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Microbial production of neryl- α -D-glucopyranoside from nerol by *Agrobacterium* sp. M-12 reflects glucosyl transfer activity

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ABSTRACT

Terpene alcohol is widely used in perfumes and is known to possess antibacterial activity. Moreover, in its glycosylated form, it can be applied as a nonionic surfactant in food, and in the pharmaceutical, chemical, cosmetic, and detergent industries. Presently, chemical production of terpene glucosides is hampered by high costs and low yields. Here, we investigated the microbial glucosylation of nerol (*cis*-3,7-dimethylocta-2,6-dien-1-ol), a component of volatile oils, by *Agrobacterium* sp. M-12 isolated from soil. A microbial reaction using washed cells of *Agrobacterium* sp. M-12, 1 g/L of nerol, and 100 g/L of maltose under optimal conditions yielded 1.8 g/L of neryl- α -D-glucopyranoside after 72 h. The molar yield of neryl- α -D-glucopyranoside was 87.6%. Additionally, we report the successful transglucosylation of other monoterpene alcohols, such as geraniol, (-)- β -citronellol, and (-)-linalool, by *Agrobacterium* sp. M-12. Thus, microbial glucosylation has potential widespread applicability for efficient, low-cost production of glycosylated terpene alcohols.

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Terpene alcohol glycosides are known components of grape, tea, rose, and other plants. They are utilized mainly in perfumes [1], whereas their glycosides have wider uses as flavor precursors, antimicrobials, anti-fungal agents, and detergents. Not surprisingly, they have attracted increasing attention and a search for efficient production methods [2,3].

In plants, glycosides are synthesized via phosphorylation of a sugar. The aglycone and the sugar are biosynthesized separately, and the sugar is phosphorylated by adenine triphosphate. Then, the phosphorylated sugar reacts with uridine triphosphate to produce uridine diphosphate (UDP) sugar, which is characterized by an energy-rich anhydride. Eventually, this UDP sugar is coupled with the aglycone to produce the glycoside and a free UDP. Based on these mechanisms, several approaches for UDP sugar-driven glucosylation have been developed, including those employing quercetin 3-*O*-rhamnoside [4] and apigenin 7-*O*-glucoside [5]. However, these techniques are hampered by low productivity, which has limited their practical use.

Glycosides are also known to be synthesized by chemosynthesis, via the Koenigs-Knorr reaction and Schmidt glycosylation. These techniques, however, require expensive reagents such as silver (I) carbonate, as well as complex, multistage protecting and deprotecting steps.

Enzymatic glucosylation (reversed hydrolysis and transglucosylation) catalyzed by glycosidases

represents an attractive method to form glycosidic bonds without the need for costly cofactors, such as UDP, or detailed knowledge of chemical synthesis [6,7]. Preparation of glycosides by transglucosylation benefits from a short reaction time and higher yields. Some glycosides, such as hesperidin glycosides, 2-*O*- α -D-glucopyranosyl-L-ascorbic acid and arbutin, are already produced at an industrial scale by transglucosylation using bacterial glycosidases [8–10].

A few reports have described the enzymatic production of terpene alcohol glycosides by transglucosylation. Terpene glucosides have been synthesized by *Escherichia coli* expressing *Pichia etchellsii* β -glycosidases starting from terpenes, such as nerol, geraniol, and citronellol, plus cellobiose [11]. However, the structure of these terpene glucosides has not been determined. Gunata et al. [12] have described the glucosylation of the above terpene alcohols by β -glycosidases from *Aspergillus niger*, *Trichoderma reesei*, *Candida molischiana*, and almond, using cellobiose as carbohydrate donor. However, the yield of monoterpene glucoside from monoterpene was unattractively low (< 4%). The low yield occurred due to the nature of the enzyme and the accumulation of glucose in the reaction mixture following hydrolysis of cellobiose. The enzymatic transfer of the glucosyl group from cellobiose to terpene alcohols is less efficient than that of phenolic alcohols, such as benzyl alcohol and 2-phenylethyl alcohol. Donho et al. [13] showed that

β -galactosidase from *Aspergillus oryzae* exhibited glucosylation activity towards nerol, using lactose as carbohydrate donor and leading to the accumulation of neryl- β -D-galactopyranoside. However, the amount and yield of the product obtained by the enzymatic reaction was not reported.

The α -glucosidase activities of soil bacteria during the synthesis of α -D-glucopyranoside by transglucosylation have been described previously [14]. We reported that *Ensifer* sp. M-26 could glycosylate 6-gingerol, the active ingredient in ginger, to 5-O- α -glucosylgingerol using maltose as a less costly glycosyl donor [15]. While screening for microorganisms with alkyl alcohol glycosylation activities [16], we discovered a strain, *Agrobacterium* sp. M-12, capable of glycosylating terpene alcohol.

The present study reports the synthesis of nerol glucoside via glucosylation of nerol by *Agrobacterium* sp. M-12 and describes some of the resulting glucoside's characteristics. We also examine the transglucosylation activity towards other terpene alcohols, such as geraniol, (-)- β -citronellol, and (-)-linalool.

Materials and methods

Chemicals

α -Glucosidase from yeast, β -glucosidase from almond, nerol (*cis*-3,7-dimethylocta-2,6-dien-1-ol), and geraniol were purchased from Wako Pure Chemical Industries (Osaka, Japan). (-)- β -Citronellol was purchased from Tokyo Chemical Industry (Tokyo, Japan). (-)-Linalool was purchased from Sigma-Aldrich (St. Louis, MO, USA). Transglucosidase from *A. niger* and cyclodextrin glucosyl transferase (CGTase) from *Bacillus macerans* were supplied by Amano Enzyme (Nagoya, Japan). Other chemicals were commercially available products and were used without further purification. The glucosides of nerol and geraniol used as standards for high-performance liquid chromatography (HPLC) were prepared by microbial transglucosylation.

Glucosylation of nerol using transglucosidase and CGTase

A reaction mixture (300 μ L) containing 0.15 mg nerol, 30 mg maltose, 20 mM potassium phosphate buffer (pH 7.0), and 2 units of transglucosidase or 0.03 units of CGTase were incubated at 30°C for 24 h. The enzymatic activity was determined by thin-layer chromatography (TLC) or HPLC. One unit of transglucosidase was defined as the amount of enzyme that hydrolyzed 1 μ mol of maltose per minute at 30°C. One unit of CGTase was defined as the amount of enzyme that produced 1 μ mol of β -cyclodextrin per minute.

Culture media and cultivation

Basal medium consisting of 30.0 g maltose, 2.0 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g KH_2PO_4 , 1.0 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g yeast extract, and 2.0 g polypeptone in 1000 mL distilled water (pH 7.0), was used for a jar fermenter culture, unless otherwise stated. In the case of flask cultures, the maltose content was 50.0 g/L. For the seed culture, *Agrobacterium* sp. M-12 was inoculated onto 50 mL of medium in a 200-mL flask and incubated at 30°C for one day with shaking at 180 rpm. The cell suspension from the seed culture was then inoculated in 50 mL of medium in a 200-mL baffled flask and grown on a rotary shaker at 180 rpm and 30°C. The cells precultured in flasks were inoculated in a 1-L jar fermenter (Able Co., Tokyo, Japan) containing 400 mL of basal medium. There, cells were grown under controlled conditions of pH 7.0 adjusted with 2 N NaOH, 30°C, and 400 rpm. The air flow rate was 1 vvm. Bacterial growth was measured at 600 nm using an Amersham Biosciences Ultrospec 3300 Pro (GE Healthcare, Little Chalfont, UK).

Reaction conditions for *Agrobacterium* sp. M-12

Cells harvested from a 0.5-mL aliquot samples from the jar fermenter were washed once with 0.5 mL of 20 mM potassium phosphate buffer (pH 7.0). The washed cells were incubated with 0.5 g/L terpene alcohols such as nerol, 100 g/L maltose, and 20 mM potassium phosphate buffer (pH 7.0) in a total volume of 0.5 mL. The mixture was incubated at 30°C with vigorous shaking and then an equal part of methanol was added to the reaction solution and centrifuged at 15,000 rpm for 4 min. After that, the supernatant was analyzed by TLC and HPLC. A time course for the conversion of nerol to neryl- α -D-glucopyranoside was carried out using 8.0 mL of harvested cells in culture medium and 8.0 mL of reaction mixture composed of 20 mM potassium phosphate buffer (pH 7.0), 100 g/L maltose, and 1.0 g/L nerol. The reaction was performed at 40°C with vigorous shaking.

Analysis of transglucosylation products

The reaction solution was analyzed by TLC on silica gel 60 F₂₅₄ plates of 0.2-mm thickness (Merck, Darmstadt, Germany). Development was performed with a solvent system consisting of 2:2:1 (v/v/v) 1-butanol/2-propanol/water. Separated compounds were detected by spraying 10% H_2SO_4 -methanol on the TLC glass plate and heating at 160°C for 1–2 min. Reaction products were analyzed quantitatively by HPLC using a COSMOSIL® 5C18-AR column (5 μ m, 4.6 mm \times 250 mm; Nacalai Tesque, Inc.,

Kyoto, Japan). Conditions included a solvent:water/methanol ratio of 3/7, a flow rate of 0.5 mL/min, temperature of 40°C, and detection at 215 nm. Under these conditions, nerol and neryl- α -D-glucopyranoside were eluted at 11.3 min and 17.3 min, respectively. The amount of neryl- α -D-glucopyranoside was calculated from a standard calibration curve made using the compound isolated in this study. The product was obtained by extraction with ethyl acetate from the reaction mixture. Ethyl acetate was then distilled away from the extract layer and the crude product was dissolved in methanol. The solution was fractionated by reverse-phase HPLC using a YMC-Pack ODS-A column (10 μ m, 10.0 mm \times 250 mm; YMC co., LTD., Kyoto, Japan) at a solvent:water/methanol ratio of 3/7, flow rate of 1.0 mL/min, room temperature, and detection at 215 nm. Methanol present in the fraction was distilled away, and neryl- α -D-glucopyranoside contained in water was extracted with ethyl acetate. The extract layer was then dehydrated with anhydrous magnesium sulfate, and ethyl acetate was distilled. Finally, the glucoside was obtained as a colorless and transparent liquid. 13 C nuclear magnetic resonance (NMR) and 1 H NMR spectra of the products were recorded with a JNM-ECXII (JEOL, Tokyo, Japan) spectrometer at room temperature in CDCl₃ solution and chemical shifts were recorded in δ (parts per million). 13 C NMR (400 MHz, CDCl₃) shifts were as follows: 140.2 (7-C), 131.5 (2-C), 123.7 (4-C), 121.3 (9-C), 97.6 (1'-C), 73.8 (3'-C), 72.5 (2'-C), 72.2 (5'-C), 70.4 (4'-C), 63.4 (6'-C), 61.3 (10-C), 31.8 (6-C), 26.4 (5-C), 24.6 (1-C), 22.4 (8-C), and 16.4 (3-C); 1 H NMR (400 MHz, CDCl₃) shifts were as follows: 5.37 (t, 1H; H-9), 5.09 (t, 1H; H-4), 4.78 (d, 1H; H-1'), 4.10 (dd, 2H, H-10), 3.77 (dd, 1H; H-6'a), 3.66 (dd, 1H; H-2'), 3.61 (t, 1H; H-3'), 3.54 (m, 1H; H-5'), 3.35 (dd, 1H; H-6'b), 3.29 (t, 1H; H-4'), 2.10 (t, 2H; H-5), 2.04 (t, 2H; H-6), 1.73 (s, 3H; H-8), 1.65 (s, 3H; H-3), and 1.59 (s, 3H; H-1).

Properties of neryl- α -D-glucopyranoside

To confirm the glucosidic linkage between glucose and nerol, 2.9 g/L neryl- α -D-glucopyranoside and 0.5 units of glucosidase were incubated at 30°C for 2 h. The enzymatic activity of glucosidases was determined by HPLC. One unit of α -glucosidases was defined as the amount of enzyme that hydrolyzed 1 μ mol of maltose per minute at 25°C. One unit of β -glucosidases was defined as the amount of enzyme that hydrolyzed 1 μ mol of *p*-nitrophenyl- β -D-glucopyranoside per minute at 37°C. To evaluate its solubility, purified neryl- α -D-glucopyranoside was diluted 10 times with water and agitated at 30°C for two days. After centrifugal separation, the supernatant was analyzed by HPLC.

Results and discussion

Glucosylation of nerol

We previously screened for microorganisms in the soil that could accumulate glucosylated nerol from nerol and maltose [16]. After screening a large number of bacteria, *Agrobacterium* sp. M-12, which can grow on a medium containing maltose as the sole carbon source, was found to exhibit high glucosylation activity towards nerol when maltose was added to the reaction mixture. Supporting our finding, an earlier study revealed α -anomer-selective glucosyl transfer from maltose by a maltose-assimilating bacterium [14]. The 16S rDNA sequence (Figure S1) revealed 99.8% identity to *Agrobacterium larrymoorei* strain AF3.10 (type strain). *Agrobacterium* sp. M-12 was deposited at the National Institute of Technology and Evaluation (NITE) under accession number NITE AP-02189.

In the present study, we compared the synthesis of nerol glycoside using transglucosidase, CGTase, or *Agrobacterium* sp. M-12. As shown by TLC analysis, a clear spot thought to be a transglucosylation product (indicated by an arrow in Figure 1) was detected in the reaction mixture of *Agrobacterium* sp. M-12. Other oligosaccharides such as malto-oligosaccharides, or other glucosylated derivatives such as nerol

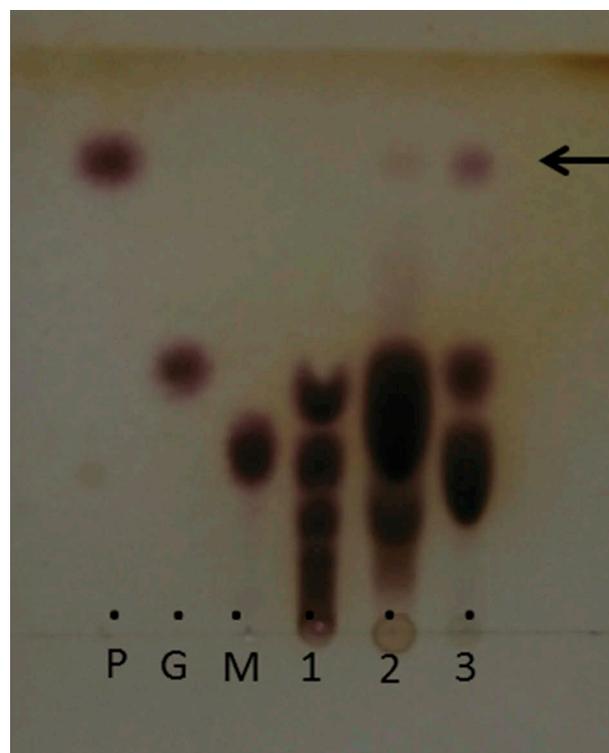


Figure 1. Thin-layer chromatography showing reaction products obtained from nerol and maltose using CGTase (lane 1), transglucosidase (lane 2), and washed cells of *Agrobacterium* sp. M-12 (lane 3).

The arrow indicates the reaction product derived from nerol. Spots of lower mobility than maltose indicate malto-oligosaccharides. P, nerol glucoside; G, glucose; M, maltose.

oligoglucosides (for example, nerol α -maltoside) were not detected in the reaction mixture, indicating that *Agrobacterium* sp. M-12 had strong transglucosylation activity towards nerol. Transglucosidase produced only small amounts of the product, as revealed by TLC analysis. The accumulation of glucose and oligosaccharides was considerably more pronounced in the reaction with transglucosidase than with *Agrobacterium* sp. M-12. Maltose was preferred over nerol as an acceptor substrate for the transglucosylation reactions. Glucosylated compounds from nerol were not observed in the reaction with CGTase.

Isolation and identification of the product obtained by glucosylation of nerol by *Agrobacterium* sp. M-12

The product obtained by glucosylation of nerol by *Agrobacterium* sp. M-12 was a colorless and transparent liquid; it was identified as neryl- α -D-glucopyranoside (Figure 2) via ^1H NMR and ^{13}C NMR. The specific chemical shifts of the product are listed in Materials and methods. ^{13}C NMR singlet signal at δ 97.6, which is assignable as an anomeric carbon of glucose, indicated that *Agrobacterium* sp. M-12 selectively produced a stereoisomer of an anomer. Furthermore, ^1H NMR doublet signal at δ 4.78 was derived from an anomeric proton of glucose, its small coupling constant ($J = 3.00$ Hz) suggested an α -glucosyl linkage to nerol. Moreover, we confirmed the glycosidic linkage between glucose and nerol following

hydrolysis by α - and β -glucosidase. In that reaction, the glucosylated nerol was hydrolyzed by α -glucosidase, with the concomitant release of glucose. In contrast, β -glucosidase did not catalyze the hydrolysis of glucosylated nerol (Figure S2).

Optimization of culture and reaction conditions for *Agrobacterium* sp. M-12

To obtain active cells of *Agrobacterium* sp. M-12 for neryl- α -D-glucopyranoside production, *Agrobacterium* sp. M-12 was cultured in a jar fermenter. The time course of the fermentation is illustrated in Figure 3. As enzymatic activity appeared similar during growth and stationary phase, the glucosylation reaction was evaluated on cells harvested when optical density at 600 nm was nearly 5.0.

The conditions of glucosylation by *Agrobacterium* sp. M-12 were examined in detail, including the effect of maltose concentration and temperature. The process of nerol α -glucosylation was positively affected by increasing the starting concentration of glycosyl donor (maltose). We also confirmed that the washed cells of *Agrobacterium* sp. M-12 could not form neryl- α -D-glucopyranoside when glucose replaced maltose as an α -glucosyl donor. After investigating the rate of the reaction using a range of maltose concentrations up to 300 g/L, we selected 100 g/L of maltose as the optimal glycosyl donor concentration, as it resulted in the highest reaction rate (Figure S3). Figure 4 shows the effect of reaction temperature. At 60°C, the activity decreased rapidly and therefore

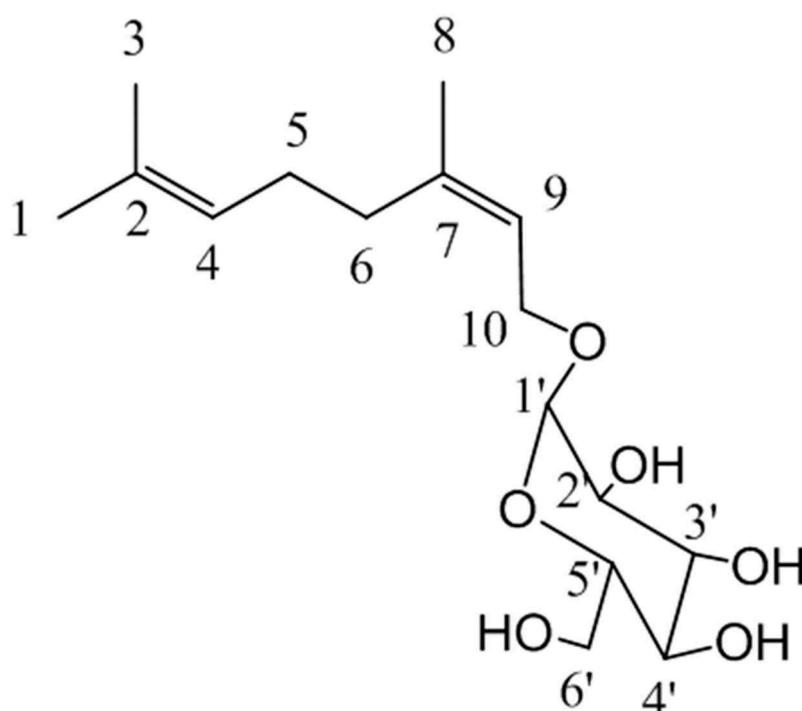


Figure 2. Structure of the transglucosylated product of nerol obtained by reaction with *Agrobacterium* sp. M-12. Numbers correspond to the assigned NMR signals described in the text.

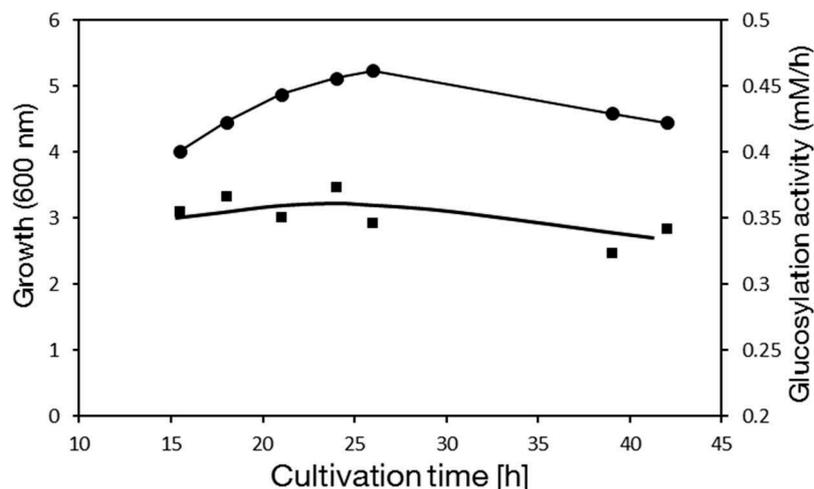


Figure 3. Time course showing growth and transglucosylation activity of *Agrobacterium* sp. M-12 in a 1-L jar fermenter. Closed circles, turbidity at 600 nm; closed squares, transglucosylation activity. Values represent means of three independent experiments.

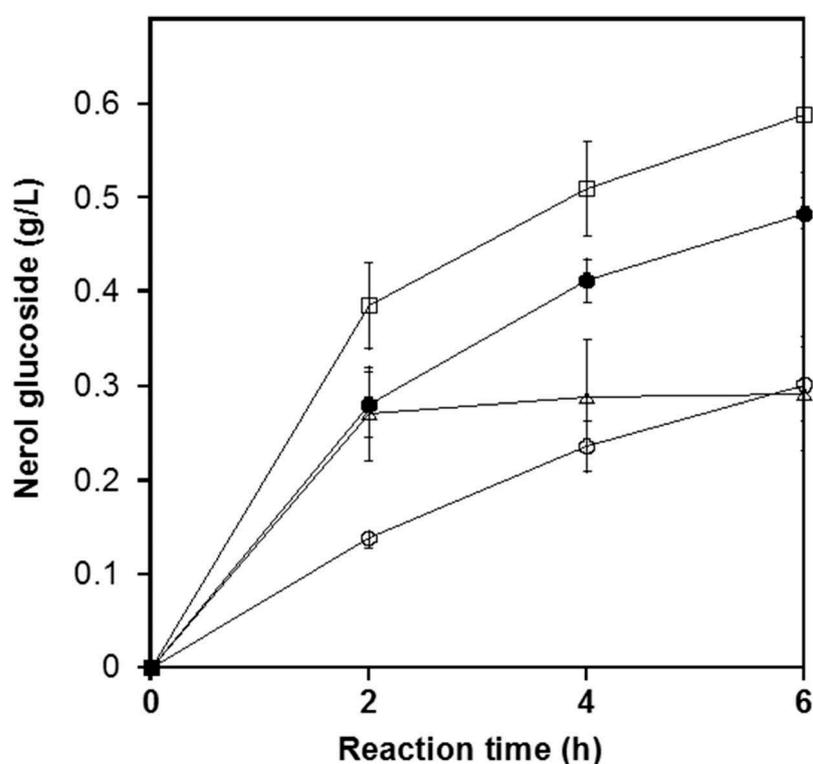


Figure 4. Effect of reaction temperature on the production of neryl- α -D-glucopyranoside.

Open circles, 30°C; closed circles, 40°C; open squares 50°C; open triangles, 60°C. Values represent means of three independent experiments. Bars represent standard derivations.

optimal temperature was set to 50°C, which allowed the reaction to be completed within 6 h. Nevertheless, it should be noted that the largest amount of neryl- α -D-glucopyranoside accumulated at 40°C in 48 h. These results may be explained by a gradual loss of activity at temperatures above 50°C.

Production of neryl- α -D-glucopyranoside

Figure 5 shows the time course for the synthesis of neryl- α -D-glucopyranoside under optimal conditions. These allowed for 1.0 g/L of nerol to be converted to

1.8 g/L of neryl- α -D-glucopyranoside after 72 h, corresponding to a molar yield of 87.6%. Other oligosaccharides such as maltotriose and maltotetraose, or neryl maltoside were not detected. However, in the time course monitored by HPLC, other small peaks appeared in addition to those for nerol and neryl- α -D-glucopyranoside. These were subsequently identified as geraniol and geraniol glucoside (geranyl- α -D-glucopyranoside) by HPLC and NMR analyses (data not shown). Zhou et al. [17] reported endogenous dehydrogenation and isomerization of geraniol into nerol in *E. coli*. Thus, we suspected that the formation of geraniol and the

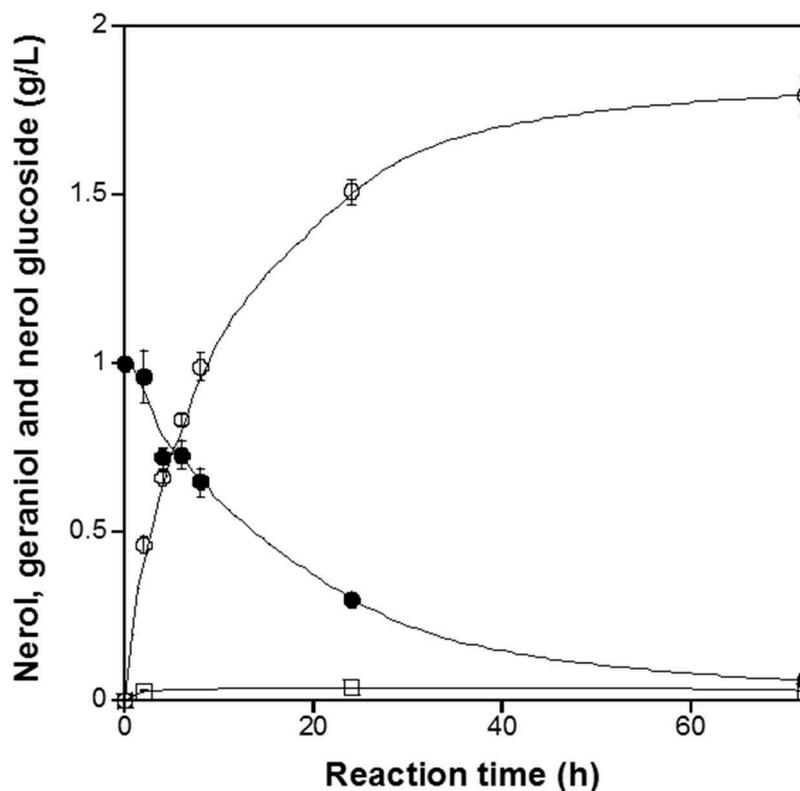


Figure 5. Time course showing the conversion of nerol by *Agrobacterium* sp. M-12.

Cells harvested from an 8.0-mL aliquot sampled from the jar fermenter were washed once with 8.0 mL of 20 mM potassium phosphate buffer (pH 7.0). The washed cells were incubated with 1.0 g/L nerol, 100 g/L maltose, and 20 mM potassium phosphate buffer (pH 7.0) in a total volume of 8.0 mL. The reaction was carried out in a 15.0-mL conical tube at 40°C with vigorous shaking. Closed circles, nerol; open circles, neryl- α -D-glucopyranoside; open squares, geraniol.

glucoside was due to endogenous isomerization of nerol to geraniol and its subsequent glucosylation in the *Agrobacterium* sp. M-12 reaction mixture. This hypothesis is supported by the accumulation, after a 72-h reaction period, of 0.03 g/L of geraniol and 9.3 mg/L of geraniol glucoside. According to our knowledge, neither chemical nor enzymatic preparation of neryl- α -D-glucopyranoside has been published to date. Recently, various glucosyltransferases identified in plant genomes, have been expressed in other organisms by heterologous recombination. β -D-Glucosides of terpene alcohols, such as geraniol, nerol, and citronellol, were synthesized by a microbial-based whole cell biotransformation system [3]; however, the accumulation of glucosides was low. Selection of the active enzyme and construction of an efficient UDP-glucose regeneration system are necessary for any future practical applications.

Properties of neryl- α -D-glucopyranoside

In general, glycosylation of non-sugar compounds increases their solubility; this was confirmed in the present case. The amount of neryl- α -D-glucopyranoside dissolved in water was 22.0 g/L, substantially higher than the 0.53 g/L of nerol (at 25°C) [18]. Neryl- α -D-glucopyranoside had also a slightly

sweeter smell compared to nerol, which explains its use in fragrances.

Glucosylation of other alcohols

Agrobacterium sp. M-12 exhibited a high and efficient transglucosylation activity towards nerol, which prompted us to investigate the glucosylation of other terpene alcohols, such as geraniol, (-)- β -citronellol, and (-)-linalool. Transglucosylation products were detected in all the reactions tested (Figure S4). When geraniol was used as substrate, nerol and neryl- α -D-glucopyranoside appeared in the reaction mixture (Figure S5), in agreement with nerol transglucosylation and the consequent accumulation of geraniol and the glucoside. (-)-Linalool, which has a sterically hindered hydroxyl group, was used as an acceptor in this reaction. It should be noted that the order of reactivity of glycosidases toward hydroxyl groups is primary alcohols > secondary alcohols > phenols; whereas tertiary alcohols are unreactive [5]. Therefore, it will be interesting, in the future, to confirm the substrate specificity of transglucosylation by *Agrobacterium* sp. M-12.

On the basis of our recent discovery of α -anomer-selective glucosylation of terpene alcohol by an intracellular enzyme of *Agrobacterium* sp. M-12, which

uses maltose as the α -glucosyl donor, we expect future work to focus on identification and cloning of the enzyme responsible for transglucosylation in this species. This will proceed in parallel with characterization of its acceptor specificity for terpene alcohols and related compounds, including enantioselectivity of chiral alcohols.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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