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Isolation and characterization of piceatannol producing bacteria from soil in Erzurum, Turkey

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Abstract

Piceatannol, resveratrol's derivative, and a valuable polyphenol has managed to become one of the most remarkable candidate molecules for drug development research, with its high bioactive properties and higher stability. On the other hand, the very low amount of piceatannol in plants which are its natural source increases the cost and limits the commercialization possibilities of the product. To overcome this bottleneck, a limited number of studies have recently shown that it is possible to produce piceatannol from the resveratrol precursor much cheaper by regioselective hydroxylation catalyzed by bacteria isolated from the soil, and the search for new bacteria of similar nature in new ecosystems has gained popularity. The aim of our study, which was prepared within this framework, is the bacterial isolate with regioselective hydroxylation potential obtained as a result of selective isolation steps; determination of resveratrol hydroxylation potentials and piceatannol product yields, investigation of possibilities to increase piceatannol yield with optimization trials and identification of isolates with the highest yield. For this purpose, 200 bacterial isolates capable of resveratrol hydroxylation were obtained from soil samples taken from Erzurum (Turkey) and its surroundings by using selective media. In the continuation of the study; resveratrol hydroxylation trials were carried out with these isolates and 55 active isolates capable of producing piceatannol by regioselective hydroxylation were selected. Then, yield improvement studies of active isolates were carried out by using different carbon sources and optimizing the culture conditions. As a result, a culture collection was created by identifying the 6 most active bacterial isolates with commercialization potential using conventional and molecular methods. These are 4 Gram-positive (*Rhodococcus sp., Rhodococcus erythropolis, Paeniglutamicibacter sp., Arthrobacter sp.*) and 2 Gram-negative (*Shinella sp., Ensifer adhaerens*) bacterial isolates. As a result of the optimization studies, three of these isolates used phenol as a biocatalyst, while the other three increased the production yield of piceatannol by using 4-hydroxyphenylacetic acid.

Keywords: HPLC, Hydroxylation, Piceatannol, Resveratrol, Rhodococcus, Shinella.

1. Introduction

Piceatannol is a compound commonly found in fruits such as passion flower (*Passiflora edulis*), white tea, sugarcane, grapes, and blueberries. Despite the similarity of resveratrol with its molecular structure, piceatannol has been shown in recent studies to be more effective than resveratrol, as well as to exhibit beneficial properties for health [1-9]. This compound is known to be a powerful antioxidant as well as having cell cycle arresting, antitumor, antiobesity, antileukemic, antithrombotic, antihyperlipidemic, antiparasitic, and immunosuppressive properties in cancer, and is accepted as a potential antiarrhythmic agent [2-4, 7, 8, 10-13]. But piceatannol, like resveratrol, is found in very low concentrations in post-harvest grape canes. It has been reported in recent studies that piceatannol is approximately 0.78 µg/g in the edible part of the grape plant [12,14]. However, recent studies have shown that piceatannol is more stable than resveratrol, has higher bioavailability, meaning that it stays in vivo longer and thus has a more efficient scavenging activity of free radicals [5, 7, 9,15].

The suitability of genetic manipulation and the tolerance of heterologous enzymes enable microorganisms to produce a variety of nutraceuticals using their natural metabolic networks. Microbial production methods make it possible to produce complex natural products on an industrial scale from simple carbon sources. Microbial production methods have been developed as an environmentally friendly alternative approach for the production of value-added nutraceuticals from simple carbon sources [16]. In current studies on this subject, it is stated that the fermentation and production of an anti-cancer agent for regiospecific hydroxylation of resveratrol is carried out by a microbial process [17]. Among the strains examined in

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this current study, the effective one showed high regiospecific hydroxylation activity to produce piceatannol, and *Streptomyces* sp. It is reported that it produces 205 mg of piceatannol (60% yield) from 342 mg of resveratrol in 20 hours for the strain [17].

Biotechnological methods for the production of piceatannol have shown promise for their cost-effectiveness and environmental friendliness. In particular, biocatalysts that provide the hydroxylate resveratrol to piceatannol are useful in the production of piceatannol. While the biocatalytic method provides regioselective hydroxylation of resveratrol to piceatannol in only one step, several steps are required for chemical conversion of resveratrol to piceatannol (Figure 1) [18-20]. The enzymes that catalyze this reaction are cytochrome P450 enzymes, tyrosinases and bicomponent flavin-dependent monooxygenases [20-27].

1.1. Resveratrol hydroxylation

Natural stilbenes such as resveratrol and piceatannol are secondary metabolites found in plants. The hydroxylation of secondary metabolites increases the solubility, stability, structural variation, various pharmacological and biological activities of the compounds. On the other hand, it is known that the site-specific hydroxylation of complex aromatic compounds is quite difficult for chemical synthesis. Various studies have reported that biocatalytic hydroxylation of various plant components such as resveratrol has identified some cytochrome P450 hydroxylases, but the biotransformation efficiency and production scale are still low [26, 27].

Although chemical hydroxylation approaches have been widely used to activate, derivatize and functionalize natural compounds, regioselective hydroxylation on complex aromatic compounds is still challenging for chemical treatments due to low selectivity. In contrast, biocatalytic hydroxylation is known to provide an easy and environmentally friendly way to transfer specific oxygen. Various studies conducted in previous periods also drew attention to P450 hydroxylases as the enzyme group was designed and researched for this purpose. It has been reported in recent studies that the enzyme that catalyzes the conversion of resveratrol to piceatannol is piceatannol synthase (cytochrome P450 enzyme, CYP1B1) [3, 10, 26].

The ability of bacterial tyrosinases to catalyze o-hydroxylation reactions plays a role in the biocatalytic conversion of resveratrol to piceatannol. Various studies have been carried out using enzymes (P450 monooxygenases) to convert resveratrol to piceatannol, but it is known that this often results in poor efficiency. In a study, an alternative approach was investigated in the regioselective hydroxylation of trans-resveratrol by the tyrosinase enzyme obtained from the *Streptomyces avermitilis* MA4680 strain and instead of using a reducing agent that could be costly on an industrial scale, they used phenolic compounds as inhibitor compounds. As a result of this study, they concluded that the highest relative production was obtained in the presence of 1 mM catechol (100%), followed by 1 mM hydroquinone (97.1%), 1 mM NADH (64.2%) and 1 mM L-ascorbic acid (63.7%) [28, 29].

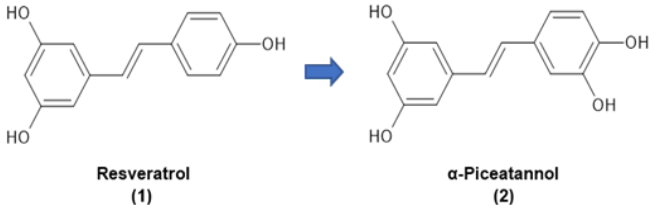

Fig. 1. Biotransformation of resveratrol to piceatannol.

In this study, the potential of bacterial organisms obtained from soil samples taken from Erzurum and its surroundings using selective medium to produce piceatannol from resveratrol was investigated. In this context, microorganisms that use resveratrol, phenol or 4-hydroxyphenylacetic acid as carbon sources were first isolated. These isolated microorganisms were used for the hydroxylation of resveratrol and various bacterial isolates that provide the biotransformation of resveratrol were obtained. Consequently, two Gram-negative bacteria and one Gram-positive bacteria using 4-hydroxyphenylacetic acid as a carbon source, and three Gram-positive bacteria using phenol as a carbon source, exhibited high resveratrol-hydroxylation activity. These isolates showing high activity were identified and saved into the National Center for Biotechnology Information (NCBI) gene bank.

2. Material and Methods

2.1. Procurement of organisms used in the study

Bacterial isolates capable of producing piceatannol from regioselective hydroxylation from resveratrol were obtained from 150 soil samples taken from Erzurum and its surroundings, using selective media according to the method suggested by Furuya *et al.* (2019) [20]. According to this; Soil samples were first suspended in physiological saline and then each sample was inoculated into KG Medium supplemented with 4-hydroxyphenylacetic acid, which is a selective medium used for the isolation of bacteria with hydroxylation potential.

2.2. Chemicals and cultivation medium

Resveratrol was purchased from abcr (Karlsruhe, Germany) and piceatannol was purchased from Thermo Scientific (USA). Tween 80 and Triton X-100 were purchased from Sigma-Aldrich (USA). KG medium contained (per liter) $(NH_4)_2SO_4$ (3 g), KH_2PO_4 (1.4 g), Na_2HPO_4 (2.1g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeCl_2 \cdot 5H_2O$ (10.6 mg), $CaCl_2 \cdot 2H_2O$ (8 mg), $ZnSO_4 \cdot 7H_2O$ (4 mg), $MnCl_2 \cdot 4H_2O$ (2 mg), $CuSO_4 \cdot 5H_2O$ (0.02 mg), KI (0.2 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.2 mg), $CoCl_2 \cdot 6H_2O$ (0.2 mg), H_3BO_3 (0.4 mg), and NaCl (10 mg) (pH 7.2) [20].

2.3. Isolation of microorganisms and Resveratrol hydroxylation assay

The six isolates were cultivated in KG medium supplemented with various organic compounds as different carbon sources. Cultures were allowed to grow for 7 days at 30 °C with a shaking speed of 240 rpm. At the end of the period, new inoculations were made from the cultures in which turbidity and precipitation were observed into fresh media, and subcultures were created and the 7-day incubation process was repeated under the same conditions. Serial dilutions were made of the subcultures that developed at the end of the period, and inoculations were made on solid media prepared by adding 15 g/L agar to KG Medium with 4-Hydroxyphenylacetic acid from these dilutions. Each colony was observed on the solid medium after incubation of the cultures at 30 °C; It was evaluated as a candidate isolate that can produce piceatannol from

resveratrol by regioselective hydroxylation.

In the preliminary study, the "resveratrol-hydroxylation test" will be used to confirm the piceatannol-producing capacity of the selected isolates and to select the most active ones [20]. According to this;

a. Each isolate is inoculated into 3 different KG media supplemented with 5 mM resveratrol, 5 mM phenol and 5 mM 4-Hydroxyphenylacetic acid, respectively, as the sole carbon source and allowed to grow at +30 °C.

b. After observing colony formation in solid cultures, the reaction medium required for the hydroxylation activity assay is prepared: [live bacterial cells (10-30 mg wet wt/mL), resveratrol (5 mM), DMSO (1% v/v), Tween 80(%) 1 v/v) and potassium phosphate buffer (200 mM, pH 7.5) containing 10% glycerol].

c. The reaction medium is left to incubate at +30 °C with vigorous shaking and 24 hours.

d. At the end of the period, the reaction products are determined by HPLC analysis in terms of content and amount [30, 31]. These data provide reference for validation of bacterial piceatannol production and selection of the most active isolates. In addition, yield information from previous sources in the literature was used for the selection of active isolates [17, 20, 21, 28].

2.4. Conditions of the HPLC method for analysis

Studies in which resveratrol and piceatannol can be analyzed in standard solutions and biological materials by HPLC method have been found in the literature [30, 31]. However, no study could be found for the simultaneous determination of regioselective hydroxylation from resveratrol in a medium-producing piceatannol. For this reason, for simultaneous analysis of Resveratrol and piceatannol by HPLC method; Ace C18 column (5µm, 250 x 4.6mm), methanol-water (60:40, v/v) mobile phase composition, flow rate 1 ml/min, 320 nm wavelength parameters were used.

3. Results

3.1 Isolation of microorganisms

Microorganisms obtained from soil samples were cultivated in KG medium supplemented with 5 mM resveratrol, phenol, or 4-hydroxyphenylacetic acid as a carbon source. After subcultivation of microorganisms from 150 soil samples, we isolated 30, 76, and 94 candidate strains that degrade and utilize resveratrol, phenol, or 4-hydroxyphenylacetic acid, respectively. Microorganisms obtained from soil samples were cultivated in KG medium supplemented with 5 mM resveratrol, phenol, or 4-hydroxyphenylacetic acid as a carbon source. After subculturing microorganisms from 150 soil samples, we isolated 30, 76, and 94 candidate strains that degrade and utilize resveratrol, phenol, or 4-hydroxyphenylacetic acid, respectively. With the hydroxylation experiment performed with these isolates, 55 bacterial isolates producing piceatannol from resveratrol were obtained. With the hydroxylation experiment performed with these isolates, 55 bacterial isolates producing piceatannol from resveratrol were obtained in Table 1. Optimization studies, which is the next step of the research, continued with the bacterial isolates that we

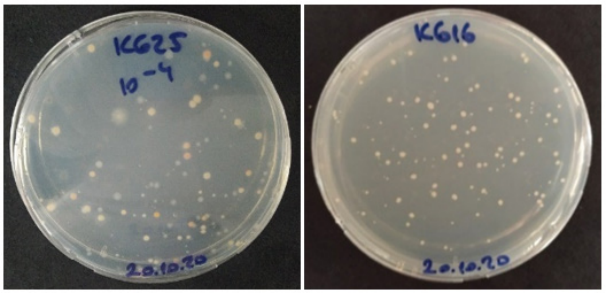

Fig. 2. Bacterial isolate samples obtained using selective media.

Table 1. Piceatannol producing of bacterial isolates.

No	Isolate Code	Amount of Piceatannol (mg/mL)	No	Isolate Code	Amount of Piceatannol (mg/mL)	No	Isolate Code	Amount of Piceatannol (mg/mL)
1	TK-11	0.231	**21**	TK-67	0.003	**41**	TK-135	0.034
2	TK-19	0.002	**22**	TK-69	0.003	**42**	TK-141	0.002
3	TK-23	0.029	**23**	TK-70	0.004	**43**	TK-143	0.003
4	TK-29	0.041	**24**	TK-72	0.011	**44**	TK-144	0.063
5	TK-31	0.052	**25**	TK-73	0.002	**45**	TK-151	0.003
6	TK-36	0.390	**26**	TK-76	0.003	**46**	TK-170	0.001
7	TK-37	0.047	**27**	TK-78	0.033	**47**	TK-173	0.001
8	TK-40	0.891	**28**	TK-80	0.003	**48**	TK-174	0.022
9	TK-42	0.048	**29**	TK-85	0.003	**49**	TK-177	0.002
10	TK-43	0.002	**30**	TK-91	0.042	**50**	TK-179	0.002
11	TK-45	0.732	**31**	TK-92	0.003	**51**	TK-182	0.023
12	TK-46	0.002	**32**	TK-95	0.004	**52**	TK-186	0.010
13	TK-50	0.002	**33**	TK-97	0.002	**53**	TK-189	0.004
14	TK-52	0.003	**34**	TK-100	0.002	**54**	TK-194	0.013
15	TK-53	0.007	**35**	TK-106	0.070	**55**	TK-199	0.004
16	TK-54	0.008	**36**	TK-107	0.022			
17	TK-59	0.005	**37**	TK-109	0.003			
18	TK-60	0.226	**38**	TK-110	0.002			
19	TK-64	0.005	**39**	TK-116	0.012			
20	TK-65	0.690	**40**	TK-133	0.003			

determined to produce piceatannol with high efficiency. The six isolates grown in KG medium were supplemented with various organic compounds as carbon sources. Data for this phase of the study are shown in Table 2. Here, while there was growth in four carbon sources for six isolates, no growth was observed in the remaining six media (Figure 2.).

3.2. Resveratrol Hydroxylation and HPLC Analysis

Findings regarding HPLC analysis after resveratrol hydroxylation are summarized below.

The accuracy and precision of the HPLC method were determined by intraday and interday variables. Intra-day and inter-day studies were carried out by reading the peak areas of the solutions prepared at three different concentrations (75, 2500, 4500 ng mL-1) falling within the calibration curves of resveratrol and piceatannol three times on the same day and on different days (maximum 3 days). By determining the averages and standard deviations of the readings, the precision of the methods as a percentage relative standard deviation (%BSS) and accuracy as a relative error are given in Table 2.

A stability study was carried out to determine the period during which the stock and working solutions of resveratrol and piceatannol remained stable during the study using the HPLC method. For this purpose, solutions of resveratrol and piceatannol prepared at three different concentrations were kept at room temperature, +4 and -20 °C for 24 and 72 hours. At the end of these periods, the peak areas of the solutions were measured and the obtained values were compared with the immediate readings of the standard solutions, and the results are given as percentage recovery in Table 3.

Calibration curves were created by plotting the peak area values against the concentrations of solutions prepared in different concentration ranges of resveratrol and piceatannol. Information about this data is given in Figures 3 and 4. Additionally, the chromatogram of the standard solution mixture of Resveratrol (5 ppm) and piceattannol (5 ppm) [Retention time of resveratrol (3.6 minutes), piceattannol (4.7 minutes)] is given in Figure 5.

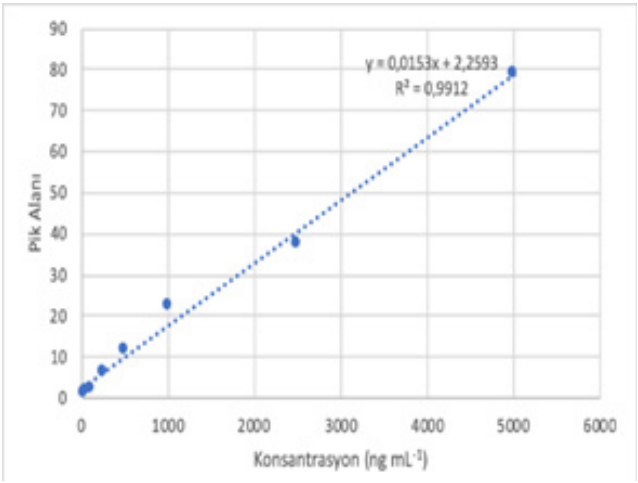

Fig. 3. Resveratrol calibration curve.

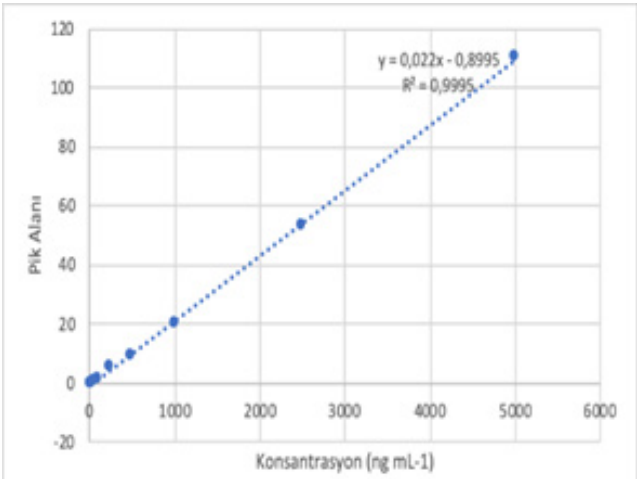

Fig. 4. Piceatannol calibration curve.

Table 2. Accuracy and precision values of the method.

		During the day			Between days		
	Added (ng mL^{-1})	Situated±std. deviation	% relative fail	% SSD	Situated±std. deviation	% relative fail	% SSD
Resveratrol	75	73.4 ± 1.62	-2.13	2.20	72.9 ± 1.57	-2.80	2.15
	2500	2480 ± 86.1	-0.80	3.47	2475 ± 83.2	-0.10	3.36
	4500	4470 ± 172.3	-0.67	3.85	4516 ± 193.4	0.36	4.29
Piceatannol	75	73.9 ± 1.97	-1.47	2.67	73.2 ± 1.84	-2.40	2.51
	2500	2472 ± 89.4	-1.12	3.62	2531 ± 92.7	1.24	3.67
	4500	4516 ± 162	-0.36	3.59	4489 ± 171	-0.24	3.81

Table 3. Stability values of resveratrol and piceatannol.

	Added (ng mL^{-1})	Room Temperature 24 h	Room Temperature 48h	4 °C 24 h	4 °C 72 h	-20 °C 24 h	-20 °C 72 h
Resveratrol	1000	100.2 ± 3.62	98.6 ± 3.55	99.7 ± 3.16	101.3 ± 3.11	101.6 ± 2.67	99.5 ± 3.46
	3000	101.3 ± 2.27	98.5 ± 4.60	102.1 ± 4.08	99.4 ± 3.71	99.5 ± 2.47	98.1 ± 2.67
	5000	101.1 ± 2.96	98.7 ± 2.47	101.4 ± 3.14	97.8 ± 3.01	101.3 ± 4.17	101.4 ± 4.47
Piceatannol	1000	102.6 ± 3.44	98.6 ± 3.12	99.1 ± 2.93	100.4 ± 3.79	98.6 ± 1.84	99.6 ± 3.18
	3000	99.3 ± 3.24	98.9 ± 2.76	100.9 ± 1.98	99.3 ± 2.36	101.2 ± 1.76	99.5 ± 2.97
	5000	100.5 ± 3.46	99.5 ± 1.93	99.6 ± 3.17	99.8 ± 4.42	99.4 ± 3.73	99.3 ± 3.41

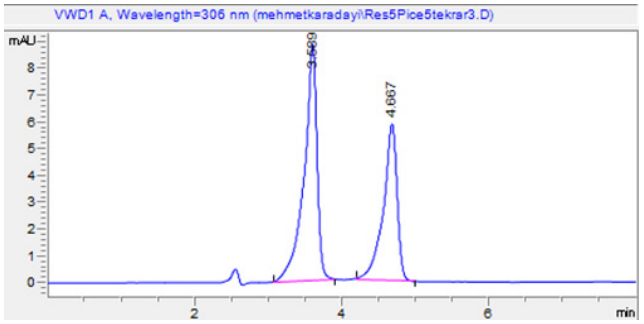

Fig. 5. Chromatogram of standard solution mixture.

3.3. Optimization studies

3.3.1. Effects of different carbon sources on piceatannol producing

The contributions of different carbon sources to piceatannol production in six isolates were discussed in this study. When the study data were examined, it was observed that TK-11, TK-36, and TK-45 produced piceatannol in high yield in the presence of phenol, and TK-40, TK-60 and TK-65 produced piceatannol in high yield in the presence of 4-hydroxyphenylacetic acid (Table 4). No production of isolates was observed in other carbon sources.

3.3.2. Effect of detergents on Resveratrol hydroxylation

In a study conducted in the last decade, it was observed that adding Tween 80 or Triton X-100 detergents to the reaction medium significantly increased the production of piceatannol from resveratrol [21]. Therefore, in this study, the effect of various Tween 80 or Triton X-100 concentrations on piceatannol production was also examined (Table 5). In the absence of detergents, neither isolate was able to convert resveratrol to piceatannol. However, although all isolates efficiently converted resveratrol to piceatannol in the presence of Tween 80, none of the isolates could convert resveratrol to piceatannol in the presence of Triton X-100.

3.3.3. Effect of cultivation time

In the reaction time, which is another optimization condition on yield, piceatannol production was not observed in all bacterial isolates at the 4th hour, and it was observed that the yield results at the 8th and 16th hours were lower than the yield results at the other hours. However, it was observed that the yield results of the TK-11 coded isolate reached the highest level at the 20th hour, the TK-60 coded isolate at the 12th hour, and the other 4 isolates at the 24th hour. Information about this optimization study is given in Table 6.

3.4. Identification of isolates

DNAs of active isolates were isolated using the Promega Wizard® Genomic DNA Purification Kit. Afterwards, the molecular identification process was completed with 16S rRNA PCR application and sent for sequence analysis

Table 4. Effects on piceatannol producing yield of various carbon sources.

Isolates Code	Amount of piceatannol (g/mL)									
	4H	R	F	G	3-H	3,4-H	4-A	B	3HB	4HB
TK-11	0.231	0.183	0.551	0.013	0.003	0	0	0	0	0
TK-36	0.390	0.151	0.497	0.021	0.004	0	0	0	0	0
TK-40	0.891	0.229	0.102	0.007	0.005	0	0	0	0	0
TK-45	0.732	0.576	0.923	0.015	0.004	0	0	0	0	0
TK-60	0.326	0.129	0.024	0.009	0	0	0	0	0	0
TK-65	0.690	0.017	0.011	0.064	0.003	0	0	0	0	0

Table 5. Role of detergents.

Isolates Code	Tween 80				Triton X-100			
	%0,5	%1	%1,5	%2	%0,5	%1	%1,5	%2
TK-11	0.127	0.551	0.113	0.085	0	0	0	0
TK-36	0.159	0.497	0.133	0.064	0	0	0	0
TK-40	0.378	0.891	0.401	0.266	0	0	0	0
TK-45	0.923	0.732	0.311	0.023	0	0	0	0
TK-60	0.048	0.226	0.061	0.025	0	0	0	0
TK-65	0.369	0.690	0.422	0.231	0	0	0	0

Table 6. Effect on cultivation time.

Isolate code	Cultivation time					
	4h	8h	12h	16h	20h	24h
TK-11	0	0.072	0.196	0.375	0.551	0.482
TK-36	0	0.002	0.024	0.106	0.332	0.497
TK-40	0	0.022	0.102	0.321	0.648	0.891
TK-45	0	0.043	0.107	0.353	0.792	0.923
TK-60	0	0.031	0.362	0.312	0.265	0.226
TK-65	0	0	0.062	0.125	0.428	0.690

and bacterial isolates were identified. The data of the PCR application performed are shown in Figure 6. Additionally, these identified isolates were recorded in the NCBI database. Isolates recorded in the NCBI database are shown in Table 7.

3.5. Nucleotide sequence accession number

The nucleotide sequences of the 16S ribosomal DNAs of the 6 isolates (*Rhodococcus* sp., *Rhodococcus erythropolis*, *Paeniglutamicibacter* sp., *Arthrobacter* sp., *Shinella* sp., *Ensifer adhaerens*) obtained were submitted to GenBank under the assigned accession numbers OQ726411, OQ726412, OQ726413, OQ726414, OQ726415, OQ726416, respectively.

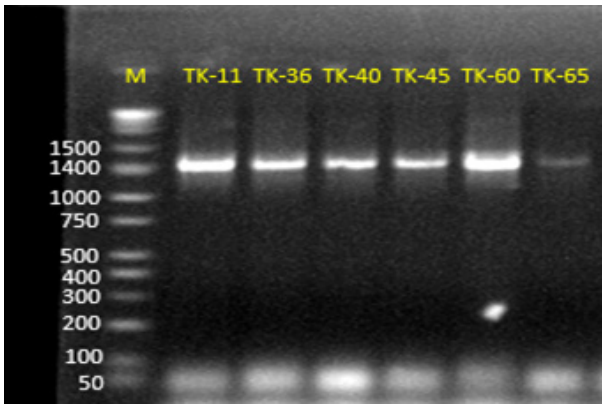

Fig. 6. Results of PCR application of bacterial isolates.

4. Discussion

Natural polyphenols are abundant in fruits, vegetables, whole grains and foods and beverages derived from them, such as chocolate, wine, olive oil or tea, making it the most important source of phytochemicals found in the human diet. It is also known that these compounds are very diverse and include several subgroups of phenolic compounds, ranging from simple substances such as phenolic acids and stilbenes to complex polymerized molecules such as tannins. Natural stilbenes are secondary metabolites produced by plants to protect themselves against stressful conditions such as UV, radiation, extreme heat, and fungal or bacterial infections. These compounds exist as monomers such as piceatannol and resveratrol. Natural stilbenoids such as resveratrol and piceatannol are well-known anti-inflammatory compounds with various biological activities through in vitro and in vivo studies. These two compounds stand out as the most researched plant secondary metabolites so far [1, 32-35].

It is known that resveratrol is widely used in medicine and pharmacy. Today, many plant species contain resveratrol in high concentrations, making resveratrol available relatively cheaply. However, the bioavailability of resveratrol is below 1% and its low stability limits the use of this compound and increases the tendency towards its derivatives such as piceatannol, which is more stable and has high bioavailability. On the other hand, piceatannol is found at very low levels in plants and when obtained through extraction from plants, it becomes a very expensive natural commercial product. For this reason, there is a need for a "more economical piceatannol production process" that has increasingly increased research and commercial application areas and can meet the needs in these areas. At this point, he states that oxidation catalysts that can regioselectively hydroxylate resveratrol to piceatannol can provide a new, efficient and cost-effective synthetic approach.

It is an important fact that screening different microorganisms known to live in various areas is an effective approach for the industrial production of new drugs and bioactive compounds via biocatalytic reactions. For this reason, it has been reported in various studies that microbial processes have become a good model system for developing an industrial biotransformation system to produce drugs and biologically active compounds [17].

From this perspective, the production of piceatannol using biotechnological methods is a promising approach both economically and environmentally. In particular, biocatalysts that hydroxylate resveratrol to piceatannol are very useful in the production of piceatannol by biotechnological methods [20, 36, 37].

It is known that this reaction, which is very promising from an industrial point of view, is catalyzed by cytochrome P450 enzymes, tyrosinases and two-component flavin-dependent monooxygenases. In this respect, soil bacteria, which constitute the microbial source of our project, stand out with their high research and use potential. Furuya *et al.* In a recent study conducted by (2019) [20]; From 135 soil samples, candidate isolates capable of producing piceatannol from a total of 158 resveratrol were selected using selective media in which resveratrol, 4-hydroxyphenylacetic acid and phenol were used as the sole carbon source, and 41 of them were shown to successfully produce piceatannol. Bacterial isolates obtained from this and similar studies are not only valuable natural biocatalysts for the production of biotechnological technologies aimed at the production of piceatannol from resveratrol but also offer unique genetic resources for the development of recombinant strains. For example, it has been shown that recombinant *E.coli* expressing the two-component flavin-dependent monooxygenase HpaBC can effectively produce piceatannol (49 mM, 12 g/L) from resveratrol [36].

Table 7. Molecular diagnostic information and NCBI GenBank® accession numbers of isolates that efficiently produce piceatannol by regioselective hydroxylation from resveratrol.

Isolate Code	Number of Bases Read	Isolates Name	Similarity (%)	Access number	Piceatannol Yield (mg/mL)
TK-11	1053	*Rhodococcus* sp.	99	OQ726411	0.551
TK-36	1012	*Rhodococcus erythropolis*	100	OQ726412	0.497
TK-40	973	*Paeniglutamicibacter* sp.	99	OQ726413	0.891
TK-45	1079	*Arthrobacter* sp.	100	OQ726414	0.923
TK-60	1188	*Shinella* sp.	100	OQ726415	0.362
TK-65	1256	*Ensifer adhaerens*	100	OQ726416	0.690

Current literature information indicates that recombinant microorganisms may be more advantageous for high-yield production applications. However, due to the biosafety and ethical problems that arise in commercial-scale production processes of these organisms, their use in industrial production, especially in the medical, pharmaceutical and food sectors, is quite limited. In such a case, research on the discovery of natural organisms that can do the same job offers more practical and hopeful approaches. Moreover, when the production process is desired to be carried out through the use of recombinant microorganisms, the gene source that will be needed in the production of the recombinant microorganism is the microorganisms to be isolated from nature [20].

However, when the subject is reduced to the production of piceatannol from resveratrol by natural microorganisms, it is clearly seen that there are only a few studies in the literature and there is a very important gap in this field. The problem of low efficiency was encountered in two studies that are considered the pioneers of these studies. Lee and his colleagues succeeded in producing piceatannol with 78% yield from 100 μM resveratrol solution with *Streptomyces avermitilis* in 2012 [28]. Roh and Kang isolated *Streptomyces* sp. from soil. They managed to produce 280 μM piceatannol from 500 μM resveratrol with the SD-14 strain. This experiment was carried out in a 3L scale trial and the molar conversion efficiency was determined as 60% [17]. In the most recent study in this field, Furuya and his colleagues isolated 41 Gram (+) and Gram (-) natural bacteria that can hydroxylate resveratrol to piceatannole from soil samples and found the two with the highest efficiency (*Ensifer* sp. KSH1: 3.6 mM - 0.88 g/L piceatannol yield; *Arthrobacter* sp. KSH3: 2.6 mM – 0.64 g/L piceatannol yield) were used to develop recombinant *E.coli* strains expressing HpaBC. This study is quite remarkable in terms of obtaining natural microorganisms with the highest production efficiency recorded to date on the production of piceatannol from resveratrol with biotechnological approaches and the successful development of effective recombinant strains based on them [20].

In the project study we carried out in the light of all these data, 150 soil samples from 25 different points in Erzurum and its surroundings were brought to the laboratory environment. A total of 200 bacterial isolates with the potential to regioselectively produce piceatannol from resveratrol were obtained from these soil samples using selective media as stated in the literature. These bacterial isolates obtained were used as active isolates required in the next steps of the study. With the subsequent hydroxylation experiment, 55 isolates that regioselectively produced piceatannol from resveratrol were obtained. Among these isolates, studies continued with 20 isolates that showed high yields, and optimization studies were subsequently carried out. As a result of optimization studies, 6 bacterial isolates showed yields equal to or higher than the piceatannol product yield obtained in literature studies. These identified bacteria were recorded in the NCBI database.

5. Conclusion

In this study, we isolated *Rhodococcus* sp., *Rhodococcus erythropolis*, *Paeniglutamicibacter* sp., *Arthrobacter* sp., *Shinella* sp., *Ensifer adhaerens* bacteria that produce piceatannol from resveratrol. For this purpose, microorganisms that can use resveratrol, phenol or 4-hydroxyphenylacetic acid as carbon sources were first isolated.

With this study, when microorganisms using these carbon sources were incubated with resveratrol, microorganisms that could produce piceatannol from resveratrol without the formation of any detectable by-products were obtained. The isolation of both Gram-negative (*Shinella* sp., *Ensifer adhaerens*) and Gram-positive (*Rhodococcus* sp., *Rhodococcus erythropolis*, *Paeniglutamicibacter* sp., *Arthrobacter* sp.) bacteria is noteworthy in that they are different isolates, considering previous studies. Although there were bacteria that convert resveratrol to piceatannol through regioselective hydroxylation, *Rhodococcus* sp., *Rhodococcus erythropolis*, *Paeniglutamicibacter* sp. and *Shinella* sp. have been shown to exhibit such activity for the first time [17, 28]. Moreover TK-11, TK-36, and TK-45 exhibited high hydroxylation activity for resveratrol in the presence of phenol as a carbon source, and TK-40, TK-60, and TK-65 exhibited high hydroxylation activity for resveratrol in the presence of 4-hydroxyphenylacetic acid as a carbon source (Table 4).

Non-ionic detergents such as Tween 80 and Triton In the absence of detergent, neither strain was able to hydroxylate resveratrol. In the screening for resveratrol hydroxylate microorganisms, we added 1% (v/v) Tween 80 to the reaction mixture, which allowed us to isolate the targeted microorganisms. On the other hand, no formation of piceatannol was observed in all experiments using Triton X-100.

With the optimization studies, piceatannol production was not observed in all bacterial isolates at the 4th hour, and it was observed that the yield results at the 8th and 16th hours were lower than the yield results at other hours. However, it was observed that the yield results of the TK-11 coded isolate reached the highest level at the 20th hour, the TK-60 coded isolate at the 12th hour, and the other 4 isolates at the 24th hour.

With the optimization studies, piceatannol production was not observed in all bacterial isolates at the 4th hour, and it was observed that the yield results at the 8th and 16th hours were lower than the yield results at other hours. However, it was observed that the yield results of the TK-11 coded isolate reached the highest level at the 20th hour, the TK-60 coded isolate at the 12th hour, and the other 4 isolates at the 24th hour.

When the study data is examined, it is seen that the isolates produce 0.551 (mg/mL), 0.497 (mg/mL), 0.891 (mg/mL), 0.923 (mg/mL), 0.362 (mg/mL) and 0.690 (mg/mL) from resveratrol, respectively. These production efficiencies are the highest levels reported for the biotechnological production of piceatannol by natural microorganisms, but the *Shinella* strain has been observed to produce piceatannol at lower levels than literature data [17, 28]. Therefore, it is thought that these microorganisms exhibiting high resveratrol-hydroxylation activity may be useful biocatalysts for the bioproduction of piceatannol. Therefore, more studies are needed in this regard, as well as research studies including cloning studies for industrial production.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for

publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Taha Yasin Koc, Medine Gulluce, Mehmet Karadayi did all the steps in the research work, HPLC analyzes were made by Bilal Yilmaz.

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