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MICROBIAL BIOTRANSFORMATION OF α -(+) - PINENE BY NEWLY IDENTIFIED STRAIN OF *GLUCONOBACTER JAPONICUS* MTCC 12284

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ABSTRACT

A newly identified acetic acid bacterial strain *Gluconobacter japonicus* MTCC 12284 isolated from decayed yellow orange citrus fruits (mandarins) collected from distant locations in Navi Mumbai fruit market biotransformed the terpene hydrocarbon α-(+)-pinene and produced valuable aromatic chemicals, verbenol, verbenone, alpha terpineol and trans-sobrerol. Products were identified by mass spectrometry and quantified by gas chromatography. Under reaction conditions, from 1%v/v of α-(+)-pinene the valuable aromatic chemical verbenone concentration observed was 6.2mg in 30ml of production media (equivalent to a molar yield of 2.18 %) showing 95.6% substrate conversion in 7 days of incubation. The optimal conditions in the biotransformation medium were 1% glucose concentration, a pH of 7.0 and 30°C temperature.The products obtained have considerable industrial potential.

INTRODUCTION

The production of desired chemicals by microbial biotransformations can offer several advantages over chemical production methods. 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.The most significant advantage of microbial biotransformation is the ability of the biocatalyst to be regio, stereo or enantioselective (De Carvalho & Da Fonseca 2006). This selectivity proves to be very useful as many bioconversions involve the synthesis of products with one or more chiral centres (Zambianchi *et al*., 2004). In chemical synthesis, multi-step procedures are used to obtain enantiomericaly pure products, where as biocatalysts have the ability to produce pure products in a single step (Gibbon & Pirt 1971, Krings & Berger 1998). Consumers prefer to avoid synthetic products, and there has been a great demand for natural products (Welsh *et al*., 1989). Biotechnology has the potential to generate these natural products through biotransformation carried out by microorganisms and their enzymatic systems (Santos *et al*., 2003).

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Terpenes represent the largest group of natural products. Research on various chemical and biological aspects of monoterpenes was stimulated mainly due to their commercial importance in flavor and fragrance industry (Croteau 1988). Among monoterpenes more than 160,000 tonnes/year of alpha pinene accumulate world wide as major byproduct during citrus oil and wood processing. During kraft wood pulping process, crude sulphate turpentine (CST) is released as the industrial waste from the large paper producers. This crude sulfate turpentine is sold to other companies for further distillation and purification to obtain constituent products. Several companies derive terpenes from crude sulfate turpentine (Chinn, 1989).

Terpenes and other compounds derived from turpentine are used as raw materials or submaterials for products such as tires, plastics, adhesives, flavors and fragrances, cosmetics, paints, and pharmaceuticals. Separation by process-scale chromatography can yield α-pinene (purity up to 99%) and β-pinene, 3-carene, α-terpinene, limonene, and phellandrene (Scott & Karen 2002). Among these terpenes, alpha pinene represents an ideal starting material for biocatalysis because of their almost unlimited availability and the fact that they already bear the desired chiral mono and bicyclic C_{10} hydrocarbon lead structures 'pre-synthesized' by nature. In the present work alpha pinene purified from crude sulphate

turpentine using sulfate adsorption technology was used as substrate for the biotransformation to produce a valuable aromatic chemical verbenone. One of the potential biotechnological natural flavour and fragrance products formed from biotransformation of alpha pinene is the valuable verbenone, reminiscent of frankincense, with an average market price of approximately US$3,000/ kg. Verbenone with molecular formula $C_{10}H_{14}O$ is an aromatic bicyclic ketone terpene, with pronounced camphor and mint flavour notes. Verbenone is a major flavour constituent of strawberry, raspberry, rosmarinus and spearmint flavour mixtures (Ravid *et al*., 1997). Verbenone is used in perfumery, aromatherapy, herbal teas, and herbal remedies. Verbenone is also used to control harmful insects, and hence has potential for use in agriculture, and is an intermediate in the synthesis of valuable perfumes and medicinal substances. Production of verbenone by biotransformation of alpha pinene which costs only U.S. $ 0.0075/kg is expected to be inexpensive and profitable. A preliminary report on the possibility of biotransforming alpha pinene into verbenone (Fig.1) was made earlier by Bhattacharya *et al*.,(1960) using *Aspergillus niger* cultures, and this work provided a basic insight into the mechanism.

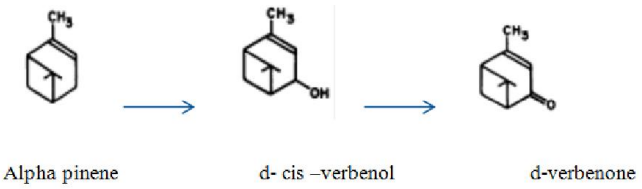

Fig.1. pathway of verbenone formation from alpha pinene

Based on the above mentioned aspects, during a screening programme to isolate microrganisms with the capability of converting readily available monoterpene hydrocarbon α-(+)-pinene to oxygenated products, α-(+)- pinene transforming acetic acid bacterium from decayed yellow orange citrus fruits was isolated and coded as 24B1 (Deepthi priya *et al*., 2014 and Deepthi priya *et al*., 2015). The strain was identified *as Gluconobacter japonicus* (Fig. 2) which belongs to the class Alpha proteobacteria and family Acetobacteriaceae. Under culture conditions the microorganism yielded verbenone, trans sobrerol and alpha terpineol as the major products from alpha pinene.

MATERIAL AND METHODS

Gluconobacter japonicus MTCC 12284, Potato dextrose broth, α-(+)-pinene (>98% purified from CST) was used as substrate, α-(+)-pinene (>98% Fluka) and (IS)-(-)-verbenone (99% Aldrich) were used as internal standards. All other chemicals or solvents were of analytical grade.

Inoculum Development

A loop full of bacterial culture was transferred aseptically to Erlenmeyer flasks containing 40 ml of PDB and incubated at 30ºC at 275rpm for three days.

Biomass harvesting

After reactivation of the microorganism, the culture was grown in 500ml of PDB, using an active inoculum of 1ml in the exponential phase with an initial optical density of 1.2 at 660 nm for a period of 24 to 72 hrs on a rotary shaker at 275 rpm at 30°C at an initial pH of 7.After the growth of culture, the entire culture was centrifuged at 8,000 rpm for 30min, and the supernatant was withdrawn. Sterile distilled water was added to a final volume of 300 ml. Flasks were then stirred to resuspend the cells and centrifuged again. The supernatant was then discarded and the precipitated cells were used for the biotransformation.

Biotransformation

The methodology was based on Ieda Rottava *et al.,* (2010). Approximately 2g of biomass was transfered to an 100ml Erlenmeyer flask containing 30ml of mineral medium with composition $(NH4)_2SO_4$: 5.00g/L; KH_2PO_4 : 0.9g/L; NaCl: 0.50g/L; $MgSO_4.7H_2O$: 0.40g/L; $CaCl_2$: 0.60g/L; KCl: 2.15g/L; $FeSO_4.7H_2O$: 0.01g/L; $ZnSO_4$:0.01g/L; $CuSO_4$: 0.01g/L; $NaNO_3$: 3.6g/L of pH 7 with 1%v/v of α-(+)-pinene. Experiments of biotransformation were started after inoculation of biomass and the flasks were kept in orbital shaker at 30°C and 275 rpm for 8 days. The experiment was also carried out in parallel with controls, in the same conditions without the presence of microorganism. All the experiments were performed in closed cotton plugs in order to avoid the substrate and product evaporation. Flasks were harvested for every 24hrs and the product recovery was performed by liquid – liquid extraction with ethyl acetate. The final solution was dried over anhydrous sodium sulphate, concentrated and analysed in GC and GC/MS.

Products analysis

The reaction products were identified by GC/MS Agilent 5975C, using a HP5 column. The column temperature was programmed to 50°C for 3 min, increased at 5°C/min at 130°C and then increased at 15°C/min at 210°C by 5 min. Helium was the carrier gas, the injection and detector temperatures were 250°C. The dried solution of 1µl was injected into the GC/MS system. The apparatus operated with a flow rate of 1 ml/min in electronic impact mode of 70 eV and in split mode (split ratio 130:1).The identification of the compounds was accomplished by comparing the mass spectra with those from the NIST library and by additional comparison of the GC retention time of standard compounds. The quantitative analysis was carried out in a GC Agilent 7890A with automatic injector and flame ionization detector. The column HP5 (30m×0.32mm) was used at the same experimental conditions described above for GC/MS analysis. The compounds were identified by injection of the external standards compared to the retention times. The quantification was carried out by the standard calibration curve of the interest compound, evaluating the relative area from the interest compound.

RESULTS AND DISCUSSION

Biotransformation products of α-(+) - pinene with *Gluconobacter japonicus* MTCC 12284

On biotransformation of α-(+)-pinene with *Gluconobacter japonicus* MTCC 12284 culture the aroma compounds

detected were of great significance. From (Fig. 3), the products detected with their respective retention times were camphene (8.1), limonene (10.6), verbenol (14.5), isoborneol (15.05), alpha terpineol (15.987), verbenone (16.6), and trans sobrerol (21.032). The linear and theoretical retention indices confirmed the detection of verbenone and the NIST library search of GC-MS in addition confirmed the formation of verbenone (Fig. 4) and other products from α-(+)-pinene. Under experimental conditions, verbenol, verbenone, and sobrerol were detected in 24hrs of incubation. The kinetic study of concentration of verbenone produced was carried out for eight days where maximum verbenone production was achieved in 7 days of incubation and there after verbenone concentration started declining. (Fig. 5).

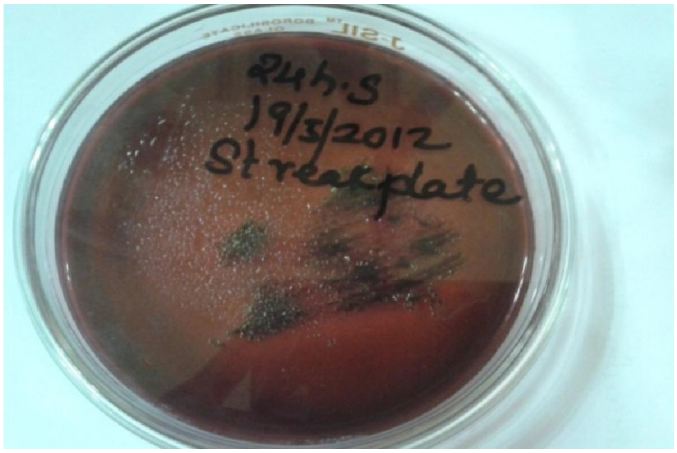

Fig.2. Green metallic sheet colonies of *Gluconobacter japonicus* MTCC 12284 isolated from decayed yellow orange citrus fruits in 24hrs of incubation on EMB agar

On biotransformation of 1%v/v α-(+)-pinene, 6.2 mg (molar yield of 2.18%) of verbenone was produced with 95.6% substrate conversion in seven days of incubation where as 4.946mg (molar yield of 1.76%) of verbenone was observed with 96% substrate conversion in eight days of incubation in 30ml of production media. Our results indicate that verbenone is one of the major end products of α-(+)-pinene metabolism by *Gluconobacter japonicus* MTCC 12284. The majority of α-(+)-pinene was utilised by *Gluconobacter japonicus* culture whereas in concurrent controls only 52% α-(+)-pinene was utilised. The strain was screened from citrus fruits, with the expectation that such cultures would be highly likely to show relevant biotransformation potential.

The screening procedure involved the following two steps: (i) culture development in a growth medium and (ii) use of the biomass in a reaction mixture for the biotransformation. *Gluconobacter japonicus* MTCC 12284 strain isolated from decayed citrus fruits (Fig. 2) was found to be potential in carbon source study and biotransformation study. From (Fig's 3&4) the strain was also found to be potential to biotransform the monoterpene α-(+)-pinene to verbenone and other aromatic chemicals like alpha terpineol, verbenol and sobrerol. Under reaction conditions, it was observed that microbe free control experiments also accumulated sobrerol, both cis and trans-verbenol and verbenone with more undegraded alpha pinene which indicates auto oxidation, whereas drastic decrease in the α-(+)-pinene concentration was observed in 24hrs in biotransformation flask which predicts the efficiency of culture to biotransform alpha pinene. From (Fig. 5) prolonged incubation resulted in decreased yield of verbenone that may have been due to further conversion of verbenone to other degradative products.

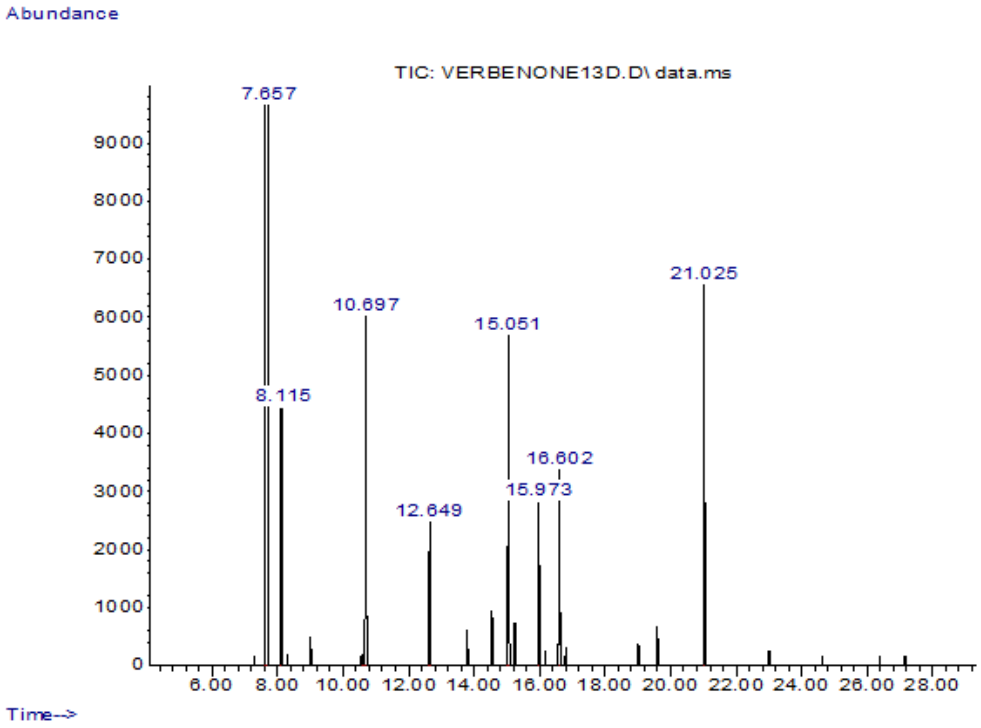

Fig.3. GC-MS chromatogram of products formed on α-(+)- pinene biotransformation with *Gluconobacter japonicus* MTCC 12284 culture

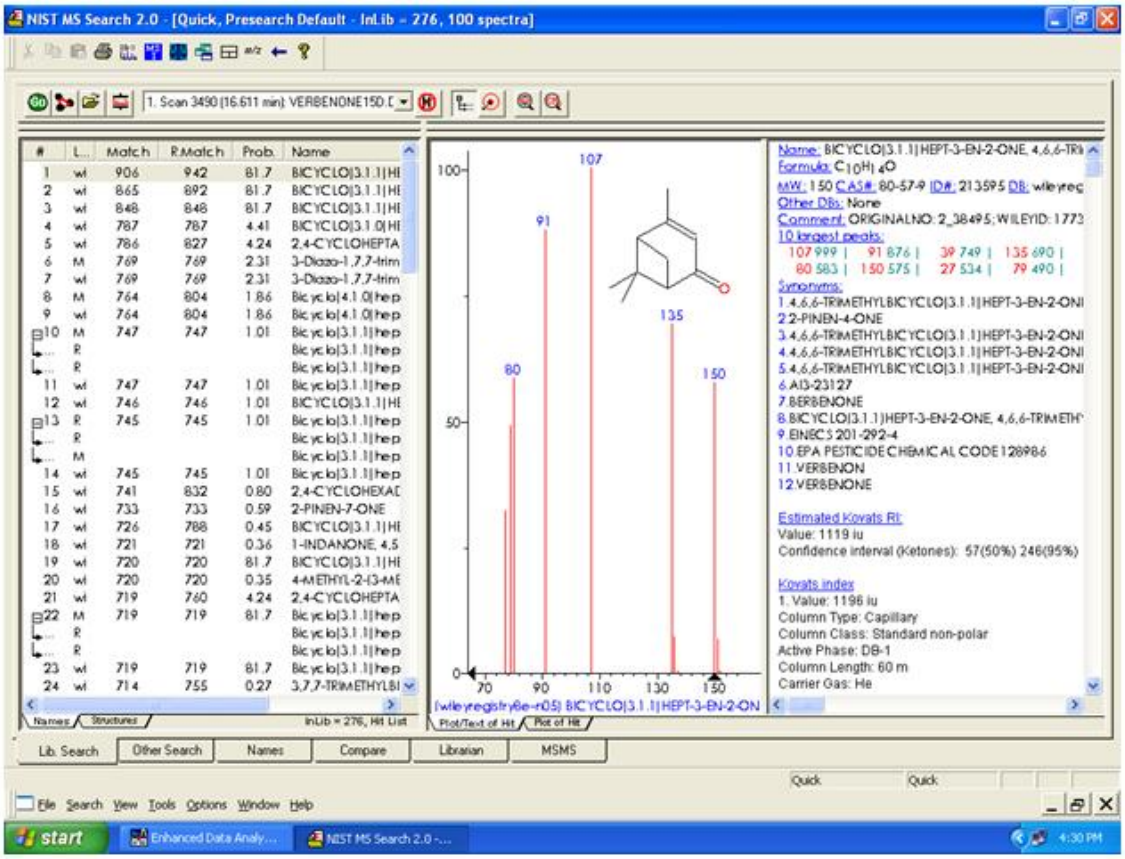

Fig: 4.NIST search of verbenone detected on biotransformation of α-(+) - pinene with *Gluconobacter japonicus* MTCC 12284

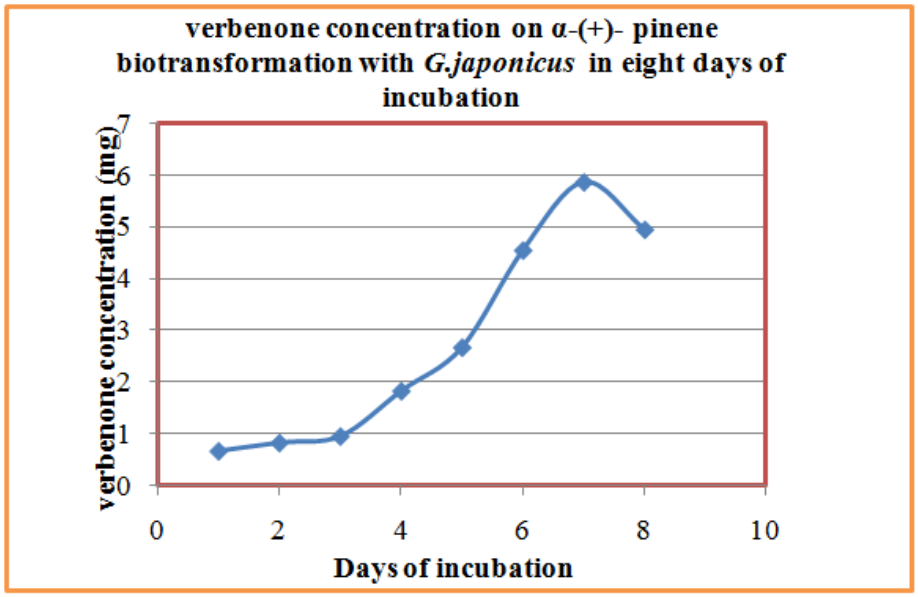

Fig:5. kinetic curve of concentration of verbenone produced on biotransformation of α-(+)- pinene with *Gluconobacter japonicus* MTCC 12284 in eight days of incubation

The present study focuses on previous studies of α-pinene biotransformation where Van Dyk *et al*., (1998) used *Hormonema* sp., freshly isolated from pine forest litter, which hydroxylated mono and bicyclic monoterpenes on the cyclohexane ring, and converted (-)-α-pinene. The two products formed from (-)-α-pinene were identified as verbenone (0.3 g/L) and trans-verbenol (0.4g/L) after 96h. Busmann and Berger (1994) described verbenone and trans-verbenol, together with myrtenol and trans pinocarveol, as major biotransformation products of α-pinene formed by basidiomycetes fungi. Susan *et al*., (1986) used a strain of the bacterium *Serratia marcescens*, isolated from sewage sludge, which oxidized the terpene hydrocarbon α-pinene to produce trans-verbenol., as the major product, with verbenone and trans-sobrerol as minor products in 24hrs. Agrawal and joseph (2000) used resting cells of a locally isolated strain of

Aspergillus niger for the bioconversion of alpha pinene to verbenone in which formation of verbenone was raised from trace amounts under screening conditions to 3.28 mg/100 ml in 6hrs. Despite the fact that alpha-pinene degradation has been extensively studied with different strains (Shukla and Bhattacharyya, 1968; Afghan Farooq *et al*., 2002; Michael Pescheck *et al*., 2009), in present work, in contrast, *Gluconobacter japonicus* MTCC 12284 identified as novel strain which belongs to the family Acetobacteriaceae (Malimas *et al*., 2009; Lidia & stanislaw 2009) is been primarily reported in biotransformation studies of alpha pinene.

In consideration to the metabolic pathway, there are probably a number of mechanisms involved in the biotransformation of alpha pinene to verbenone signifying the action of a variety of enzymes. In the present study since we employed acetic acid bacterium which ought to possess the property of oxidation (Juraj *et al*., 2006; Adachi *et al*., 2003 and Jintana Kommane *et al*., 2012), it can be assumed that α-(+)-pinene must have undergone oxidation step to produce verbenone. This mechanism focuses on the preliminary report of Bhattacharya *et al*., (1960) in which alpha pinene undergoes two distinct biochemical reactions, i.e. a hydroxylation to produce verbenol and dehydrogenation, to form verbenone (Fig. 1). Hence the strain was further subjected to optimisation studies to elucidate the pathway of α-(+)-pinene degradation by the strain, to improve the yields of verbenone.

Conclusion

The bacterial strain *Gluconobacter japonicus* MTCC 12284 was found to be novel strain isolated from decayed yellow orange citrus fruits shown to be capable of transforming α-(+)-pinene to produce verbenol,verbenone, alpha terpineol and sobrerol ,which has great industrial value and may be useful for the preparation of other terpene alcohols and ketones. Further study and optimization of culture conditions may enable other useful products to be obtained. Since we have employed acetic acid bacterium it is reasonable to assume that the reaction mechanism of bio transformation can be an oxidation for verbenone production. The biotransformation reaction was further subjected to optimisation process to improve the yield of verbenone and also to elucidate the pathway and parametres.

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REFERENCES

Adachi, O., Moonmangmee, D., Toyama, H., Yamada, M., Shinagawa, E., Matsushita, K. 2003. New developments in oxidative fermentation. *Appl Microbiol Biotechnol* 60: 643–653.

Afghan Farooq, B., Satoshi Taharab, M Iqbal Choudharya, Atta-ur-Rahmana, Zafar Ahmeda, K Husnu Can Baser, Fatih Demircic Z. 2002. Biotransformation of alpha-Pinene by *Botrytis cinerea. Naturforsch.* 57c, 303-306.

Agrawal, R, and Joseph, R. 2000. Bioconversion of alpha pinene to verbenone by resting cells of *Aspergillus niger. App Microbiol Biotechnol*. 53:335-337.

Bhattacharyya, P.K., Prema, B.R., Kulkarni, B.D., Pradhan, S.K. 1960. Microbiological Transformation of Terpenes: Hydroxylation of alpha Pinene. *Nature* 187:689—690.

Busmann, D and Berger, R.G. 1994. Zeitschrift für Naturforschung C. *A Journal of Biosciences*, 49 545–552.

Chinn, H. 1989. Turpentine-United States (CEH Data Summary). In: Chemical Economics Handbook, SRI International, pages 596.5000A-596.5000O.

Croteau, R.B. 1988. Metabolism of plant monoterpenes. Opportunities in microbial biotransformation of terpenes. ISI Atlas of Science: *Biochem*. 1, 182-184.

De Carvalho, C.C.R., Da Fonseca, M.M.R.. 2006. Biotransformation of terpenes. *Biotechnol Adv.*24 (2): 134-142.

Deepthi priya, K., Manish petkar, Chowdary, G.V. 2014. Isolation, screening and identification of terpene resistant microorganisms from decayed yellow orange citrus fruits. *Research and reviews; journal of pharmacy and pharmaceutical sciences*, vol.3, p. 12-21.

Deepthi priya, K., Manish petkar, Chowdary, G.V. 2015. Bioproduction of aroma compounds from alpha pinene by novel strains. AASCIT. *International journal of biological sciences and applications* ISSN: 2375-3811.vol 2(2) 15-19.

Gibbon, G.H., Pirt, S.J. 1971. Degradation of alpha-Pinene by Pseudomonas PX1. *FEBS Letters* 18 (1): 103—105.

Ieda Rottava, Priscilla F Cortina , Camila A Zanella, Rogerio L Cansian , Geciane Toniazzo, Helen Treichel, O.A.C Antunes, Enrique G Oestreiche and Debora Oliveira. 2010. Microbial Oxidation of (-)-α-pinene to Verbenol Production by Newly Isolated Strains. *Appl Biochem Biotechnol* .162:2221–2231.

Jintana Kommane, Somboon Tanasupawat, Pattaraporn Yukphan, Duangtip Moonmangmee, Nuttha Thongchul, Yuzo Yamada and Emeritus 2012. Identification and Oxidation Products of *Gluconobacter* Strains Isolated from Fruits and Flowers in Thailand. *International Journal of Biology* Vol. 4, No. 1.

Juraj, S vital, Jan Tkac., Igor Vostiar., Marian Navratil., Vladimr stefuca., Marek Bucko., Peter Gemeiner 2006. Gluconobacter in biosensors: applications of whole cells and enzymes isolated from gluconobacter and acetobacter to biosensor construction. *Biotechnol Lett* 28:2003–2010.

Krings, U., Berger, R.G. 1998. Biotechnological production of flavours and fragrances, *Appl. Microbiol. Biotechnol.* 49, pp. 18.

Lidia Stasiak and Stanislaw Błażejak. 2009. acetic acid bacteria – perspectives of application in biotechnology. *Journal of food and nutrition sciences*– a review, vol. 59, no. 1, pp. 17-23.

Malimas, T., Yukphan, P., Takahashi, M., Muramatsu, Y., Kaneyasu, M., Potacharoen, W., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M. and Yamada, Y. 2009. *Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the *Alphaproteobacteria*. *Int. J. Syst. Evol. Microbiol* 59, 466-471.

Michael Pescheck, Marco Antonio Mirata, Bianca Brauer, Ulrich Krings, Ralf Gunter Berger, Jens Schrader. 2009.

Improved monoterpene Biotransformation with *Penicillium* sp by the use of a closed gas loop bioreactor. *J of Ind Microb Biotechnol.*36:827–36.

Ravid, U., Putievsky, E., Katzir, I., Lewinsohn, E., Dudai, N. 1997. Identification of (1R)-(+)-verbenone in essential oils of Rosmarinus officinalis *L. Flav Frag J* 12: 1109±1112.

Santos, A.S, Pereira, Jr, N Silva, I.I. and Antunes, O.A.C. 2003. Microbiological Oxidation of Isosafrole into Piperonal. *Applied Biochemistry Biotechnology*, 107, 649-658.

Scott Masten, Karen, E Haneke. 2002. Turpentine (Turpentine Oil,Wood Turpentine, Sulfate Turpentine, Sulfite Turpentine). Review of Toxicological Literature, Toxicological Summary for Turpentine [8006-64-2].

Shukla, O.P., and Bhattacharyya, P.K. 1968. Microbial Transformations of Terpenes. Part XI Pathways of Degradation of alpha & beta Pinenes in a Soil Pseudomonad (PL-Strain). *Ind J Biochem* 5: 92—101.

Susan J wright, Philip Caunt, David carter, Peter B Baker. 1986. Microbial oxidation of α- pinene by *Serratia marcescens*. *Applied Microbial Biotechnology* 23: 224-227.

Van Dyk, M.S., van Rensburg, E., Moleleki, N. 1998. Hydroxylation of (+)-limonene, (−) α-pinene and (−) β-pinene by a Hormonema Sp. *Biotechnol Lett* 20:431–436.

Welsh, F.W., Murray, W.D., Williams, R.E. 1989. Microbiological and Enzymatic Production of Flavor and Fragrance Chemicals. *Critical Reviews in Biotechnology*, 9(2), 105-169.

Zambianchi, F., Raimondi, S., Pasta, P., Carrea, G., Gaggero, N. and Woodley, J.M. 2004. Comparison of cyclohexanone monooxygenase as an isolated enzyme and whole cell biocatalyst for the enantio selective oxidation of 1,3 dithiane. *Journal of Molecular Catalysis. B, Enzymatic* 31:165.

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