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Using Eucalypt Oil as a Carbon Source for Deriving Other Compounds – Development of a microbial library

A report for the RIRDC/ Land & Water
Australia/ FWPRDC/ MDBC
Joint Venture Agroforestry Program

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Foreword

Land damage by salinity is a serious issue for Australian landowners and agricultural producers. To address this, farmers are planting trees – in many cases oil mallees – which will gradually resolve the increasing salinity in the area. Such large plantations of *Eucalyptus* will result in an abundance of leaf oil. This represents an opportunity for Australia to discover and improve on eucalypt oil applications. Correspondingly, development of new commercial uses for eucalypt oil could assist with encouraging planting for land rehabilitation, and diversification of farm income.

This publication describes the use of a proprietary device, Evolver[®], to develop a library of microbes that consume 1,8-cineole as the sole carbon source. It also provides a preliminary analysis of some of the by-product derivatives produced by these discovered microbes and suggests future directions as to their application.

The project found that it was able to multiply (enrich for) microbes that can survive and grow in the presence of relatively high and constant concentrations of 1,8-cineole – and thus produce 1,8-cineole derivatives in a manufacturing setting. Fourteen (14) strains of bacteria were isolated from samples obtained from the Evolver[®], nine of which may be independently different strains. These bacteria can be used for further multiplication and production of cineole derivatives. Preliminary characterization of the bacteria suggests that additional species, not previously reported to grow on 1,8-cineole, may have been isolated. Four cineole derivatives were discovered of which three are thought to be intermediate compounds and one may be a new product. A range of other compounds were observed in low quantities. Further research is required to identify the microbes, and to increase quantities of the cineole derivatives, screen their activity as potential anti-cancer, anti-microbial and herbicide compounds, and investigate chemical modification of the derivatives for use as surfactants and solvents.

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This report is an addition to RIRDC's diverse range of over 1600 research publications. It forms part of our Agroforestry and Farm Forestry R&D program, which aims to integrate sustainable and productive agroforestry within Australian farming systems. The JVAP, under this program, is managed by RIRDC.

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Peter O'Brien

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Abbreviations

DM = Defined Media
DRBC = Dichloran-Rose Bengal Chloramphenicol Agar
FID = Flame Ionisation Detector
FW = Formula Weight
GC = Gas Chromatography
GC-MS = Gas Chromatography – Mass Spectrometry
IUPAC = International Union of Pure and Applied Chemistry
NIST = National Institute of Standards Technology
NMR = Nuclear Magnetic Resonance
OD = Optical Density
PBS = Phosphate Buffered Saline
YNB = Yeast Nitrogen Base

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Executive Summary

What the report is about

Microbes can cheaply and environmentally break down abundant carbon sources. This presents an opportunity to derive new applications for eucalypt oil which may have broadscale, commercial application. This publication describes the use of a proprietary device to develop a library of microbes that consume 1,8-cineole as the sole carbon source. It also provides a preliminary analysis of some of the by-product derivatives produced by these discovered microbes and suggests future directions as to their application. This project is the first phase of a longer-term project on adding value to eucalypt oil.

Who is the report targeted at?

The report is for researchers and industry investors interested in the scope for new chemical products from eucalypt oil, that may have broadscale commercial or industrial application.

Background

It is estimated that around 6 million hectares are affected by dryland salinity and this will increase to 17 million hectares in the coming decades. As a result, *Eucalyptus* trees are being planted at a large scale to address this issue. This will lead to an abundance of eucalypt oil available for world markets and hence presents an opportunity for Australia to discover and improve on eucalypt oil applications. Incorporating added value products derived from biocatalysis of key eucalypt oil constituents with schemes such as the integrated tree crop system of Western Australia, would assist in the exploitation of these potential opportunities.

Novel applications for the eucalypt oil constituents in the chemical, solvent, perfumery, pharmaceutical, agricultural and paint industries are possible using biocatalysis. Environmentally, microbial or enzymatic transformations are preferable to traditional chemical synthetic approaches since they are less polluting and often require less energy consumption. Microbial transformations are also more specific in their catalysis and can often carry out a complex multi-step synthesis in a single transformation. This biological approach to chemistry has already been championed recently by chemical companies such as DuPont and Cargill-Dow. The more applications found for eucalypt oil the greater the positive impact on the environment through providing an incentive for farmers to plant trees to try to address the dryland salinity issue, and improve carbon sequestration.

The long term outcome for this project (i.e. beyond this proposal) is to derive new chemical entities with new applications using biocatalysis (such as microbial biotransformations or enzymatic transformations) from *Eucalyptus* leaf oil constituents, with a view to addressing the large surplus forecast for eucalypt oil. As the first phase of such a project, the establishment of a microbial library is required and is the subject of this project.

Aims/Objectives

The aims for this specific project were:

1. Establish a microbial library that can utilize 1,8-cineole as a sole carbon source either in a purified form or from crude leaf oil from *E. polybractea*.
2. Preliminary identification of chemical products derived from microbial transformations of 1,8-cineole using GC-mass spectrometry analysis.

Methods used

Methods were:

- 1) Enrichment of microbes using 1,8-cineole as the sole carbon source. Enrichment took place using a proprietary enrichment device developed at CSIRO.
- 2) GC Mass spectrometry analysis of enriched supernatant
- 3) Obtaining pure cultures by monoculturing onto plates of defined media, with the 1,8-cineole as the carbon source.

- 4) Purified cultures (scaled up on 1,8-cineole as the sole carbon source), metabolic byproducts from supernatants, and metabolic intermediates from cell extracts analyzed on the GC Mass spectrometer.
- 5) Pure cultures which produced potentially useful products from 1,8-cineole as the sole carbon source, were adapted to grow on crude eucalypt oil from *E. polybractea*.
- 6) Finally microbes were catalogued accordingly and stored appropriately depending on their identification.

Results/Key findings

The findings of this project were as follows:

A) Enrichment of microbes using 1,8-cineole as the sole carbon source:

A proprietary device, the Evolver[®] invented at CSIRO was used to enrich for microbes that can grow on 1,8-cineole as the sole carbon source. By enriching for 1,8-cineole consumers, it was anticipated that 1,8-cineole derivatives would form either as terminal by-products or as intermediates of microbial metabolic function.

Using the Evolver[®], a consortia of microbes were enriched to use 1,8-cineole as the sole carbon source. Unlike previous literature which reports 1,8-cineole microbial metabolism, the Evolver[®] allowed for the enrichment of microbes at a constantly controlled concentration of 1,8-cineole. This constant concentration of 1,8-cineole was 0.6 to 0.8g l⁻¹ and such conditions have not previously been reported. Such conditions are unique in that we have now enriched for microbes that can survive and indeed grow in the presence of relatively high and constant concentrations of 1,8-cineole and thus produce 1,8-cineole derivatives in a manufacturing setting.

B) Obtaining pure cultures by monoculturing onto plates of defined media with the 1,8-cineole as the carbon source:

Enriched microbes that could grow on 1,8-cineole as the sole carbon source (obtained from the Evolver[®]), needed to be purified to monocultures, to allow for further analysis. Fourteen (14) strains of bacteria were isolated from samples obtained from the Evolver[®], nine of which may be independently different strains. Although the strains isolated in this study have not been formally identified, preliminary characterization of the isolates suggests that additional species, not previously reported to grow on 1,8-cineole, may have been isolated.

Two isolates could grow on 2 g l⁻¹ 1,8-cineole, a level higher than previously attained in other studies. This is a significant achievement in a short time frame and using the Evolver[®] to further acclimate microbes to even higher concentrations would be strongly recommended.

One purified bacterial isolate (B2) was also grown on eucalypt oil (90% pure 1,8-cineole) to evaluate its ability to consume 1,8-cineole in the presence of other carbon sources. In this instance it was observed that B2 could grow readily in eucalypt oil.

All microbes isolated after 1,8-cineole growth have been catalogued.

C) GC-Mass Spectrometry analysis of 1,8-cineole derivatives:

Purified microbes isolated during the monoculturing phase of the project were scaled up in the presence of 1,8-cineole as the sole carbon source. The supernatants and microbial lysates from the purified microbes, as well as from the Evolver[®], were analyzed on a GC mass spectrometer to identify any 1,8-cineole derivatives that may have resulted during growth of the microbes on 1,8-cineole. Four compounds were consistently observed. Three of these are considered to be intermediates as they are present whilst the bacteria were growing, while the other one was considered as product because it appeared at the final stages of bacterial growth.

Mass spectral library matching using the National Institute of Standards Technology (NIST) database did not indicate any firm matches with the 4 cineole derivatives discovered, and our initial proposed definition would have indicated entirely new chiral compounds. However, on the basis of published literature, the three ‘intermediates’ are postulated to be two isomers of 2-hydroxy-1,8-cineole and 2-oxo-1,8-cineole. The ‘product’ has not been formally identified. In one case, there is also evidence consistent with the formation of a di-hydroxy-1,8-cineole. Several other compounds were observed when eucalypt oil (crude cineole) was used as substrate, but their concentrations were too low to allow mass spectra to be obtained in the time available.

This project has thus delivered on the outcomes stated in the initial application by:

- 1) Establishing a microbial library that can utilize 1,8-cineole as a sole carbon source in a purified form and from crude leaf oil from *E. polybractea*.
- 2) Identifying chemical products derived from microbial transformations of 1,8-cineole using GC-mass spectrometry analysis.

Implications for relevant stakeholders:

This project represents a preliminary investigation of the scope to derive new chemical entities, and will require further investment to determine its commercial prospects.

Recommendations

Further extension of this work is now necessary if the microbes and chemicals discovered are to be applied for commercial advantage. The following recommendations are made:

- A) Identify the isolated microbes to species level in order to assess for any new species discovered.
- B) Given the rapid manner by which microbes can be enriched using the Evolver[®], the microbial library should be expanded. This is recommended since only a single pH and temperature was used as enrichment parameters in the Evolver[®]. Expanding the library through use of a range of pH and temperature conditions, would also allow for isolates such as B2 to acclimate to even higher 1,8-cineole concentrations.
- C) Make sufficient quantities of 1,8-cineole derivatives to isolate and confirm the structures of 1,8-cineole derivatives using Nuclear Magnetic Resonance.
- D) Screen 1,8-cineole derivatives for potential anti-cancer, anti-microbial and herbicide activities. Esterification with carboxylic acids may also lead to perfumery products.
- E) Evaluate the chemical modification of 1,8-cineole derivatives for use as surfactants and solvents.
- F) Isolate, characterise, clone and express the enzyme(s) responsible for the production of the 1,8-cineole derivatives in 1,8-cineole tolerant microbes. Using directed evolution, improve the enzyme activity to productivity levels acceptable for manufacturing.

Chapter 1: Introduction

Land damage by salinity is a serious issue for Australian landowners and agricultural producers. Current estimates suggest that the 6 million hectares affected will increase to more than 17 million hectares by 2050 (National Land and Water Resources Audit, 2001). Extensive plantations of certain tree species such as *Eucalyptus* sp. have the potential to ameliorate and reverse the incursion of salt in many areas. The Western Australian government has for example planted 20000 ha of timber belt plantations of *Eucalyptus* to control salinity and produce pulpwood. Such large plantations of *Eucalyptus* will result in an abundance of leaf oil. This represents an opportunity for Australia to discover and improve on eucalypt oil applications. The long term aim for this project is therefore to derive new chemical entities with new or improved applications using biocatalysis (such as microbial biotransformations or enzymatic transformations) and *Eucalyptus* leaf oil constituents with a view to addressing the large surplus forecast for eucalypt oil.

The Oil Mallee Company estimates that the harvesting of *Eucalyptus* trees by farmers will yield \$15/tonne just for biomass if the trees are planted on land with production values of around \$79/hectare (source: <http://www.oilmallee.com.au/index.html>). Under best bet scenarios it is projected that this will be commercially attractive for farmers to pursue. However under less optimal conditions, an additional income from eucalypt oil would help make plantations in dryland areas more commercially viable. Oil mallees have the greatest potential for reforestation in areas affected by dryland salinity in Western Australia. It is estimated that 10 million hectares of land in Western Australia are situated in areas where reforestation with oil mallees is realistic for purposes such as the integrated tree processing plant. A joint project with the Department of Conservation of Land Management and Oil Mallee Association plans to seed up to 30 million trees in these areas. As at 2003, they had planted 9 million trees (<http://www.oilmallee.com.au/index.html>). It is anticipated that from current plantings, 20,000 tonnes of whole tree mallee biomass will be generated per year, and 250 tonnes of oil will be extracted per year (<http://www.oilmallee.com.au/index.html>). The projected expansion towards 30 million trees would require 10 plants be set up to handle the biomass generated, with a total output of 10,000 tonnes per year of oil (<http://www.oilmallee.com.au/index.html>).

Whether a large increase in supply of eucalypt oil to the world market would lead to new market opportunities depends on finding high value uses of the oil and decreasing cost of production. The integration of value-added products derived from biocatalysis of key eucalypt oil constituents into multiple-product schemes such as the biomass/charcoal/eucalypt essential oil integrated tree crop system of Western Australia, would allow exploitation of potential opportunities arising from an impending abundance of eucalypt oil. There are also environmental benefits, as financial returns provide an incentive for farmers to plant trees to address the dryland salinity issue; this will also lead to an improvement in carbon sequestration (an annual carbon sink of 3-4 tonnes of carbon per kilometre of hedge) and a possible source of speciality chemicals such as chemicals, solvents, paints etc.

Pre-World War 2, leaves of *Eucalyptus* species were simply distilled for their 1,8-cineole, or used for extraction of piperitone as a starting material for laevo-menthol and production of geranyl acetate. A small amount of oil was also used for bactericidal properties (Lassak, 2002). More recently, applications for eucalypt oils have been extended to include non-prescription medicinal purposes, perfumes and flavouring oils, industrial oils and sources of chemicals such as piperitone. The current world market for pharmaceutical eucalypt oil is only 3,500 tonnes per year (Lassak, 2002), with little possibility of increased Australian supply unless new value-added products are formulated and marketed (Zorzetto and Chudleigh, 1999). Eucalypt oil has also been used as a degreasing agent and fuel additive. However to date large scale industrial applications have not been developed due to small scale production, high cost, low reliability of supply and strong competition from other materials (Turner 2001). Further modification of such oil constituents via microbial or enzymatic biotransformations has not been thoroughly examined for its potential to generate new chemical entities with novel applications.

Eucalypt oil is comprised predominantly of mono- and sesquiterpenes. The monoterpenes are the major constituents and can be classified into acyclic, monocyclic and bicyclic. 1,8-cineole is an example of a bicyclic monoterpene. Sesquiterpenes are currently not the focus of this project since they are the minor constituents of eucalypt oils. 1,8-cineole, however, can comprise between 10% to 90% of the oil depending on the *Eucalyptus* species. It is the principal leaf oil constituent in one of the main Australian commercial oil species, *Eucalyptus polybractea*, making up between 60-90% of the leaf oil (Doran, 1992).

There has been a recent surge in interest for using microbial transformations for catalysing the production of novel chemicals, particularly in the chemical industry. For example, DuPont/Genencor have made a new plastic (Sorona) by combining the 1,3-propanediol made via microbial transformation, with terephthalic acid. The US based company, Cargill has made a biodegradable plastic based on lactic acid polymers, where the basic monomer is from lactic acid produced from lactic acid ferments. In collaboration with Genencor (a US based biotech firm) and the Australian National University (Professor Martin Banwell), our laboratory has made optically active dihydrodiols used as chiral building blocks by fermentation using glucose as a feedstock. Our laboratory has also been involved in the hydroxylation of small chemical entities using microbes to use as derivatization handles in further chemical synthesis.

Only a limited amount of work has been carried out using biological transformations of eucalypt oil constituents (Nishimura, 1996). Of note is the oxidative transformation of 1,8-cineole by the bacterium *Pseudomonas flava* to 2- α and 2- β -1,8, cineole (Carman *et al.*, 1986 and McRae *et al.*, 1979). Another microbial transformation is the synthesis of ($\pm$)-trans-6-hydroxy-p-menth-1-en-3-one and ($\pm$)-7-hydroxy-p-menth-1-en-3-one from piperitone using the fungal species *Aspergillus niger* and *Proactinomyces roseus* (Lassak, 1973). Urinary metabolites from the brushtail possum *Trichosurus vulpecular* have also involved a variety of cineole-derived transformations (Carman, 1994). Further esterification of these cineole derivatives using low molecular weight acids or alcohols might yield perfumery applications (Lassak, 2002). The enzymes that catalyse these reactions in microbes can also be extracted and used in-vitro for efficient transformations of oil constituents or expressed in microbes that are more tolerant to the oil constituent.

The potential for further modifications of 1,8-cineole for broadening oil applications using microbial biotransformations, has not been extensively studied. In particular, a systematic evaluation of microbial biotransformations of 1,8-cineole is an essential first step in utilizing microbes and/or their oil metabolising enzymes for discovering alternative uses for eucalypt oil. A first step in such a systematic study is establishment of a microbial library and assigning a tentative identification to the products derived from 1,8-cineole biotransformations.

This project proposed to establish a library of microbes that can metabolise 1,8-cineole from *E. polybractea*. We proposed that 1,8-cineole be fed as the sole carbon source to cultures in a unique device specifically developed for enrichment purposes at CSIRO Molecular Science. Once these cultures have been enriched they can be catalogued, with a view to further manipulation in transformation reactions directed toward tentatively identifying novel chemical compounds from 1,8-cineole. Not only will these microbes be of use for microbial transformations, they will also allow for the extraction of the enzymes responsible for improved expression and biocatalysis in other microbial systems, or simply retention for in-vitro processes at a later stage.

The long term outcome for this project is to derive new chemical entities with new applications using biocatalysis (such as microbial biotransformations or enzymatic transformations) from *Eucalyptus* leaf oil constituents with a view to addressing the large surplus forecast for eucalypt oil.

The outcomes specifically for this proposal were to establish:

- 1) A microbial library that can utilize 1,8-cineole as the sole carbon source either in purified form and from crude *E.polybractea* leaf oil.
- 2) A list of chemical products derived from microbial transformations of 1,8-cineole tentatively identified using GC-mass spectrometry analysis.

Chapter 2: Enrichment for microbes that utilize 1,8-cineole as the sole carbon source

2.1 Summary

To enable isolation of 1,8-cineole-utilising microbes, populations of 1,8-cineole-utilising microorganisms were established using a novel microbial enrichment device referred to as the Evolver[®]. A slow-growing 1,8-cineole-utilising microbial population was also established using the same system. The 1,8-cineole tolerance of the microbial population was evaluated by observing growth in the presence of 0.6-0.8 g l⁻¹ residual 1,8-cineole; the first time such an observation has been reported. Growth of a cineole-degrading isolate (designated B2) with eucalypt oil as the sole carbon source is also reported.

2.2 Introduction

The study of microbial metabolic pathways and their intermediates requires pure cultures of microorganisms with the appropriate metabolic capability. Whilst some studies of metabolism of 1,8-cineole in microorganisms have been published, the use of the Evolver[®] enables the rapid isolation of new types of microorganisms (from an extremely diverse mixture) which may produce unique metabolic intermediates derived from 1,8-cineole. These intermediates may have use directly or after further derivatization, in a variety of applications. Furthermore, the Evolver[®] can be used not only to evaluate some of the properties of microbial populations, but also to enhance any of a number of desired characteristics. The following sections describe the processes involved in the selection of 1,8-cineole-utilising microorganisms and the first steps in the elucidation of some of the properties of mixed populations and pure cultures.

2.3 Materials and methods

2.3.1 Source of microorganisms

An environmental sample from plants containing a diverse range of microbial flora was used as the starting material for enrichment of 1,8-cineole-utilising microbes.

2.3.2 Media

Defined media were used as the nutrient sources for the Evolver[®]. Initially, a nutrient source containing plant fertiliser was used. To enable more consistent operation and to overcome a requirement for pH control, plant fertiliser was replaced with a buffered defined medium (DM). The composition of DM is shown below:

		Final concentration
	KH ₂ PO ₄	0.5 g l ⁻¹
	NH ₄ Cl	1 g l ⁻¹
A	Trace Metals solution	1.0 ml l ⁻¹
	10% Na ₂ SO ₄	2.0 ml l ⁻¹
Adjust to pH7 with NaOH and autoclave		
B	Ca/Mg Stock Solution	10.0 ml l ⁻¹

- A. Trace Metals solution:
- 1 g l⁻¹ FeSO₄·7H₂O
 - 0.2 g l⁻¹ CoSO₄·7H₂O
 - 0.1 g l⁻¹ MnSO₄·H₂O
 - 0.1 g l⁻¹ NiCl₂·6H₂O
 - 0.05 g l⁻¹ NaMoO₄·2H₂O
 - 0.062 g l⁻¹ H₃BO₃
 - 0.07 g l⁻¹ ZnCl₂
 - 0.02 g l⁻¹ CuSO₄·5H₂O
- B. Ca/Mg stock solution: (sterile where required)
- 17 g l⁻¹ MgCl₂·6H₂O
 - 1 g l⁻¹ CaCl₂·2H₂O

Defined medium (DM) was found to be problematic in that after storage for several days, some of the metal ions started to precipitate which had a negative affect on growth. Furthermore, the low level of phosphate in DM resulted in a poorly buffered medium. This was a particular problem when it was used for batch cultivation. A new medium formulation designated 461S was devised; its composition is shown below:

The defined medium 461S is a modification of a minimal medium described by Nagel and Andreesen as cited by DSMZ (German culture collection – www.dsmz.de/media). The preparation of 461S is as follows:

Autoclave Salts (B) and Trace Elements (C) together
Add sterile Phosphates (A) after autoclaving

Standard 461S = pH7 once Phosphates (A) added
Low Phosphate 461S requires adjustment with NaOH.

		ml l ⁻¹ Stock		
		Standard	1/20 phosphate	1/50 Phosphate
A	100× Phosphates	10 ml	0.5 ml	0.2 ml
B	100× Salts	10 ml	10 ml	10 ml
C	Trace Elements	0.7 ml	0.7 ml	0.7 ml

- A. 100× Phosphates:
- 1.45 g l⁻¹ Na₂HPO₄
 - 0.25 g l⁻¹ KH₂PO₄
- B. 100× Salts:
- 0.01 g l⁻¹ CaCl₂
 - 0.5 g l⁻¹ MgSO₄·7H₂O
 - 0.01 g l⁻¹ MnSO₄
 - 0.3 g l⁻¹ NH₄Cl
 - 0.05 g l⁻¹ NaCl
- C. Trace Elements: (Dissolve FeSO₄ in HCl before adding other components)
- 6.56 g l⁻¹ FeSO₄·7H₂O
 - 0.14 g l⁻¹ ZnCl₂
 - 0.12 g l⁻¹ MnSO₄·H₂O
 - 0.01 g l⁻¹ H₃BO₃
 - 0.45 g l⁻¹ CoSO₄·7H₂O
 - 0.004 g l⁻¹ CuCl₂·2H₂O
 - 0.048 g l⁻¹ NiCl₂·6H₂O
 - 0.072 g l⁻¹ NaMoO₄·2H₂O
 - 1000ml 5M HCl

2.3.3 Growth conditions

For simplicity the growth conditions used for each experiment will be described as part of the results and discussion. The carbon source (1,8-cineole) is poorly soluble in water therefore nutrients and 1,8-cineole were added to the Evolver[®] separately.

2.3.4 Chemicals and reagents

Where possible all reagents were of analytical grade or better and sourced from reputable suppliers. Purified 1,8-cineole, specification number E98OIL, was supplied by Felton Grimwade and Bickford Pty Ltd and eucalypt oil extracted from *E. polybractea* (batch number 12020202) was supplied by G.R. Davis Pty Ltd.

2.4 Results and Discussion

2.4.1 Discovery of 1,8-cineole-utilising microorganisms

Discovery of 1,8-cineole-utilising microorganisms was performed using a proprietary device designated the Evolver[®], a system developed at CSIRO Molecular Science for the isolation of microorganisms with useful properties.

An environmental sample from plants was used as the source of microorganisms and as the inoculum for the Evolver[®]. After 37 hours in batch culture with 1,8-cineole and plant fertiliser as the nutrient sources, growth was observed. After growth had been established the Evolver[®] was operated in a mode designed to enable discovery of 1,8-cineole-utilising microorganisms. With plant fertiliser as the nutrient source consistent operation was established after 44 hours; the nutrient source was then changed to a minimal salts medium (DM, refer to Section 2.3.2), which has a defined composition and results in improved operation. After 69.5 hours under these operating conditions a sample was taken for isolation and purification of 1,8-cineole-utilising microorganisms. The Evolver[®] was operated in this mode for a further 11 days with samples being taken on day 5 and day 14 for further isolation and purification of 1,8-cineole-utilising microorganisms. Several samples of the microbial population were mixed with glycerol (cryopreservative) and transferred to -80°C for storage. Should any problems such as loss of viability or loss of phenotype arise during isolation and purification of the 1,8-cineole-utilising microorganisms, the preserved mixed populations are available to re-establish pure cultures.

2.4.2 Air-stripping of 1,8-cineole from the Evolver[®]

As the boiling point of 1,8-cineole is 175-179°C some 1,8-cineole may have been lost from the Evolver[®] due to evaporation. To determine the loss of 1,8-cineole during normal operation, 0.69 g of 1,8-cineole (final concentration of 0.91 g l⁻¹) was added to a stationary phase mixed culture. The solubility of 1,8-cineole in water is low and 1,8-cineole dissolves slowly in water therefore after the addition of 1,8-cineole to the Evolver[®] no samples were taken for 30 minutes to ensure complete dissolution. After 30 minutes samples were then taken periodically for measurement of residual 1,8-cineole. After the initial 30-minute period the measured 1,8-cineole concentration was 0.61 g l⁻¹ (Figure 2.1) suggesting that some 1,8-cineole had evaporated from the Evolver[®]. However, because 1,8-cineole is hydrophobic some of the losses may be attributable to interaction with components of the Evolver[®] and/or the hydrophobic membranes of microorganisms present in the bioreactor.

In another experiment, 0.69 g of 1,8-cineole was added to an Evolver[®] (final concentration of 0.91 g l⁻¹) inoculated with a stationary phase mixed culture. Before the addition of 1,8-cineole the aeration was turned off. To enable complete dissolution of 1,8-cineole no samples were taken until 30 minutes after the addition of 1,8-cineole. The 1,8-cineole concentrations were estimated using gas chromatography. The starting concentration (arrow labelled “1,8-cineole added”) was calculated from the amount of 1,8-cineole added because the slow dissolution of 1,8-cineole in water was expected to have resulted in a large sampling error.

After 4 hours, 99% of the added 1,8-cineole had been stripped from the Evolver[®] (Figure 2.1) with the loss due to evaporation slowing as the residual 1,8-cineole concentration decreased (data not shown). The loss of 1,8-cineole was due to air stripping and not microbial activity (data not shown) with a maximum rate of 1,8-cineole stripping of 0.34 g l h⁻¹. The loss of 1,8-cineole from the Evolver[®] was not considered to be a major problem and the issue was resolved by simply increasing the amount of 1,8-cineole added to the reactor.

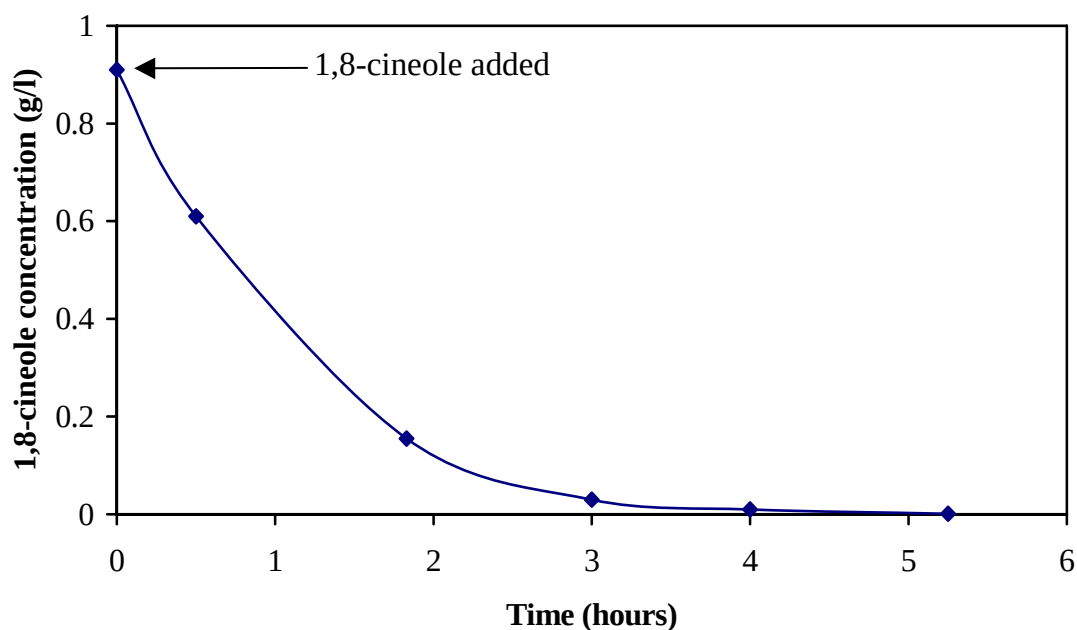


Figure 2.1. 1,8-cineole stripping from the Evolver[®] over a 5.25 hour period.

2.4.4 Discovery of slow growing 1,8-cineole-utilising microorganisms

Eukaryotic microorganisms such as yeast and fungi are generally slower growing than prokaryotes and the previous discovery process (section 2.4.1) may not have enabled their isolation. The Evolver[®] operation was therefore modified to facilitate isolation of yeast and fungi. Some problems with DM were identified (see Section 2.3.2) and were overcome with the use of a different medium formulation designated 461S (refer to Section 2.3.2). The phosphorus content of 461S medium was reduced from 0.43 g l⁻¹ to 0.021 g l⁻¹ to enable the subsequent evaluation of the 1,8-cineole tolerance of the newly isolated microorganisms.

Once consistent operation of the Evolver[®] had been established, a sample was removed to enable isolation and purification of any slow-growing 1,8-cineole-utilising microorganisms.

Results for this section are given in section 3.4.2.

2.4.5 1,8-cineole-tolerance of 1,8-cineole-utilising microorganisms

The phosphorus level in the nutrient source used for the Evolver[®] was reduced 50-fold to 0.009 g l⁻¹. Under these conditions microbial populations could be established with a residual 1,8-cineole concentration varying from 0.05-0.09 g l⁻¹ to a maximum level 0.6-0.8 g l⁻¹. Higher concentrations than these resulted in a rapid decline in the viability of the microorganisms. Although these concentrations are not significantly higher than those reported in the literature, it should be noted that the growth in the Evolver[®] exerts significant more pressure on cell survival than conventional growth conditions. Furthermore, even after exposure to a 1,8-cineole concentration of 3.6 g l⁻¹, the microbial population was able to recover and re-establish itself, albeit at a lower residual 1,8-cineole concentration.

This is the first report of growth of 1,8-cineole-utilising microorganisms under such conditions with residual levels of 1,8-cineole greater than 0.5 g l⁻¹.

2.4.6 Growth of mono-isolate B2 on eucalypt oil

Mono-isolate B2 had demonstrated an ability to grow in relatively high concentrations of 1,8-cineole (Section 3.4.2). It was thus chosen for further evaluation of its ability to grow on eucalypt oil. A 100 ml culture of isolate number B2 (for details relating to B2 refer to Section 3.4.2) was grown in a 250 ml Erlenmeyer flask shaking at 200 rpm and 30°C. After a 16 hour incubation the culture was centrifuged and the pellet resuspended in a small volume of residual supernatant. The resuspended pellet was used to inoculate an Evolver[®] which had previously been sterilised.

After inoculation, the addition of eucalypt oil was used to establish a growing culture. Growth was established almost immediately, an observation that was expected given the inoculum had been grown in 461S medium (refer to Section 2.3.2) with 1,8-cineole as the carbon source. After consistent operation had been established the Evolver output and the turbidity of the culture were high demonstrating that mono-isolate B2 was able to grow on eucalypt oil. On day 4 the eucalypt oil addition was increased and under these conditions the residual 1,8-cineole concentration ranged from trace levels to a maximum of 0.02 g l⁻¹. The addition of eucalypt oil was increased further, which resulted in a residual 1,8-cineole concentration of 0.085 g l⁻¹. Two further increases in the flow rate of eucalypt oil resulted in residual 1,8-cineole concentrations of 0.32-0.46 g l⁻¹ and 1.0-1.2 g l⁻¹. A residual 1,8-cineole concentration of 1.0-1.2 g l⁻¹ resulted in a marked reduction of the output from the Evolver[®] which was coupled with a corresponding decline in the turbidity of the culture. These observations were indicative of a reduction in the metabolic output of the population. However, the culture appeared to regain consistent operation, which suggested that the micro-organisms might have become restricted by the toxicity of the eucalypt oil. This was not investigated further.

The composition (as determined by GC analysis – refer 4.4.4.1) of the significant components of the eucalypt oil being used for this experiment was:

1.6%	α -pinene
3.5%	cymene
1.5%	limonene
93.4%	1,8-cineole

Interestingly, none of the minor components of the eucalypt oil (α -pinene, cymene and limonene) were detected in the culture supernatants from the Evolver[®] suggesting that these compounds were also being used for growth. However, it is possible that these components are lost from the Evolver due to air stripping and without further study this problem remains unresolved.

Clearly mono-isolate B2 can use eucalypt oil for growth with its tolerance for 1,8-cineole being at least 1.0 g l⁻¹. The isolate may also be capable of using the minor constituents of eucalypt oil but at this stage loss due to evaporation cannot be ignored.

2.5 Future Directions

To enable isolation of microorganisms which produce metabolic intermediates distinct from those found during this study, isolations could be performed under different growth conditions. Variation to parameters such as pH and temperature may result in the isolation of other microorganisms with unique metabolic pathways and intermediates. Similarly, alternative sources of microorganisms might result in the isolation of microbes with new metabolic processes. A full elucidation of metabolic pathways involved will enable the identification of other intermediates which are not produced at detectable levels.

From a manufacturing perspective (should one of the intermediates identified as part of this study prove to be useful) enhancing the 1,8-cineole and/or eucalypt oil tolerance of any isolate would be useful, a process that can be performed using the Evolver[®]. As part of this process the ability of each isolate to grow on eucalypt oil should also be evaluated. Alternatively, crude leaf pressings or milled leaves could be used as feedstock.

Other new metabolites may be formed from some of the minor components in eucalypt oil. Existing isolates could be grown on the minor components to establish whether these compounds can be used as a carbon source and if so, what, if any, metabolites are produced. If another component of eucalypt oil (or any essential oil) is far more valuable than 1,8 cineole, a microbe which exclusively utilises 1,8 cineole, could be used to recover the more valuable compound by removal of the 1,8 cineole.

The loss of 1,8-cineole (or any other volatile) from the Evolver[®] whilst not be problematic during this study may need to be addressed. Recycling effluent air may help to reduce the loss of volatile organic molecules from the reactor.

Chapter 3: Isolation, purification and preliminary characterisation of 1,8-cineole utilising microorganisms

3.1 Summary

A selection of individual microorganisms able to utilise 1,8-cineole as a sole carbon source was isolated from a mixed population. Preliminary characterisation of the monoisolates (colony morphology; Gram stain microscopy) was carried out resulting in the preliminary identification of approximately 9 different strains and a total of 14 isolates. Subjecting the monoisolates to increasing 1,8-cineole concentrations showed that at least 2 isolates could grow in the presence of 2 g l^{-1} 1,8-cineole, a level higher than that previously attained in other studies. Supernatant from exponential and stationary phase growth cultures was obtained and analysed by GC. All monoisolates showed the same characteristic profile of 'intermediates' and 'products' formation. 'Intermediates' increase in the exponential phase, then decrease as the culture reaches stationary phase; 'products' increase at stationary phase. A microbial library of organisms able to metabolise 1,8-cineole has been initiated.

3.2 Introduction

The potential for changing population dynamics in a mixed culture may create additional issues when dissecting the metabolic pathways involved in utilisation of 1,8-cineole as a sole carbon source. In addition, different species may have evolved unique pathways for the metabolism of 1,8-cineole resulting in different metabolic intermediates and end products. Therefore, the major goal of this section of the project was to isolate monoisolates that had been enriched for growth on 1,8-cineole within the mixed Evolver[®] population. Once isolated, the metabolic pathway leading to utilisation of 1,8-cineole of each individual strain can be examined.

3.3 Materials and Methods

3.3.1 Media

Nutrient Agar: Pre-mixed powder (28 g l^{-1}) (Oxoid Australia)

Dichloran Rose-Bengal Chloramphenicol Agar (DRBC): Purchased pre-poured (Oxoid Australia)

Minimal Media:

Carbon source (usually 1,8-cineole) was added after media sterilisation at the concentrations indicated in Results and Discussion (refer 3.4).

DM - Refer section 2.3.2.

Solid media - Add 1% Agar no.1 (Oxoid Australia).

461S - Refer section 2.3.2.

Solid media –Autoclave Salts (B) and 1.5% agar (Oxoid Australia);

Add sterile Phosphates (A) and sterile Trace Elements (C) after autoclaving.

YNB – Yeast Nitrogen Base

1.7 g l⁻¹ YNB powder (Difco Laboratories)

10 mM (NH₄)₂SO₄

3.3.2 Growth conditions

All cultures were incubated at 30°C for 1-7 days. Plates were placed either in a plastic sleeve, or a sealed container including an open vial of 1,8-cineole. Small-scale batch liquid cultures (5-10 ml) were carried out in screw-capped bottles (~30 ml). 500 µl of a stationary phase 1,8-cineole grown culture was inoculated into 5-10 ml minimal media + 1,8-cineole. Larger scale batch cultures were grown as either 100 ml in a 250 ml Erlenmeyer flask, or 500 ml in a 2 l Erlenmeyer flask. 10% (v/v) of a stationary phase 1,8-cineole grown culture was used as inoculation. Erlenmeyer flasks were closed with a tight cotton bung and a double foil cap sealed with tape to prevent 1,8-cineole loss from the system. Liquid cultures were shaken at 200 rpm until they had reached the appropriate growth phase.

The detection of fungal isolates is discussed in section 2.4.4. Starter cultures of fungal isolates K-1 and K-2 were initiated by scraping ¼ DRBC plate into 50 ml YNB + 1% glucose and incubated for 16 h (allowing conditioning of K-1). 1 ml of this starter culture was used to inoculate YNB + 0.25 or 0.5 g l⁻¹ 1,8-cineole.

3.3.3 Culture growth measurement

1 ml samples were removed from shake flasks and the optical density (OD) measured at a wavelength of 600 nm on a Shimadzu UV-1601 UV-visible spectrophotometer.

3.4 Results and Discussion

3.4.1 Isolation of Monocultures from Evolver®

It was expected that a mixed population of organisms would result due to the initial inoculum source in the Evolver® (refer 2.3.1). Therefore in order to isolate individual strains, three temporally independent samples from the Evolver® were collected and either spread-plate or streaked for single colonies onto minimal media (DM or 461S – refer 2.3.2) containing 1,8-cineole as the sole carbon source. Due to the relative insolubility of 1,8-cineole, the carbon source was spread over the surface of plates (5, 10 or 20 µl per plate). Subsequently, 1,8-cineole was added prior to pouring of plates (0.5, 1.0 or 2.0 g l⁻¹).

Growth on solid minimal media (DM or 461S) containing 1,8-cineole as sole carbon source was found to be very poor – small micro-colonies after 3-7 days. Poor growth could be due to a number of reasons: Relatively low levels of 1,8-cineole are used due to the potential toxic nature of the compound (MacRae et al, 1979; Azerad, 1993); 1,8-cineole is volatile (Figure 2.1) and hence the actual concentration remaining available in the solid media may be low. In order to try to overcome these problems a 1,8-cineole-saturated environment was created by incubating plates within a sealed container that included a sample of 1,8-cineole in an open vial. However, the resulting levels of 1,8-cineole saturation appeared to be inhibitory, as isolates subsequently identified as being able to grow in liquid 1,8-cineole, showed inhibition of growth on solid media when placed in the 1,8-cineole-saturated chamber.

There still remained the issue of isolating single colonies to ensure purity of monoisolates. Therefore micro-colonies from the minimal media + 1,8-cineole primary isolation plates were streaked for single colonies onto Nutrient agar or DRBC Agar (refer 3.3.1). Single colonies were then inoculated back into minimal media + 1,8-cineole liquid cultures to identify those that showed the ability to use 1,8-cineole as the sole carbon source. Only a relatively small subgroup (14 isolates of 94 total) of the initial single isolates subsequently grew in liquid culture with 1,8-cineole as the sole carbon source (Table 3.1). There are a number of explanations for this low recovery rate. It has previously been suggested (Hawkes et al, 2002) that if selection is not maintained, strains may not retain the ability to use 1,8-cineole as the sole carbon source (perhaps due to loss of plasmids bearing genes responsible for 1,8-cineole utilisation). In addition, there are population dynamics within the Evolver© resulting in 1,8-cineole being degraded by some microorganisms to intermediates prior to further degradation (refer section 4.2). However, there may be secondary microorganisms present that are not able to utilise 1,8-cineole, but are able to utilise the intermediates as sole carbon source. When monocultured, these secondary microbes will not be able to grow on 1,8-cineole as sole carbon source. Also, at the time of sample collection from the Evolver©, 1,8-cineole was limiting and thus in very low/absent concentrations. There may have been microorganisms present in the mixed population that are not tolerant to 1,8-cineole, but can grow in the absence of 1,8-cineole on other carbon sources present in trace amounts in the Evolver©. Once again these microbes would not show growth in the presence of 1,8-cineole when monocultured. Taken together, these results suggest that the practice of monoculturing (including non-selective propagation) may limit outcomes, and that the use of mixed populations to produce 1,8-cineole derivatives should be incorporated into future experimental design.

3.4.2 Preliminary characterisation of 1,8-cineole utilising monocultures

Colony morphology on Nutrient agar and Gram stain characteristics for 14 monoisolates are listed in Table 3.1. Some monoisolates may be independent representatives of the same strain (A-3, B-1, F-6 and G-2; B-2 and B-3; G-3 and I-8), resulting in the probable identification of at least nine different bacterial species. Several species have previously been identified as being able to utilise 1,8-cineole as a sole carbon source (MacRae et al, 1979; Nishimura et al, 1982; Williams et al, 1989; Liu et al, 1990; Miyazawa et al, 1991; Hawkes et al, 2002). Abraham's study (1994), examining taxonomic distribution of selected strains in relation to their biotransformation capability, suggests that in general, fungal strains are more likely than bacteria to have hydroxylation activity towards terpenoids (including 1,8-cineole). This can further be broken down suggesting that Gram-positive bacteria are more active than Gram-negative towards terpenoids. This type of study does not, however, reflect the relative distribution of species within the environment and therefore cannot be taken to represent the distribution of species likely to be isolated here. In fact, the majority of isolates identified in this study are Gram-negative bacteria (6 of 9 predicted bacterial species). While the strains isolated in this study have not been identified down to the species level, preliminary characterisation of these isolates suggests additional species not previously reported may have been identified in this study.

The lack of fungal representatives present in the isolates identified in this study so far might be explained by the conditions used in the Evolver©. A minimal defined media containing no additional carbon source is required for these experiments - these are possibly lacking the added vitamins that fungal species often require. The pH of the media was maintained at pH 7 ($\pm$ 0.1), whereas fungal species generally prefer a slightly acidic environment. Fungal species in general have a longer doubling time than bacteria, however the Evolver© at the times of sample collection was not operating under conditions for fungal growth to be encouraged. A subsequent growth experiment in which conditions were encouraging for fungal growth was carried out and samples collected and plated onto media more suitable for fungal growth (DRBC – section 3.3.1). Two fungal monoisolates were identified on DRBC plates. K-1 appears to be a filamentous fungus producing green conidia, while K-2 appears to be a fission yeast. Yeast Nitrogen Base (YNB - refer 3.3.1) is a defined minimal media suitable for fungal organisms. While strong growth was seen for both fungal isolates in

YNB + glucose, after 7 days incubation there was little or no apparent growth in YNB + 1,8-cineole (refer 3.3.2). These findings highlight the concept that the initial Evolver© culture media and conditions can be adapted to bias outcomes in favour of certain classes of micro-organism (bacterial versus fungal; slow versus fast growers; pH optimum). These conditions may be chosen to reflect those most suitable for downstream processing.

3.4.3 Growth in increasing 1,8-cineole concentrations

The highest concentration of 1,8-cineole allowing growth for each of the 14 bacterial isolates was examined. Previous reports suggest that bacterial strains are not tolerant to 1,8-cineole concentrations above approximately 0.5 - 1.0 g l⁻¹ (MacRae et al, 1979; Azerad, 1993; Williams et al, 1989). However, none of these reports address the issue of loss of 1,8-cineole from growth medium due to air stripping (refer 2.4.3), thus the actual 1,8-cineole concentration is probably below that of initial inoculation. Hence tolerance levels cited previously may be an overestimate.

Small-scale batch cultures were used with initial 1,8-cineole concentrations ranging from 0.5 g l⁻¹ to 2.5 g l⁻¹. Growth was qualitatively monitored. After 6 days, growth in the maximum concentration of 1,8-cineole was determined (Table 3.1) (NB. The maximum possible concentrations of cineole would be used for commercial purposes). The majority of isolates showed growth in the range 0.5 g l⁻¹ to 1.5 g l⁻¹, isolates I-8 and I-10 requiring a number of days to show signs of growth even at 0.5 g l⁻¹. Isolates B-2 and G-5 were determined to have the highest tolerance, showing growth in initially 2.0 g l⁻¹ 1,8-cineole. It should be remembered however, that these are batch cultures and levels will drop from the initial concentration once the microbes start to utilise the 1,8-cineole. The level of tolerance to increasing concentrations of 1,8-cineole, while examined initially here, would best be addressed in a continuous culture situation where the actual 1,8-cineole concentration can be maintained at the desired level. Nevertheless, the levels seen for isolates B-2 and G-5 indicate a substantially higher tolerance to 1,8-cineole than that previously documented for bacterial species under similar culture condition (MacRae et al, 1979; Azerad, 1993).

3.4.4 Exponential and Stationary phase monocultures

It was predicted that during growth of the monoisolates, products from the biotransformation of 1,8-cineole might be secreted into the culture supernatant, which could be detected by GC analysis (Chapter 4). When the carbon source is exhausted (stationary phase), metabolic intermediates may not be present as the 1,8-cineole may be completely degraded to CO₂ and H₂O. Hence both stationary and exponential growth phase cultures were examined for the presence of metabolic intermediates and end products in the culture supernatant.

Monoisolates were grown in 100 ml of 461S + 0.5 g l⁻¹ 1,8-cineole in 250ml flasks, sealed with a cotton bung and taped foil cap. An examination of loss of 1,8-cineole due to air stripping from this type of culture vessel showed there was less than 5% over an initial 7.5 h period, and approximately 20% over a 24 h period (Figure 3.1). In a separate experiment, 100 ml 461S + 0.5 g l⁻¹ 1,8-cineole in 250ml flasks, sealed with a cotton bung and taped foil cap. 1 ml of isolate B-2 1,8-cineole grown stationary phase culture was used as inoculum, while the blank flask remained uninoculated. 1,8-cineole concentrations were analysed by GC and growth rate (OD₆₀₀) of isolate B-2 was monitored over a 24 h period.

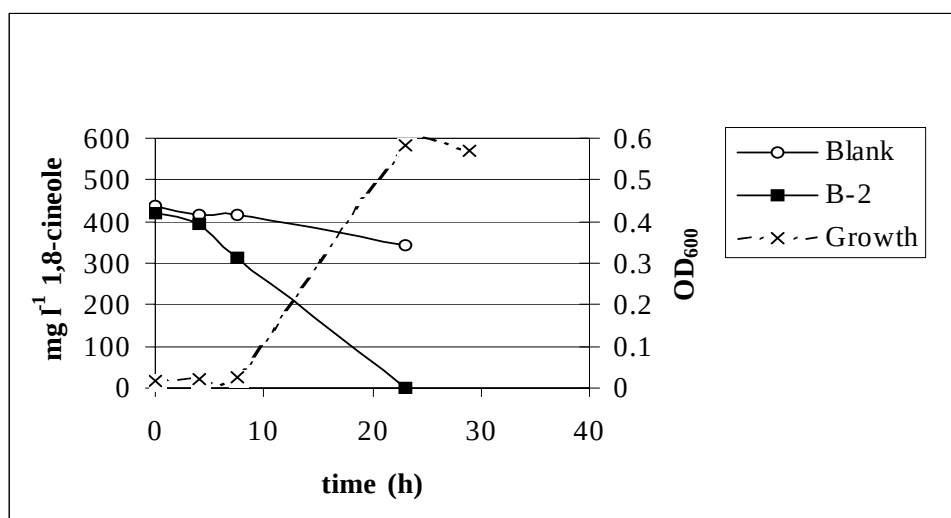


Figure 3.1. 1,8-cineole stripping in flasks

As described above, flasks were inoculated with 1 ml of stationary phase 1,8-cineole grown cultures and their growth rate monitored by OD₆₀₀. When an OD₆₀₀ of 0.2-0.4 had been reached (approximately 3-5 doublings) cells were considered in exponential phase. The cultures were continued until stationary phase had been reached as indicated by the growth curve (Figure 3.2). Lag phase and growth rate varied between strains resulting in variation in time to reach exponential phase (less than 8.3 h for isolates A-3, B-1,2,3,4,9, F-3,6, G-5 and E-12; approximately 24 h for isolates G-2,3 and I-8; and greater than 24 h for isolate I-10). 100 ml 461S + 0.5 g l⁻¹ 1,8-cineole in 250ml flasks were inoculated with 1 ml of stationary phase 1,8-cineole grown cultures and their growth rate monitored by OD₆₀₀. Samples for GC analysis were collected at time points indicated representing exponential (1) and stationary (2) phase growth.

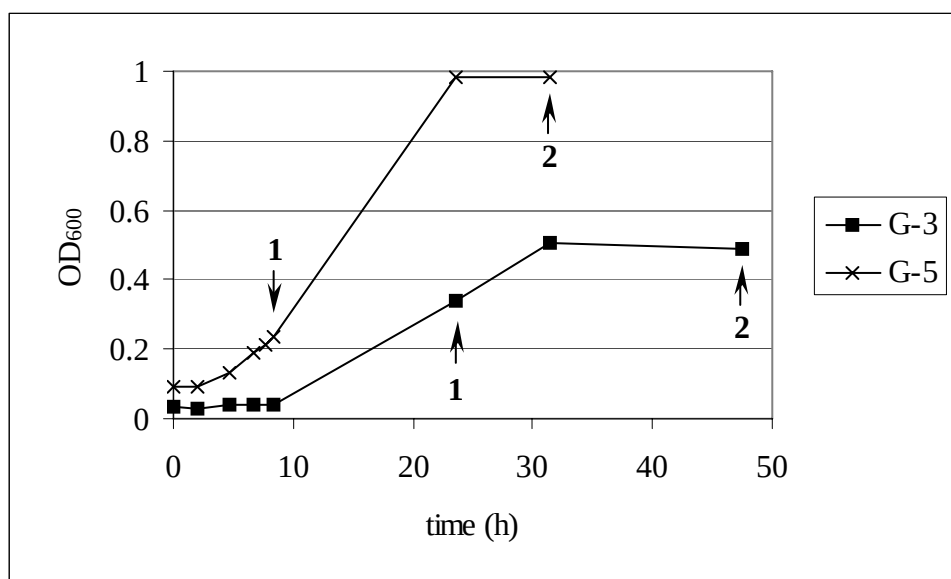


Figure 3.2. Exponential and Stationary phase growth (example growth curves)

3.4.5 GC analysis

3.4.5.1 Culture supernatants

Approximately 1-2 ml samples from either the Evolver© or 100 ml shake flasks (exponential or stationary phase growth) were passed through a 0.45 μ m filter to clarify the sample. The resultant supernatant was subjected to GC analysis using the method described in 4.2.2. The GC profile for all isolates in exponential phase was similar, as were the profiles from stationary phase cultures. The monoisolate profiles basically also mirror those from samples taken from the Evolver© at various stages of growth phase. Exponentially growing cultures showed moderate levels of 1,8-cineole and usually the presence of five 1,8-cineole derivatives (refer Figure 4.1). Once the culture reached stationary phase, 1,8-cineole and ‘intermediates’ levels became undetectable while ‘product’ levels generally rose. Samples from the Evolver© generally showed high levels of intermediates when 1,8-cineole was still detectable, the level of ‘products’ only rising when 1,8-cineole became depleted. This suggests that the microbes were utilising 1,8-cineole in preference to the ‘intermediates’, only degrading the ‘intermediates’ when 1,8-cineole availability has been exhausted.

3.4.5.2 Cell extracts

Cell extracts were prepared as described in 4.2.1. Several independent experiments collecting extracts from exponential and stationary phase cultures of isolate B-2 gave inconclusive results, probably due to insufficient cell mass from the 500 ml cultures. Culture conditions and cell extraction methods will need to be modified before any further conclusions regarding intracellular metabolic products can be made.

3.4.6 Catalogue and storage

A 1,8-cineole-utilising microbial library has been initiated (Table 3.1) with the storage of the 14 bacterial strains isolated to date. Cultures growing in 461S + 0.5 g l⁻¹ 1,8-cineole were harvested in exponential phase and frozen at -80°C in the presence of 15% glycerol.

3.4.7 Future Directions

Classification down to the species level of the monoisolates isolated to date will determine whether new species that are able to utilise 1,8-cineole as a sole carbon source have been characterised in this study. Selection in continuous culture for isolates able to utilise 1,8-cineole may not only differentiate species, but identify strains of the same species more suited to the environment. Hence, the strains isolated here may have already been ‘evolved’ for growth on higher levels of 1,8-cineole. Modification of the initial selection conditions may result in additional species being identified, thus expanding the 1,8-cineole-metabolising microbial library initiated here. In particular, conditions more favourable for fungal growth should be examined in order to increase their representation within the library. The use of mixed populations to produce 1,8-cineole metabolic products should also be incorporated into future experimental design to cover the possibility of a dynamic population interaction.

Table 3.1: Summary of preliminary characterisation of bacterial isolates.

Isolate ^a	Evolver [®] sample isolation number	Primary isolation plate	Colony morphology on NA	Microscopy	Growth in maximum concentration 1,8-cineole ^b
A-3	1	NA	Yellow; small; shiny	Gram -'ve; short rod; granular	1.5 g l ⁻¹
B-1	1	0.5 g l ⁻¹ 1,8-cineole	Pale yellow; small; shiny	Gram -'ve; short rod; granular	1.0 g l ⁻¹
F-6	2	5 µl 1,8-cineole	Pale yellow	Gram -'ve; short rods	1.0 g l ⁻¹
G-2	2	5 µl 1,8-cineole	Very pale yellow; small; shiny	Gram -'ve; rods	1.0 g l ⁻¹
B-2	1	0.5 g l ⁻¹ 1,8-cineole	Yellow; medium; shiny	Gram -'ve; narrow rod	2.0 g l ⁻¹
B-3	1	0.5 g l ⁻¹ 1,8-cineole	Very pale yellow; small; shiny	Gram -'ve; narrow rod	1.5 g l ⁻¹
G-3	2	5 µl 1,8-cineole	Bright yellow; shiny	Gram -'ve; medium rods	1.0 g l ⁻¹
I-8	3	2.0 g l ⁻¹ 1,8-cineole	Bright yellow; shiny	Gram -'ve; medium rods	0.5 g l ⁻¹
B-4	1	0.5 g l ⁻¹ 1,8-cineole	Pale yellow; small; shiny	Gram +'ve; variable rod	1.5 g l ⁻¹
B-9	1	0.5 g l ⁻¹ 1,8-cineole	Very pale yellow; large; non-uniform colonies	Gram +'ve; variable rod; ??spores	1.5 g l ⁻¹
E-12	2	5 µl 1,8-cineole	Yellow; shiny	Gram -'ve; very small cocci	1.0 g l ⁻¹
F-3	2	5 µl 1,8-cineole	Pink; very small	Gram +'ve; large coccobacilli	1.5 g l ⁻¹
G-5	2	5 µl 1,8-cineole	Pink; very small	Gram -'ve; long thick rods; granular; ?branched	2.0 g l ⁻¹
I-10	3	2.0 g l ⁻¹ 1,8-cineole	Bright yellow; very small	Gram -'ve; very long narrow rods; cell aggregation; granular areas within cells; ?filamentous	0.5 g l ⁻¹

^aIsolates are grouped according to similarity, suggesting possible independent representatives of the same species.

^bStarting concentration in small scale batch cultures (refer 3.4.3). Growth after 6 days incubation.

Chapter 4: Identification of Metabolites

4.1 Summary

This Chapter covers analysis of substrate and products in pure cultures and in Evolver© experiments with the aim of establishing the identity of any metabolites found. GC techniques were used, so only volatile and semi-volatile compounds are included. Five compounds were consistently observed. Three of these are considered to be intermediates as they are present only during exponential growth phase of the microorganisms in the presence of 1,8-cineole, whether in the Evolver© or in pure culture shake flasks. The remaining two have been called ‘products’ because they appear during stationary phase (refer section 3.4.5.1). Mass spectral library matching was inconclusive. However, on the basis of published literature, the three ‘intermediates’ are postulated to be two isomers of 2-hydroxy-1,8-cineole and 2-oxo-1,8-cineole. The ‘product’ has not been identified. There is also evidence in one case (from many tens of samples) consistent with the formation of a di-hydroxy-1,8-cineole. Several other compounds were observed when eucalypt oil (crude cineole) was used as substrate, but their concentrations were too low to allow mass spectra to be obtained. Future work towards identification of all compounds requires production and purification of sufficient amounts to enable Nuclear Magnetic Resonance (NMR) spectra to be measured. The availability of authentic samples, probably only via complex synthetic routes, would also be highly desirable to enable confirmation of the proposed identities.

4.2 Introduction

The initial products of metabolism of 1,8-cineole have been previously studied for only a limited number of microorganisms (refer chapter 1) although somewhat more extensive studies have investigated compounds in the urine of the eukaryote *Trichosurus vulpecular* (brush-tail possum) – a species which has been studied because of its diet (Carmen et al., 1994). As in previous studies, only gas chromatography has been used in this work for the separation of biotransformation products. Gas chromatography is a high resolution technique applicable to both volatile and semi-volatile compounds and therefore is capable of recovering a wide spectrum of compounds in one relatively simple analysis in a short period of time (< 1 hr). This also assists with sample throughput. A potential alternative, HPLC, is less amenable to recovering a broad range of compounds on any one given column and solvent system, even when gradient elution is used. As well, the resolution is inferior to GC and coupling to a mass spectrometer for potential identification of compounds is more difficult. Nevertheless, use of HPLC should be considered in any future and more in-depth work.

4.3 Materials and Methods

4.3.1. Sample pretreatment

4.3.1.1. Aqueous samples

Culture supernatants from the Evolver© or from shake flasks of pure cultures were filtered through a 0.45 µm cellulose acetate membrane filter prior to storage in 1 ml vials ready for injection. A control experiment showed that this did not affect the result. If analysis was not possible within 18 hrs, the samples were stored at 4°C until required.

4.3.1.2. Cell extractions

The cell mass from a 500 ml shake flask culture was collected by centrifugation (16,000 rpm/ 10 min/ 4°C), and washed by resuspending in PBS (250 ml) and re-centrifuging. The pellet was then suspended in 4 ml of a chloroform:methanol (2:1 v/v) solution and vortexed for 2 min at ambient temperature (ca. 20°C). The mix was transferred to 1.5 ml microfuge tubes and spun at 16,100 rpm/5 mins/ ambient temperature. In cases when the mixture was not uniphaseic other than for undissolved cell residue, the lower chloroform layer was taken for GC analysis (i.e. if the residual water after the washing step was unavoidably high).

4.3.2. GC analysis

All samples were analysed on a Varian Model 3600CX instrument equipped with a Model 8200CX automatic injection system using a Phenomenex Zebron ZB5 capillary column, Part No. 7HG-G002-11 (30m × 0.25mm × 0.25 µm film) and FID detector. Injection temperature was 250°C, the detector temperature was 300°C and helium at a constant pressure of 20 psig was used as carrier gas. At column temperatures of both 65° and 70°, the split ratio for injection was 57 as measured on the GC. Varian Star Version 5.51 software was used for instrument control and data collection and analysis.

For rapid analysis of 1,8-cineole, the column was run isothermally at 70°. The 1,8-cineole eluted at approximately 9.5 mins (new column).

For profiling of the products a GC oven temperature programme commencing at 65°C was used. After a hold time of 5 mins, the temperature was raised to 80° at 2.5°/min, held for 4 mins, raised at 5°/min to 240° and finally held for a further 3 mins for a total run time of 50 mins. Under these conditions, the 1,8-cineole eluted at approx. 10.0 mins (new column) and was quantified when required.

4.3.2.1. Calibration

The GC was calibrated with standard solutions of 1,8-cineole in ethanol and was found to be linear up to 9550 mg/l, the upper limit tested. 1,8-cineole has only a limited solubility in water, but a standard solution of ca. 2000 mg/l lay on the same calibration line. The calibration solution also contained known quantities of limonene, the hydrocarbon equivalent of 1,8-cineole which is typically found as a contaminant, especially in raw oils though seldom in refined 1,8-cineole (eucalyptol).

In the case of the unknown compounds found as metabolites, the same calibration factor as for 1,8-cineole was used to estimate their concentrations. In other words, the numbers quoted are cineole-equivalent values. Accurate quantitation, and indeed, identification, requires availability of authentic samples.

4.3.3. GC-MS for potential identification of metabolites

GC-MS analyses were conducted on a Finnigan Model Thermoquest MAT 95XL high resolution mass spectrometer interfaced to an Agilent Model 6890 GC and run under Thermoquest ICIS Executive Ver 8.2.1 software. The GC column and conditions were identical to those used in the profiling method described above. Mass spectra were compared with the NIST 62k library.

4.4. Results and Discussion

4.4.1. Compounds formed during microbial growth on 1,8-cineole

Examples of the chromatograms obtained at different stages of bacterial growth on 1,8-cineole are shown in Figure 4.1. These particular chromatograms were determined early in this project and formed the basis for much of the direction of the project despite the fact that they also show a relatively high background blank above ca. 30 mins retention time. This blank is believed to derive mainly from impurities (possibly oil) in the carrier gas, but septum- and column-bleed are other possibilities. As the work progressed, the blank was virtually eliminated by installing a hydrocarbon trap and conditioning the column daily at 280°C for ca. 30 mins. It was also found that as the column aged, the retention time of the peaks decreased, but it was readily shown by MS that the peaks could still be correctly identified, although not necessarily the identity of the compounds themselves. Thus, the 29.6 min peak, which was just large enough to be integrated in the case of the A-3 sample is part of the background blank (just visible) in the other 5 chromatograms.

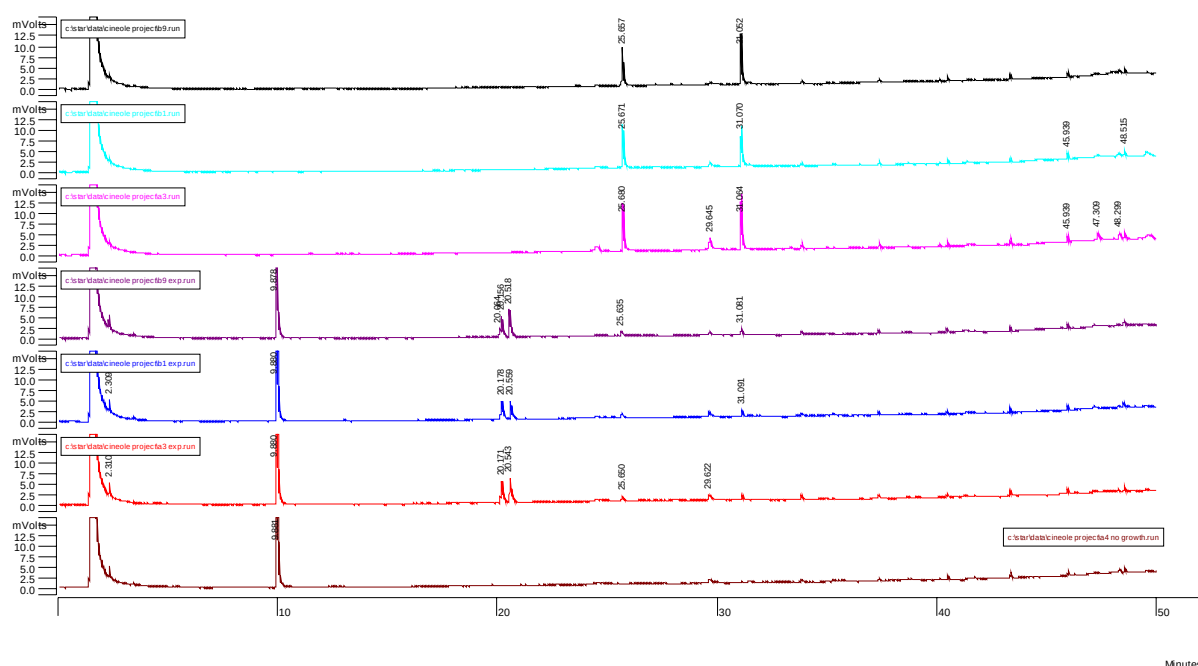


Figure 4.1. Representative GC chromatograms of aqueous phase of microorganisms grown on 1,8-cineole.

Culture supernatants from selected monoisolates (Table 3.1) grown to exponential or stationary phase were subjected to GC analysis. From the bottom, A-4 (no growth), A-3 (exponential), B-1 (exponential), B-9 (exponential), A-3 (stationary), B-1 (stationary) and B-9 (stationary).

The monoisolate A-4 did not grow and the trace shows only the residual 1,8-cineole (at approximately 10 min.). The three isolates at exponential phase show a lower level of residual 1,8-cineole, a triplet of peaks near 20 mins and small peaks at 25.7 and 31.0 mins. By the time the growth reached stationary phase, the cineole had all been consumed, the 20 min triplet had also disappeared

and the 25.7 and 31.0 min peaks have increased. This pattern is typical for all the microorganisms we studied, including mixed culture samples taken from the Evolver©, although the ratios of the various peaks varied. Indeed, the state of growth in the Evolver© could be determined by inspection of the GC profile.

4.4.1.1. Nomenclature

In one sense, all peaks not present in the blank are biotransformation products. However, for the sake of convenience, the 20.0 min triplet will be designated as 'Intermediates' (I1, I2 and I3) since they form and are then apparently consumed, whilst the term 'Products' will be reserved for end products. Thus the 31.0 mins peak will be designated as P2.

4.4.2. Identification of GC peaks by MS

Representative samples containing the 5 consistently observed peaks described above and shown in Figure 4.1 have also been analysed by GC-MS in an endeavour to identify the chemical compounds concerned. Because MS is generally less sensitive than Flame Ionisation Detector (FID), these samples were usually taken from the Evolver© because of the higher concentrations which were present.

As a first step, the mass spectra were compared with the National Institute of Standards technology (NIST) 62,000 compound library, but except for the known 1,8-cineole peak, no or relatively poor matches were obtained. The best result obtained was for the compounds designated as I1 and I3, and both gave 2-exo-hydroxy-1,8-cineole as the best match. This obviously cannot be correct for both. It is possible, of course, that the compounds formed in this study are so specialised that their mass spectra are not contained in either this library or probably all others, despite the large number of compounds which some of these libraries contain.

As part of previous studies of 1,8-cineole metabolism – see particularly the work of MacRae, Carman, Flynn, Williams, Liu, Miyazawa and Nishimura – complex chemical syntheses of the potential candidate metabolites have been described and the mass spectra measured and listed. These have been assembled in order to compare them with the mass spectra which have been measured in this work (manual comparison). Such a manual evaluation of the mass spectra goes beyond the level of analysis proposed in the initial project methodology but was deemed necessary since the search of the NIST database proved unproductive.

The combined results of previous work (MacRae et al (1979); Carman et al (1986); Williams and Trudgill (1989); and Williams et al (1989)) have also led to an understanding that the main chemistries concerned in the early stages of the microbial metabolism of 1,8-cineole are hydroxylation, oxidation, ring opening, hydrolysis and dehydration. This is summarised in Table 4.1 and Figure 4.2. Table 4.1 also includes other possibilities proposed by Carman's group (1984 – 2001) and Flynn and Southwell (1979). It should be noted that there is some inconsistency in nomenclature arising from the use of systematic IUPAC nomenclature, particularly in Aust. J. Chem., and the more trivial system based on substitution into 1,8-cineole without renumbering the ring afterwards according to IUPAC. The latter approach is used in this report.

Table 4.1. Summary of potential products from 1,8-cineole
(source: published literature, especially Carmon's group 1984-2001, and Flynn and Southwell 1979)

Formula Weight. (Potential Parent ion of Mass Spec)	Molecular Formula.	Modification to cineole. (Note: In most cases, isomers are possible.)
154	C ₁₀ H ₁₈ O	Cineole (no modification)
170	C ₁₀ H ₁₈ O ₂	Substitution with single -OH
168	C ₁₀ H ₁₆ O ₂	Oxidation of the -OH compound (above): Formation of ketone. Formation of double bond.
186	C ₁₀ H ₁₈ O ₃	Substitution with two -OH
184	C ₁₀ H ₁₆ O ₃	Oxidation of the dihydroxy derivative: Conversion of one of the -OH groups to ketone. Formation of double bond.
184	C ₁₀ H ₁₆ O ₂	Formation of cineolic acid: (substitution with -OOH on one of the three methyl groups.).
202	C ₁₀ H ₁₈ O ₄	Baeyer-Villiger oxidation of mono hydroxy or mono keto derivative with ring opening and acid formation.
184	C ₁₀ H ₁₆ O ₃	Loss of water to form keto-lactone after ring opening (above).

The mass spectra obtained for the compounds identified in this study were compared with those assembled from the literature, together with MS first principles. The literature reported GC retention times (Carman et al, 1986) on a slightly polar column similar to the one used in this work and knowledge of GC principles leads to the following conclusion about the three intermediates observed in this work:

I₁ is probably (95%) 2-*exo*-hydroxy-1,8-cineole,
I₂ is probably (99%) 2-oxo-1,8-cineole,
and I₃ is probably (95%) 2-*endo*-hydroxy-1,8-cineole.

It has not been possible to identify the 'product' P2 with such certainty. P2 has a likely FW of 184 and may be the keto-lactone derivative reported by MacRae et al (1979). P2's mass spectrum is similar, although not identical, to that reported for the keto-lactone derivative and its GC retention time is not also inconsistent.

The proposed identities have been indicated in Figure 4.2, but further work is required to confirm them.

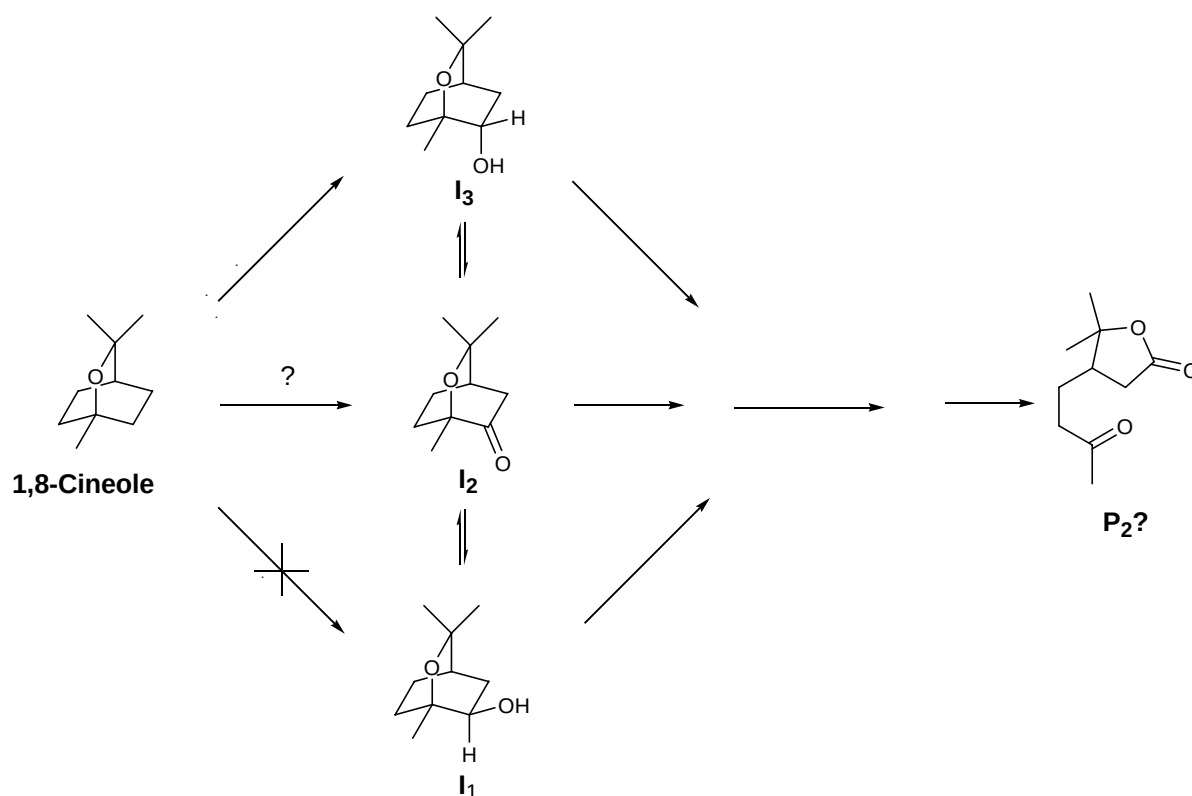


Figure 4.2. Summary of reaction scheme for microbial degradation of 1,8 cineole (after Carman et al,1986; and Williams and Trudgill 1989). The assigned identities of I1-3 and P2 (this work) are tentative.

4.4.3. Stability of Intermediates I₁, 2 and 3

During the course of this work, it was observed that the intermediates were unstable in the samples and, in some cases, they had completely disappeared within 3 days in filtered samples (0.45 μ m).

A larger scale culture (500 ml of organism B-2) was set up. Storage of aliquots of the supernatant obtained by centrifuging showed that 4°C was sufficient to maintain the concentration of residual 1,8-cineole and intermediates for at least 28 days. Treatment with nitrogen did not offer any advantage. The samples which were stored at room temperature, whether under nitrogen or not, commenced growth due to the ineffectiveness of centrifuging cf. filtration for removing organisms. Both the residual 1,8-cineole and all three intermediates were consumed within 7 days, but in this experiment they were all present after 4 days, albeit at reduced amounts.

4.4.4. Minor Compounds

In addition to the 5 compounds discussed above, some samples showed very small amounts of other compounds when chromatographed on the GC with FID detection. In none of the 5 or 6 cases has it been possible to obtain a satisfactory mass spectrum. In most cases, the compound was not detected at all on the less sensitive GC-MS system. The compound may have been unstable or was an analytical artefact because it was also not seen when the same sample was subsequently re-analysed on the GC-FID. In one case, a very weak mass spectrum was obtained which suggested the presence of a

compound of FW = 186, possibly a dihydroxy derivative of 1,8-cineole, but it could not be confirmed in any other samples.

4.4.4.1. Eucalypt oil as substrate

As well as the usual five compounds, there are also several additional small peaks which are probably metabolites present in the supernatant of a B-2 culture growing in the Evolver[®] on crude cineole (93.4% by GC area in the Eucalypt oil sample used). It was, however, demonstrated that the major impurities present in the crude cineole feed – α -pinene, cymene and limonene – are not detectable in the Evolver[®] supernatant. GC-MS analyses have been run on three samples, but in this early phase of the project, have not yet been successful in identifying any compounds. The concentrations are so low that with splitless injection the peaks are too broad, whereas with the minimum practical split ratio of 10 they are too small.

4.5. Conclusion and Future Directions

This project has shown the consistent formation of five distinct compounds when 1,8-cineole is metabolised by microorganisms (Table 4.2). Three of these, which appear to be intermediates, have been reasonably confidently identified and although not present in the NIST database have been previously reported in the literature. One of the two others has probably been reported previously, but the fifth product awaits identification and has not been found in either the NIST database or in the surveyed literature. All 5 products will still require NMR elucidation to confirm their structures

Further work is required to confirm the identity of the proposed compounds described in this report (section 4.4.2) and to identify other potential compounds. This will require the availability of authentic samples, but it will help considerably to separate and purify sufficient material from the microbial syntheses to obtain NMR spectra, mass spectra, microanalysis and physical data such as melting point and/or boiling point.

Attempts to confirm the reality of minor components and identify them should also be made. This will require the culture of further isolates, and techniques to concentrate and preserve the samples. Solvent extraction and/or solid phase adsorption techniques should be applicable.

Work on the eucalypt oil substrate should also be continued to determine whether any more metabolites can be isolated and identified.

Analyses of the Evolver[®] samples showed that quite high concentrations of the intermediates (up to 1400 mg/l[†] for I₃, 120 mg/l of I₁ and 170mg/l of I₂) and products (up to 130 mg/l[†] for P₂) could be achieved. It would be necessary to devise a separation and purification scheme. Solvent extraction (continuous) and column chromatography (batch) may be applicable. An evaluation of the economics of such a scheme would be necessary.

The long term aim of this project is to identify new chemical products and derivatives from 1,8-cineole, which can be used for potential new products, thereby allowing expansion of the eucalypt oil market. This will assist *Eucalyptus* plantations to be more commercial, particularly where they are needed for environmental reasons in low rainfall and saline regions. Industry is interested in screening these 1,8 cineole derivatives for potential herbicide, anticancer, and antibacterial activities. Because these molecules have now been biotransformed, mostly to hydroxylated derivatives, they can also be considered as building blocks for further derivatization and thus screened for the same industrial purposes mentioned above.

[†] Estimated concentration expressed as 1,8-cineole equivalent.

Table 4.2. Summary of chemical library of compounds observed in this study.

DESIGNATION	GC RETENTION TIME*. (mins).	PROPOSED FW (via MS)	PROPOSED IDENTIFICATION.
I ₁	19.7	170	2- <i>exo</i> -hydroxy-1,8-cineole
I ₂	19.9	168	2-oxo-1,8-cineole
I ₃	20.2	168	2- <i>endo</i> -hydroxy-1,8-cineole
P ₂	30.8	184	Keto-lactone derivative after ring opening.
Minor peaks (Euc. Oil as substrate).	18.1, 18.6, 18.9. 19.8, 25.0, 26.0, 27.0, 27.5.		Concentration too small to obtain satisfactory mass spectra. Further work required to concentrate and/or isolate these.

* On a well-used, but still satisfactory column, using the profiling conditions described in Section 4.3.2. These times decrease slightly as the column ages.

Chapter 5.0 References.

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