCV; μL mL^{-1}) of each mother culture ($n = 5$).[24] The PCV of a cell suspension was determined by centrifugation at 1000 × *g* for 5 min using hematocrit TPP tubes.[27] All nutrient media and glassware had been previously autoclaved at 120 °C for 20 min to avoid contamination. Culture preparation and sampling were carried out in a laminar flow hood sterilized with ethanol and a UV lamp using a sterile pipette tip.

Biodegradation mixture

C. reinhardtii was collected by centrifugation at 2500 × *g* for 5 min as soon as the mother cultures reached the end of stationary phase. Each cell pellet was resuspended into fresh TAP medium. Then *C. reinhardtii* (10 μL mL^{-1}) was incubated in 500 mL Erlenmeyer flasks containing 100 mL of grape pomace extract. Erlenmeyer flasks were shaken at a rate of 140 min^{-1} under a light intensity of 70–80 μmol photons m^{-2} s^{-1} (28 °C). Three replicates were analyzed, while each resulting solution was measured three times. The cell growth, the amount of catechin and epicatechin as well as the amount of total polyphenolic compounds after biodegradation were measured at different time points (0, 8, 24, 48, 72, 96, 120 and 144 h).

Determination of total polyphenolic compounds

The total polyphenols content (mg L^{-1}) was determined spectrophotometrically using the Folin–Ciocalteou (FC) method, according to Ainsworth and Gillespie,[28] and expressed as gallic acid equivalent (GAE). In more detail, 2 μL of sample was pipetted into a well of a 96-well microplate containing 158 μL of water, and 10 μL of FC reagent was added. After 5 min, 30 μL of Na_2CO_3 solution was added. During oxidation with FC reagent phenolic compounds are decreased to blue-colored molybdenum and tungsten oxides. After incubation for 30 min at 40 °C, the absorbance of the solution was measured at $\lambda = 765$ nm (Multiscan Sky, ThermoScientific) against a blank sample consisting of 160 μL of deionized water. The measurements were compared to a standard curve of prepared gallic acid solutions (0–500 mg L^{-1}) and expressed as milligrams of GAE per gram of grape pomace extract (before and after biodegradation). All measurements were performed in triplicate.

Determination of catechin and epicatechin

The qualification and quantification of catechin and epicatechin were performed using a high-performance liquid chromatography system (Agilent 1260 Infinity II) with a quaternary pump, diode array detector and autosampler. Separation was carried out on a C-18 column (GraceSmart; length: 250 mm; inner diameter: 4.6 mm). The gradient elution was accomplished using water (solvent A) and acetonitrile (solvent B) as presented in Table 1. The parameters of the chromatographic process were a flow rate of 0.8 mL min^{-1}, an injection volume of 10 μL and an analysis time of 23.00 min. Quantification of phenolic compounds was performed using internal standards (catechin hydrate and epicatechin; Sigma-Aldrich) and the absorbance was measured at 280 nm.

Determination of minerals

At the end of the cultivation, the culture was centrifuged and the biomass was removed. In the supernatant, sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), copper (Cu), manganese (Mn), iron (Fe) and zinc (Zn) were measured using atomic

Table 1. Gradient of elution solvents

No.	Time (min)	Solvent A	Solvent B
1	0.00	90.0	10.0
2	10.00	85.0	15.0
3	12.00	80.0	20.0
4	14.00	60.0	40.0
5	16.00	40.0	60.0
6	18.00	5.0	95.0
7	20.00	40.0	60.0
8	22.00	75.0	25.0
9	23.00	90.0	10.0

absorption spectroscopy.[29] Additionally, phosphorous (P) was determined colorimetrically according to Kitson and Mellon[30] with minor modifications. Specifically, 10 mL of sample was placed into a 50 mL volumetric flask and 10 mL of ammonium vanadomolybdate was added. The solution was incubated at room temperature for 1 h in order for the reaction to be completed and the color to be developed. After dilution to volume with water and mixing, the color intensity was measured in a photometer (DR 2800 spectrophotometer, Hach) at 470 nm. Meanwhile, blank was prepared by adding 10 mL of ammonium vanadomolybdate in a 50 mL volumetric flask containing 10 mL of deionized water. The solution was diluted to volume accordingly. The quantification of phosphorus took place by comparing to a standard curve of prepared phosphorus solutions (0.002–0.02%) and was expressed as grams of phosphorus per 100 mL of grape pomace extract (before and after cultivation). All measurements were conducted in triplicate.

Statistical analysis

For the statistical analysis of experiments results, the reported mean and standard deviation (SD) were calculated using IBM SPSS Statistics 24 software.

RESULTS AND DISCUSSION

Effect of grape pomace on *C. reinhardtii* cell growth

Grape pomace extract (100 mL) was combined with the *C. reinhardtii* mother culture (100 mL) when it reached the end of stationary phase (total cell concentration: 10 μL mL^{-1}). The beginning of the cultivation started at the zero time point and the cell concentration was measured at different time points (0, 8, 24, 48, 72, 96, 120 and 144 h of cultivation) in order to study the growth of *C. reinhardii* in the presence of grape pomace extract. It was observed that cells increased linearly (R^2 = 0.99; Fig. 1) and their maximum concentration (10.2 ± 0.2 μL mL^{-1}) was reached 144 h after the beginning of cultivation. This finding is directly in line with the growth behavior of *C. reinhardii* in the regular medium (TAP), where it also reached its maximum concentration of 10 μL mL^{-1} at its stationary phase. Moreover, results from Fig. 1 demonstrate that *C. rheinhardii* was able to grow in an environment containing grape pomace extract from *Vitis vinifera* Assyrtiko in a final concentration of 15% (w/v).

Biodegradation of polyphenols

Initially, the concentration of total polyphenolic compounds was measured at various cultivation time points. According to the results, the microalga of choice managed to decrease the total polyphenolic concentration by 43% (Fig. 2).

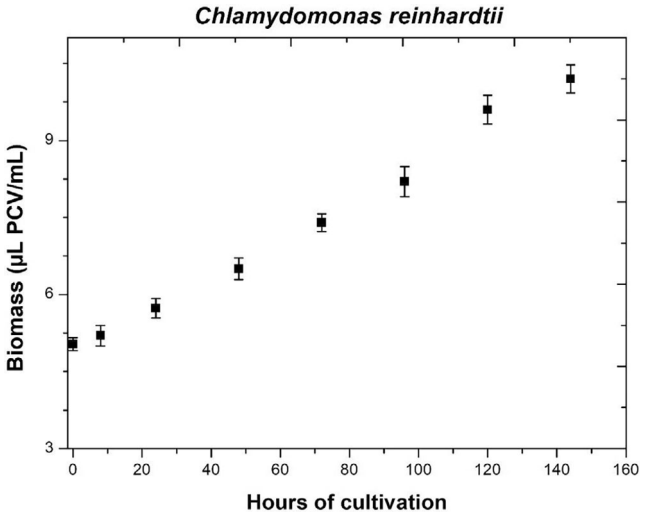

Figure 1. Concentration of *C. reinhardtii* during biodegradation at different time points.

Catechin and epicatechin are the most abundant polyphenols in *V. vinifera* Assyrtiko grape pomace (Fig. 3). As mentioned before, they are phytotoxic and inhibit seed germination.[9] Figure 3 demonstrates the chromatograms of grape pomace extract at different points of cultivation (0, 75 and 144 h), which also suggests the decrease of catechin and epicatechin. Furthermore, Fig. 4 reveals that both polyphenols decreased with increasing time. The total decrease of catechin and epicatechin was 100%. These findings are in accordance with results reported by Nazos *et al.* about phenol biodegradation by *C. reinhardii*.[23] According to previous works, catechins, mainly those extracted from tea, exhibit antimicrobial properties against some pathogenic bacteria such as *Serratia marcescens*, *Escherichia coli*, *Proteus mirabilis*, *Bacillus cereus*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Candida albicans*.[6,31] However, this is not the case in our study, as *C. reinhardii* resists both catechin and epicatechin and reaches a concentration of 10 μL mL^{-1} at its stationary phase (Fig. 1). This is an important finding in the understanding of polyphenol behavior in microorganism growth, since research into the

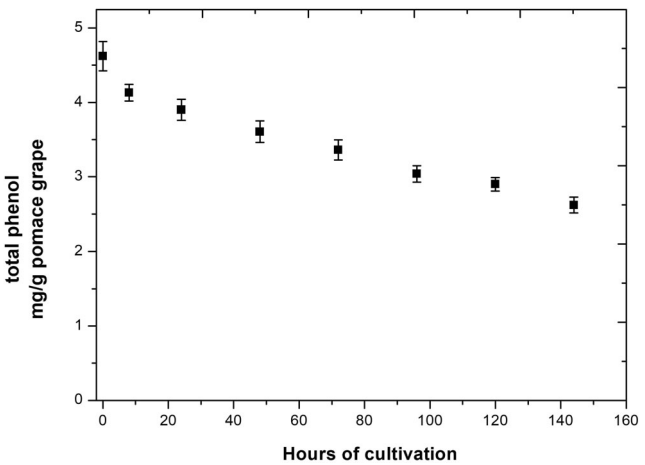

Figure 2. Total polyphenolic compounds of *V. vinifera* Assyrtiko grape pomace extract at various cultivation time points.

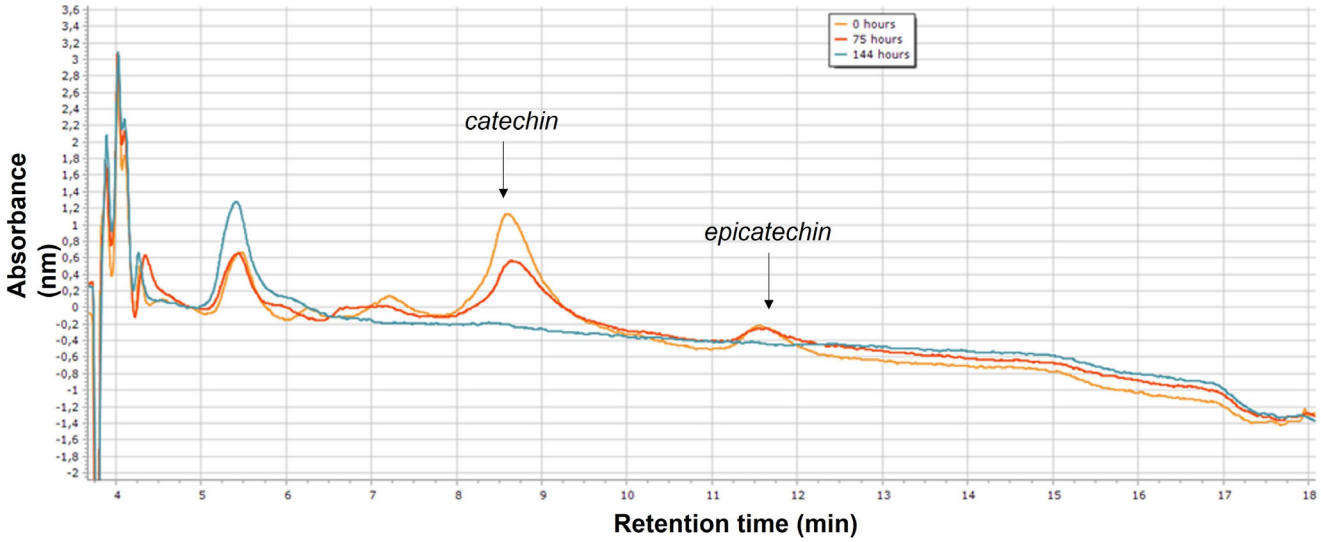

Figure 3. Chromatograms of grape pomace extract at different point of cultivation (0, 75 and 144 h).

interaction between polyphenolic compounds and microorganisms has been focused only on their antimicrobial properties against pathogenic bacteria,[6,32,33] while just a few studies[31] have investigated the positive effect of polyphenols in microorganism growth.[34] Researchers have shown that green alga *Schenedesmus obliquus* biodegrades phenolic compounds.[35,36] Also, they have found that the selection of appropriate conditions was the key for increasing biodegradation. The strategy of microalgae is to invest in energy growth instead of biodegradation. Specifically, higher toxicity levels lead to lower growth and higher biodegradation, whereas low toxicity levels lead to higher growth and lower biodegradation.[37] Moreover, Nazos *et al*. observed biodegradation in *C. reinhardtii* only in limited carbon cultures.[24] We observed similar behavior in our study; catechine and epicatechine were used by the microalga as an alternative carbon source.

Mineral determination

Six days after cultivation, when *C. reinhardii* reached its stationary phase, removal of the biomass by centrifugation took place and the aqueous supernatant was analyzed further, in order to evaluate its potential for being used as a soil improver. According to the results (Table 2), the final product obtained after biodegradation is rich in minerals, a fact that makes it a good candidate for being used in fields as a soil improver. According to Regulation 2019/1009 of the European Parliament and of the Council,[38] soil improvers constitute promising raw materials for the production of innovative fertilizing products in a circular economy. More specifically, copper, manganese, iron and zinc were present at higher concentrations, while the concentrations of calcium and manganese were very low in the grape pomace extract after the cultivation of *C. reinhardii*. Soil improvers shall be defined as an EU fertilizing product the function of which is to maintain, improve or protect the physical or chemical properties, the structure or the biological activity of the soil to which it is added.[38] Furthermore, soil improvers can be divided in two different categories: organic and inorganic. In the case of our study, the final product of bioremediation can be characterized as an inorganic soil improver as the only carbon source in the solution was polyphenols, which were consumed by *C. reinhardii* during the biodegradation procedure. Based on Regulation 2019/1009 of the European Parliament and of the Council, the requirements of an inorganic soil improver refer to copper and zinc concentration. Specifically, Cu content must not exceed 300 mg kg^{-1} dry matter

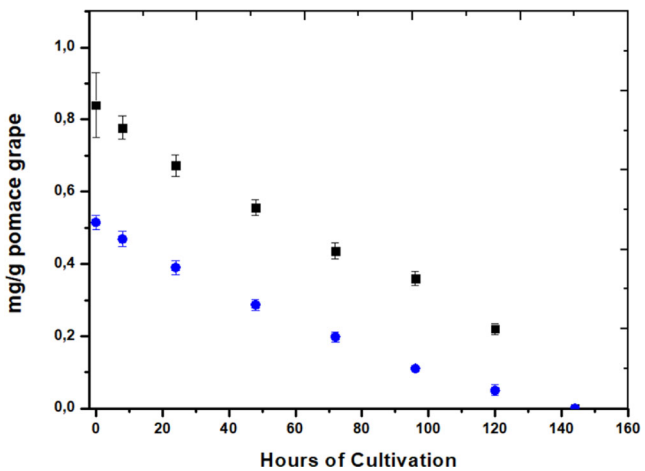

Figure 4. Catechin (■) and epicatechin (●) of *V. vinifera* Assyrtiko grape pomace extract at different cultivation time points.

Table 2. Composition of minerals (mg $(100\ mL)^{-1}$) in *V. Vinifera* Assyrtiko grape pomace extract after cultivation

Mineral	Measurement (mean ± SD)
Phosphorus (P)	11.4 ± 0.1
Potassium (K)	5.1 ± 0.3
Calcium (Ca)	0.3 ± 0.0
Magnesium (Mg)	0.1 ± 0.0
Copper (Cu)	1.1 ± 0.2
Manganese (Mn)	8.8 ± 0.2
Iron (Fe)	10.0 ± 0.2
Zinc (Zn)	26.3 ± 0.1
Sodium (Na)	1.0 ± 0.1

and Zn content must not exceed 800 mg kg^{-1} dry matter. According to Table 1, the mineral analysis of the final product meets the expectations. In any case, more studies should be conducted in order to make a safe conclusion about the ability of the final product to act as a soil improver.

However, more studies are required in order to investigate how this biodegradation affects the total nitrogen amount as well as the BOD and COD content of the product.

SUMMARY

Our data indicate that *C. reinhardii* is a microorganism capable of biodegrading polyphenols in *Vitis vinifera* Assyrtiko grape pomace extract, making this winemaking waste environmentally friendly when it is disposed on fields. In addition, the findings of this study demonstrate the potential of the biodegraded product to be used as a soil improver. These products can have a great positive impact on several crops, considering the necessity of improving soil quality. This soil improver is supported by the latest principles of the circular economy of wastes applied in the European Union.[39]

ACKNOWLEDGEMENTS

This research has been co-financed by the European Regional Development Fund of the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurships and Innovation, under the call RESEARCH-CREATE-INNOVATE (project code T2EDK-00523).

The authors would like to acknowledge PhD candidate Napoleon Stratigakis for his excellent assistance.

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