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Natural Melanin Synthesized by *Aureobasidium pullulans* Using Food Wastes and its Characterization

Gamze Nur Mujdeci

Faculty of Food Engineering, Hitit University, Corum, Turkey

Abstract

Background and Objective: Food wastes cause economic losses and environmental problems. Hence, ability to transform food wastes into high-value added products is highly attractive. The aim of this study was to produce melanin pigments by fermentation that include wide potential uses in agriculture, cosmetics and pharmaceutical industries using domestic wastes such as melon peel, watermelon peel and carrot peel and industrial by-products such as whey and molasses.

Material and Methods: Two *Aureobasidium pullulans* strains were assessed for melanin production. Fourier-transform infrared spectroscopy, scanning electron microscope, zeta potential, ultraviolet absorbance and solubility assays were carried out to characterize produced melanin nanoparticles.

Results and Conclusion: The highest intracellular (0.19 g l^{-1}) and extracellular (3.52 g l^{-1}) melanin concentrations were produced by *Aureobasidium pullulans* NBRC 100716 using carrot peel extracts as fermentation media. Results of characterization were compared with those of synthetic melanin used as standard and the produced nanoparticles were validated. Particle sizes of the nanoparticles ranged 10-760 nm with negative charges, as suggested by previous literature. Results showed that carrot peel was a good candidate, which could be used for the production of high value-added melanin. When carrot peel extract was used as a fermentation medium, characteristics of the melanin produced by *Aureobasidium pullulans* NBRC 100716 strain were similar to those of synthetic melanin.

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***Corresponding author:**

Gamze Nur Mujdeci*
Food Engineering, Hitit University, Corum, Turkey

Tel: 00905446963446
E-mail: gnurkara@hitit.edu.tr

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1. Introduction

Melanin, one of the most widespread pigments in the animal kingdom, is described as a high-molecular weight hydrophobic polymer formed by oxidative polymerization of phenolic and indolic compounds [1]. Typically, melanin, a dark-brown or black pigment, is negatively charged, insoluble in water and organic solvents and structurally includes a high molecular weight. This structure makes the pigment stable and resistant to various physicochemicals such as oxidants, drying agents, extreme temperatures, UV light, heavy metals and drugs [2]. Melanin is an important resistance material in fungi, which help the fungi survive under various stress conditions [3]. Several fungal species can synthesize 1,8-dihydroxynaphthalene (DHN)-melanin from acetyl-coenzyme A via polyketide pathway [4]. The structural formula of DHN-melanin is presented in Figure 1. Melanin can be found inside or outside the cells, depending on the type of fungi [5]. *Aureobasidium* (*A.*) *pullulans*, one of the melanin-producing fungi, produces this pigment intra and extracellularly. Although *A. pullulans* is generally known in literatures for the production of biopolymer pullulan, this study focused on the melanin production of this species. In general, a little data are published on this metabolic pathway. Recently, interests have increased in pigments produced by microorganisms instead of synthetic pigments [6-8].

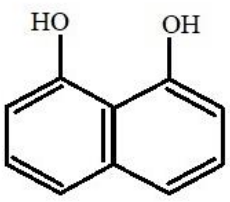

Figure 1. Structural formula of DHN-melanin

The reason for this is that the pigments from natural sources are safer, environmentally friendly and easily degradable with no harmful effects. Santhanalakshmi et al. [9] have shown that synthetic pigments include harmful effects on humans, animals and environments.

Since melanin includes functional characteristics such as photosensitivity, light barrier quality, free radical scavenging ability, antioxidant activity and binding to metal ions and certain organic compounds (e.g. drugs and toxins) characteristics, it is used in the preparation of optical biomimetics, cosmetics, UV protective lenses, food dyes, anti-melanoma treatments and metallic nanoparticles. Furthermore, melanin is used to strengthen various biopolymer-based films [10]. Considering the wide potential uses and the increased demands for melanin pigments, studies on melanin production using microorganisms clearly include significant potencies [11,12]. Agroindustrial raw materials and byproducts have been suggested as low-cost alternative carbohydrate sources for the microbial metabolite production. This also minimizes environmental problems and those linked to disposal of such substances [13]. In a study by Tarangini and Mishra, fruit wastes (pineapple and orange wastes) were used for melanin production by *Bacillus safensis*. In another study vegetable waste was used for melanin production using *Pseudomonas* spp. [14]. Overall, melanin production from various microbial species, particularly *A. pullulans*, is an attractive research subject. Since there are a few studies on the production of melanin using *A. pullulans*, the major aim of the current study was to produce melanin from *A. pullulans*. In addition, another aim of this study was to fill gaps in literatures using food wastes that have not been studied previously. Hence, specific research objectives included 1) assessing the best melanin-producing *A. pullulans* strain within the experiments, 2) investigating food industry byproducts (whey and molasses) and food wastes (melon, watermelon and carrot peels) for melanin production and 3) characterizing the produced melanin.

2. Materials and Methods

2.1. Aureobasidium pullulans strains

In this study, *A. pullulans* AZ-6 and *A. pullulans* NBRC 100716 strains were used for melanin production. The strains were kindly provided by Prof. Z. Yesim Ozbas from Hacettepe University, Ankara, Turkey. While *A. pullulans* AZ-6 was isolated from fresh Gemlik olives in Turkey [15], *A. pullulans* NBRC 100716 was isolated from strawberries in Japan. Strains were stored at 4º C on yeast extract malt extract (YM) slants, including (g l^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; and agar, 15. Cultures in YM agar were recovered in YM broth through continuous subcultures. For long-term preservation, cultures were stocked in yeast extract peptone dextrose (YEPD) media with 20% glycerine at -70 ºC.

2.2. Collection and pretreatment of food wastes and industrial byproducts

In this study, whey, molasses solution, melon extract, watermelon and carrot peel were used as natural fermentation media. Whey was supplied by a cheese factory in Corum City, Turkey, and used directly as the fermentation media with no pretreatments.Molasses was sourced from a sugar factory in Corum City, Turkey, and diluted at a ratio of 1:10 (v v^{-1}) using a method by Israilides et al. [16]. Melon, watermelon and carrot peel were used as fermentation media after separate preprocessing using a method by Tarangini and Mishra [14]. In this method, 2 L of distilled water were added to 1000 g of peel. After boiling the mixture at 100 °C for 30 min, the extract was separated by filtration using cellulose filter papers. Sterilization of the fermentation media was carried out at 121 °C for 15 min using autoclave.

2.3. Preparation of inocula

For the preparation of inocula, *A. pullulans* strains were cultured on YM agar slants at 28 °C for 2 days and then cultures were inoculated into 250-ml cotton-plugged Erlenmeyer flasks containing 100 ml of sterile tryptic soy broth (TSB), including (g l^{-1}) casein peptone, 17.0; soy peptone, 3.0; D(+)glucose, 2.5; NaCl, 5.0; and K_2HPO_4, 2.5. Culture was incubated at 30 ºC for 48 h at 100 rpm using shaking incubator to achieve cell counts of 1-5×10^7 cells per mL. Five percent (v v^{-1}) of each inoculum were used for inoculations in fermentation media.

2.4. Batch fermentation experiments

Fermentation experiments were carried out in 300-ml cotton-plugged Erlenmeyer flasks containing 150 ml of fermentation media. Flasks were incubated at 30 °C using shaking incubator with a shaking speed of 100 rpm.

2.5. Analytic assays

2.5.1. Assessment of biomass concentration

Assessment of biomass concentration was carried out based on a procedure suggested by Mujdeci [17]. During the experiments, 10 ml of culture sample were collected from the fermentation media every 24 h. Sample was centrifuged at 2,599 g for 20 min; then, supernatant was separated. Pellet (biomass) was dried at 60 ºC until constant weight using oven. Biomass concentration of each *A. pullulans* strain was calculated in dry cell weight (DCW, g l^{-1}).

2.5.2. Assessment of intracellular and extracellular melanin concentrations and purification

Concentration of melanin produced by *A.pullulans* AZ-6 and *A. pullulans* NBRC 100716 strains in fermentation media was measured by collecting 10 ml of the culture samples every 24 h of fermentation under aseptic conditions. Collected samples were centrifuged at 3,743 g for 10 min and the supernatant containing extracellular melanin was separated from the pellet. Then, 1 N NaOH in a volume of

10 ml was added to the pellet containing the intracellular melanin and the resulting mixture was autoclaved at 121 °C for 20 min. Mixture was centrifuged at 3,743 g for 10 min and the supernatant containing intracellular melanin was gently separated and transferred into a sterile tube. Then, HCl 2 M was added to the supernatants containing intracellular (iMNP) and extracellular melanin nanoparticles (eMNP) until their pH was 2 and melanin was precipitated. The precipitation process was carried out twice. Precipitated melanin was separated using centrifuge (3,743 g, 10 min) and then washed repeatedly with distilled water until pH reached to 7. Melanin was dried at 60 °C until reached a constant weight and stored at -18 °C [11]. Total melanin concentration was expressed as the sum of iMNP and eMNP concentrations.

2.6. Characterization of melanin

To characterize MNPs, a series of assessments were carried out. First, extracted melanin particles were characterized using Fourier-transform infrared spectroscopy (FTIR) and Thermo Scientific/Nicolet IS10 (Thermo Fisher Scientific, Waltham, MA, USA), within the range of 4,000-600 cm^{-1} with 40 scans at a resolution of 4.0 cm^{-1}. The zeta potential of melanin particles was measured using zeta-potential analyzer (Malvern ZetaSizer Nano ZSP, Malvern Instruments, Malvern, UK) and the molecular structures were assessed using a scanning electron microscope (SEM) (Quanta FEG] 450, FEI, Amsterdam, Netherlands). Furthermore, spectroscopic analysis of the extracted melanins was carried out. Briefly, 0.5 mg of each iMNP and eMNP samples was separately dissolved in 10 mL of KOH solution (1 mol l^{-1}) and UV-visible absorption spectra at 220–800 nm were measured using UV-VIS spectrophotometer (Shimadzu, UV-1800, Kyoto, Japan). Wavelength (λ_{max}) with the highest absorbance was recorded. Then, 1 mol l^{-1} KOH solution was used as blank [18]. For solubility assessment, each of iMNP and eMNP samples (0.1 g) was dissolved in 10 mL distilled water, 1 mol L^{-1} KOH and 1 mol l^{-1} NaOH solutions as well as various organic solvents (chloroform, ethyl acetate, ethanol, methanol, acetic acid, ether, petroleum ether, hexane and acetone). After stirring at 25 °C for 1 h, solution was filtered through coarse filter papers. Absorbance measurement of the filtrates was carried out at λ_{max} [19]. All characterizations were carried out for standard synthetic melanin (Sigma Chemical, St. Louis, MO, USA) and data were compared.

2.7. Statistical analysis

The SPSS Software v.13.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were presented in mean ±SD (standard deviation). The means of each group were analyzed using 2-way analysis of variance (ANOVA) and Tukey's test to report significant differences between the strains and wastes. In general, $p < 0.05$ was considered statistically significant in the two replicates.

The average values of duplicate assessments for maximum biomass concentrations of *Aureobasidium pullulans* AZ-6 and *Aureobasidium pullula*ns NBRC 100716 and the maximum iMNP and eMNP and the total MNP concentrations produced by these strains are presented in Table 1. Statistical analysis using two-way ANOVA for each maximum biomass, eMNP and total MNP concentrations showed that population means of the strains and food wastes were significantly different and interactions between the food wastes and strains were significant ($P < 0.05$). Tukey's test revealed no significant difference between the groups. In experiments with carrot peel extract as fermentation media, the highest biomass concentration at 44.80 g l^{-1} ±1.36 was recorded for *Aureobasidium pullulans* NBRC 100716. Fermentation media that mostly stimulated growth of *Aureobasidium pullulans* AZ-6 included watermelon peel extract with the highest biomass concentration of 41.80 g l^{-1} ±2.01. In experiments using whey, biomass concentrations of the *A. pullulans* strains were quite low and their changes over time were similar (Figure 2). When molasses solution was used as fermentation media, the maximum biomass concentrations of *Aureobasidium pullulans* AZ-6 and *Aureobasidium pullulans* NBRC 100716 were closely similar. As seen in Figure 2, when melon or watermelon peel extracts were used as fermantation media, biomass concentrations of the *A. pullulans* strains decreased sharply and the death phase began. When melon peel extract was used, the biomass concentration decreased after Day 13 of fermentation. In watermelon peel extract, *Aureobasidium pullulans* AZ-6 and *Aureobasidium pullulans* NBRC 100716 biomasses respectively decreased after Days 12 and 13 of fermentation due to the depletion of nutrients in the environment. In experiments; in which, carrot peel extract and molasses solution were used, stationary phase of the microorganisms started at the end of the fermentation.

Concentrations of eMNP produced by the *A. pullulans* strains over time are shown in Figure 3. In general, changes over time of eMNP produced by *Aureobasidium pullulans* AZ-6 and *Aureobasidium pullulans* NBRC 100716 strains using melon peel extract and changes of eMNP produced by *Aureobasidium pullulans* NBRC 100716 strain using molasses extract were similar. As seen in Figure 3, the maximum eMNP concentration of the study (3.52 g l^{-1}) was achieved using *Aureobasidium pullulans* NBRC 100716 with carrot peel extract. The maximum eMNP of 0.92 g l^{-1} produced by *Aureobasidium pullulans* AZ-6 strain was achieved when carrot peel extract was used as fermentation media. The eMNP production of *Aureobasidium* ck compared to its original color. In experiments where only molasses was used, the color difference caused by the eMNP production could not be seen because molasses was dark brown (Figure 4). Of the strains from this study, only *Aureobasidium pullulans* NBRC 100716 strain could produce iMNP.

3. Results and Discussion

Table 1. Effects of food waste or by-products on the maxima of the biomass, extracellular melanin, and intracellular melanin concentrations of *A. pullulans* AZ-6 and *A. pullulans* NBRC 100716, determined in culture taken at every 24-hour interval*

Food waste/by product	Maximum biomass concentration (g l^{-1})		Maximum extracellular melanin concentration (g l^{-1})		Maximum intracellular melanin concentration (g l^{-1})	Total melanin concentration (g l^{-1})	
	AZ-6	NBRC 100716	AZ-6	NBRC 100716	NBRC 100716	AZ-6	NBRC 100716
Melon peel extract	15.50±2.01^{ab}	6.70±0.43^{ab}	0.16±0.02^{ab}	0.22±0.06^{ab}	0.07±0.01	0.16±0.02^{ab}	0.29±0.07^{ab}
Watermelon peel extract	41.80±2.01^{ab}	19.30±0.00^{ab}	0.51±0.07 ^{a}	0.60±0.10^{a}	0.18±0.02	0.51±0.07^{a}	0.78±0.12^{a}
Whey	2.50±0.49^{b}	1.90±0.23^{b}	0.00±0.00 ^{c}	0.00±0.00^{c}	0.00±0.00	0.00±0.00^{b}	0.00±0.00^{b}
Carrot peel extract	27.70±1.56^{ab}	44.80±1.36^{ab}	0.92±0.18^{abc}	3.52±0.28^{abc}	0.19±0.02	0.92±0.18^{ab}	3.71±0.32^{ab}
Sugar beet molasses	28.20±3.36^{a}	27.30±0.36^{a}	0.00±0.00 ^{c}	0.03±0.01^{c}	0.02±0.00	0.00±0.00^{ab}	0.05±0.01^{ab}

*: Results represent the mean ± SD (n=2); the value in the same column followed by the same letter is not significantly (P > 0.05) different; no significant (P > 0.05) difference between the strains were determined.

Changes of iMNP concentration produced by *Aureobasidium pullulans* NBRC 100716 strain in various fermentation media over time are presented in Figure 5.

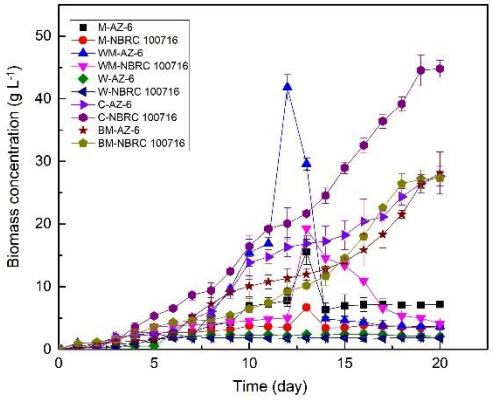

Figure 2. Variations of biomass concentrations with time (M-AZ-6: Food waste (FW); melon peel, strain (S); *Aureobasidium pullulans* AZ-6, M-NBRC 100716: FW; melon peel, S; *Aureobasidium pullulans* NBRC 100716, WM-AZ-6: FW; watermelon peel, S; *Aureobasidium pullulans* AZ-6, WM-NBRC 100716: FW; watermelon peel, S; *Aureobasidium pullulans* NBRC 100716, W-AZ-6: FW; whey, S; *Aureobasidium pullulans* AZ-6, W-NBRC 100716: FW; whey, S; *Aureobasidium pullulans* NBRC 100716, C-AZ-6: FW; carrot peel, S; *Aureobasidium pullulans* AZ-6, C-NBRC 100716: FW; carrot peel, S; *Aureobasidium pullulans* NBRC 100716, BM-AZ-6: FW; sugar beet molasses, S; *Aureobasidium pullulans* AZ-6, BM-NBRC 100716: FW; sugar beet molasses, S; *Aureobasidium pullulans* NBRC 100716

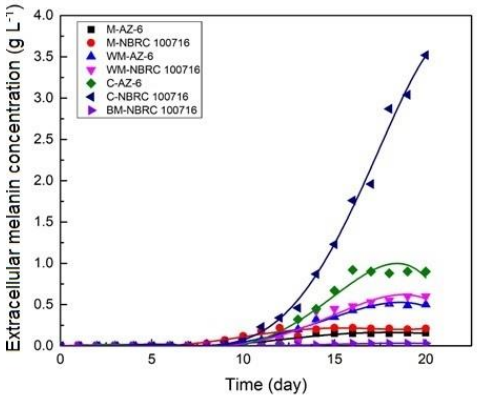

Figure 3. Variations of extracellular melanin concentrations with time (**M-AZ-6**: Food waste (FW); melon peel, strain (S); *Aureobasidium pullulans* AZ-6, **M-NBRC 100716**: FW; melon peel, S; *Aureobasidium pullulans* NBRC 100716, **WM-AZ-6**: FW; watermelon peel, S; *Aureobasidium pullulans* AZ-6, **WM-NBRC 100716**: FW; watermelon peel, S; *Aureobasidium pullulans* NBRC 100716, **C-AZ-6**: FW; carrot peel, S; *Aureobasidium pullulans* AZ-6, **C-NBRC 100716**: FW; carrot peel, S; *Aureobasidium pullulans* NBRC 100716, **BM-NBRC 100716**: FW; sugar beet molasses, S; *Aureobasidium pullulans* NBRC 100716

Figure 4. Comparison of the color changes due to extracellular melanin production on Day 10 of fermentation

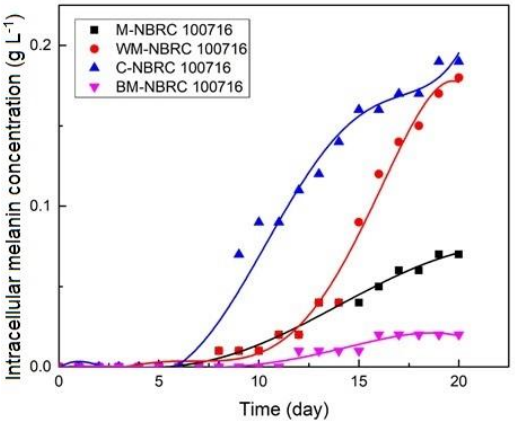

Figure 5. Variations of intracellular melanin concentrations with time (**M-NBRC 100716**: FW; melon peel, S; *Aureobasidium pullulans* NBRC 100716, **WM-NBRC 100716**: FW; watermelon peel, S; *Aureobasidium pullulans* NBRC 100716, **C-NBRC 100716**: FW; carrot peel, S; *Aureobasidium pullulans* NBRC 100716, **BM-NBRC 100716**: FW; sugar beet molasses, S; *Aureobasidium pullulans* NBRC 100716

This strain did not produce iMNP in experiments using whey. The maximum iMNP concentrations in watermelon and carrot peel extracts were closely similar to each other as 0.18 and 0.19 g l^{-1}, respectively. In this study, iMNP production began on Day 8 of fermentation in melon and watermelon peel extracts, Day 9 of fermentation in carrot peel extract and Day 12 of fermentation in molasses solution. Liu et al. [3] reported that melanin synthesis by *A. pullulans* did not occur in the early stage of cultivation. Similar results were reported in the present study.

Overall, these results showed that the highest quantity of total melanin (3.71 g l^{-1}) produced by *Aureobasidium pullulans* NBRC 100716 in carrot peel extract (Table 1). El-Gamal et al. [11] set up a series of fermentation experiments using wastes of tomato paste to produce melanin*pullulans* NBRC 100716 strain started on Day 8 of fermentation for melon and watermelon peel and Days 9 and 12 of fermentation for carrot peel and molasses, respectively. As seen in in Figure 2, melanin was produced in logarithmic phases of the strains. Color of the fermentation media; in which, the eMNP production was observed, changed to darken brownish-bla using *A. pullulans*. The authors reported that the maximum level of pigment included 300 mM on Day 9 of incubation. Santhanalakshmi et al. [9] investigated several wastes and concluded that banana stalk, coconut husk, rice husk, dark millet and wheat and rice flours were the best agricultural wastes for the pigment production. Guo et al. [20] reported that the maximum melanin quantity produced by *Streptomyces kathirae* under optimum conditions (3.3 g l^{-1} amylodextrine, 37 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 0.1 g L^{-1} $CaCl_2$ and 54.4 µM $CuSO_4$) was 13.70 g l^{-1}. Studies have reported that organisms produce melanin to protect themselves against the environmental stress [21-24]. In this study, melanin production increased immidiately when sugar of the media was utilized (data not shown). In general, three various cell morphologies of *A. Pullulans* are described as polymorphic fungi with long, branched septate filaments, large chlamidospores and smaller elliptical yeast-like cells. It is described that yeast-like cells do not produce melanin. Approximately half of the melanin produced by *A. pullulans* is stored in the cell wall of chlamydospore cells and the rest is released into the environment as black granules. The melanin-storing chlamidospore form of *Aureobasidium pullulans* NBRC 100716 cells photographed in this study is shown in Figure 6.

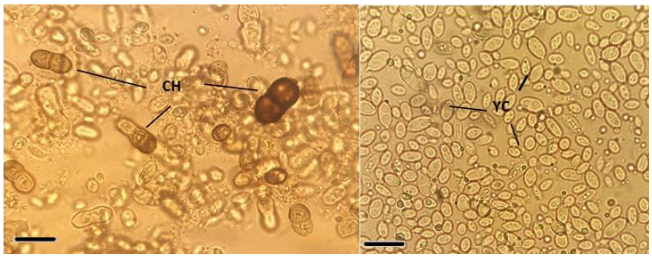

Figure 6. Light micrograph of the morphological forms of melanin producing *Aureobasidium pullulans* NBRC 100716 at Day 20 of fermentation in carrot peel extract (scale bar = 10 µm). CH, chlamydospore

3.1. Characterisation of melanin

The first step in characterisation of melanin included FTIR analysis. The FTIR transmittance spectra of the extracted iMNP, eMNP and synthetic melanin are demonstrated in Figure 7.

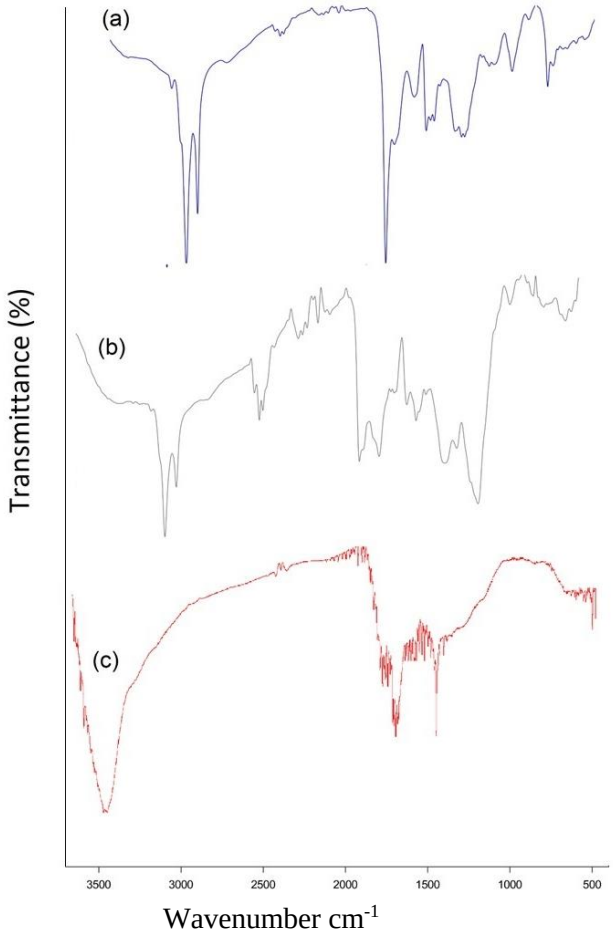

Figure 7. Fourier-transform infrared spectroscopy spectra of (a) intracellular, (b) extracellular melanins produced by *Aureobasidium pullulans* NBRC 100716 in carrot peel extract, and (c) synthetic melanin

Signals in 3600-2800 cm^{-1} area are attributed to stretching vibrations (O-H and N-H) of the amine, amide or carboxylic acid, phenolic and aromatic amino functions in indolic and pyrrolic systems. Results verified presence of these chemical groups in spectra of iMNP, eMNP and synthetic melanin by the peaks at 3007, 3212.14 and 3421.16 cm^{-1}, respectively. The N-H stretching of iMNP, eMNP and synthetic melanin origin peaks can be seen at 2918.77, 2922.64 and 2910.00 cm^{-1}, respectively. Appearance of the peaks at 1706.63, 1740.45 and 1717.00 cm^{-1} in FTIR spectra of iMNP, eMNP and synthetic melanin, respectively can be linked to the stretching of C=O. At 1500–1400 cm^{-1}, peaks refer to the presence of N-H vibration and C-N stretching. At 800–600 cm^{-1}, weak peaks correspond to the substitution of aromatic rings by aromatic hydrogens. The FTIR data of the extracted, synthetic and fungal melanin samples in the literature are listed in Table 2.

Table 2. Comparison of F-IR data of extracted melanin, synthetic melanin and some fungal melanin samples in the literature

Assignments	Wave number (cm^{-1})						
	Synthetic melanin	Intracellular melanin	Extracellular melanin	Melanin produced by *A. pullulans* [11]	Melanin produced by *O. sinensis* [25]	Melanin produced by *Phyllosticta capitalensis* [26]	Melanin produced by *Aspergillus bridgeri* [27]
−OH and –NH_2 str	3421.16	3272.18	3212.14	3421.66	3421.98	3352.50	3419.64
N-H str.	2910.00	2918.77	2922.64	2921.70	2929.74	2924.99	2925.47
C=O str	1717.00	1706.63	1740.45	1716.22	1706.49		
C=C str. and C=O str.	1636.04	1654.56	1620.54	1651.11	1618.56	1639.80	1633.20
N-H bend.	1540.57	1533.14	1548.61	1543.72			
N-H str. and C-N str.	1457.24	1460.31	1452.07	1456.12		1401.39	1459.26
C-H str and O-H bend.	1384.51	1433.95, 1376.94	1395.45, 1377.05	1386.33			
O-H str.	1245.00	1245.98	1220.64				1258.50
C-H str.			1019.08				1020.30
C=C bend.		721.68	773.02				

A. pullulans = Aureobasidium pullulans, O. sinensis = Ophiocordyceps sinensis

As seen in this table, FTIR results of the melanin produced by *A. pullulans* showed the presence of peaks at 3421.66 cm^{-1} (-OH and -NH_2 stretching), 2921.70 cm^{-1} (N-H stretching), 1716.22 cm^{-1} (C-O stretching), 1651.11 cm^{-1} (C=O and C=C stretching), 1543.72 cm^{-1} (N-H bending), 1456.12 cm^{-1} (N-H and C-N stretching) and 1386.33 cm^{-1} (C-H stretch and O-H bending) [11]. Dong and Yao [25] reported that FTIR spectra of melanin produced by *Ophiocordyceps sinensis* included a strong broad band at 3421.98 cm^{-1} as well as strong bands at 1716.22 and 1618.56 cm^{-1}. Moreover, it was reported that melanin from *O. sinensis* included a peak of 2929 cm^{-1} [25]. The FTIR spectra of melanin from *Phyllosticta capitalensis* was characteristic, including peaks linked to -OH and -NH conjugated carbonyl bonds [26] (Table 2). Kumar et al. [27] reported that FTIR spectra of melanin pigments from *Aspergillus bridgeri* included bands at 3419.4, 2925.47, 1633.20, 1459.26, 1258.50 and 1020.30 cm^{-1}. As seen in Table 2, FTIR spectroscopy characteristics of the pigment from *Aureobasidium pullulans* NBRC 100716 strain were correlated to synthetic melanin. The FTIR spectroscopy characteristics of melanin produced by various microorganisms have been reported previously. Based on these results, it was concluded that the pigment was melanin.

In this study, the second assay for the characterisation of melanin included investigation of the molecular structure using SEM and zeta analyser. It has been reported that SEM is a powerful method for the morphological characterization and particle size distribution of various types of melanin [1]. When studies were assessed, it was clearly seen that the granule morphology and size range (30-1000 nm) of melanin depended on the source. Figure 8a shows an SEM image of the reference synthetic melanin, showing that the synthetic melanin possesses an amorphous (irregular) shape pattern in range of 100-700 nm.

At a higher magnification, the synthetic melanin showed the substructure of irregular units with variable sizes. Similar SEM structures of the synthetic melanin were reported by Correa et al. [28]. In the present study, purified iMNP synthesized by *Aureobasidium pullulans* NBRC 100716 included amorphous materials with no definable structures, composed of tightly gathered particles of various sizes (Figure 8b). Size of the iMNPs ranged 45-500 nm. The eMNPs demonstrated a 3-D network structure and presence of macro porosity. Pores of eMNPs are shown with arrows in Figure 8c.

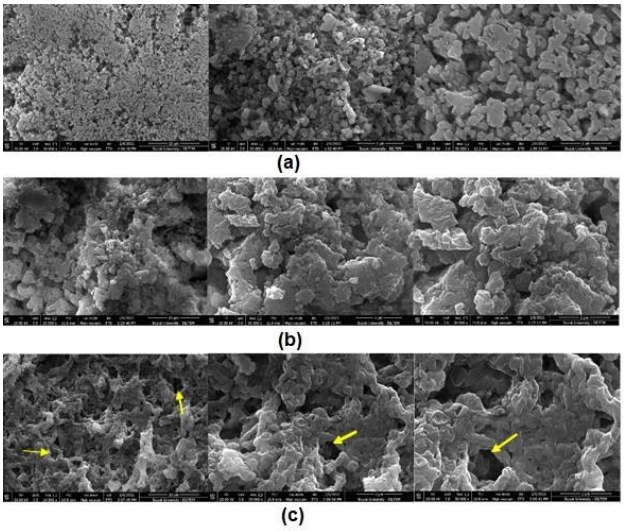

Figure 8. Scanning electronmicrograph of (a) synthetic, (b) intracellular, and (c) extracellular melanin nanoparticles at 10.0, 30.0 and 50.0 K× magnifications

Size of the eMNP particles was quite variable, ranging 10–760 nm. To the best of the author's knowledge, this was the only available study in the literature that investigated melanins produced by *A. pullulans* with SEM. Potential analyser was used to assess electro kinetic surface potential of the MNPs (Figures 9a, b, c).

Values of the zeta potentials for sMNP, iMNP and eMNP included -20.8 ±4.6, -13.0 ±5.17 and -20.3 ±3.74 mV, respectively. These results were similar to those of Rageh et al. [29], who reported the zeta potential of chemically synthesized MNPs as -20.6 ±1.8 mV. Normally, magnitude of the zeta potential reveals stability of the nanoparticles against aggregation. It has been reported that melanin includes a highly negative surface due to zeta potential, which confers stability to the particles by electrostatic repulsion, avoiding aggregation [30].

It has been stated that UV-visible spectral analysis is useful for the preliminary characterization of melanin pigments and nanomaterial forms [31]. Figure 10 shows the UV-visible absorbance spectra of purified eMNP and iMNP with that of synthetic melaning pigment used as reference. The maximum absorbance was observed under UV-C region of 220 nm for iMNP, eMNP and synthetic melanin. The absorbance decreased at longer wavelengths. This decrease was a characteristics of the melanin. Similar absorbance profiles were observed for melanin from *O. sinensis* and eumelanin nanoparticles [25,32]. A recent systematic literature review stated that the maximum absorption wavelength of alkali solutions ranged 196-300 nm, depending on the melanin source [1].

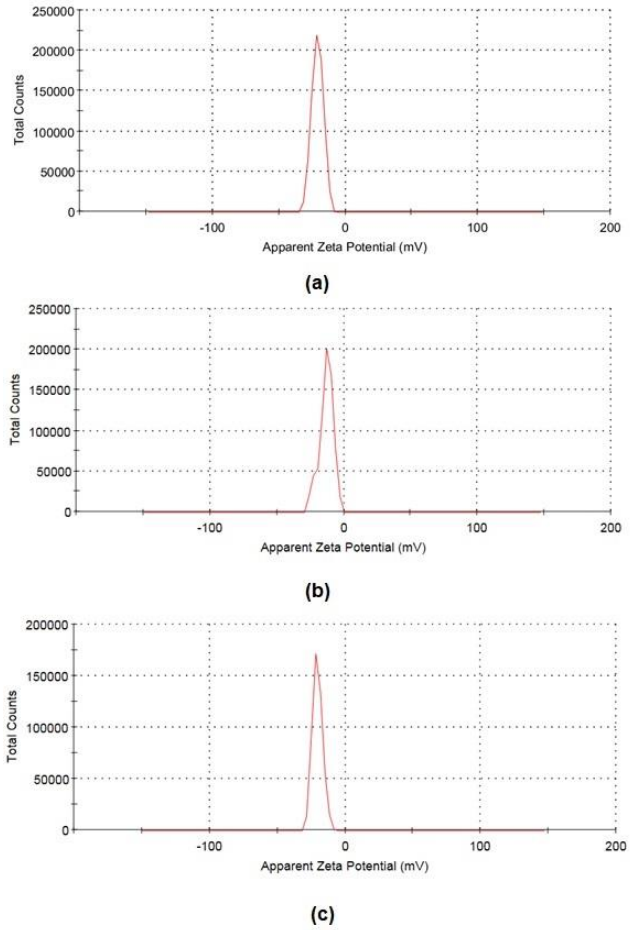

Figure 9. Zeta potential distribution of (a) synthetic, (b) intracellular, and (c) extracellular melanin nanoparticles

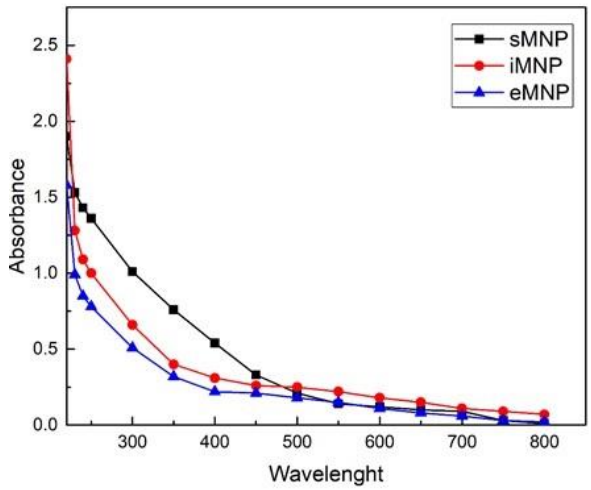

Figure 10. Ultra-violet absorbance spectra of the synthetic (sMNP), intracellular (iMNP) and extracellular (eMNP) melanin nanoparticles

For example, melanin pigments synthesized by *Auricularia auricula* showed maximum UV absorption at 215 nm [33] while the maximum absorption of purified melanin pigments from *Actinoalloteichus* MA-32 and

Chroogomphus rutilus was observed at 300 nm [34,35]. Low solubility of melanin in distilled water in most organic and inorganic solvents, except-aqueous alkali, and resistance to degradation by concentrated acids are distinctive characteristics of this pigment. In this study, a significant similarity was reported between the extracted and synthetic melanin pigments. All these pigments were insoluble in water and organic solvents (chloroform, ethyl acetate, ethanol, methanol, acetic acid, ether, petroleum ether, hexane and acetone) and soluble in alkali only (Table 3).

4. Conclusion

Nowadays, disposal of food waste is one of the problems in industries. Fruit and vegetable wastes support microbial growth as they are rich in soluble sugars and micronutrients. In this study, potential use of melon, watermelon and carrot peels as well as whey and molasses were investigated for supporting microbial growth and producing melanin. In summary, carrot and watermelon peel extracts were the fermentation media that most favored growth of *Aureobasidium pullulans* AZ-6 and *Aureobasidium pullulans* NBRC 100716 strains, respectively. Only *Aureobasidium pullulans* NBRC 100716 could produce intracellular and extracellular melanin. Of the food wastes in the study, the highest melanin production was linked to carrot peel extract. It is important to transform food wastes into high value-added products. The major reasons for this importance are environmental pollution and economic losses caused by the wastes. This study contributed to the transformation of carrot peel, one of the carrot processing industry wastes, into a product with high added values. Although studies have been carried out on characterization of fungal melanin, a little scientific understanding of melanin synthesized by *A. Pullulans* is reported. This study has a critical role in molecular and physicochemical characterizations of melanin nanoparticles extracted from this species. The FTIR results in this study showed that intracellular and extracellular nanoparticles synthesized by *Aureobasidium pullulans* NBRC 100716 and synthetic melanin spectra were similar. In addition, UV absorbance and solubility test results of MNPs synthesized by *Aureobasidium pullulans* NBRC 100716 and those of synthetic MNPs were quite similar.

Table 3. Comparison of the physicochemical properties of melanin produced by *A. pullulans* NBRC 100716 in the carrot peel extract with synthetic melanin

Physicochemical properties	Intacellular melanin	Extacellular melanin	Synthetic melanin
Color	Black	Black	Black
Solubility			
Distilled water	-	-	-
1mol/L KOH	+	+	+
1mol/L NaOH	+	+	+
Chloroform	-	-	-
Ethyl acetate	-	-	-
Ethanol	-	-	-
Methanol	-	-	-
Acetic acid	-	-	-
Ether	-	-	-
Petroleum ether	-	-	-
Hexane	-	-	-
Acetone	-	-	-

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6. Conflict of Interest

The authors report no conflicts of interest.

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ملانین طبیعی ساخته شده توسط *آروباسیدیوم پولولانس* با استفاده از ضایعات مواد غذایی و ویژگی‌های آن

گامز نور ماج دسی

دانشکده مهندسی غذا، دانشگاه هیتیت، کوروم، ترکیه

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واژگان کلیدی

- *آروباسیدیوم پولولانس*
- تعیین ویژگی‌ها
- ضایعات مواد غذایی
- ملانین

***نویسنده مسئول**

گامز نور ماج دسی
دانشکده مهندسی غذا، دانشگاه هیتیت، کوروم، ترکیه
تلفن: ۰۰۹۰۵۴۴۶۹۶۳۴۴۶

پست الکترونیک:
gnurkara@hitit.edu.tr

چکیده

سابقه و هدف: ضایعات مواد غذایی ضررهای اقتصادی و مشکلات زیست محیطی را موجب می‌شود. از این رو، امکان تبدیل ضایعات مواد غذایی به فرآورده‌هایی با ارزش افزوده بالا بسیار قابل توجه می‌باشد. هدف این مطالعه، تولید رنگدانه ملانین که کاربرد بالقوه گسترده‌ای در صنایع کشاورزی، آرایشی و دارویی دارد، به روش تخمیر با استفاده از ضایعات خانگی مانند پوست طالبی، پوست هندوانه و پوست هویج و فرآورده‌های جانبی صنعتی مانند آب پنیر و ملاس می‌باشد.

مواد و روش ها: دو سویه *آروباسیدیوم پولولانس* برای تولید ملانین مورد بررسی قرار گرفتند. به‌منظور شناسایی نانووذرات ملانین تولید شده، آزمون‌های طیف بینی مادون قرمز تبدیل فوریه[۱]، میکروسکوپ الکترونی روبشی[۲]، پتانسیل زتا، جذب فرابنفش و حلالیت انجام شد.

یافته‌ها و نتیجه‌گیری: بالاترین غلظت درون سلولی ($^{-1}$0/18g l) و خارج سلولی ($^{-1}$3/52 g l) توسط *آروباسیدیوم پولولانس* NBRC 100716با استفاده از عصاره پوست هویج به‌عنوان محیط کشت تخمیر تولید شد. نتایج نشان داد در مقایسه با ملانین مصنوعی، به‌عنوان استاندارد، نانوذرات تولید شده مورد تایید قرار گرفت. اندازه ذرات نانوذرات ۷۶۰-۱۰ نانومتر وبدون بار منفی بود، همان‌طور که در منابع علمی قبلی پیشنهاد شده بود. نتایج نشان داد پوست هویج انتخاب مناسبی برای تولید ملانین با ارزش افزوده بالاست. هنگامی‌که عصاره پوست هویج به‌عنوان محیط کشت تخمیر مورد استفاده قرار گرفت، ویژگی‌های ملانین تولید شده توسط سوش *آروباسیدیوم پولولانس* NBRC 100716شبیه ملانین سنتزی بود.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[۱] Probiotic agents

[۱] Fourier transform infrared spectroscopy or FTIR

[۱] Scanning electron microscope or SEM