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Microbial Conversion of (+)-Limonene by an *Enterobacter agglomerans* Isolate

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Abstract *Enterobacter agglomerans* 6L was isolated from citron (*Citrus junos*) peel by using an enrichment culture containing (+)-limonene. It was able to metabolize limonene and grew well (A_{600}: 4.5) on limonene as a sole carbon source. *E. agglomerans* 6L was highly resistant to limonene toxicity, and grew to 1.0 optical density (A_{600}) even at 5% (v/v) of limonene in Luria-Bertani media. γ-Valerolactone and cryptone were detected as the major metabolic products of limonene by *E. agglomerans* 6L.

Key words: Microbial conversion, citron peel, *Enterobacter agglomerans*, (+)-limonene

The monocyclic terpenoid (+)-limonene (4-isopropyl-1-methylcyclohexene) is an attractive terpene starting material for microbial bioconversions to synthesize higher value flavor constituents which are utilized in flavor and perfume applications [10]. This terpene is commonly found in many essential oils and is the major component of oils derived from citrus peel waste [1]. Several investigators have found that limonene can be converted into more valuable flavor compounds such as α-terpineol, perillyl alcohol, perillyl aldehyde, and carvone by using microorganisms [2, 4, 5, 10]: these converted flavor products may be used as flavorings and antimicrobial agents in foods and pharmaceuticals [1, 8]. Such studies have not yet been successful to attain the commercial production scale, due to relative toxicity of limonene to most microorganisms and multiplicity of limonene metabolites formed [3, 7, 14]. Chang *et al.* demonstrated [4, 5] that *Bacillus stearothermophilus* BR388 and its clone *E. coli* EC 409A were able to grow on limonene as the sole carbon source. However, the amount of desirable monoterpene products produced by the strains BR388 and EC 409A are too low to stimulate any commercial interest. It is presumed that the major reason for this is due to relative toxicity of limonene or its metabolites, resulting in low biomass and low levels of the resultant monoterpene products. Therefore, the development of strains highly resistant to limonene and yielding high biomass levels on limonene as the sole carbon source are necessary to carry out the limonene bioconversion successfully.

The objectives in this study were to isolate microorganisms, which are highly resistant to (+)-limonene and can utilize this monoterpene efficiently as the sole carbon source, and subsequently to identify the major limonene metabolites.

Microorganism Isolation

Microorganisms capable of growing on limonene were isolated by inoculation of small pieces of citron (*Citrus junos*) peel into 50 ml of M9 salt media (Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g per liter) with 1 ml of (+)-limonene (97%, Sigma-Aldrich Co., U.S.A.) in a 125 ml screw-cap bottle, and incubation at 30°C while shaking. After 24 h incubation, 100–200 μl of the culture were plated on a M9 salt agar media (noble agar, Difco, Inc., U.S.A.) in Petri dishes, containing 100 μl of limonene in a small glass tube attached to the cover, and incubation was continued at 30°C. Cultures showing growth on repeated transfers were retained as putative limonene users.

Several microorganisms capable of degrading limonene were isolated from limonene enrichment culture by using a citron peel inoculum. To confirm growth on limonene as the sole carbon source, the isolates were repeatedly cultured on solid or liquid minimal media with limonene. Finally, three colonies, designated as 6L, 6S, and 15, were isolated as limonene-degrading microorganisms. Three isolates produced small creamy colonies at 28°C in 2–3 days on M9 minimal plates and utilized limonene as the sole carbon source. The isolates were rod shaped, and Gram-negative strains by Gram staining and microscopic observation. Metabolic fingerprinting of the isolates was carried out by using a Microlog III system (Biolog Inc., U.S.A.), and the

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three isolates, 6L, 6S, and 15, were identified as *Enterobacter aggromerans*, *Xanthomonas maltophilia*, and *Klebsiella pneumoniae*, respectively.

Resistance to Limonene

To determine their relative resistance to limonene, the isolates were cultured in LB broth (bacto-tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g per liter) containing (+)-limonene in the range of 0-5% (v/v) concentrations. Growth was measured by determining turbidity level at 600 nm in a spectrophotometer (corrected for medium absorbance). *E. coli* TG1, which is not able to degrade limonene, was used as a control microorganism.

Limonene is toxic to many microorganisms, even at low concentrations of the flavor compound [3-5, 9, 11]. To test the ability of the strains to tolerate limonene, we observed their growth rate in LB media containing limonene at concentrations in the range of 0.0-5.0% (v/v) (Fig. 1). It is noted that the concentration of limonene shown in Fig. 1 was not corrected for losses due to volatilization. The growth of *E. coli* TG1, which has no limonene-degrading pathway, dramatically decreased upon exposure to limonene vapor, indicating that the strain was very sensitive to limonene. However, the growth of the three isolates (6L, 6S, and 15) was maintained over the 0.0-5.0% (v/v) range of limonene, thus demonstrating that these strains were highly resistant to limonene. In particular, *E. aggromerans* 6L showed relatively high growth (A_{600}: 1.0) at 5% (v/v) limonene concentration. In contrast to the previous observation that the growth of *B. stearothermophilus* BR388 rapidly decreased at above 0.15% (v/v) limonene concentration and eventually stopped growing (A_{580}: 0.0) at 0.2% (v/v) concentration [5], the above three isolates in this study were highly resistant to limonene.

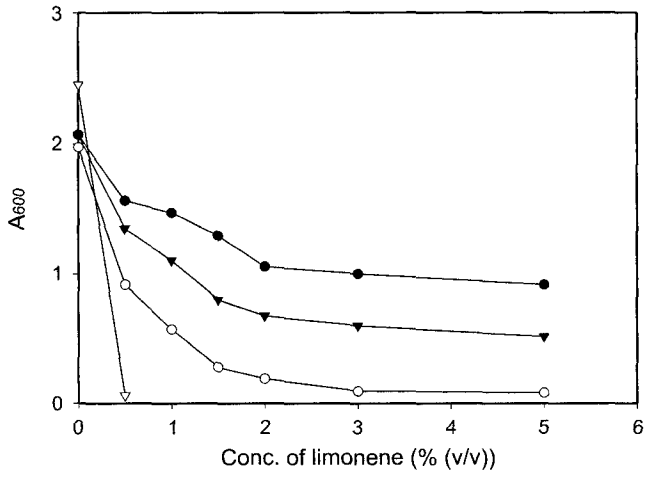

Fig. 1. Growth inhibition of the three isolated strains 6L (●), 6S (○), and 15 (▼) by (+)-limonene.
E. coli TG1 (▽) was tested as a control. Limonene was added directly to LB media at concentrations of 0-5% (v/v).

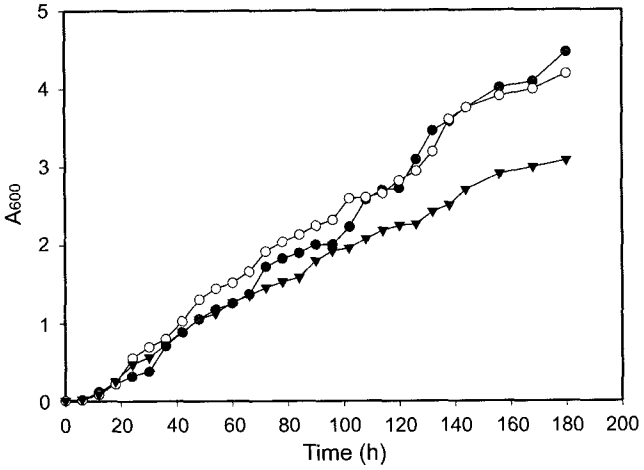

Fig. 2. Growth of three isolated microorganisms in M9 salt media using limonene as the sole carbon source.
Cells were cultured in 250 ml naphelo flasks with a side arm. (+)-Limonene vapor was introduced into the culture from liquid state limonene in the side arm. Symbols: ●, Strains 6L; ○, 6S; ▼, 15.

Growth in Liquid Minimal Media

Triple-baffled 300 ml of nephelo culture flasks with cleanout port and depressed side arm (Bellco Inc., Vineland, U.S.A.) were used for studies on growth and limonene metabolism of the isolate. These flasks made it possible to introduce (+)-limonene as a vapor from liquid limonene into the culture in the side arm.

Growth of the three isolates in M9 salt medium containing limonene was measured, and high levels of biomass were archived by all three isolates (A_{600}: 3.0-4.5, Fig. 2). Without any additional nutrient supplementation, the three isolates could grow well only on limonene as a sole carbon source. But microorganisms in other reports [2, 4, 5], which contained limonene metabolism, were able to grow on limonene as a sole carbon source, where biomass levels were modest. To enhance their growth, the above authors reported that some additional nutrient (yeast extract) or high level inoculum was needed. *B. stearothermophilus* BR388 [5] and its clone *E. coli* EC409A [4] showed 0.07-0.08 at A_{580} cell growth with 0.125 g/l and 0.06 g/l yeast extract supplementation, respectively, and *Pseudomonas gladioli* [2] showed 10^9 CFU with 17% (v/v) inoculum in minimal salt medium containing limonene. These results demonstrate that these three isolates in the present study had much improved limonene metabolism compared with those referred to in the previous reports [2, 4, 5].

Production of Monoterpenes

For the studies on microbial conversion of limonene, 1% (v/v) inoculum of cells grown in M9 salt medium on limonene vapor was introduced into 50 ml of the M9 salt medium with limonene, in the side arm of a 300 ml closed culture flask. Cultures were incubated at 28°C in a shaking

incubator, and limonene metabolites were analyzed after 12-24 h, 96 h, and 120 h of incubation. Control flasks contained 50 ml of the M9 salt medium and 1% inoculum. Duplicate fermentation and control flasks were used with duplicate assays per flask.

Cultures were centrifuged at 12,800 ×g for 20 min at 4°C, and passed through a Millipore 0.45 mm filter. The filtrates were extracted with ether (3×0.5 vol.), and the ether fraction was evaporated to ~10 ml under a gentle nitrogen stream, dried over 3 g of anhydrous sodium sulfate, and further concentrated to 100 µl. Control flasks were extracted by the same procedure as above. Products were then analyzed by gas chromatography-mass spectrometry (GC-MS), using ethylmaltol (Sigma-Aldrich Co., U.S.A.) as an internal standard. The GC-MS system consisted of a mass spectrometer coupled with gas chromatograph QP-5000 (Shimadzu, Japan), and a DB-wax fused silica capillary column (60 m length×0.32 mm i.d., J&W Scientific Co.) was used for separations. The conditions used were; 1 µl injection, He carrier gas, injection port at 230°C and detector port at 250°C, and column temperature was programmed from 40-230°C at 2°C/min up to 150°C and then at 4°C/min up to 230°C with a 3 min initial hold time. Compound identifications were made by comparing GC-retention indices (RI) [12] and mass spectral data (Wiley 139 and NIST 12, 62) with those of authentic standards.

One µl each sample was taken from the culture extracts for GC-MS analysis, and an olfactory test (a sniffing test) was performed for the overall sample aroma. Grassy and fresh floral aromas were smelt in the culture extract of 6L and a floral aroma with chemical reagent note from 6S and 15. Based on these findings, 6L was selected for the studies of limonene metabolism. Partial 16s rDNA sequence determination confirmed 6L as *E. aggromerans* with 99% identity.

Various metabolites were produced by *E. agglomerans* 6L from limonene. Their types and amounts varied with culture time (Table 1): γ-valerolactone and cryptone were produced as the major conversion products of limonene. The microbial conversion products in the medium were detected after 96 h of cultivation and they accumulated at peak levels after 120 h of cultivation. Fresh floral and woody flavors were strong in the 120 h culture, whereas control cultures did not produce these metabolites.

Table 1. Some metabolites produced by *E. agglomerans* 6L from limonene.

Cultivation time (h)	Metabolites and concentration (g/l)	
	γ-Valerolactone	Cryptone
12-24	Not detectable	Not detectable
96	3.0	0.4
120	2.9	1.5

The microbial conversion of limonene by *E. agglomerans* 6L provides a high potential for natural production of more valuable flavor compounds, such as γ-valerolactone and cryptone. A stable and inexpensive sources of γ-valerolactone and cryptone may facilitate further research on their potential usefulness to the flavor and fragrance industries.

The metabolic ability of *E. agglomerans* 6L, including limonene, resulted in the production of monoterpenes of biotechnological interest. *E. agglomerans* 6L was isolated from citron peel, which contains limonene as well as many other terpenoid compounds [6, 13]. Further studies on the metabolic capability of *E. agglomerans* 6L to digest citron oil, and to identify the major conversion products will allow us to target potential biotechnological applications for the *E. agglomerans* 6L in the citrus industry.

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