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Response of *Oenococcus oeni* PsuI to Extracted Phenolic Pigments from Apple Peels

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Apple peels contain various pigments responsible for the red color. Among which are the phenolic pigments including anthocyanin, flavonols, and proantho cyanidins. Phenolics are important components of cider and potentially affect the metabolism of *Oenococcus oeni*. The capability of *O. oeni* to interact with phenolic pigments extracted from apple peels was investigated. Phenolic pigments were extracted from apple peels using acidified methanol, then mixed with cultures of *O. oeni*. By using reversed phase C18 HPLC, several peaks at 318 nm corresponding to flavonols were detected in cell-free supernatants at retention times between 2 to 35 min. As a result of bacterial treatment, one of the flavonols peak at retention time of 14.06 was reduced in concentration from 130 μg/μl to 111 μg/μl, indicating a considerable bacterial removal of the pigment from the extract. This observation was confirmed by Transmission electron microscopy which showed that *O oeni* is capable of pigment absorption, thereby affecting the color of apple peels pigment extract. The growth of *O. oeni* PsuI in the presence of phenolic pigments was investigated and it was found that their presence enhanced the bacterial growth by 1.68 fold increase.

Key words: *O. oeni* PsuI , Phenolic pigments, HPLC, TEM, Flavonols.

Apple cider contains various phenolic compounds including phenolic pigments [1.] Phenolic compounds are ubiquitous in plant foodstuffs and therefore they are a significant component of the human diet. In addition, they influence sensorial food properties such as flavor, astringency, and colors. They are classified according to their structure as phenolic acid derivatides, flavonoids, and tannins[2].

Apple skin color is caused by the pigments chlorophyll and carotenoids located in plastids and by the phenolic pigments (anthocyanin, flavonols and proanthocyanidins) located in the vacuole[3]. Apples contain many types of phenolic derivatives and flavonoids (flavan-3-ols, flavonols, procyanidins, chalcones, and anthocyanins)[4,5,6].

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Apples are fruits consumed worldwide in different forms, *i.e.* cooked, fresh, in juices and in cider. Their beneficial properties to human health are related to the high content of phenolic compounds[7].

A fermentation process such as cider making consists of two main steps: alcoholic fermentation by yeasts, followed by a process whereby lactic acid bacteria transform malic acid into lactic acid. This so-called malolactic fermentation decreases acidity and improves microbial stability[8]. Although many lactic acid bacterial species are present, *Oenococcus oeni* is the dominant species among those triggering the malolactic fermentation.

Studies on the influence of phenolic compounds on lactic acid bacteria (LAB) have focused on LAB associated with vegetable fermentations[9]. During fermentation, some components (ethanol, pH and sulphites) are known to affect the physical and chemical properties of

the cytoplasmic membrane of LAB [10]. Phenolic content may have a positive or negative effect on lactic acid bacteria depending on the nature and concentration of the compound and on the bacterial strain [11].

Yeast in the fermentation has the ability to remove pigments leading to the clarification and improvement of beverages color [12]. It is known that yeast strains influence the production and the quality of fermented beverages, and that individual strains therefore may be suited to different uses [13]. Yeasts were evaluated to find a suitable culture for fermentation of cloudy apple juice into cider. The turbidity decreased during fermentation when plant debris and yeast cells settled, as reflected by the accumulation of lees at the bottom of the bottles.

During processing of apples, several steps are involved which can have significant effects on the content of polyphenols [14]. The ciders had a lighter color than the juices. A change to brighter appearance during the fermentation has been reported previously [15] .The change in color is caused by chemical modifications of the phenols. Yeasts are very reductive and the chromophore is therefore removed from some pigments during the fermentation, thus reducing the color intensity [15]. The phenolic content of the cider is not only important for the color, but also for the flavour and proposed health benefits. Polyphenols are secondary plant metabolites that influence the flavour, aroma, color and clarity of processed apple products [14].

Therefore, the main objective of the present study is to investigate if *O. oeni* PsuI has the same capability, which is to remove the phenolic pigments extracted from apple peels. To our knowledge no work has been done on the removal of phenolic pigments by this organism.

MATERIALS AND METHODS

Microorganism

The bacterial strain *O. oeni* PsuI was obtained from David Mills Laboratory, Department of Viticulture and Enology, University of California, Davis, CA, USA. The strain was grown on AR medium (2% Tryptone, 0.5% glucose and 0.5% yeast extract and supplemented with 20 ml/l of apple juice). The strain was stored by maintaining it on the same medium with 15% glycerol at –20 °C.

Extraction of phenolic pigments from Apple peels

Pigments were extracted using the conventional method with polar solvents [16, 17]. Ten red apples were purchased from a local market in Alexandria, Egypt in March, 2012. The peels were cut from the fruit surface and carefully freed from the pulp, twice washed with distilled water for 1 min, and dried between sheets of filter paper. Ten disks of peels (11 mm in diameter) were cut out with a cork borer, twice washed with distilled water for 1 min, and dried between sheets of filter paper. Extracts were obtained by adding the peels in solvent of 99 ml Methanol: 1 ml HCl. The extracts were evaporated till dryness to determine its weight and re-dissolved in methanol. The concentration was estimated as 260 µg/µl. The extract was stored at – 20 °C until analysis.

Media and cultivation

Portions (5ml) of AR medium in test tubes were autoclaved for 20 min at 121 °C. Tubes were inoculated with 100 µl of a 7-day-old culture and incubated for 5 days at 30°C under static conditions.

Measurement of Phenolic pigments

After centrifugation of *O. oeni* PsuI cultures, cell pellets were washed twice. In Eppendorf tubes, washed pellets were suspended in 600 µl methanol and 600 µl extracted pigments at a final concentration of 130 µg/µl and kept at room temperature for 48 hrs, during which the cells were colored due to uptake of the colored extract. The residual concentration of the colored extracted pigments in the supernatant was monitored by HPLC[18] and UV spectroscopy in comparison to the control samples (130 µg/µl of extracted pigments only).

HPLC analysis

Cells of *O. oen*i PsuI incubated with extracted pigments (final concentration of 130 µg/µl) for 24 hrs were prepared. Samples were centrifuged and cell-free supernatants of samples were analyzed by HPLC with UV detector (Central lab of Faculty of Science, Moharem Bek, Alexandria University).

The analysis was performed by reverse-phase HPLC. Conditions: detection at 318 nm (Agilent 1200 PDA detector); Eclipse plus C_{18} column (3.5 µm 4.6x250 mm); linear gradient over 45 min (0 to 100 % CH_3CN in H_2O/ 0.1% TFA); flow rate 1.0 ml/min.

Analysis by UV Spectroscopy

The cell-free supernatant samples were subjected to spectroscopic analysis using the UV detector of the above mentioned HPLC located in the central lab of Faculty of Science, Moharem Bek, Alexandria University.

Transmission Electron Microscope (TEM)

To study the effect of extracted phenolic pigments on *O. oeni* PsuI structure, the TEM technique was applied to observe changes in cells after the cell pellets were colored due to uptake of colored extracted pigments as mentioned in the previous section. Freshly taken *O. oeni* PsuI samples were fixed using a universal electron microscope fixative [19]. A series of dehydration steps were followed using ethyl alcohol and propyline oxide. The samples were then embedded in labeled beam capsules and polymerized. Thin sections of cells were cut using an LKB 2209-180 ultramicrotome and stained with a saturated solution of urinyl acetate for a half hour and lead acetate for 2 min. The procedure was also applied to control cells. Electron micrographs were taken using a transmission electron microscope (JEM-100 CX Joel), at the Electron Microscope Unit, Faculty of Science, Alexandria University.

Growth measurement

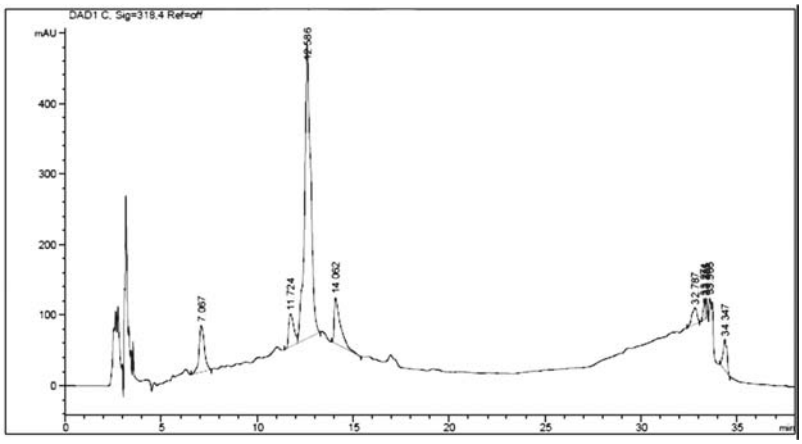
Two ml of the AR broth were dispensed in eppendorf tubes. The broth was inoculated with 100 µl of *O. oeni* PsuI culture and 5 µg/µl phenolic pigments were added. The culture was incubated at 30 °C for 5 days and optical density was monitored in comparison to control culture.

RESULTS AND DISCUSSION

Analysis of phenolic pigments by HPLC and UV Spectroscopy

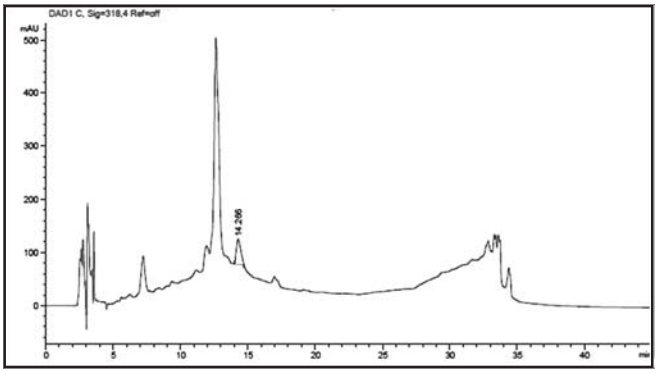
In cider making, Yeast cells perform primary alcoholic fermentation process and it interacts with phenolic pigments resulting in clarification process. In this study, *O. oeni* PsuI which is known for its secondary malolactic fermentation would be investigated for its ability to assist yeast cells in the same way.

First, the extract from apple peel will be



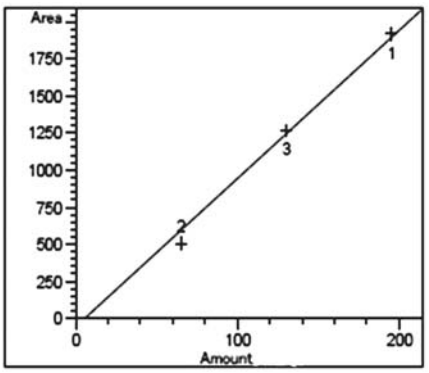
Peak	Ret Time (min)	Width (min)	Area (mAU*s)	area
1	7.067	0.2983	1274.81580	8.8118
2	11.724	0.2899	864.05499	5.9725
3	12.586	0.3041	9340.41895	64.5630
4	14.062	0.3240	1485.41638	10.2675
5	32.787	0.2608	436.51697	3.0173
6	33.271	0.1041	198.05803	1.3690
7	33.385	0.0796	146.87155	1.0152
8	33.565	0.0932	101.59781	0.7023
9	34.347	0.1935	619.38916	4.2814

Fig. 1.HPLC chromatogram of nine peaks of the extracted phenolic pigments of 130 µg/µl concentration (control) at 318 nm



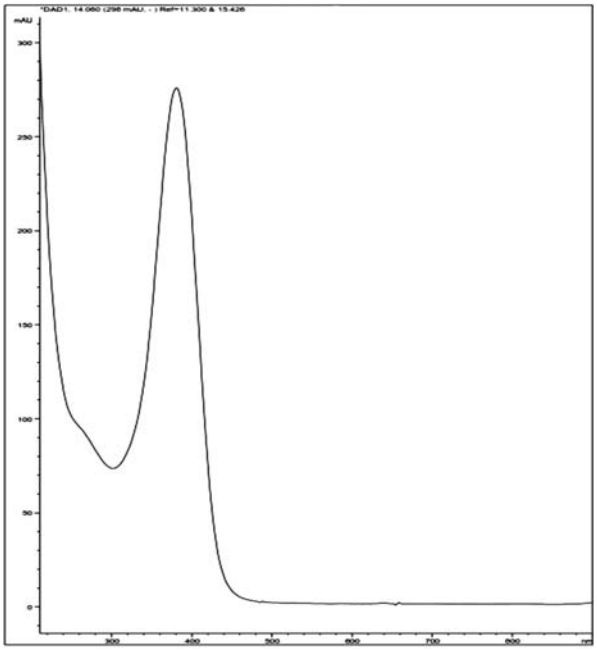
(A)

Ret Time (min)	Area [mAU*s]	Amt/ Area	Amount [µg/ul]
14.266	1060.57666	1.05154e-1	111.52379 ←



(B)

Ret Time (min)	Level	Amount µg/µl	Area	Amt/Area
14.062	2	65.00000	505.46628	1.28594e-1
	3	130.00000	1261.66406	1.03039e-1
	1	195.00000	1914.30200	1.01865e-1



(C)

Fig. 2. HPLC chromatogram showing the same peaks within the sample supernatant after pigment removal by *O.oeni* PsuI cells. Analysis of the peak separated at retension time of 14.06 (A),incomparison to calibration curve (B). The absorption range at 400 nm was also monitored using the UV detector (C)

explored for its kind of phenolic pigments present. Apple peels were prepared and extracted with acidified methanol as described in Methods. Since the HPLC technique has become the method of choice for chromatographic separation and rapid quantification of phenolic compounds, therefore this method was selected to evaluate the content of apple peel methanolic extracts.

An HPLC equipped with a UV detector was used to analyze the different kinds of phenolic pigments found in the extract. The primary spectral HPLC analysis of the methanolic extract from the control sample at 130 µg/µl concentration showed that most of the peaks contributing to light absorption in the wavelength range of 318 nm are between a retention time of 2 to 35 min (Fig 1).

Apple skin contains mainly anthocyanin cyanidin -3-galactoside [20] and a high concentration of flavonols and proanthocyanin[21]. These compounds were previously measured by spectrophotometer and each was detected at different ranges of wavelength. Anthocyanin was detected at a 530 nm wavelength, Proanthocyanin at 280 nm, and flavonols at 350 nm [3]. The extracted phenolic pigments in this study appeared yellow to brownish after dryness, suggesting that they are flavonols [22]. The use of wavelength measurement at 318 mm focused on flavonols range and not other kind of phenolic pigments. This indicates that the peaks in our data were in the range of flavonols.

The content of phenolics in apple fruits has been studied extensively [23]. Absorption techniques commonly involve spectrophotometric measurement on solutions obtained after crushing the tissues and removal of solids. Traditional methods for its determination consisted in measurement of total phenolics because of their association with browning reactions. Such measurements estimate the soluble pigments and are usually performed near 400 nm[24].

In order to determine which flavonols were removed by *O. oeni* PsuI cells, the apple peel extract was incubated with the bacteria, followed by HPLC analysis of the cell-free extract as described in Methods. Four clear peaks were chosen to be analyzed in the sample supernatant after bacterial treatment to determine which peak was affected by *O. oeni* PsuI cells. Peaks at retention time of 7.067, 12.58, 34.34 and 14.062 were analyzed separately by HPLC using calibration curves to determine their final concentration and the absorption of each peak was also determined using UV detector equipped in the HPLC device (data not shown). The data revealed (not shown) that for peaks separated at retention time 7.067, 12.58 and 34.34, the concentration didn't change from the initial concentration which was 130 µg/µl indicating that these peaks were not absorbed by *O.oeni* PsuI cells, while for peak at 14.062 retention time, the concentration was reduced to 111.52 µg/µl as shown in Fig 2A&2B, suggesting that this peak was absorbed by *O. oeni* PsuI cells. The UV detector results in Fig 2C, shows that maximum absorption of this peak was at 400 nm.

Microorganisms such as yeast cells which perform primary fermentation in beverages can adsorb both flavans and anthocyanins in polymeric form, showing a great affinity for flavonols [25.]

Flavonols are generally easier to study than the anthocyanins since they are more stable. Most of the early studies were limited because they were based on the techniques of thin-layer chromatography (TLC) and column chromatography. Separation and identification of these glycosides by conventional chromatographic methods is very difficult due to the similarity of their molecular weight and polarity. The development of HPLC methods for the separation and detection of the flavonols has made the task much simpler and more reliable [22].

The experiment here showed the capability of flavonols removal by *O. oeni* PsuI cells, indicating that during fermentation process, *O. oeni* PsuI can assist yeast in pigment removal, resulting in clarification of beverages such as cider making.

Transmission electron microscopy

Because extensive pigment removal was observed, the transmission electron microscopic technique was applied to observe changes in cell structure after incubation of the *O. oen*i PsuI without and with phenolic pigments.

Micrographs corresponding to control cells (Fig 3A) and those incubated with phenolic pigments (130 µg/µl) are shown in Fig 3B. Control cells (a) appeared with regular cell wall and no dense areas are seen. Cells exposed to pigments (b) showed small tiny dense dark areas absorbed and distributed inside the cell. Similarly, it has been demonstrated that *E.*coli are able to absorb amino acid derivative pigment[26].

In beverages such as wine, a very simple method, based on evaluation of the biomass color of yeast colonies to determine the wine yeast aptitude to adsorb pigments from grape skins and seeds has been developed [27]. The assumption was that biomass color reflects the binding of grape pigments to the biomass. *O. oeni* have the capability of removal of color from solutions [28]. The data presented here reveal that *O. oeni* PsuI which is known to undergo the secondary fermentation process(malolactic fermentation) in beverages, is also involved in pigment removal indicating that it can assist yeast cells in the

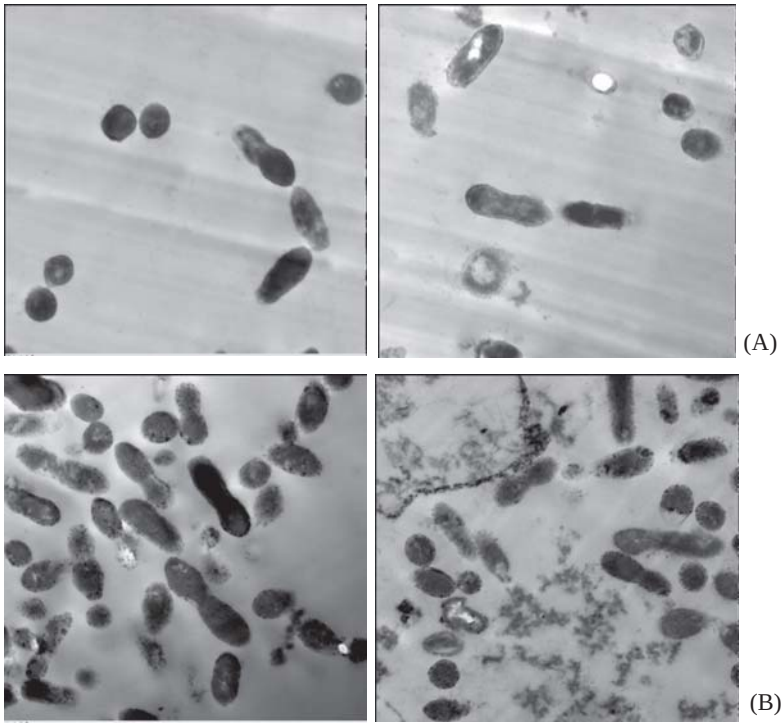


Fig. 3. Transmission electron microscopy showing the free cells at magnification power of 7500X for *O. oeni* PsuI(A) and pigment accumulating within the cells appearing as tiny dots (B)

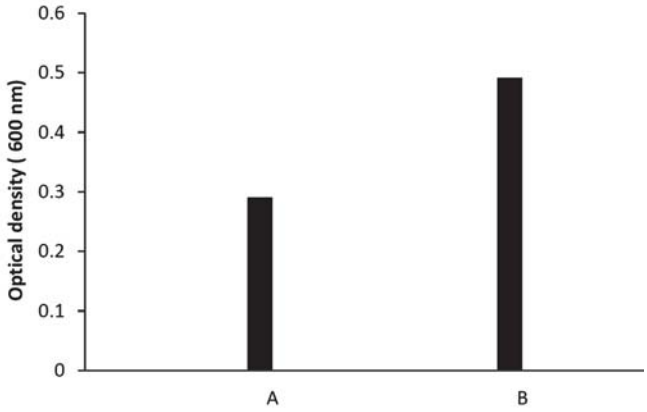


Fig. 4. Showing the effect of phenolic pigments on growth of *O. oeni* PsuI cells (B) in comparison to control cells (A)

adsorption process thereby improving the quality of most beverages.

For other phenolic pigments, it is well known that yeasts can influence beverage color [29] by anthocyanin adsorption on yeast cell walls [30]. Adsorption of Phenolic pigments such as anthocyanins and polymeric anthocyanins by the yeast cells is commonly observed in winemaking. The yeast lees of red wines following fermentation are typically tinted red or purple. Several studies have documented anthocyanin adsorption onto yeast cells [31, 32].

Micrographs also revealed that the growth and viability of *O. Oeni* PsuI cells could have been affected on exposure to phenolic pigments. The control cells seem fewer in number

than the exposed cells, indicating that phenolic pigments might have enhanced the cell growth. To confirm our claim, the growth was monitored after 5 days in cultures supplemented with and without the extracted phenolic pigments (Fig 4). The results showed a 1.68 fold increase in growth.

In agreement to our expectations, flavonoids and carotenoid pigments increased the cell multiplication and metabolic activity of *Saccharomyces cerevisiae* [33]. Also the growth of different strains of *O. oeni* was stimulated in the presence of flavonoids and phenolic acids [34].

In various plants such as leguminous plants, some of the flavonoids exuded by legume seeds and roots induce transcription of rhizobial nodulation genes, which allow these bacteria to establish a symbiotic association with their host plant. In addition, flavonoids enhance the growth rates of bacterial cells and promote bacterial movement toward the plant [35].

Plants such as Alfalfa (*Medicago sativa* L.) release different flavonoids which increase the growth rate of *Rizobium meliloti* in a defined minimal medium [36].

CONCLUSION

O. oeni PsuI is capable of absorption of phenolic pigments (expected flavonols) extracted from apple peels at a retention time of 14.06 nm as its concentration is reduced from 130 µg/µl to 111 µg/µl. Absorption was confirmed by TEM and pigments were found distributed within *O. oeni* PsuI cells. These results indicate that during cider making, *O. oeni* PsuI assist yeast cells in the removal of phenolic pigments leading to the clarification of beverage. During which the cell growth and viability of *O. oeni* PsuI was enhanced by these pigments.

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