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The Use of Biotechnology for the Production of Flavor and Fragrance

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Abstract: Bacterial strains isolated from *Ferula galbanum* plant, galbanum gum, surrounding terpene soaked soils, were screened for their ability to transform β-pinene to α-pinene. One bacteria, was found. The morphological characteristics and biochemical reaction results led to the tentative identification of the strain as *Micrococcus* sp. strain PIN. Based on these results, bacterial strain was chosen for further studies. In order to optimize growth condition of bacterial strain in complex medium, the growing condition i.e., nitrogen source, pH, temperature, aeration rate were optimized. The optimum growth of bacterial strain was at the present (7 g L^{-1} urea, 30°C, 120 rpm, pH 6.5). The optimum conversion β-pinene to α-pinene was obtained when 7 g L^{-1} urea applied as nitrogen source for bacterial strain.

Key words: Biotransformation, biotechnology, flavor, fragrance, β-pinene, α-pinene

INTRODUCTION

Flavoring compounds are of considerable importance to the food, perfumery and pharmaceutical industries (Abraham and Berger, 1994; Chang *et al*., 1995; Gatfied, 1988; Guenther, 1966; Hagedorn and Kaphammer, 1994; Molinari *et al*., 1995; Tripathi *et al*., 1997; Van Rensburg *et al*., 1997). The major sources of natural flavoring in general are plants and some of these are also chemically synthesized. Recent developments in biotechnology have enabled the production of natural flavorings to be carried out economically and more efficiently. Although enzymes are used in the biotransformation, microbial whole cells have shown great potential for biotransformation owing to the ease with which the microorganisms can be cultivated and used in the bioreactors (Ravid *et al*., 1997; Agrawal and Joseph, 2000).

The classification of biotechnological generated compounds as natural has increased the attention to biotransformation systems (De Carvalho *et al*., 2000).

Recent market surveys have indicated that consumers prefer products labeled as natural the term giving customers a positive connotation while artificial has a negative impact (Sharpell, 1985; Tyrrel, 1990). Terpenes are the largest class of plant secondary metabolites (Harborn, 1991).

β-pinene and α-pinene are bicycle monoterpens hydrocarbons and are precursors of many flavors and fragrances (Pinder, 1960; Yoo *et al*., 2001; Yoo and Day, 2002).

Monoterpenes as substrate of microbial transformations are extensively used by flavor and fragrance industry. Bioconversion or biotransformation can be used to convert monoterpene precursors into the more valuable natural flavors and fragrances (Toniazzo *et al*., 2004). Plants such as *Ferula gummosa* Boiss are great source of monoterpens particulary, β-pinene. *Ferula galbanum* Boiss, is a wild plant indigenous to Iran. It grows in the northern and western parts of country. β-pinene is a grat substrate 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 of bacteria and culture conditions: The strain used in this study was isolated by selective medium from galbanum gum, *Ferula galbanum* plant and surrounding terpene soaked soil. Samples were taken from greenhouse of Kashan and Khorasan, Iran 2006. Samples, 10 g, were aseptically transferred into 250 mL flask containing 100 mL of sterile saline, also was added 0.1 g tween 80 to galbanum gum and these suspension was used for direct isolation of microorganisms. For direct isolation, appropriate dilutions of suspension were plated on selective mediums. Bacteria were characterized by biochemical and morphological tests (Fordes *et al*., 2002).

Preculture in Erlenmeyer flasks: Erlenmeyer flasks of 100 mL volume, containing 50 mL complex medium 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 were inoculated with colonies picked out on

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nutrient agar. After 20 h growth in a rotary shaker (model 3528 Environ-shaker (lab-line Instrument, Inc., Melrose park, IL, USA) operated at 100 rpm and 37°C, the optical density at 620 nm (OD_{620}) of the media was close to 0.1. These broths were then used to inoculate the Erlenmeyer flasks.

Biocatalyst production: One milliliter sample of the preculture were used as inoculums in the Erlenmeyer flasks 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 weighted and then stored frozen at -20°C.

Biotransformation performance: 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 suspended in 1 mL buffer phosphate (Na_2HPO_4, NaH_2PO_4), pH 8.

Physical treatment by sonication was applied to a concentrated suspension using a sonicator (Lab Sonic, B. braun Biotech International, Samamicro Compay). Seven cycles of 1 min, at 1 min intervals, were carried out for all samples. Cell free extracts were prepared by sonication, 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. Extracts were dried over anhydrous sodium sulphate and concentrated under a steam of nitrogen about 1 mL. Concentrated extracts were transferred into 2 mL amber vial equipped with Teflon-lined caps and stored at -20°C until analysis. Biotransformation products were 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.

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 galbanum gum, *Ferula galbanum* plant and surrounding terpene soaked soil.

Among the microorganisms, one bacterial strain capable of transforming β-pinene to α-pinene. Based on this result, bacterial strain was chosen for further studies.

This strain was a nonspore forming, Gram positive and non motile cocci, which was also catalase positive. This strain was not lysed with lysostaphin, resistant to the antibiotic furazolidone, susceptible to 0.04 U of bacitracin and microdase-positive; it usually will only grown aerobically.

The results of biochemical tests of strain are presented in Table 1. These morphological characteristics and biochemical reaction results led to the tentative identification of the strain as *Micrococcus* sp. strain PIN (Fordes *et al*., 2002).

To obtain enough products for identification and further testing, preliminary optimization studies may be necessary.

Table 1: Taxonomic characteristics of *Micrococcus* sp. strain PIN

Characteristics	Description
Gram strain	+
Catalase	+
Modified oxidase	+
Motility	-
Aerotolerance	A[b]
Bacitracin (0.04 U)[a]	S
Furazolidone (100 μg)	R
Lysostaphin (200 μg mL^{-1})	R[c]
Predominant arrangement of cells (other than single cells), most common appearance listed first	Clusters, tetrads
Cytochromes present	+
Acidity from utilizable carbohydrates	Often acid
Growth at 45°C	-

[a]:For bacitracin, susceptible ≥10 mm; for furazolidone, susceptible ≥15 mm. [b]: Kocuria (Micrococcus) kristinae is facultatively anaerobic. [c]: Some strains os Micrococcus, Artbrobacter (Micrococcus) agilis and Kocuria are susceptible to lysostaphin. +: ≥90% of species or strains positive; -: ≥90% of species or strains negative; R, Resistant; S, Sensitive

Yields can be improved substantially by systematic studies of environmental and nutritional parameters. For quantitative estimations of catalytic efficiency, it may be necessary to estimate biomass, the mount of biocatalyst present.

Therefore, the growth condition of bacterial strain in complex medium was optimized. The experiments were performed in triplicates.

Effect of nitrogen sources: The determination of the effect of different nitrogen sources on cell growth were established using 5 g L^{-1} concentration of yeast extract and urea and 2.5 g L^{-1} concentration of ammonium nitrate, ammonium sulphate. As it is shown in Fig. 1, the highest growth cells were obtained when urea was used as nitrogen source.

Effect of urea concentration: The determination of the effect of different concentration of urea on bacteria strain growth were established using 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 g L^{-1} concentration of urea in the same manner as above The highest growth cell was obtained when 7 g L^{-1} urea was added in complex culture (Fig. 2).

α-pinene is important natural fragrance and production of α-pinene by biocatalysts with enzymes or whole cells is attractive so that we decided to examine their formation mediated by microorganisms more closely.

Transformation treatments were prepared in the same manner above. Biotransformation products were analyzed by GC.

Although, all the variables in culture medium influenced growth rate and biomass production at the end of the 54 h cultivation period, the biomass produced in complex medium with 7 g L^{-1} urea caused the maximum bioconversion (Fig. 3). Since the bioconversion reaction mixture contained the same a mount of biomass (1 g), the only difference to be considerable was the physiological state of the bacterial strain biomass cultivated in different levels of urea. Studies with microorganism culture have shown that nitrogen source concentration in the cultivation medium influence not only the growth but also the route of metabolism. It may have some adverse effect on the growth as well as catalytic activity of the microorganisms. Therefore the concentration of nitrogen source was also found to influence it bioconversion β-pinene to α-pinene. It was noteworthy that above this urea concentration. The bioconversion efficiency was drastically affected for instance at 2 g L^{-1} urea it was only 0.0052% α-pinene and at 6 g L^{-1} urea it was 0.1886.

Earlier research the optimum conversion β-pinene to α-pinene was obtained when 20 g L^{-1} glycerin applied

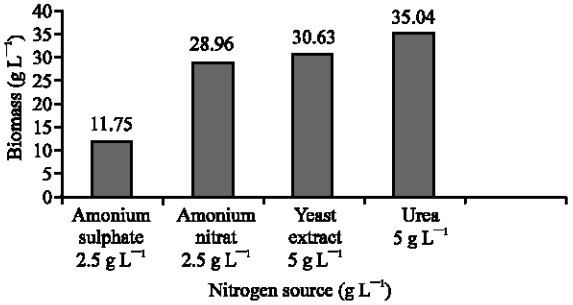

Fig. 1: Effect of nitrogen sources on cell growth. Growth was conducted in a shaker at 37°C and 100 rpm, 54 h

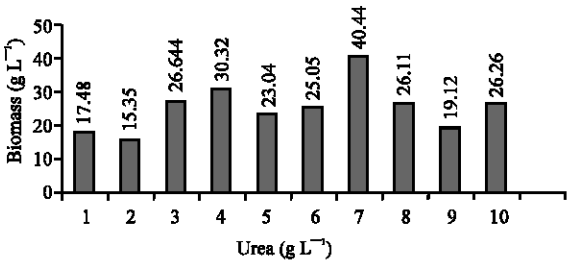

Fig. 2: Effect of urea concentration on cell growth. Growth was conducted in a shaker at 37°C and 100 rpm, 54 h

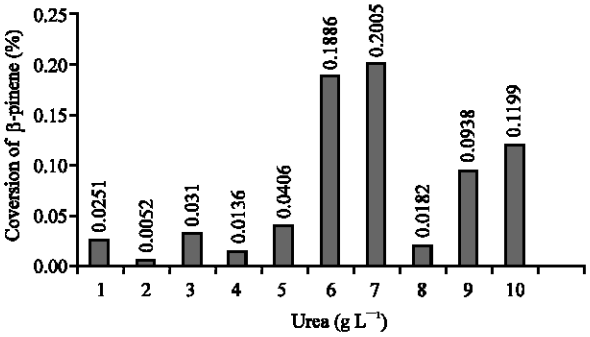

Fig. 3: The effect of different concentrations of urea on biotransformation β-pinene to α-pinene

as carbon source and it was 0.1327% α-pinene compared with optimum conversion β-pinene to α-pinene was obtained when 7 g L^{-1} urea as nitrogen source and it was 0.2005% α-pinene.

Effect of temperature: One milliliter sample of the preculture as inoculums was transferred into 500 mL flasks containing 100 mL of complex medium. The flasks were incubated in shakers at different temperature 27 to 45°C and 100 rpm. After 54 h, cells were harvested by

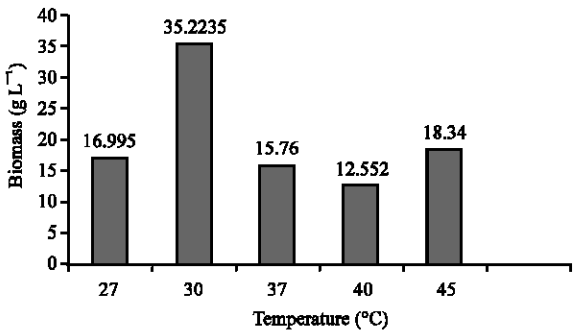

Fig. 4: Effect of temperature on cell growth. Growth was conducted in a shaker at different temperature and 100 rpm, 54 h

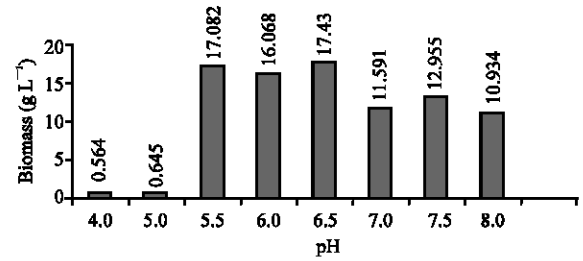

Fig. 5: Effect of different pH on cell growth were measured. Growth was conducted in a shaker at 30°C and 100 rpm, 54 h

centrifuging. As it can be seen from Fig. 4, highest cells growth was obtained at 30°C. It was found that the optimum temperature was 30°C (Fig. 4). Increase of incubation temperature resulted in a decrease in the cell growth.

Effect of pH on cell growth: Effect of different pH ranging from 4 to 8 pH on cell growth were measured and it was found the optimum growth cell of bacterial strain was at the present pH 6.5 (Fig. 5). The pH effect in growth cell was striking in that at the optimum of pH 6.5, 17.43 g L^{-1} was produced. At pH values above or below this optimum, biomass was quite low.

Effect of aeration rate: One milliliter sample of the preculture as inoculums was transferred into 500 mL flasks containing 100 mL of complex medium, pH 6.5. The flasks being placed in a rotary shaker operated at different aeration rate ranging from 90 to 120 rpm at 30°C. After 54 h cells were harvested by centrifuging. The optimum growth of bacterial strain was at 120 rpm (Fig. 6).

Variation in the environmental and nutritional parameters have tremendous effect on growth cell and also the routes of metabolism.

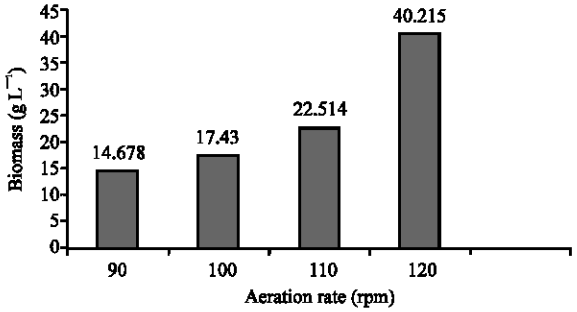

Fig. 6: Effect of aeration rate on cell growth. Growth was conducted in a shaker at different aeration rate and 30°C for 54 h

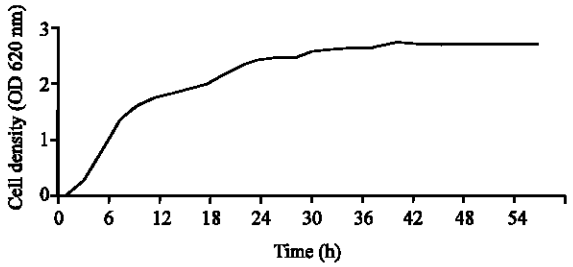

Fig. 7: The curve growth of bacterial strain isolated from galbanum gum, *Ferula galbanum* plant and surrounding terpene soaked. The experiment was performed in Erlenmeyer flasks at 30°C and 120 rpm, 54 h

The variables adopted in the culture medium could have had a more direct effect on bioconversion than the physiological state of the biomass. For all the variables in the case (e.g., pH, aeration rate, temperature, nitrogen source), the biomass grown for 54 h was employed. The biomass was obtained in optimum condition (e.g., optimum pH, aeration rate, temperature and nitrogen source) can be considered as suitable biocatalyst for bioconversion. Although these experiment are only preliminary they have shown that physiological state of the biomass direct effect on bioconversion.

The curve growth: To determination the curve growth, 1 mL sample of the preculture as inoculums was transferred into 500 mL flasks containing 100 mL of complex medium containing 10 g L^{-1} peptone, 9 g L^{-1} urea, 20 g L^{-1} glycerin, 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 6.5 prior to sterilization. The flasks were sampled at the following time interval, 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 54 h. The flasks were run in duplicate. The flasks were sampled in duplicate at the same time interval give above. All flasks were shaken at 30°C, 120 rpm under optimal

growth conditions. At the indicated sampling times, flasks were moved from the shaker and sampled. Growth was followed by measuring the absorbance at 620 nm (Fig. 7).

It suggested the microbial cells are used in biocatalysts. Therefore, the cell membrane may severely hamper. In order to improve the rate of exchange between intra- and extra cellular media, several methods have been described for this purpose (Felix, 1982). Microbial cells can also be effectively broken by physical means.

Sonication is convenient and effective on the laboratory scale. Therefore, it is necessary that cell free extracts were prepared by sonication followed by centrifugation.

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.

The present study again demonstrates the use of whole cell microorganisms as biocatalysts for their intrinsic to catalyse specific and selective reactions under mild reaction conditions. The added advantage of using whole cells is that the addition of a purified cofactor is not required, since it already contained within the cell.

Moreover, this is the first report of biotransformation β-pinene to α-pinene by a bacterial strain.

CONCLUSION

Bacteria isolated by selective medium from galbanum gum, *Ferula galbanum* plant and surrounding terpene soaked soil were presumptively identified as *Micrococcus* sp. This bacterium capable transforming β-pinene to α-pinene. The optimal growth cell was identified. this is the first report of biotransformation β-pinene to α-pinene by a bacterial strain. The optimum growth of bacterial strain was at the present (7 g L^{-1} urea, 30°C, 120 rpm, pH 6.5). The optimum conversion β-pinene to α-pinene was obtained when 7 g L^{-1} urea applied as nitrogen source for bacterial strain.

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