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Application of Biotransformation in Flavor and Fragrance Industry

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Abstract: The present study describes the isolation of microorganisms capable of producing α-pinene from β-pinene. 24(15 fungi, 9 bacteria) microorganisms were isolated from galbanum, gum and soil, were screened for their ability to transform β-pinene to α-pinene. Biotransformation products were extracted with n-hexan and analyzed by gas chromatography. One microorganism (bacterial strain) were found. The biotransformation medium involved, phosphate buffer pH 6, 100 μL β-pinene, 1 g biomass of microorganism, 37°C, 150 rpm and 22 h. The experiments were performed in conical flasks. The optimum cell growth were obtained when 30 g L^{-1} glycerin applied. The optimum conversion β-pinene to α-pinene was obtained when 20 g L^{-1} glycerin applied as carbon source for bacterial strain.

Key words: Biotransformation, β-pinene, α-pinene, flavor and fragrance industry

INTRODUCTION

Biotransformation or biocatalysis is the use of biological agents to effect specific chemical change on compounds that are not part of their normal biochemistry. These conversions of compounds can be carried out by intact microbial cell; plant cells, isolated enzymes and they result in the formation of novel useful products that are often difficult or impossible to obtain by conventional chemical means. This definition differentiates biotransformation from biosynthesis or metabolic chemistry. Both of which are concerned with reactions carried out by biological systems on components of their natural biochemistry. The distinguishing feature of biotransformation is its use for the preparation of products of defined chemical structure that are related to the substrate or starting material for the reaction by only a small number of chemical changes and in many cases by changes brought about by the action of only a single enzyme (Herbert, 2000).

Many essential oils contain compounds that resemble in their structure flavor and aroma compounds. These compounds can be converted by microorganisms or enzymes into desired aroma chemicals. The products of such bioconversions are considered natural, since the EEC legislation incorporate products that are produced from biological sources by living cells or their components under the term natural products (Shimoni *et al*., 2000).

Given the profitability of the conversion of low priced, commercially available monoterpens to high priced flavoring and fragrance agent, considerable effort has gone into exploring microbial transformations for this purpose (Van Rensburg *et al*., 1997).

Flavor and fragrance are extremely important for the food, feed, cosmetic, chemical and pharmaceutical industries.

Pinene (α and β) are bicyclic monoterpenes hydrocarbons. α-pinene is the principal constituent of turpentine from most confiers and is a component of the wood and leaf oils of a wide variety of other plants (Pinder, 1960; Yoo *et al*., 2001; Yoo and Day, 2002).

β-pinene is isomeric with α-pinene and accompanies the latter in most source of the hydrocarbon (Erman, 1986; Yoo *et al*., 2001). Pinenes (α and β) are major components of commercial turpentine and of great value as precursors for making flavors and fragrances (Rassmann, 1990; Mckibben, 1989). Of the 13761 metric tons of turpentine produced as by-products in the pulp and paper industry of the United State, between 20 and 25% is used by the flavored fragrance industries (Mattson, 1984; Yoo *et al*., 2001; Yoo and Day, 2002).

This trend has forced companies to direct their attention towards flavours of natural origin. For this reason, the microbial transformation of α-and β-pineneis of considerable potential interest for application to the flavour and fragrance industries (Griffiths *et al*., 1987; Misra *et al*., 1996; Yoo *et al*., 2001; Yoo and Day, 2002). One such treasure, whose name and fragrance is today little know, is galbanum. Galbanum (*Ferula* sp.) is an oleoresin that exudes from an umbelliferous plant similar

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to fennel. The Egyptians imported it from ancient Iran, still virtually the sole source for galbanum today. A milder galbanum has historically come from Afghanistan. *Ferula gummosa* boiss, Umbelliferae, is a wild plant indigenous to Iran. It grows in the northern and western parts of the country Chemical composition of galbanum contain 6% volatile oil, 67% resin, 19% gum and 8% foreign matter. The volatile oil consists mainly of a hydrocarbon of the terpene series. Components were identified, constituting 94.6% of the essential oil in which the principal components were β-pinene (50.1%), α-pinene (18.3%), 3-carene (6.7%), α-thujene (3.3%) and sabinene (3.1%). Therefore *galbanum* is a great source of β-pinene. β-pinene is a great substrat for convert to α-pinene (Zargari, 1989; Sayyah *et al*., 2001).

MATERIALS AND METHODS

Chemicals: α-pinene and β-pinene were obtained from Bell GmbH company (Germany).

Isolation and screening of microorganisms: Microorganisms used in this study were isolated by selective medium from galbanum gum, *Ferula* galbanum plant and surrounding terpene soaked soil. Soil samples and *Ferula* galbanum plant and gum galbanum were taken from greenhouses of Khorasan and Kashan, ten grams of each sample was suspended in 100 mL saline, also was added 0.1 g tween 80 to gum and these suspensions were used for both direct isolation of microorganisms. For direct isolation, appropriate dilutions of soil suspension were plated on selective medium and different microorganisms were isolated based on their morphology. Isolate colonies were transferred into NA (Nutrient agar) slants (bacteria strain), SD (Subrodexterose agar) slants (fungi). Pure cultures were stored at 4°C and transferred bimonthly. Working cultures were maintained at room temperature and transferred monthly. Microorganisms were characterized by biochemical and morphological tests.

Biomass production for biotransformation: Erlenmeyer flasks of 250 mL volume, containing 50 mL medium complex containing 10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 20 g L^{-1} glucose, 2 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4$, 0.5 g L^{-1} K_2HPO_4. The medium was adjusted to pH 5.3 (fungi), pH 6.8 (bacterial strain) prior to sterilization. The medium were inoculated with colonies picked up on NA for bacteria and SD for fungi. After, growth at 37°C in a rotary shaker in a model 3528 Environ-shaker (lab-line Instrument, Inc., Melrose park, IL, USA) operated at 100 rpm for 20 h. Absorbance at 620 nm (OD_{620}) was monitored for evaluation of culture growth by spectrophotometer (spectro UV-VIC Double s Beam PC 8 Scanning Auto cell LABOMED, INC). The optical density at 620 nm (OD_{620}) of the media was close to 0.1. These broths were used to inoculate 1 mL in Erlenmeyer flask of 500 mL volume containing 100 mL complex medium, Erlenmeyer were shaken at 100 rpm and 37°C for 54 h.

Cells were harvested by centrifuging. The culture broth for 20 min at 11300 x g in a refrigerated 4°C centrifuge (Sorrall RC5C Dupont, Newtown, CT, USA). After aseptically removing the supernatant from each bottle, cell pellets were washed twice with about 30 mL of sterile saline solution and then cell pellet were pooled and re-centrifuged. Cell pellets were stored at -20°C.

Biotransformation medium: Bioconversion was carried out by adding 100 μL β-pinene and 1 g biomass in 100 mL buffer phosphate (Na_2HPO_4, NaH_2PO_4), pH 6 and the flasks were shaken at 150 rpm at 37°C for 22 h. The substrate control consisted at sterile medium incubated under same condition, but without microorganism. After, 22 h cells were harvested by centrifuging. The culture broth for 20 min at 11300 x g in a refrigerated 4°C centrifuge. After aseptically removing the supernatant from each bottle, cell pellets were suspend in 1 mL buffer phosphate (Na_2HPO_4,NaH_2PO_4), pH 8, cell free extracts were prepared by sonication (1 min, interval 1 min, 7 folds, tip power level 4, 50% cycle, 4°C). (Lab Sonic, B. Braun Biotech International, Samamicro Company) followed by centrifugation (11300 x g, 20 min, 4°C). The supernatant as biotransformation products were extracted with n-hexan, n-hexan as an internal standard. n-hexan was dried over anhydrous sodium sulphate and concentrated under a steam of nitrogen a bout 1 mL. Concentrated extracts were transferred into 2 mL amber vial equipped with Teflon-lined caps and stored at -20°C until analysis. The extract of production was analyzed by GC (Gas chromatography).

Analysis: The identification of monoterpenes metabolites were obtained by the previous reported method. One microliter of each extract was injected into capillary column Hp5 5% phenyl methyl siloxan 30 m, 530×150 μm nominal (Agilent 6890 series GC system. Helium with a linear velocity of 25 cm sec^{-1} was used as GC carrier gas. The injector temperature was set at 50°C. Column oven temperature programmed as follows 50°C for 1 min and then a rise 5°C min^{-1} to 130°C and then a rise 30°C min^{-1} to 280°C where it was held for 5 min. To prepare a retention index, n-hexan containing each of the following standard compounds, β-pinene and α-pinene were injected under the same conditions.

Optimize growth condition: In order to optimize growth condition of microorganisms in complex medium, carbon sources were optimized.

For inoculum preparation, one loopful of cells stored on NA (bacterial strain) slants were aseptically transferred into erlenmeyer flasks of 250 mL volume, containing 50 mL of complex medium. Microorganisms were cultured in a shaker at 37°C and 100 rpm for 20 h. One milliliter of culture was transferred into 500 mL flasks containing 100 mL of complex medium. The determination of the effects of different carbon sources (glycerin, *galbanum* gum, glucose) on cell growth was established using 20 g L^{-1} concentration of each carbon sources. One milliliter aliquots of inoculums were transferred to 500 mL flasks containing 100 mL of complex medium. The flasks were incubated in shakers at 37°C and 100 rpm for 54 h in the same manner as above. Culture broth was evenly dispensed into 8×250 mL sterilized centrifuge bottles. Cells were harvested by centrifuging the culture broth for 20 min at 11300x g in a refrigerated (4°C) centrifuge. After aseptically removing the supernatant from each bottle, cell pellets were washed twice with about 30 mL of sterile saline solution and then cell pellets were pooled and re-centrifuged. The pooled cell pellets was washed with about 100 mL of saline solution. Finally, the supernatant was decanted and the combined weight of bottle plus cell pellets was measured. Transformation treatments were prepared using flasks of 500 mL volume containing 100 mL buffer phosphate pH 6, 1 g cell pellets obtained from each of carbon sources and 100 μL β-pinene. The biotransformation product was extracted in the same manner as above. The extract of production was analyzed by GC (Gas Chromatography). Biotransformation products were extracted with n-hexan as internal standard. The n-hexan was dried over anhydrous sodium sulphate and concentrated under a stream of nitrogen to about 1 mL. Concentrated extracts were transferred into 2 mL amber vials equipped with Teflon-lined caps and stored at -20°C until analysis.

Growth on different concentrations of glycerin (optimal carbon source): For inoculums preparation, one loopful of cells stored on NA (bacteria strain) slants were aseptically transferred into erlenmeyer flasks of 250 mL volume, containing 50 mL of complex medium. Microorganisms were cultured in a shaker at 37°C and 100 rpm for 20 h. The determination of the effects of different concentration of glycerin on microorganisms growth was established using 5, 10, 15, 20, 25, 30 and 35 g L^{-1} concentration of glycerin. One milliliter aliquots of inoculum were transferred to 500 mL flasks containing 100 mL of complex medium. The flasks were incubated in shakers at 37°C and 100 rpm for 54 h. Culture broth was evenly dispensed into 8×250 mL sterilized centrifuge bottles. Cells were harvested by centrifuging the culture broth for 20 min at 11300 × g in a refrigerated (4°C) centrifuge. After aseptically removing the supernatant from each bottle, cell pellets were washed twice with about 30 mL of sterile saline solution and then cell pellets were pooled and re-centrifuged. The pooled cell pellets was washed twice with about 30 mL of sterile saline solution finally, the supernatant was decanted and the combined weight of bottle plus cell pellets was measured. Transformation treatments were prepared using flasks of 500 mL volume containing 100 mL buffer phosphate pH 6, 1 g cell pellets obtained from each of carbon sources and 100 μL β-pinene. The flasks were incubated in a shaker at 37°C, 150 rpm, 22 h. After 22 h cells were harvested by centrifuging the culture broth for 20 min at 11300 × g in a refrigerated 4°C centrifuge. The culture broth for 20 min at 11300 × g in a refrigerated 4°C centrifuge. After aseptically removing. The supernatant from each bottle, cell pellets were suspend in 1 mL buffer phosphate, pH 8, cell free extracts were prepared by sonication (1 min, interval 1 min, 7 folds followed by centrifugation (11300 × g, 20 min, 4°C). The supernatant as biotransformation products were extracted in the same manner as above. The extract of production were analyzed by GC (Gas chromatography).

RESULTS AND DISCUSSION

This present study is a part of a long-term project for developing efficient microbial bioconversion processes of essential oil components to flavor and fragrance chemicals. The aim of the present study was to isolate microorganisms capable of producing α-pinene from β-pinene. Various microorganisms were isolated from soil samples taken from aromatic plants greenhouses such as gum of galbanum using direct isolation. Based on their morphology, microorganisms had isolated from galbanum gum, *Ferula gummosa* boiss and surrounding terpen soaked soil with expectation that such culture would be highly likely to shown relevant biotransformation potentials. The screening procedure involved the following two step (I) culture development in a growth medium and (ii) use of the biomass (as resting cells) in a reaction mixture for biotransformation. Twenty four microorganisms different (14 fungi, 10 bacterial strains) were isolated using direct isolation. Fifteen microorganisms (5 bacterial strain, 10 fungi) isolated from *Ferula gummosa* boiss and surrounding terpene soaked soil.

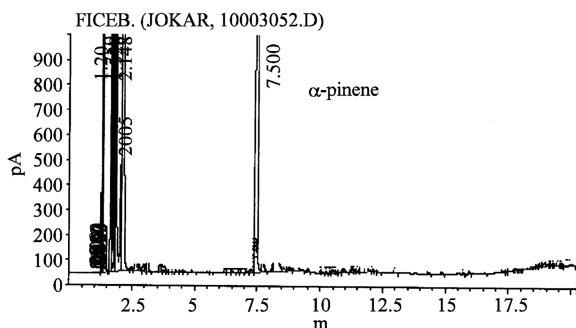

Fig. 1: GC chromatogram of α-pinene standard

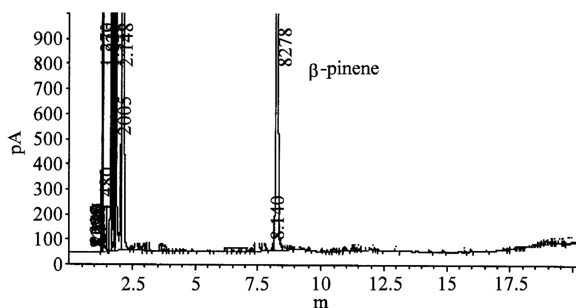

Fig. 2: GC chromatogram of β-pinene standard

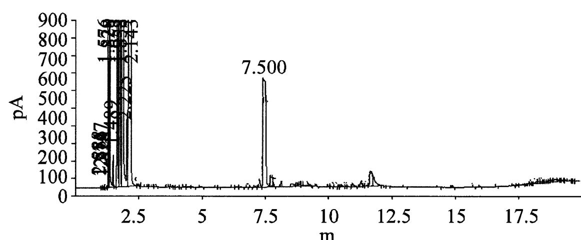

Fig. 3: GC chromatogram of conversion products by bacterial strain. Bioconversion was carried out by adding 100 μL β-pinene and 1 g biomass in 100 mL buffer phosphate pH 6 and the flasks were shaken at 150 rpm at 37°C for 22 h. The biotransformation products were extracted in the same manner as above. The biotransformation products were analyzed by GC

Microorganisms (5 bacterial strains, 4 fungi) isolated from galbanum gum. Microorganisms were tested for their ability to produce α-pinene in the presence of β-pinene. The bioconversion products of bacterial strain were identified by GC and the spectral data (Fig. 3) were compared with that of standard data (Fig. 1 and 2).

Of 24 microorganisms, one microorganism (bacteria strains) capable of transforming β-pinene to α-pinene (Fig. 3). Biotransformation medium were prepared using flasks of 500 mL volume containing 100 mL buffer phosphate with the optimum pH 6, 1 g biomass and 100 μL β-pinene, the flasks were incubated in a shaker at 37°C and 150 rpm for 22 h. After 22 h all flasks were extracted with n-hexan as internal standard. The n-hexan was dried over anhydrous sodium sulphate and concentrated under a stream of nitrogen to about 1 mL. Concentrated extracts were transferred into 2 mL amber vials equipped with Teflon-lined caps and stored at -20°C until analysis. But also there was no bioconversion detectable. It suggested, substrate (β-pinene) transported into cell, enzymes capable of transforming β-pinene to α-pinene, there were into cytoplasm, after biotransformation β-pinene to α-pinene, product (α-pinene) and substrate and other product not capable to transport from cytoplasm. Because, transport of product and substrate is greatly hindered by the membrane barrier.

It is necessary that cell free extracts were prepared by sonication followed by centrifugation (11300x g, 20 min, 4°C). The supernatants were used as cell free extract.

Supernatant used as biotransformation products, were extracted with n-hexan and analyzed by GC. Therefore, of 24 microorganisms; one microorganism is capable of transforming β-pinene to α-pinene.

In order to increase biotransformation β-pinene to α-pinene, cell growth condition, i.e., carbon sources were optimized.

Glucose, glycerin and galbanum gum were used as carbon sources, the cell growth, biomass were measured after 54 h incubation of flasks containing 100 mL complex medium. Growth was conducted in a shaker at 37°C, 100 rpm, pH 6.8 (bacterial strain), for 54 h. The determination of the effects of different carbon sources (glycerin, galbanum gum, glucose) on cell growth was established using 20 g L^{-1} concentration of each carbon sources.

The optimum growth of bacterial strain was at the present glycerin as carbon source (Fig. 4).

The effect of different concentrations of glycerin on cell growth were measured after 54h incubation of flasks containing 100 mL complex medium at 37°C, 100 rpm, pH 6.8 (bacterial strain).

The determination of the effects of glycerin concentration on cell growth was established using 5, 10, 15, 20, 25, 30 and 35 g L^{-1}. The optimum growth of bacterial strain was at the present 30 g L^{-1} glycerin as carbon source (Fig. 5).

Biomass obtained of each of glycerin concentrations (bacterial strain) used for biotransformation medium.

Biotransformation medium were prepared using flasks of 500 mL volume containing 100 mL buffer phosphate

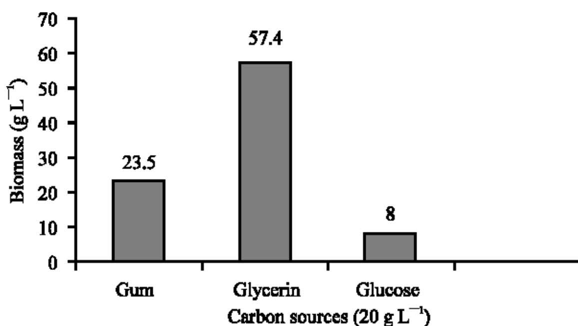

Fig. 4: Glucose, glycerin, galbanum gum were used as carbon source. The cell growth, biomass were measured after 54 h incubation of flasks containing 100 mL complex medium. Growth was conducted in a shaker at 37°C, 100 rpm, 54 h, pH 6.8 (bacterial strain)

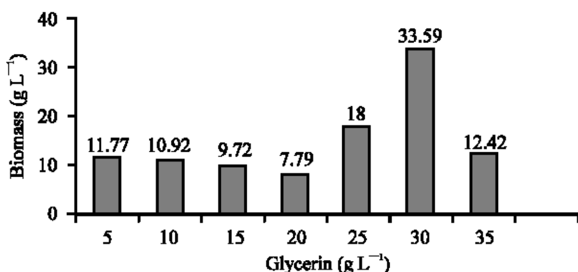

Fig. 5: The optimum growth was obtained when glycerin was applied as carbon source. The effect of different concentrations of glycerin on cell growth was measured after 54 h incubation of flasks containing 100 mL complex medium. Growth was conducted in a shaker at 37°C, 100 rpm, 54 h, pH 6.8 (bacterial strain)

with the optimum pH 6, 1 g biomass and 100 μL β-pinene, the flasks were incubated in a shaker at 37°C and 150 rpm for 22 h. After 22 h, cells were harvested by centrifuging the culture broth for 20 min at 11300x g in a refrigerated 4°C centrifuge. After aseptically removing. The supernatant from each bottle, cell pellets were suspend in 1 mL buffer phosphate (Na_2HPO_4, NaH_2PO_4), pH 8, cell free extracts were prepared by sonication (1 min, interval 1 min, 7 folds) followed by centrifugation (11300x g, 20 min, 4°C). The supernatant as biotransformation products were extracted with n-hexan, n-hexan as an internal standard. n-hexan was dried over anhydrous sodium sulphate and concentrated under a steam of nitrogen a bout 1 mL. Concentrated extracts were transferred into 2 mL amber vial equipped with Teflon-lined caps and stored at -20°C until analysis. The extract of production was analyzed by GC (Gas chromatography).

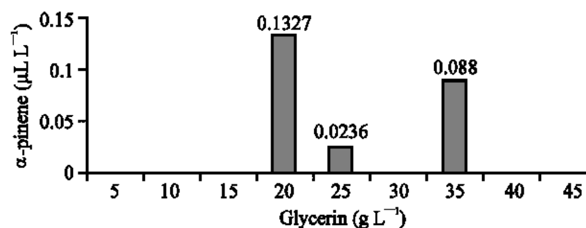

Fig. 6: The effect of different concentrations of glycerin on biotransformation β-pinene to α-pinene. Transformation treatments were prepared using flasks of 500 mL volume containing 100 mL buffer phosphate pH 6, 1 g cells pellets obtained from each concentration glycerin and 100 μL β-pinene. The flasks were incubated in a shaker at 37°C, 150 rpm, 22 h, the biotransformation products were extracted in the same manner as above. The biotransformation product were analyzed by GC

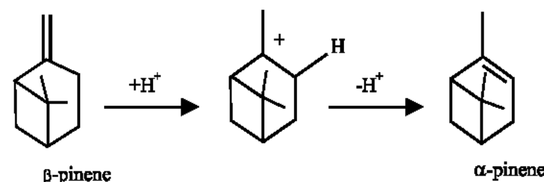

Fig. 7: Suggested procedure for the production of natural α-pinene by bacterial strain

Under the conditions used, bacterial strain grown on 20 g L^{-1} glycerin produced, the highest a mount of α-pinene, even compared to bacterial strain grown on 30 g L^{-1} glycerin (Fig. 6). It suggested at the present 20 g L^{-1} glycerin, product of enzyme capable of transforming β-pinene to α-pinene is the highest. While the highest cell growth was with 30 g L^{-1} glycerin. Studies with microorganisms culture have shown that carbon source concentration in the cultivation medium influence not only the growth but also the routes of metabolism. Therefore, the concentration of carbon source was also found to influence it bioconversion to α-pinene. The optimum conversion β-pinene to α-pinene was obtained when 20 g L^{-1} glycerin applied as carbon source. It was noteworthy that above this glycerin concentration. The bioconversion efficiency was drastically affected for instance and at 35 g L^{-1} glycerin it was only 0.0888% α-pinene. And at 15 g L^{-1} glycerin, there was no bioconversion detectable.

The reaction sites of β-pinene, which are highly susceptible to chemical reactions are also easily attacked

by corresponding enzyme systems. In this regard, the first chemical or enzyme attack may occur at the double bond of β-pinene. Chemical attack of electrophilic reagents on β-pinene proceeds preferentially from the less hindered side of the molecule to produce a cation, elimination of the proton from the more hindered carbon produced more stable alkene, i.e., α-pinene in this condition (Fig. 7).

CONCLUSIONS

In the present study, 24 microorganisms isolated from galbanum gum, *Ferula* galbanum plant and surrounding terpene soaked soil. One microorganism capable of transforming to α-pinen. The growth conditions of microorganisms optimized. Glycerin, glucose, galbanum gum used as carbon sources. The growth bacterial strain with glycerin source was highest. The determination of the effects of glycerin concentration on microorganisms growth was established using 5, 10, 15, 20, 25, 30 and 35 g L^{-1}. The growth cell bacterial strain was highest with 30 g L^{-1} glycerin.

The indicates that bacterial strain conduct bioconversion of β-pinene with a feasible reaction with cell growth on 20 g L^{-1} glycerin.

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