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Conversion of Citron (*Citrus junos*) Peel Oil by *Enterobacter agglomerans*

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Abstract Citron peel oil was extracted from citron (*Citrus junos*) fruit by steam distillation, and was used as starting material for microbial conversion to synthesize attractive flavor compounds by using *Enterobacter agglomerans* 6L. *E. agglomerans* was isolated from citron peel and was able to metabolize the citron peel oil and grew well (A_{600}: 3.0) on the citron peel oil as the sole carbon source. Multiple terpene metabolites were produced by *E. agglomerans* 6L on M9 salt media with citron oil vapor. The identified bioconversion products from the citron peel oil included trans-2-decenal, octanol, δ-valerolactone, γ-valerolactone, cryptone, hydroxycitronellol, cuminol, and γ-dodecalactone.

Key words: Microbial conversion, citrus byproduct, citron peel oil, *E. agglomerans*

Citron (*Citrus junos*) is a type of citrus fruit, which grows throughout China, Korea, and Japan. It looks much like a small grapefruit and can be either yellowish or greenish. Its flavor is similar to that of a grapefruit, but it has definite overtones of Mandarin orange. Citron has a fresh pleasing scent and a tangy-taste. Since it is not consumed as a raw fruit like orange or grapefruit, it has become very popular as a processed food (i.e. citron tea, seasoning) or as an ingredient for Chinese herbal medicine in Korea. The majority of harvested citron is just pressed by using a cold-press extractor, and the extract is kept for further food processing [5]. However, the yield from fruit pressing is 10–15%, and about 90% of the original fruit on a weight basis becomes waste. Because this waste contains many terpenes, which are toxic to most microorganisms [4, 10], it cannot be readily decomposed [7], causing contamination of the environment. In the present study, we tried to use this waste as a renewable natural resource.

The major volatile components of citron peel oil, extracted by cold-pressing, are *d*-limonene, γ-terpinene, linalool, camphene, myrcene, and phellandrene; *d*-limonene predominates and accounts for 70–75% (on a total weight percent) of citron volatile components, although many other terpenes are present in small quantities [14]. These naturally occurring materials in citron oil are commonly used as flavoring constituents for food and other applications [6].

Increasingly, consumers prefer food products containing natural flavors over those containing artificial (synthetic) flavors, and this has led to an increased demand for natural flavors and fragrances [1]. In this context, the use of a microbial bioconversion process to produce more valuable flavor chemicals from relatively cheap compounds may have economic significance [9]. The resulting products should compete favorably with products derived from essential oils, and offer the advantage over synthetic chemicals in being considered and labelled "natural." Therefore, much research has been conducted on the microbial conversion of terpenes, and consequently well-identified and useful terpene oxidation products have been obtained [2–4, 8, 11]. The microbial conversion of low-valued terpenes to high-valued derivatives has been recognized as a commercial opportunity, however, enthusiasm has been dampened by the multiplicity of products produced and the low product yields due to the relative toxicity of terpenes to most microorganisms [8]

We previously reported that *Enterobacter agglomerans* 6L, which was isolated from citron peel, efficiently utilized limonene as a sole carbon source [10]. Citron peel oil, which contains many terpenes such as limonene, can cheaply be obtained from pressed citron waste. In this study, our objective was to examine the possibility of microbial bioconversion of the constituents of citron peel

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oil to other terpenes of interest. *E. agglomerans* 6L was inoculated to a citron oil containing medium, and the major citron oil conversion products were identified.

MATERIALS AND METHODS

Citron Peel Oil

Citron peel oil was prepared from pressed citron peel waste by steam distillation. Steam was generated by a steam generator (Miura Co., Seoul, Korea), and distillation was carried out for 30 min at 115°C (2 kg f/cm^2).

Microorganism and Its Growth

E. agglomerans 6L was grown in 300-ml nephelo flasks with cleanout port and depressed side arm (Bellco Inc., Vineland, U.S.A.) containing 50 ml of M9 salt medium (Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g per liter, pH 7.0), 0.5 ml of inoculum and citron oil vapor introduction. These flasks were able to allow vapor introduction of citron peel oil into the culture from liquid state citron oil which was contained in the side arm, and the flasks were incubated at 28°C with shaking.

Microbial Conversion

Triple-baffled 300-ml nephelo culture flask with side arm was used for the microbial conversion study (Fig. 1). A 1% inoculum of cells grown in M9 medium on citron oil vapor was introduced into each closed culture flask. The flasks were then cultivated for 12–24 h, 96 h, or 120 h with shaking at 28°C. After incubation, the cells were removed by centrifugation, and the terpene products in the supernatant were analyzed, as described below. Bacterial control flasks contained M9 medium on the bacterial (6L) inoculum without citron peel oil. Citron oil control flasks contained M9 medium without the bacterial inoculum, and citron oil vapor was supplied from the flask side arm.

Product Analysis

After cell removal by centrifugation, the culture supernatant was passed through a Millipore 0.45-μm filter, and the filtrate was extracted with ether (3×0.5 vol). The ether fraction was then evaporated and concentrated under a stream of nitrogen. Control was extracted by using the same procedure. Extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactometry (GC/O).

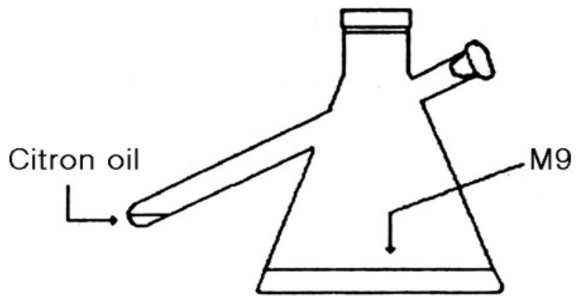

Fig. 1. Bioconversion culture of citron peel oil with triple-baffled naphelo flask.

The GC-MS system used consisted of a mass spectrometer coupled with gas chromatograph QP-5000 (Shimadzu, Japan). A DB-Wax fused silica capillary column (60 m length×0.32 mm i.d.; J&W Scientific Co.) was used for the separation. Conditions were 1 μl injection; He carrier gas; injection port at 230°C and detector port at 250°C; column temperature programmed from 40–230°C at 2°C/min up to 150°C and at 4°C/min up to 230°C with a 3 min initial hold time. Ethyl maltol was used as an internal standard, and positively identified compounds were quantified by using calibration of amount ratio (compound/internal standard) vs peak area ratios (compound/internal standard) under identical experimental conditions [12].

GC/O system consisted of a Varian 3350 (Varian instrument group, Walnut Creek, CA, U.S.A.) equipped with a flame ionization detector (FID) and a sniffing port. GC/O was performed by one trained panelist familiar with the aroma of citron oil and its metabolites. A sample was injected into a fused silica capillary column (DB-wax, 30 m length× 0.32 mm i.d.×0.25 μm film thickness; J&W Scientific Co.). Conditions used were 1 μl injection; He carrier gas; injection port at 200°C and detector port at 250°C; column temperature programmed from 40–200°C at 8°C/min with initial and final hold times of 5 and 30 min, respectively.

Identification of Compound

Compounds were identified by comparing GC-retention indices (RI) [13] and mass spectral data (Wiley 139 and NIST 12, 62) with those of authentic standards.

RESULTS

Flavor Components of Citron Peel Oil

Citron peel oils were extracted from pressed citron peel waste by steam distillation, and its volatile components were analyzed by GC-MS (Table 1). Twenty-two compounds (hydrocarbons and alcohols) were identified. *d*-Limonene was the major component (ca. 54%), and γ-terpinene, linalool, β-myrcene, β-phellandrene, α-pinene, β-pinene, α-farnesene, β-farnesene, and ρ-cymene constituted minor components present at levels exceeding 1% of the total peak area of flavor components. Many other components were also present, but less than 1% level.

Growth in Liquid Culture

The growth of *E. agglomerans* 6L in M9 salt medium with citron oil was examined. Without any additional nutrient

Table 1. Volatile components of the citron peel oil extracted by steam distillation.

No.	Component	RI[a]	Authentic RI	Peak[b] (%)
1	α-Pinene	1011	1010	3.18
2	α-Thujene	1025	1023	0.01
3	β-Pinene	1086	1088	1.66
4	β-Myrcene	1154	1156	5.79
5	Limonene	1196	1196	53.69
6	β-Phellandrene	1210	1213	3.38
7	γ-Terpinene	1226	1223	14.02
8	*cis*-Ocimene	1239	1238	0.45
9	ρ-Cymene	1252	1250	1.3
10	α-Terpinolene	1263	1276	0.96
11	Sabinene hydrate	1460	1463	0.01
12	Linalool	1537	1537	4.17
13	Caryophyllane d	1564	1562	0.13
14	Germacrane b	1571	1572	0.36
15	Terpinen-4-ol	1578	1591	0.33
16	Farnesene cis-β	1639	1636	1.42
17	α-Terpineol	1671	1655	0.81
18	Humulene	1673	1672	0.3
19	α-Amorphene	1691	1691	0.04
20	Farnesene *cis*,*cis*-α-	1699	1697	1.31
21	Germacrene D	1721	1718	0.18
22	Thymol	2098	2100	0.20
23	Unknowns			5.49
sum				100

Steam distillation was carried out at 115°C (2 kg f/cm^2) for 30 min.
[a]Retention indices.
[b]Relative percentage of total peak area.

supplementation, *E. agglomerans* 6L was able to grow well on citron oil as a sole carbon source and reached a high biomass level; i.e., A_{600}: 3.2 after 120 h (Fig. 2). *E. coli* TG1, which is not able to degrade citron oil, was used as a control. *E. agglomerans* 6L was originally isolated from limonene media and a high level of biomass was achieved (A_{600}: 4.5 after 180 h) on limonene as a sole carbon source. In the previous report [10], we found that this strain was also useful for citron peel oil digestion. The observed growth of *E. agglomerans* 6L implies that its broad metabolic capability makes it suitable for digesting limonene or citron peel oil.

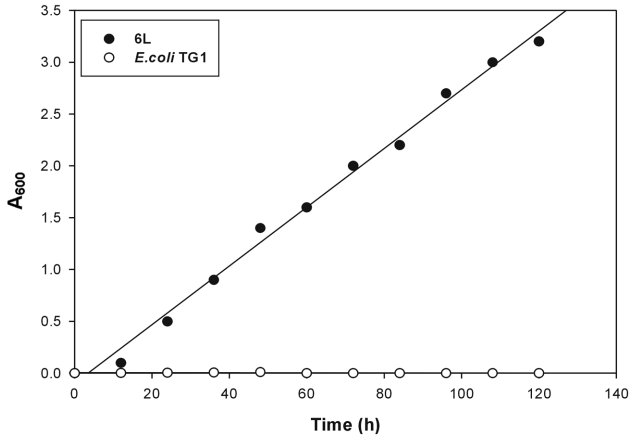

Fig. 2. Growth of *E. agglomerans* 6L in M9 minimal media citron peel oil as the sole carbon source.
Cells were cultured in 300-ml naphelo flasks with a side arm. Citron oil was introduced in the vapor state into the culture from the liquid state contained in the side arm.

Conversion Products of Citron Oil

Terpene compounds are relatively unstable (readily oxidized), and some of the compounds identified in the bioconversion flask were resulted from the atmospheric-oxidation (auto-oxidation) of terpenes. Similar to other instances where the products are produced, it is unclear whether the products were formed as the result of bioconversion or auto-oxidation [15]. Therefore, in this study, the bioconversion flask contained M9 minimal medium, *E. agglomerans* 6L, and citron oil vapor, and the control flask contained M9 minimal medium and citron oil vapor without *E. agglomerans* 6L (citron oil control flask). Another control experiment was carried out by growing *E. agglomerans* 6L in the absence of citron peel oil (bacterial control flask). This will prove that there is no *de novo* synthesis of the detected flavor compounds. The microbial conversion products were defined as the compounds detected only from microbial conversion flasks, but not from the bacterial control or the citron control flasks.

As shown in Table 2, growth of *E. agglomerans* 6L on M9 salt medium with citron oil vapor resulted in the formation of terpene metabolites, which varied in type and quantity with culture time. No accumulation of bioconversion product was observed in 12–24 h culture. Many terpene compounds were detected in the bioconversion flask after 96 h; trans-2-decenal, octanol, γ-valerolactone, cryptone, cuminol, hydroxycitronellol, and several uncharacterized compounds. The aroma of this culture

Table 2. Volatile components produced from citron oil bioconversion by *E. agglomerans* 6L.

Metabolites	Cultivation time (h) and Concentration of products (mg/l)		
	12–24	96	120
Trans-2-decenal	nd*	4.01	0.14
Octanol	nd	11.83	0.66
γ-Valerolactone	nd	1.05	0.39
Cryptone	nd	1.32	3.54
δ-Valerolactone	nd	nd	0.13
Cuminol	nd	0.12	0.16
Hydroxycitronellol	nd	1.13	1.90
γ-Dodecalactone	nd	nd	0.61

*nd : not detectable.

was mild floral, herbaceous, and turpentine. Trans-2-decenol, octanol, γ-valerolactone, cryptone, δ-valerolactone, cuminol, hydroxycitronellol, γ-dodecalactone, and several uncharacterized compounds were produced in the bioconversion flask after 120 h (Table 2). A pleasant floral flavor mixed with pine resin smells were strongly sensed after 120 h of the culture. The control cultures (bacterial control or citron control) after 96 h or 120 h did not produce these metabolites and such smells.

Aroma-Active Compounds from the Bioconversion

Sometimes, instrumental data do not necessarily correspond to sensory data; that is, a compound present at high concentration will not necessarily provide an intense aroma. Therefore, GC/O was conducted in order to verify important aroma-active compounds in the citron oil conversion products. γ-Valerolactone (cut grass smell-by GC/O), cryptone (floral smell-by GC/O) and hydroxycitronellol (floral smell-by GC/O) were likely to contribute to the characteristic flavor of the bioconversion of citron oil, and mass spectra of these three compounds by GC-MS are shown in Fig. 3. They could be smelt through a sniffing test as dominant flavors in the samples extracted from the bioconversion flasks.

The amounts of the aroma-active compounds varied with culture time, however, they were not detectable after cultivating for 12–24 h. The highest yields of γ-valerolactone were obtained after 96 h of culture, and the amount of γ-valerolactone decreased after 120 h of culture and represented only 40% of γ-valerolactone present at 96 h. The highest productions of crypton and hydroxycitronellol were obtained after 120 h of culture (Table 2).

DISCUSSION

To the best of our knowledge, this study constitutes the first report on the microbial conversion of citron peel oil. In the present study, citron oil, a byproduct of the citron processing industry, was used as a raw material for the microbial conversion to high-valued flavor chemicals such as γ-valerolactone, cryptone, and hydroxycitronellol. We demonstrated in the previous paper [10] that *E. agglomerans* 6L was converting pure R-(+)-limonene to γ-valerolactone and cryptone. Citron peel oil is a mixture of various terpene compounds (Table 1), and its major compound (ca. 54%) was confirmed as limonene. These

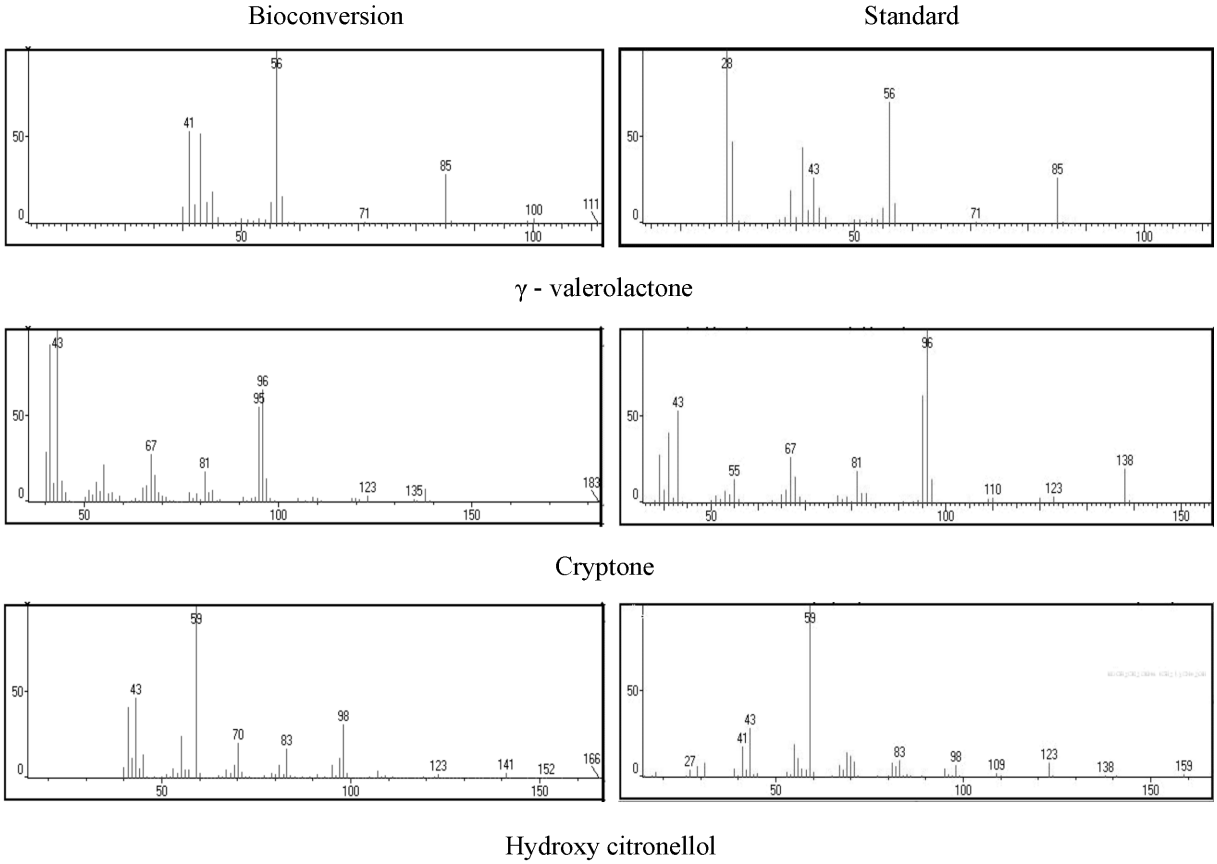

Fig. 3. Mass spectra of γ-valerolactone, cryptone, and hydroxycitronellol; left lane is mass spectra of bioconversion products, and the right lane is mass spectra of standard compounds.

results suggest that the microbial conversion products, γ-valerolactone and cryptone, were produced from limonene, and that the other compounds (trans-2-decenal, octanol, δ-valerolactone, hydroxycitronellol, cuminol, and γ-dodecalactone) should have been produced from the other terpene compounds (Table 1) present in citron oil. *E. agglomerans* 6L has a broad ability to metabolize various terpenes, which are major constituents of citron peel oil. This ability has encouraged a wide interest in *E. agglomerans* 6L for industrial applications. In recent years, commercial demands favor aroma compounds from natural sources. The microbial conversion system described herein provides the basis for the production of unique and high-valued terpenes from waste materials, such as pressed citron peel, thus offering the advantage of providing natural sources. Future strategies for controlling the number and concentration of terpene metabolites in such incubations involve the cloning of genes responsible for the citron oil-degrading pathway in *E. agglomerans* 6L.

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