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**RESEARCH ARTICLE**

# ENHANCED PRODUCTION OF VERBENONE FROM BIOTRANSFORMATION OF α -(+)- PINENE WITH PERMEABILISED CELLS OF *GLUCONOBACTER JAPONICUS* MTCC 12284

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**ABSTRACT**

Verbenone is a valuable aromatic chemical used in aromatherapy and as a food flavourant. *Gluconobacter japonicus* MTCC 12284 isolated from decayed yellow orange citrus fruits is reported for verbenone production from biotransformation of α -(+)- pinene .The present study focuses on increasing the yield of verbenone by permeabilising the cells of *Gluconobacter japonicus* with various chemical agents. The verbenone production was substantially increased by permeabilising the *Gluconobacter* cells with toluene, Kpi – n hexane, chloroform, acetone, ethanol and Cetyl trimethyl ammonium bromide respectively. The optimum working conditions for permeabilisation process to achieve maximum verbenone production was found to be 1% (v/v) of α -(+)- pinene at 33°C temperature and treatment time of 30min with 1% toluene which resulted in the production of 34.4 mg of verbenone (23.45% molar yield) at pH 6 at 275rpm in 30ml of production media in 96 hrs of incubation. Verbenone production was also found to be enhanced by ultrasonicating the bacterial cells using Tris Hcl sonication buffer. The concentration of verbenone produced by ultrasonication method was found to be 31.1mg equivalent to the molar yield of 21.1%.

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## INTRODUCTION

Cell permeabilization is a technique which is used primarily for the recovery of microbial products. These products include proteins, enzymes, pharmaceuticals etc. Cell permeabilization can be used to produce a biotransformation system which can be an inexpensive alternative to purified enzymatic systems. Conventional forms of cell permeabilization were discussed with the addition of organic solvents (Flores *et al*., 1994). The principle focus of the study on one aspect of cell permeabilization is the production of cells suitable to use as a biotransformation system. During the past several decades, much interest has been generated in aromatic chemicals produced by biotransformation using microorganisms as the products obtained by this method are considered as natural (Berger, 1995). Many investigators have endeavored to increase the production of microbial metabolites using some stimulating agents, including fatty acids, surfactants, vegetable oils, and aromatic chemicals. These stimulating agents are known to mediate cell permeabilization by disorganizing the cell membrane and/or directly affecting the level of enzyme synthesis involved in product formation, thereby contributing to enhanced production of the target products (De leon *et al.*, 2003). The use of organic solvents is known as relatively effective methods for cell permeabilization, because organic solvents are less expensive than other stimulating agents and can be removed by simple evaporation (Choi *et al*., 2004; Smet *et al*., 1978). However, little information is currently available on the effects of physicochemical properties of organic solvents on cell permeabilization processes (Liu *et al*., 2000). The representative organic solvents which are reported to enhance cell membrane permeability are toluene, chloroform, ethanol, diethyl ether etc (shwetha kumari *et al.,* 2013). A few research reports have appeared in the literature to explain the effect of toluene on the permeability of the cell membrane (De leon *et al.,* 2003; Smet *et al*., 1978). Moreover, in comparison with all microbial cells, there are limited reports concerning enhancement of permeability of bacteria to improve the productivity of specific metabolites. In particular, relatively little attention has been paid to enhance the production of verbenol using permeabilisation methods (Agrawal *et al*., 1999). However, to the best of our knowledge, no attempt has been made concerning the use of organic solvents for the enhanced production of verbenone in the process of biotransformation. Hence the present study concerns the solubilisation of *Gluconobacter japonicus* cells with organic solvents and various chemical agents and physical methods

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like ultrasonication for the improved yield of verbenone. In preliminary studies, *Gluconobacter japonicus* which belongs to the Phylum proteobacteria and the family acetobacteriaceae was identified as a new bacterial isolate which produces verbenone in significant amounts (Deepthi priya *et al*., 2014, 2015a) from biotransformation of α -(+)- pinene. In the present study, the effects of several organic solvents on *Gluconobacter japonicus* was investigated in an attempt to enhance verbenone production. The effect of permeabilisation on verbenone production was elucidated by analyzing the concentration of verbenone before and after solvent treatment.

## MATERIALS AND METHODS

*Gluconobacter japonicus* MTCC 12284, Potato dextrose broth, α-(+)-pinene (>98% Fluka) and (IS)-(-)-verbenone (>99% Aldrich) were used as internal standards, Acetone, n-Butanol, Chloroform, Ethanol, Isopropanol, Potassium phosphate-Hexane, Triton X-100, Cetyl trimethyl ammonium bromide, Toluene and Sodium cholate. All other chemicals and solvents were of analytical grade purchased from Hi media.

### Chemical permeabilisation

The bacterial cells were treated with various organic solvents to enhance the cell permeability for substrate which leads to improve the yield of verbenone. The chemicals used for permeabilisation methods include Acetone, n-Butanol, Ethanol, Isopropanol, Kpi-n-hexane, Triton X-100, Chloroform, Cetyl trimethyl ammonium bromide, Toluene and Sodium cholate.

### Permeabilisation of Gluconobacter cells with organic solvents

The methodology of permeabilisation of *Gluconobacter* cells with organic solvents is based on shwetha kumari *et al.,* (2013). The microbial inoculum of 1 ml was grown in the presence of 1% (v/v) of α-(+)-pinene in 30ml of potato dextrose broth at pH 6 at 30°C, with 1% glucose and 0.6% yeast extract for 72 hrs. The induced microbial cells were harvested by centrifugation at 8000rpm for 10min at 4°C and the cells were washed with potassium phosphate buffer (0.1M at pH 7). Permeabilisation of cells was performed by adding 10 ml of 30 % acetone to the cells. The contents were mixed on a vortex shaker and incubated for 10 min at 30°C. Biomass is harvested by centrifugation at 8000 rpm for 20 min and 1 gm of biomass was added to 30 ml of mineral media with 0.5% (v/v) of α-(+)-pinene. Biotransformation is performed at pH 6 at 33°C and 275 rpm for 168 hrs. Permeabilisation of *Gluconobacter* cells was also performed with butanol, isopropanol, ethanol, chloroform and Triton x-100 using the same methodology by keeping all the parametres constant. The product recovery was performed by liquid – liquid extraction using ethyl acetate as solvent. The final solution was dried over anhydrous sodium sulphate. The concentrated sample was analysed using GC-MS and verbenone concentration was quantified in GC for every 24 hrs using internal standard verbenone. The reaction products were identified by (GC-MS, Agilent 5975C), using a capillary column HP5 (30m×0.32mm). The column temperature was programmed to 50°C for 3min, increased at 5°C/min at 130°C and then increased at 15°C/min at 210°C by 5 min. Helium is used as the carrier gas, the injection and detector temperatures were at 250°C. The dried solution of 1µl was injected into the GC/MS system the apparatus is operated with a flow rate of 1 ml/min in electronic impact mode of 70eV and in split mode. The identification of the compounds was accomplished by comparing the mass spectra from the NIST library and by additional comparision of the GC retention time of standard compounds and GC-MS fragmentation pattern. 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 quantification was carried out by the standard calibration curve of the interest compound, evaluating the relative area from the interest compound.

### Permeabilisation of Gluconobacter cells with K-pi-n-hexane buffer

The methodology of permeabilisation with K-pi-n-hexane buffer is based on Hikmet Geckil *et al*., (2005). The microbial cells were grown for 72 hrs in 30 ml of potato dextrose broth at pH 6 at 30°C, with 1% glucose and 0.6% yeast extract in the presence of 1% (v/v) of α-(+)-pinene. Biomass harvested after centrifuging at 8000rpm for 10min at 4°C was washed once with 0.05 M potassium phosphate (K-Pi) buffer (pH 7) and resuspended in the same buffer with 1% hexane. Suspension is incubated at room temperature for 1 hour, briefly vortexing for every 10 min. The tube caps were left open for 5 min in order to evaporate volatile upper phase (hexane) prior to analysis of biotransformation ability in cell-free aqueous phase. Biotransformation of 0.5% (v/v) of α-(+)- pinene is carried out with 1 gm of permeabilised cells at pH 6 at 33°C and 275 rpm in mineral media for 168 hrs. The product recovery was performed by liquid – liquid extraction using ethyl acetate as solvent. The final solution was dried over anhydrous sodium sulphate and the concentrated sample was analysed using GC-MS. Verbenone concentration obtained on biotransformation of 0.5% (v/v) of α-(+)- pinene using cell free aqueous phase and permeabilised cells was quantified in GC for every 24 hrs.

### Permeabilisation of Gluconobacter cells with Toluene

The microbial cells were grown for 72 hrs in 30 ml of potato dextrose broth at pH 6 at 30°C, with 1% glucose and 0.6% yeast extract in the presence of 1% (v/v) of α-(+)-pinene. The cells were harvested by centrifugation at 8000 g for 30 min at 4°C and washed twice in 1% (v/v) glycerol at pH 7.0. The cell pellets were resuspended in 1% (v/v) glycerol with 10 mM EDTA/pH 7.0 and toluene was added to a final concentration of 1% (v/v) (Nicholas & Daniel, 1979). The mixture was vortexed for 2 min and then incubated for 1 hr at 30°C with rotary shaking and intermittent vortexing. The cells were harvested by centrifugation, washed in 1% glycerol at pH 7.0 and then suspended in the same buffer. The mixture was kept at 30°C for 30-45 min with rotary shaking prior to the analysis. Samples were removed and added to at least 9 volumes of ice-cold acetone. The cellular precipitates were collected by centrifugation and the pellets were washed again in ice-cold 30% (v/v) aqueous acetone. Biotransformation of α-(+)- pinene is performed at pH 6 at 33°C and 275rpm in mineral media with 1gm of permeabilised cells. The product recovery was performed by liquid – liquid extraction using ethyl acetate as solvent. The final solution was dried over anhydrous sodium sulphate. The concentrated sample was analysed using GC-MS. verbenone concentration obtained on biotransformation of

0.5% (v/v) of α-(+)- pinene using cell free aqueous phase and permeabilised cells was quantified in GC for every 24 hrs.

**Permeabilisation of Gluconobacter with Cetyl trimethyl ammonium bromide**

The microbial cells were grown for 72 hrs in 30ml of potato dextrose broth at pH 6 at 30°C, with 1% glucose and 0.6% yeast extract in the presence of 1% (v/v) of α-(+)-pinene. Approximately 1g of induced cells collected at the late exponential phase was separated by centrifugation. A permeabilized solution prepared with cetyl trimethyl ammonium bromide of 0.2% (w/v) in 0.1M citrate buffer (pH = 6.2) was added to the cells, in a proportion of 0.05g per gram of cells (Rehr *et al*., 1991). The suspension was homogenized and placed in a shaker incubator at 300 rpm for 30 min. The cells were centrifuged and washed with 0.1M citrate buffer (pH 6.2) and resuspended in water. Biotransformation of 0.5% (v/v) of α-(+)-pinene was carried out using free permeabilised cells at pH 6 at 33°C and 275 rpm in mineral media for 168 hrs. The product recovery was performed by liquid – liquid extraction using ethyl acetate as solvent. The final solution was dried over anhydrous sodium sulphate. The concentrated sample was analysed using GC-MS. verbenone concentration was quantified in GC for every 24 hrs.

**Permeabilisation of Gluconobacter with Sodium cholate**

The microbial inoculum of 1ml was grown for 72 hrs in 30 ml of potato dextrose broth at pH 6 at 30°C, with 1% glucose and 0.6% yeast extract in the presence of 1% (v/v) of α-(+)-pinene. Cells were stabilized and refrigerated at 4°C. Biomass obtained is weighed and macerated with 10ml of 0.05M phosphate buffer and sonicated for 1 minute in ice bath and the cells were solubilised with 0.05% sodium cholate and also with 0.1% sodium cholate and ultra centrifuged at 40,000rpm respectively. Supernatant was analysed for enzymatic conversion of α-(+)-pinene to verbenone. Biotransformation of 0.5% (v/v) of α-(+)-pinene was also carried out using 1gm of free permeabilised cells at pH 6 at 33°C and 275 rpm in 30ml of mineral media for 168 hrs. The product recovery was performed by liquid – liquid extraction using ethyl acetate as solvent. The final solution was dried over anhydrous sodium sulphate. The concentrated sample was analysed using GC-MS. Verbenone concentration was quantified using GC for every 24 hrs.

**Physical permeabilisation of Gluconobacter cells using Ultrasonication**

The microbial cells were grown for 72 hrs in 30 ml of potato dextrose broth at pH 6 at 30°C, with 1% glucose and 0.6% yeast extract in the presence of 1% (v/v) of α-(+)-pinene. Biomass obtained after growth under optimized conditions was subjected to ultrasonication for 20 minutes under ice in ultrasonicator. Cells were collected by centrifugation at 8000 g for 10 min at 4°C and washed twice with cold phosphate buffer saline. The cells were suspended in cold phosphate buffer saline to OD (600 nm) 1.0. Cell suspension is transferred to sonication tubes with 1M Tris Buffer and sonicated at high Power for 10 min. The cells were centrifuged at 10,000 rpm for 15 min at 4°C. Supernatant is separated from the insoluble pellet. Biotransformation of 0.5% (v/v) of α-(+)-pinene was carried out using free permeabilised cells and with crude enzyme extract at pH 6 at 33°C and 275 rpm in mineral media for 168 hrs. The product recovery was performed by liquid – liquid extraction using ethyl acetate as solvent. The final solution was dried over anhydrous sodium sulphate. The concentrated sample was analysed using GC-MS. Verbenone concentration was quantified using GC for every 24 hrs.

**Determination of degradation pathway of α-(+)- pinene by *Gluconobacter***

The metabolic profile of *Gluconobacter* isolate was studied in water–organic solvent systems using α-(+)-pinene for both growth and biotransformation processes and determined the pathway of verbenone formation from α-(+)-pinene degradation.

***a. Pre-culture preparation***

The methodology of preculture preparation is based on Bicas *et al*., (2008). Three full loops of a 24 hrs-old culture from culture plate was transferred to 500 ml conical flask with 1.0 g glucose in 250 ml of mineral media. The flasks were incubated at 30°C and 200 rpm for 24 hrs, to reach an optical density of 2.0 at 600 nm.

***b. Cell cultures***

30 ml of the pre-culture was aseptically transferred to a 500 ml conical flask containing 300ml of mineral medium with the sole carbon source of 1% (v/v) of α-(+)- pinene. The flasks were left at 30°C and 275 rpm for 24 hrs.

***c. Recovery of the biomass***

The culture was centrifuged at 8000g for 10 min, the supernatant was removed and the resulting biomass was resuspended in 25ml of 30mM phosphate buffer (pH 7.5). This biomass was either used directly (biotransformation with fresh cells) or was frozen (-18°C) for the assessments with crude enzymatic extracts.

***d. Production of the crude enzymatic extracts***

The cells were disrupted in a high-pressure homogenizer using 1000 bars pressure. The concentrated biomass was thawed and treated for 1 hr at 30°C and 200rpm with 10% (v/v) ethyl acetate (Fontanille & Larroche, 2003). Cells were also subjected to permeabilisation by distrupting the cells using ultrasonicator for 30 min to prepare the enzyme crude extracts. The suspension resulting from this operation was membrane filtered to get the viable cells and also crude enzymatic extracts.

***e. Biotransformation procedure***

During biotransformation, 30ml of the concentrated biomass and the same volume of ethyl acetate were transferred to 250 ml conical flask with 30ml of mineral media. 1% (v/v) of α-(+) - pinene was added and the flasks were incubated at 30°C and 275 rpm for 24 hrs. Samples were periodically taken from the organic and aqueous phases to follow substrate consumption and product formation. The organic phase was directly injected (1µl) in the gas chromatograph while the aqueous phase had to be acidified with 20 µl/ml of concentrated sulfuric acid and extracted with the same volume of ethyl acetate and injected (1µl) in the gas chromatograph. The procedure for the crude

enzymatic extract is performed as above with 1% (v/v) of α-(+) - pinene. The concentrated sample was analysed using GC-MS for the detection of products obtained.

## RESULTS AND DISCUSSION

The effect of different permeabilising agents on verbenone yield was studied by supplementing 10% (v/v) of each chemical/organic solvent to permeabilise the biomass. Of the various permeabilising agents examined, permeabilising the cells with toluene, ultrasonication, cetyl trimethyl ammonium bromide and kpi- n hexane displayed enhanced verbenone production by 2 fold than the control (Fig.1). Permeabilising the cells with acetone, ethanol, chloroform and triton x also resulted in increased verbenone production compared to control (with unpermeabilised cells). On permeabilising the *Gluconobacter* cells using ultrasonication method, prior to the biotransformation, 31.1mg (21.7% molar yield) of verbenone is produced with 97.3% substrate utilization in 96 hrs of incubation. On permeabilising the cells with toluene, a significant increase in verbenone production of (34.48mg) (23.4% molar yield) was achieved in 96 hrs of incubation with 100% substrate conversion (Fig.2) (17.4 Retention Time). The results have shown that toluene was the best stimulant for verbenone production. The choice of cell-permeabilizing agent depends on the organism and the composition of the cell wall and its membrane. It is obvious that the connection between permeabilization and the loss of cell viability is the result of the leakage of essential compounds from the cells. The enhanced production of verbenone with toluene in this study is the result of the secretary action of toluene, which acts by modifying the outer cell structure, thereby driving out the verbenone entrapped inside the cells. Smet *et al*. (1978) stressed the important role of the outer membrane in cell permeabilization with toluene. They reported that toluene causes considerable damage to the cytoplasmic membrane, while the outer membrane remains relatively integral. The permeability characteristics of toluene-treated cells depend on the state of the outer membrane (Felix, 1982). The present results also support the role of toluene in enhancing verbenone production by its secretary action. It was observed that alpha terpineol and limonene is not detected in any of the biotransformation flasks conducted with permeabilised cells.

Permeabilisation of *Gluconobacter* cells with butanol, isopropanol, sodium cholate resulted in decrease in verbenone production, due to the inactivation of enzyme by the solvents even though they are widely known as effective cell permeabilizing agents (Shwetha kumari *et al.,* 2013). It is notified that none of the products were detected in sodium cholate solubilisation. Interestingly permeabilisation using sodium cholate was reported in *Aspergillus niger* species for verbenol formation which resulted in improvement of the verbenol yield and conversion of α-(+)-pinene (Agrawal & joseph, 2000). When the cells were treated with organic solvents, the organic solvents dissolved the cell membrane lipids and decreased the permeability barrier due to the diminishing of lipids from the cell membrane. Cells should be permeabilized without lysis or destruction of the whole inner organization. Nevertheless, the cells are obviously no longer viable after permeabilization by treatment with organic acids. Hence the solvents were added in the late stage of growth phases in this study. To date, many investigators have studied the effectiveness of organic solvents on cell permeabilization for enhanced production of target metabolites. Nevertheless, relatively few reports have been published in the fields of verbenone production by way of permeabilized cells. In previous studies of ultrasonication during verbenone production, the dehydrogenase activity was studied in supernatant and the hydroxylase activity was studied in pellet (Agrawal *et al*., 1999; Karp *et al*., 1990). In the present study, the enzyme activity was studied in supernatant as well as in pellet therefore solubilisation of the cells was done with tris buffer followed by ultrasonication. Seperation of the crude enzyme extract after sonication of the biomass was done by ultra centrifugation at 8000rpm for 30 min. It was found that solubilisation and sonication helped in releasing enzyme into the supernatant. It was observed that verbenol, verbenone and sobrerol were detected in supernatant as well in cells extract. But it is worth noting that from GC chromatographs of Figure: 3 and Figure: 4, the concentration of verbenone (17.5 retention time) is 2 fold higher in supernatant (31.1mg) (peak area 13.3) than pellet (15.3mg) (peak area 6.7) predicting the maximum enzyme activity in the supernatant. It was interesting to note that when cells were treated with kpi –n hexane buffer, maximum amount of verbenone is obtained with permeabilised cells than with supernatant. On permeabilising the cells with k-pi n –hexane, 26.06mg **(**17.9% molar yield) of verbenone is produced with 98.6% substrate conversion in 144 hrs of incubation. A possible explanation is that the cell envelope of the bacteria is prone to hexane - k pi interactions in the polysaccharide layer which are destabilized making the solvent flow in to the membrane layer probable and thus slacken cell envelope for enzyme release (Rebeca chung *et al*., 2010).

*Gluconobacter japonicus* an acetic acid bacterium cultures is reported to produce maximum concentration of verbenone at 33°C temperature, and pH 6 with 1% glucose concentration and 1g of induced biomass isolated at log phase of the culture in production media (Deepthi priya *et al*., 2015b). There is probably a specific mechanism involved in the biotransformation of **α-(+)-** pinene to verbenone, signifying the action of a variety of enzymes of *Gluconobacter* cultures (Lidia Stasiak *et al*., 2009; Peter Raspor, 2008; Jintana Kommane *et al*., 2012). To define the metabolic pathways through which the terpene substrates were degraded and to find activities that could be exploited at a preparative scale, bioconversion experiments were carried out with either concentrated fresh cells or crude enzymatic extracts. To elucidate the metabolic pathway of *Gluconobacter japonicus* culture, the micro-organism was first tested for its ability to grow on α-(+)- pinene as sole carbon and energy sources. The growth was stopped after 24 hrs because the biomass obtained at this time was a good indicator of the ability of a given substrate to support bacterial growth. The utilization of α-(+)-pinene indicated that a metabolic pathway involving it was actually active in the bacterial strain. Results obtained were presented in Table: 1, with the main products accumulated after 24 hrs of bioconversion. As per the results shown in Figure: 5, When a fresh biomass of *Gluconobacter* culture grown on α-(+) -pinene was used to convert α-(+)- pinene, verbenol was the major product accumulated after 24 hrs with verbenone and sobrerol. Verbenol was the first intermediate in α-(+)- pinene degradation, which allowed the accumulation of verbenone. In later days of incubation, verbenol levels were decreased and increase in verbenone concentration was observed. The behaviour of the crude enzymatic extracts obtained also demonstrated that verbenol was the major product after 24 hrs along with verbenone and sobrerol (Fig.6).

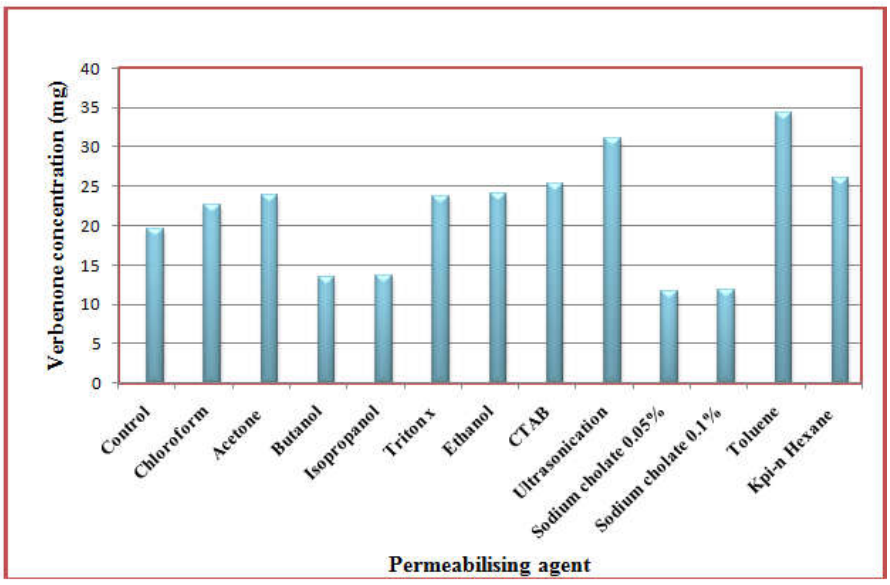


**Figure 1. Effect of permeabilising agents on verbenone concentration on biotransformation of 0.5% (v/v) of α-(+) pinene with permeabilised cells of *Gluconobacter i*solate at 33°C, at pH 6 in 168 hrs of incubation**

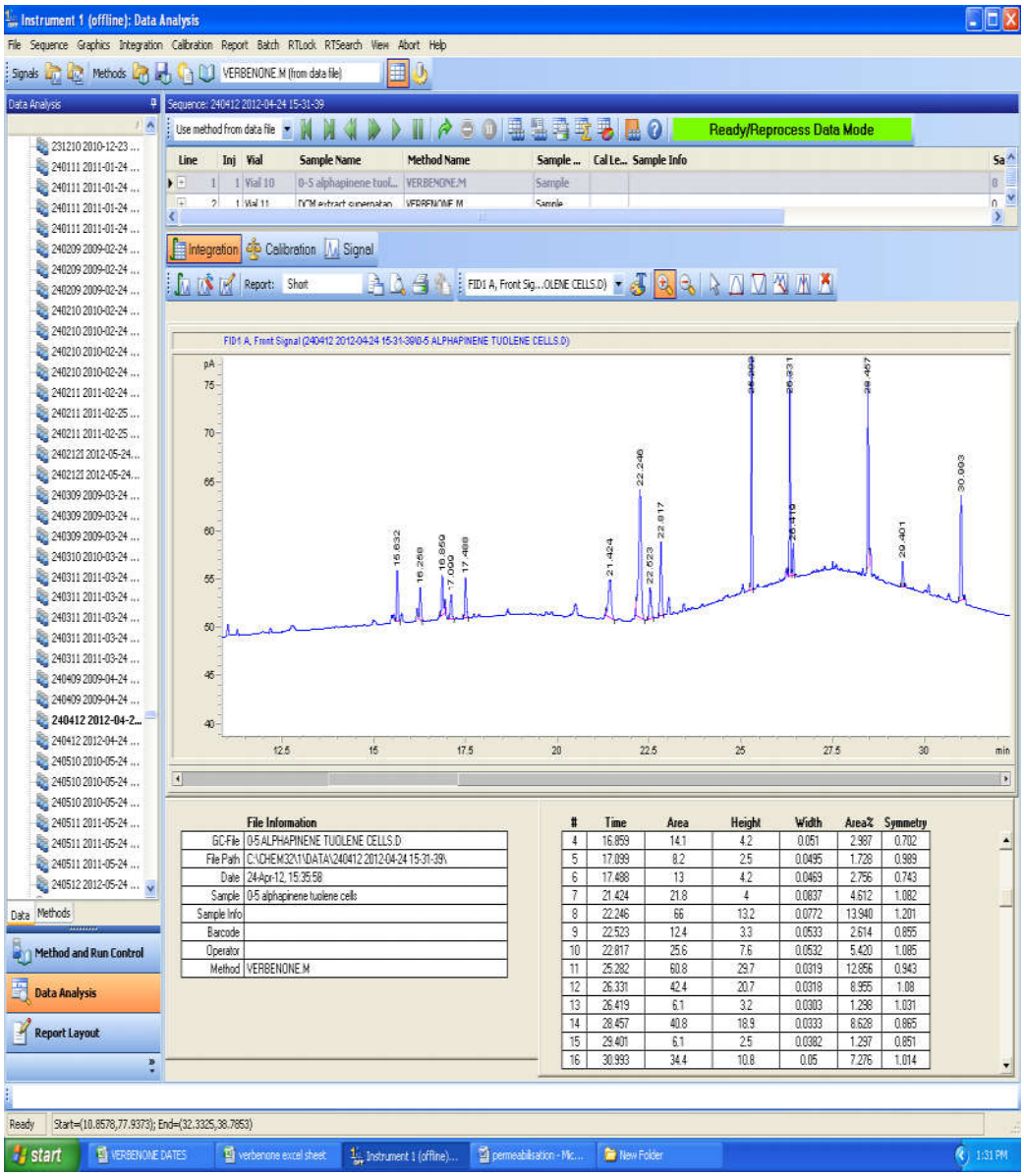


**Figure 2. GC chromatogram of products obtained on 0.5% (v/v) of α-(+)- pinene biotransformation by permeabilised cells of *Gluconobacter* isolate with toluene at 33°C at pH 6 in 96 hrs of incubation**

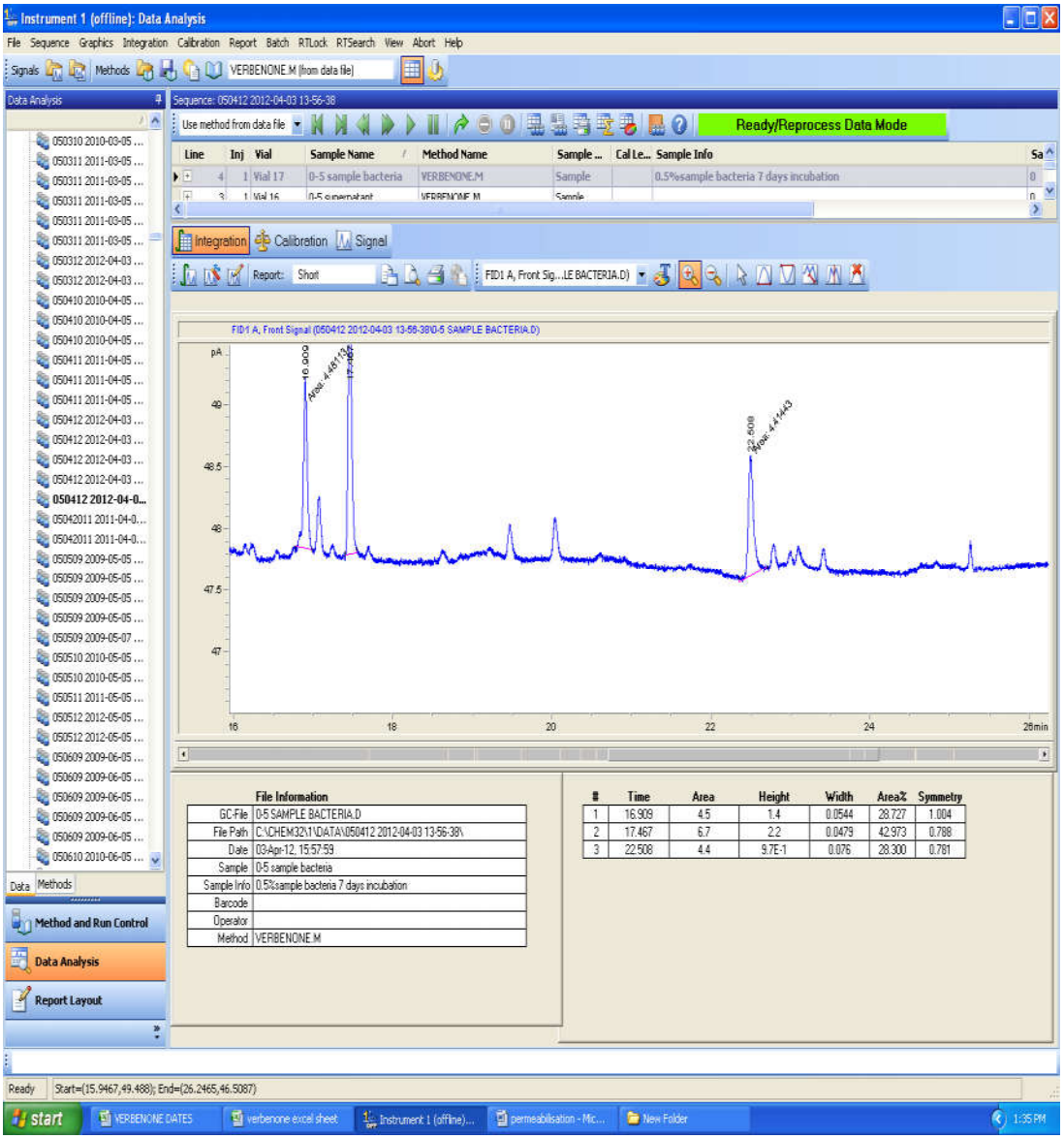


**Figure 3. GC chromatogram of products obtained on 0.5% (v/v) of α-(+)- pinene biotransformation performed with ultrasonicated cells of *Gluconobacter* isolate at 33°C at pH 6 in 96 hrs of incubation**

**Table 1. Products detected in the pathway of α-(+)- pinene biotransformation with *Gluconobacter* culture**

| Product | Retention time | Freshly grown cells | Crude enzymatic extract |
|---|---|---|---|
| Camphene | 8.11 | • | × |
| β - pinene | 9.01 | • | × |
| Limonene | 10.71 | • | × |
| Verbenol | 14.5 | • | • |
| Verbenone | 16.6 | • | • |
| Isoborneol | 15.05 | • | × |
| bornyl acetate | 15.06 | • | × |
| alpha terpineol | 15.98 | • | × |
| Sobrerol | 21.032 | • | • |

× - Product not detected
• - Product detected

Predicting the mechanism involved in the verbenone formation, *Gluconobacter* sps were reported to have FAD monoxygenases (Jintana Kommanee *et al*., 2012; Arun Gupta *et al*., 2001) in their enzymatic system of membranes. α-(+)-pinene undergoes allylic oxidation at carbon 4 through the monooxygenases to form verbenol and followed by dehydrogenation through membrane bound alcohol dehydrogenase enzyme to form verbenone (Fig:7). Biotransformation studies of α-(+)- pinene using *Gluconobacter japonicus* cultures is reported primarily in the record of α-(+)- pinene degradations (Deepthi priya *et al*., 2015b). *Gluconobacter* species are capable of incomplete oxidations which are exploited in food biotechnology. The species is also reported to involve in various ketones and acid production. The pathway of verbenone production from α-(+) -pinene has been reported in different studies of α-(+)- pinene degradations (Agrawal & joseph, 2000; Prema & Bhattacharya, 1962).

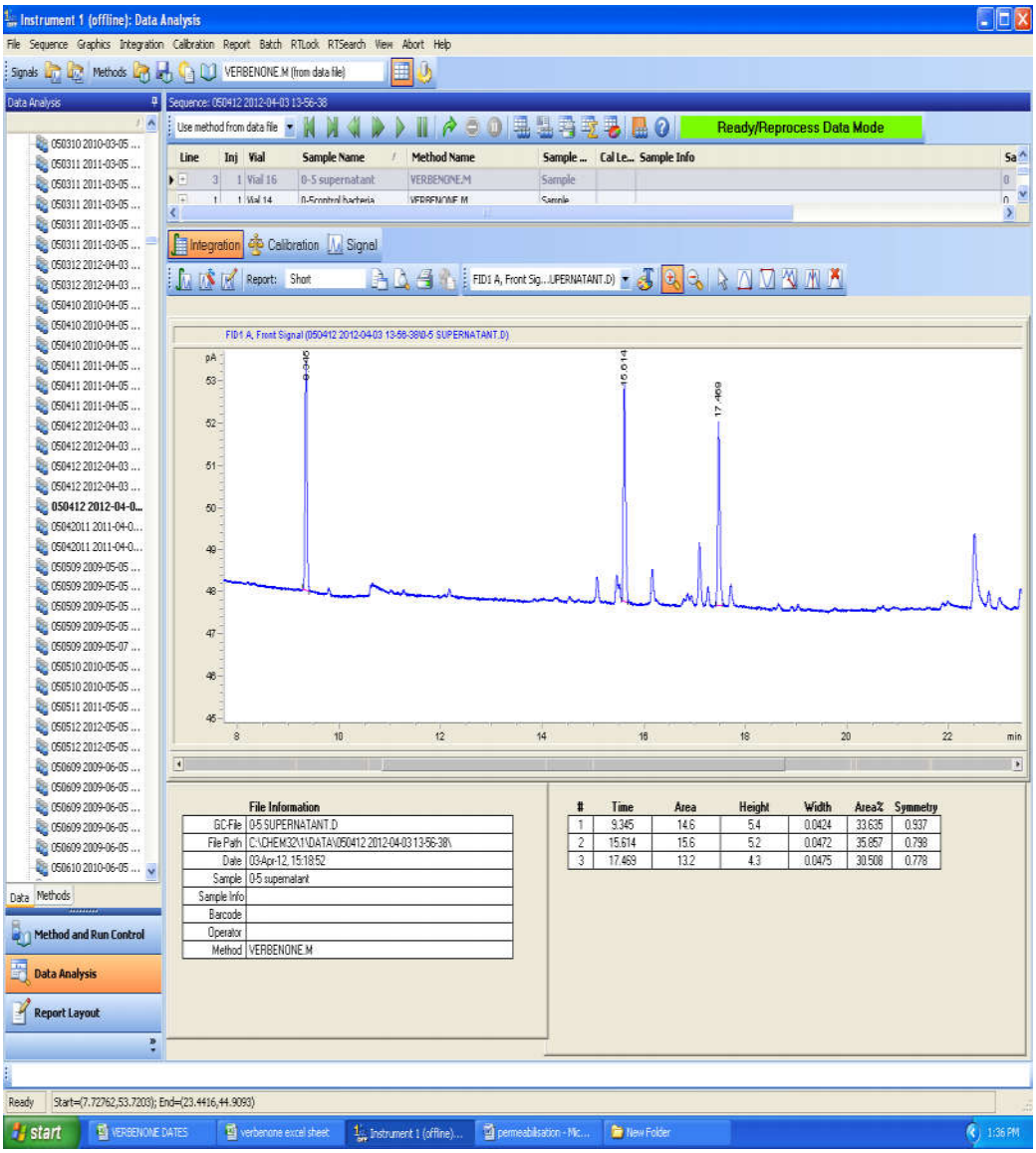


**Figure 4. GC chromatogram of products obtained on 0.5% (v/v) of α-(+)- pinene biotransformation performed with supernatant obtained during ultrasonication of *Gluconobacter* isolate at 33°C at pH 6 in 96 hrs of incubation**

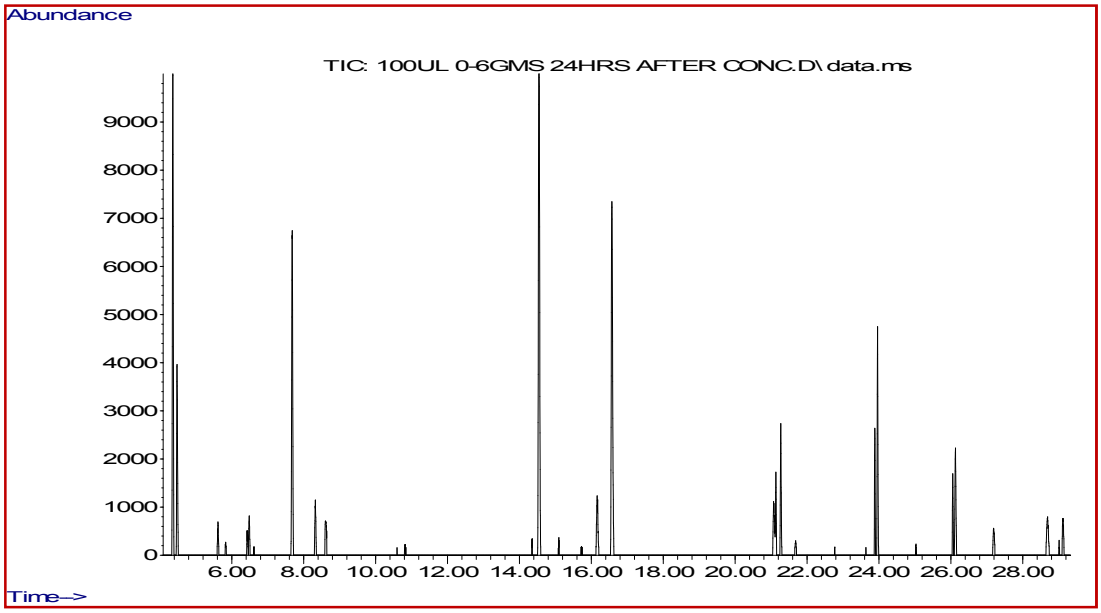


**Figure 5. GC-MS Chromatogram of products detected in the pathway of biotransformation of α-(+)- pinene with freshly grown cells of *Gluconobacter***

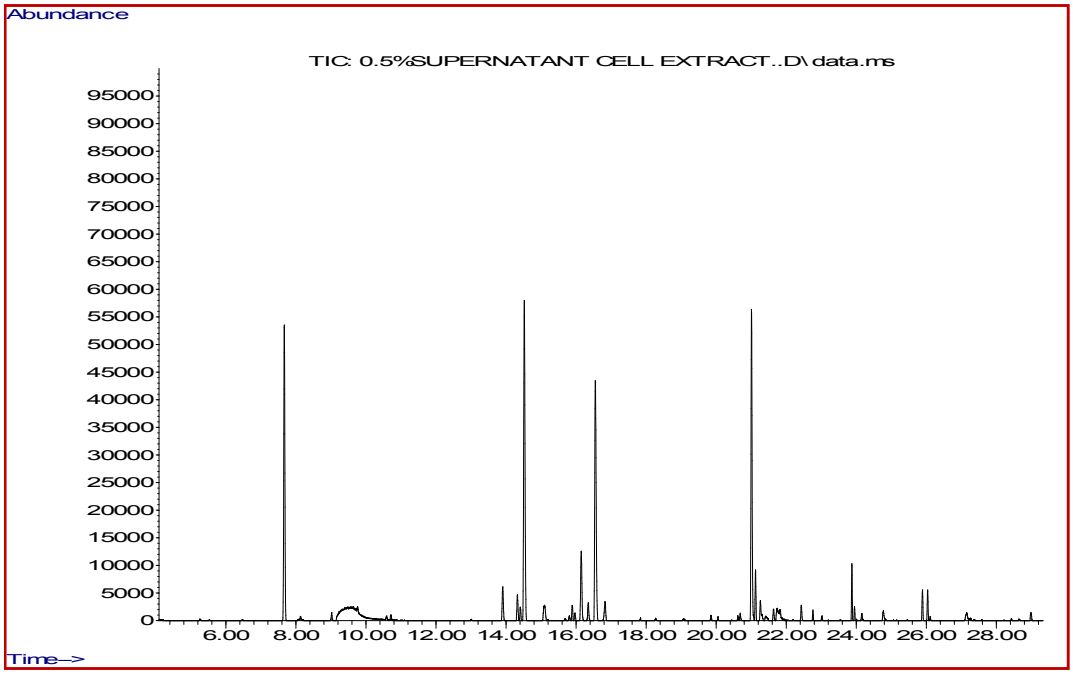


**Figure 6. GC-MS Chromatogram of products detected in the pathway of biotransformation of α-(+) - pinene with crude enzyme extract of *Gluconobacter***

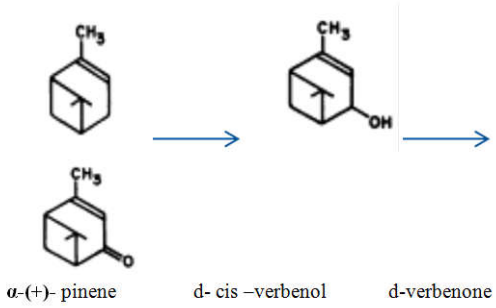


**Figure 7. Pathway of α-(+)- pinene transformation to verbenone with *Gluconobacter* culture**

## CONCLUSION

From the various permeabilising agents examined, permeabilising the cells with toluene, ultrasonication, cetyl trimethyl ammonium bromide and kpi- n hexane resulted in enhanced verbenone production by 2 fold. Permeabilising the cells with acetone, ethanol, chloroform and triton x also resulted in increased verbenone production. The present study proved the effectiveness of various organic solvents on cell permeabilization in improving the yield of verbenone. Our results also support the role of toluene in enhancing verbenone production by its secretary action. The bioconversion of α-(+)-pinene to verbenone by *Gluconobacter japonicus* cultures implicated an oxidation step to form intermediate verbenol, followed by a dehydrogenation to form verbenone. This work progress towards the biotechnological production of valuable natural flavour and fragrances compounds from cheap and abundantly available natural terpenoids.

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