Enhancement of the Microbial Biotransformation of (-)-trans-carveol to (*R*)-(-)-carvone by *Rhodococcus erythropolis* DCL14 in Various Two Phase Partitioning Bioreactor Configurations

by

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A thesis submitted to the Department of Chemical Engineering
in conformity with the requirements for
the degree of Master of Science (Engineering)

Queen's University
Kingston, Ontario, Canada
January, 2008

Abstract

Carvone is a flavor and fragrance compound that is prominent in nature and is found in the essential oils of many plants. Carvone exists as two enantiomers, (*R*)-(-)-carvone which has a spearmint aroma and (*S*)-(+)-carvone which has a caraway aroma and can be used in a variety of applications: as a common food additive, as an antimicrobial/antifungal agent and as a potato sprout inhibitor. Carvone is currently produced by the extraction of essential oils from plants where the yield and quality of the extracted oil depends largely on successful agricultural production of dill, spearmint and caraway plants. Biotechnological production can offer a constant supply of carvone that is independent of several agricultural limitations.

In this study, it was confirmed that the substrate and product of the microbial biotransformation of trans-carveol to (*R*)-(-)-carvone by *Rhodococcus erythropolis* DCL14 can be inhibitory to the cells at high concentrations. As such, a two phase partitioning bioreactor was employed where the function of the second phase (immiscible organic solvent or solid polymer beads) was to partition the inhibitory substrate into the aqueous phase at a rate governed by the metabolic demand of the cells and uptake the inhibitory product as it accumulated in the aqueous phase. Rational selection strategies were employed when determining the appropriate organic solvent and solid polymer to be used as the second phase. The performance of the reactor was evaluated based on volumetric productivity, length of biotransformation and total volume of substrate added to the reactor. The most successful reactor configuration was one in which styrene/butadiene copolymer beads were used as a second phase in the reactor and the fermentation medium was continuously circulated through an external extraction column

packed with Hytrel® 8206 polymer beads. The volumetric productivity, length of biotransformation and total volume of substrate added to this reactor were 99 mg/L·h, 48.75 h and 35 mL, respectively whereas in the single phase benchmark reactor the performance indicators were only 31 mg/L·h, 15.25 h and 5 mL, respectively. These results clearly show the advantage of employing a partitioning bioreactor configuration for the biotechnological production of high value chemical species that exhibit cytotoxicity.

Co-authorship

Ms. Emily Brennan and Ms. Helen Dry provided experimental assistance to portions of the material presented in Sections 3.4.2, 3.8.2 and 4.4.

Acknowledgements

I would especially like to thank my family for their constant support throughout my education. I would like to thank my mother and father (Stan and Sherrill Smith) for encouraging me to take on a second degree because they believed that I could do it. I would also like to thank my sister (Angela Smith) for first introducing me to Chemical Engineering at Queen's as I've truly enjoyed my time here.

I would like to thank my husband, Tim for his ever present support, and for learning to enjoy Kingston during our time here. He has been a great friend throughout my studies who helped me to laugh off the insignificant stresses and celebrate any small victories.

I would like to thank my supervisor, Dr. Andrew Daugulis for his ever positive attitude, his continual interest and enthusiasm towards my work and his motivational support. I never considered completing graduate work for anyone else and have truly enjoyed getting to know him over past couple of years.

Apart from Andrew's support, there are three people to which I owe a big thank you for helping me throughout my degree. These individuals include Steve Hodgson who was always there to answer questions and fix my equipment crises and Emily Brennan and Helen Dry for their contributions as part of their fourth year thesis project.

Last, but certainly not least, I would like to thank the lab mates that I have had the privilege of working with throughout my studies. I have to thank Dave Nielsen for introducing me to the lab and patiently supervising our lab work. I also have to thank George Prpich, Lars Rehmann, Jen Littlejohns, Matt McMahon, Fang Gao and Pedro Isaza for the fun times in the lab, their welcome advice and their continued support.

Table of Contents

List of Tables

List of Figures

Chapter 1.0 Introduction

Flavor and fragrance compounds are abundant in nature and are found as part of the essential oils of various plants. Carvone is one such flavor and fragrance compound and is produced by over 70 different plants (Burdock 1995). Carvone consists of two enantiomers, (*R*)-(-)-carvone which has a spearmint aroma and (*S*)-(+)-carvone which has a caraway aroma. Most commonly, (*R*)-(-)-carvone is produced by spearmint (*Mentha spicata*) and dill (*Anethum graveolens*) plants whereas (*S*)-(+)-carvone is produced primarily by caraway (*Carum carvi*) plants (Ravid et al. 1992).

The carvone enantiomers can be used in many applications, namely as food additives, as antimicrobial/antifungal agents and as potato sprout inhibitors. Both carvone enantiomers are widely used by the flavor and fragrance industry (Welsh et al. 1989) to flavor such things as pickles and bread (Baysal and Starmans 1999) as well as ice cream, candy, baked goods, meats, cheese, condiments, soft drinks and alcoholic beverages. Carvone has also been used to flavor pharmaceutical products and toothpaste (Baysal and Starmans 1999). Carvone, like most other oxygenated monoterpenes, exhibits some degree of antimicrobial and/or antifungal capability (Carson and Riley 1995). Carvone is currently used as a sprout inhibitor to maintain the quality of stored potatoes in the Netherlands under the name of "Talent" (Hartmans et al. 1995).

Conventionally, carvone is produced by the extraction of essential oils from plants and seeds using hydrodistillation, steam distillation and solvent extraction (Kallio et al. 1994). The product yield of each of these methods depends of course on the original quality of the essential oil in the plant and as such the carvone supply is dependent upon a

successful growing season despite several agricultural limitations. In order for the carvone supply to become independent of agricultural limitations, other production methods (namely, biotechnological routes) are being explored.

(*R*)-(-)-carvone can be produced by the microbial biotransformation of trans-carveol by *Rhodococcus erythropolis* DCL14. This microbial biotransformation is cofactor (NADH) dependent and requires the supply of an enzyme (carveol dehydrogenase) inducing carbon source (limonene). The microbial biotransformation substrate, carveol, is made up of a mixture of cis- and trans-isomers whereby only the trans-isomer is converted to carvone and the cis-isomer accumulates in the aqueous phase. The other major challenges associated with this reaction system include the low water solubility of the substrate (carveol) and the inhibitory effects of the substrate and product molecules on the organism.

An appropriate solution to the challenges associated with this microbial biotransformation is the application of a two phase partitioning bioreactor (TPPB). A TPPB is comprised of a cell-containing aqueous phase and an immiscible second phase that acts as a substrate delivery phase and a product reservoir. The TPPB is very useful in the fact that the system is self-regulating such that the substrate (whether it is poorly soluble, inhibitory or both) is slowly partitioned into the aqueous phase at sub-inhibitory levels which are governed by the thermodynamic equilibrium that exists between the two phases. Traditionally, the second phase in a TPPB is an organic solvent which is chosen based on many criteria, the most important of which include biocompatibility, non-bioavailability and affinity for the target molecule(s). More recently, the use of solid polymer beads as the second phase in a TPPB has been explored. It has been shown that

target compounds can partition between the solid polymer phase and the aqueous phase just as they partition between the two liquid phases. As with the solvent, a selection strategy is employed to choose an appropriate polymer. Most polymer beads used for this application are biocompatible and non-bioavailable and as such the most important selection criterion is the polymer's affinity for the target molecule(s).

In the present work, various TPPB configurations were employed in an effort to enhance the microbial biotransformation of trans-carveol to (*R*)-(-)-carvone by *Rhodococcus erythropolis* DCL14. The relative toxicities of the substrate (carveol) and product (carvone) were assessed. As well, the operational challenges of employing a very hydrophobic organism like *Rhodococcus erythropolis* DCL14 were addressed.

Chapter 2.0 Literature Review

2.1 Flavor and Fragrance Industries

The worldwide flavor and fragrance industry is commercially significant and was estimated at $9.7 billion USD in 1994 (Somogyi 1996). Many flavor and fragrance compounds come from natural sources such as the essential oils of plants and seeds. It is known that there are approximately 6400 natural volatiles and about 10 000 synthetic fragrance compounds, however, only a few hundred are regularly used as flavors and fragrances (Krings and Berger 1998). It is evident that aroma chemicals are high value, low volume products. For example, verbenone which can be used as a flavor compound (camphor and mint aroma) and a pharmaceutical intermediate is worth $3000 USD/kg (Agrawal and Joseph 2000). There are only about 400 aromas that are manufactured on a scale greater than 1 ton per year (Krings and Berger 1998) and due to the limited supply, they can be very costly.

Flavor and fragrance compounds can be obtained naturally from plant products or can be produced chemically. The majority (80%) of the flavors and fragrances used world-wide are produced chemically (Krings and Berger 1998). There is however a drive towards the natural production of these valuable compounds due to increasing health- and nutrition-conscious lifestyles (Abraham et al. 1994). A natural flavor is defined as:

> "the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof, whose significant function in food is flavoring rather than nutritional" (Altman 1998).

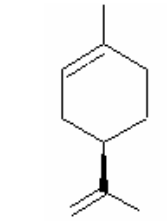
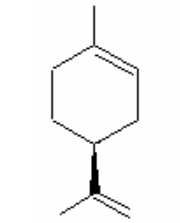
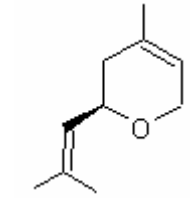
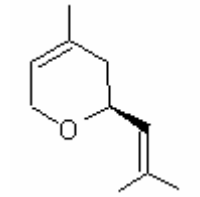
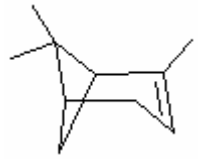
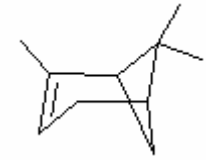
An alternative production method for aroma chemicals is their production by microorganisms whose products are considered natural (Gatfield 1988). The natural label is important for the profitability of microbiologically produced flavors (Krings and Berger 1998). For example, vanillin is an aroma chemical that has a vanilla odor and is currently produced by chemical synthesis and costs $12 USD/kg (Priefert et al. 2001). Vanillin can also be produced naturally by extraction from vanilla pods which increases the product price range to $30 USD/kg - $120 USD/kg (Priefert et al. 2001) depending on the supply and quality of vanilla beans available. In order to take advantage of these extreme increases in price for a natural product, the biotechnological production methods are of interest.

Since flavor and fragrance chemicals are of great commercial significance in the food, cosmetic and pharmaceutical industries (Krings and Berger 1998), the production costs must be carefully controlled in order to obtain a maximum profit. In order for a microbial flavor to be competitive in this industry, its price should range between $200 and $2000 USD/kg (Janssens et al. 1992).

2.2 Terpenes

Monoterpenes are branched chain 10-carbon members of the isoprenoid family that are formed from two isoprene units (van der Werf et al. 1999a). A chemically modified monoterpene is referred to as a terpenoid. A high number of terpenoids (oxygenated derivatives of terpenes) are used as flavor and fragrance chemicals in the food and perfume industries (de Carvalho and da Fonseca 2003). A few examples of the terpene family and their properties are listed in Table 2.1.

Table 2.1: Examples of terpenes that are commonly found in nature and the aromas they impart.

Terpene	Structure	Aroma
camphor		camphor tree
(*R*)-(-)-limonene		fresh citrus, orange-like
(*S*)-(+)-limonene		turpentine-like, lemon note
(*R*)-(-)-nerol oxide		floral
(*S*)-(+)-nerol oxide		geranium, spicy
(1*R*, 5*R*)-(+)-alpha-pinene		terpene-like, minty
(1*S*, 5*S*)-(-)-alpha-pinene		coniferous

Terpenes are widespread in the plant kingdom and are often responsible for the characteristic odors of plants (Bouwmeester et al. 1998). In nature, terpenes serve many ecological roles including acting as deterrents against herbivores, as antifungal defenses and as attractants for pollinators (Langenheim 1994).

Terpenes have also recently gained commercial interest due to the discovery of several functional applications, including their use in prevention and therapy of several diseases, their activity as natural insecticides and antimicrobial agents and their use as building blocks for the synthesis of many highly valued compounds (de Carvalho and da Fonseca 2006b). Krings and Berger (1998) state that more than 400 monoterpenes are distributed in nature, many of which are suitable precursor substrates. For example, α-pinene is a common monoterpene that is inexpensive and readily available and can be oxidized to verbenone, a highly priced flavoring compound (Vaněk et al. 2005, Agrawal and Joseph 2000). Verbenone has a camphor and mint aroma (Agrawal and Joseph 2000) and can also be used as a precursor for the synthesis of Taxol® (McMorn et al. 2000), a pharmaceutical drug used in chemotherapy.

2.3 Carvone

Carvone is a common terpenoid that is produced by over 70 different plants (Burdock 1995). Carvone exists as two enantiomers, (*R*)-(-)-carvone which has a spearmint aroma and (*S*)-(+)-carvone which has a caraway aroma. The fact that the nose is able to detect each enantiomer distinctively makes these compounds of great interest in the flavor and fragrance industries. The two carvone enantiomers are shown in Figure 2.1.

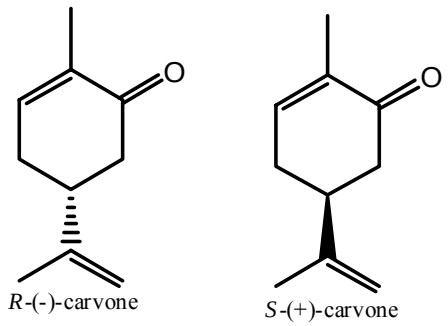


Figure 2.1: The two carvone enantiomers.

The essential oil containing carvone is produced primarily by caraway (*Carum carvi*), dill (*Anethum graveolens*) and spearmint (*Mentha spicata*) plants (Ravid et al. 1992). In caraway, the essential oil is protected in oil ducts inside the hard peel of the seed, and it is not easily accessible (Bailer et al. 2001). The wall of the seed (exocarp) is the only part of the fruit to contain the essential oil (Toxopeus et al. 1995). There are two varieties of caraway plants, annual and biennial. The annual type has lower essential oil content than the biennial variety (Bouwmeester et al. 1998). The essential oil in the seeds of the dill plants are more easily accessible because the seeds of the dill plant are much softer. However, since dill is usually used as an herb, the commercial varieties are not bred for maximum seed yields (Bailer et al. 2001). The essential oil in the spearmint plant is located within the leaves and is easily accessible.

The biosynthesis pathways for carvone in caraway and spearmint plants have been assumed to be analogous (Bouwmeester et al. 1998). The biosynthesis in caraway is a three-step reaction as shown in Figure 2.2. The biosynthesis in spearmint would follow the same reaction path, however (-)-limonene would become (-)-cis-carveol and then finally (-)-carvone (Bouwmeester et al. 1998). The reaction starts with geranyl diphosphate (GPP), a common monoterpene precursor (Bouwmeester et al. 1998), which is cyclized by a monoterpene synthase to limonene. The limonene is then oxidized to

trans-carveol by a Cyt P-450-dependent hydroxylase, and finally oxidized to carvone by an NAD^+ or $NADP^+$-utilizing dehydrogenase (Bouwmeester et al. 1998).

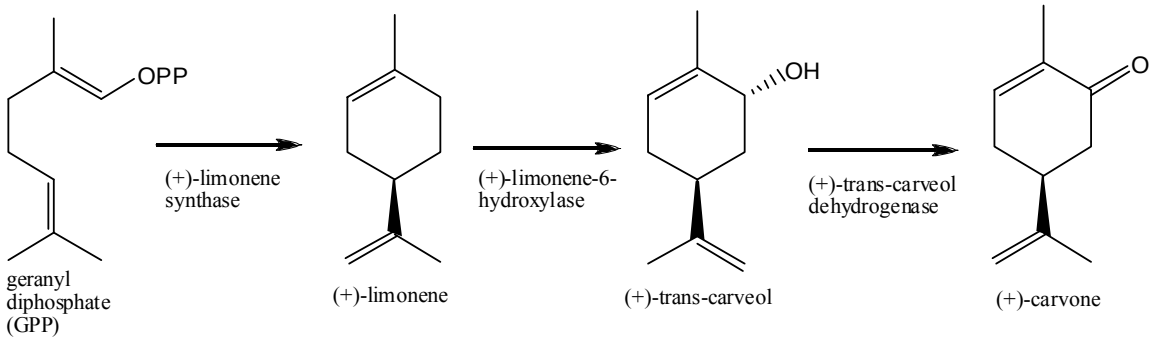


Figure 2.2: Carvone biosynthesis in caraway seeds.

During the second step, the limonene that is not converted to trans-carveol is stored in the essential oil ducts (Bouwmeester et al. 1998). The storage of the limonene intermediate clarifies why essential oil yields are determined mainly by the seeding date and harvest stage (Bowes et al. 2004). It is very important to have a well defined harvest date, where only a small amount of stored intermediates remain such that the maximum amount of carvone has been produced in the plant. Increasing efforts have focused on cross-breeding of the caraway plant varieties to increase essential oil yields. Toxopeus et al. (1995) have reported a plant population that was produced by cross-breeding and had 15% higher oil content than average.

The oil yield obtained from caraway, spearmint and dill plants varies based on production conditions such as harvesting date and cultivar or population of the various plants (Bailer et al. 2001, Galambosi and Peura 1996). The essential oil recovered from the spearmint plant (*Mentha spicata*) is extracted from the fresh shoots (aerial parts) (Aggarwal et al. 2002; Bailer et al. 2001). Generally, the spearmint oil yield ranges from 0.5 – 1.1% of which 56.6% is *R*-(-)-carvone (Aggarwal et al. 2002). Indian dill oil can be extracted from the crushed fruits (seeds) or from the leaves and tops of the Indian dill

plant (*Anethum sowa*) (Bailer et al. 2001). The oil yield ranges from 2.5 – 4% with *S*-(+)-carvone making up 50.4% of the oil (Aggarwal et al. 2002). Caraway exists in biennial and annual varieties and the essential oil content ranges between 2 – 8% (Bailer et al. 2001). The essential oil from the caraway seeds contains 50 – 70% *S*-(+)-carvone (Hornok 1992). Since the essential oils of the dill and caraway plants contain approximately the same percentage of *S*-(+)-carvone, the seed yield determines which plant can produce the most essential oil. Dill plants yield 400 – 600 kg/ha where annual caraway and biennial caraway yield 1250 kg/ha and 900 kg/ha, respectively (Bailer et al. 2001).

A number of limitations are associated with obtaining carvone via crop production. There are several factors that can influence essential oil yield and quality in crops. The most obvious limitation is that time is required for plant development, and their successful growth depends on weather conditions, soil and fertilizer composition and the available micro-nutrients (de Carvalho and da Fonseca 2006a). Other natural limitations include competition with weeds, insects and plant infections (de Carvalho and da Fonseca 2006a).

2.3.1 Applications and Commercial Value

Most essential oils that are used in food products and pharmaceuticals are used for the flavor aroma they impart (Aggarwal et al. 2002). Both carvone enantiomers, especially (*R*)-(-)-carvone, which has a spearmint flavor, are natural flavors widely used by the flavor and fragrance industry (Welsh et al. 1989). Approximately 52 000 kg carvone is consumed in the United States annually (Abbott 1999) and is currently

available from Sigma-Aldrich Canada Ltd. for approximately $82 CAD/kg. To supply the worldwide annual consumption of carvone, 100 – 150 tons of dill oil, 10 tons caraway seed oil and 1500 tons spearmint oil are produced annually (de Carvalho and da Fonseca 2006a).

As outlined in Section 1.0, carvone can be used for several applications; namely in food additives, as antimicrobial/antifungal agents and as a potato sprout inhibitor. The rising demand for natural products used in food applications has increased the necessity for the production of natural flavor compounds (Etschmann and Schrader 2006). Carvone is used to flavor several food products from bread to ice cream. This flavor compound has also been used to flavor pharmaceutical products and toothpaste (Baysal and Starmans 1999).

Another interesting potential application for carvone involves its antimicrobial activity. Several essential oils have been known to possess antimicrobial activity where most of this activity has been attributed to the oxygenated monoterpenes (Carson and Riley 1995), such as carvone. Carvone can be used to inhibit the growth of bacteria (Helander et al. 1998, Oosterhaven et al. 1996, Naigre et al. 1996), fungi (Smid et al. 1995) and as an insect repellant (Salom et al. 1996, Lee et al. 1997). Aggarwal et al. (2002) states that in vitro, both carvone isomers are active against a wide spectrum of human pathogens and bacteria.

The most important application of carvone (aside from a flavor and fragrance additive) is its use as a sprouting inhibitor in stored potatoes. Potato crops can often be stored for long periods before distribution. Sprouting is an important obstacle to overcome since it represents a loss of material, and loss of water through the permeable

surface of the sprout (Hartmans et al. 1995). It is current practice to control sprouting by employing a good temperature regime where the potatoes are stored from 2 – 4°C (Hartmans et al. 1995). However, at low temperatures, the potatoes can sweeten, leading to a high reducing sugar content which can result in brownish and bitter processed products (Hartmans et al. 1995). To avoid loss of product quality potatoes can be stored at higher temperatures, and sprouting suppressants can be applied. There are synthetic chemical sprout suppressants available; however, carvone can be applied as a natural alternative to suppress sprout growth (Hartmans et al. 1995). Studies have been conducted where carvone was able to suppress sprout growth for more than a year and also showed no negative effects on potato quality (Hartmans et al. 1995). Since its introduction in 1994, carvone has been used as a sprout inhibitor for potatoes in the Netherlands under the name of "Talent" (Hartmans et al. 1995).

The various applications of carvone as fragrance and flavor compound, antimicrobial agent and potato sprouting inhibitor justify the research aimed to provide better production methods (de Carvalho and da Fonseca 2006a).

2.3.2 Current Extraction Methods for Carvone

Currently, carvone is produced through biosynthesis in dill, caraway and spearmint plants. The essential oils of these plants (containing carvone) are then obtained using conventional extraction methods such as hydrodistillation, steam distillation and solvent extraction (Kallio et al. 1994). The steadily increasing market for flavors has forced suppliers to search for alternative extraction methods for these valuable products (Krings and Berger 1998). It is important to note that crop-processing operations,

including premature harvesting and extended storage, can cause a loss of aroma due to the volatile nature of these products (Krings and Berger 1998). It is for this reason that the production of essential oils from natural plant materials be as efficient as possible. The essential oil yield and composition (or quality) varies based on the extraction method used. This section will outline the available physical and chemical extraction methods used to obtain the essential oils from the various plant materials.

Essential oils can be extracted from natural plant materials by hydrodistillation by which carvone is typically extracted. The extraction of essential oils using this method can be very energy intensive (Moll and Uiterkamp 1997). The plant material containing the essential oil is ground to expose the essential oil-containing parts of the plant and increase the surface area for essential oil extraction. In order to minimize volatile losses, the samples are crushed under water (Bailer et al. 2001). The sample (immersed in water) is then heated at boiling temperature for extended time periods, up to 12 hours (Bailer et al. 2001). The desired essential oil components pass through a distillation column when they are volatilized, and then are condensed before being collected in the receiver vessel. Aggarwal et al. (2002) used hydrodistillation to release the essential oils from the aerial parts of *M. spicata* and the seeds of *A. sowa*. The resulting essential oils were 0.55% (w/w) from *M. spicata* and 2.5% (w/w) from *A. sowa*. The fractions of the essential oil (limonene and carvone) can be obtained at a purity of 95 – 98% using hydrodistillation (Aggarwal et al. 2002).

Another method to obtain the essential oils from plant materials is ultrasound-assisted extraction. In this case, the plant material can be crushed or left whole and then submersed in a solvent such as hexane. In the case where the plant material is ground

before treatment, the ultrasonic treatment helps to release the essential oil by exerting vibrational energy and cavitation pressure which presses the solvent into pores and ruptures the small structures within the plant material (Bailer et al. 2001). Although this method provides rapid extraction, unwanted fatty materials are also released that have to be removed from the product downstream (Chemat et al. 2005). When the plant material, such as caraway seeds, is left whole, the ultrasonic treatment assists the essential oil components in diffusing across the unbroken gland walls due to the rising temperature effect in the medium (Chemat et al. 2004). When the plant material is left whole, there is less chance for fatty deposits to be released and affect the quality of the extracted oil (Chemat et al. 2004).

The final extraction method is supercritical CO_2 extraction. This extraction method is favored because it does not require the use of organic solvents (Baysal and Starmans 1999). In this case, the caraway seeds can be packed into a column through which liquid CO_2 flows to extract the essential oil. This method can also result in the extraction of waxes, fatty acids and colorants, but these effects can be minimized through optimization of operating temperatures and pressures and CO_2 flowrates to produce a pure product (Baysal and Starmans 1999).

In general, these traditional extraction methods require long extraction times (Chemat et al. 2005) and in order to ensure that carvone can be used in a wider range of applications, extraction methods that are more energy efficient, yield pure extracts, and prevent the use of organic solvents must be developed (Chemat et al. 2004; Chemat et al. 2005).

2.3.3 Microbial Biotransformation

The production of carvone by microbial biotransformation offers several advantages. The main advantages include the ability to overcome the seasonality, essential oil content in the plant and other agriculturally related problems (de Carvalho and da Fonseca 2002a). The development of microbial biotransformation processes also allows for industrial-scale production (Krings and Berger 1998). Another advantage of biological transformation is the independence it offers from agriculture in that the natural resources in developing countries are maintained responsibly (Krings and Berger 1998). It is opportune that microbial biotransformations are characterized by high chemo-, regio- and enantioselectivity (Brunati et al. 2004). Microbial biotransformations generally operate under mild and ecologically compatible conditions (Brunati et al. 2004).

The microbial biotransformation of carveol to carvone has been studied extensively. This single-step microbial biotransformation is carried out by whole cells of *Rhodococcus erythropolis* DCL14. Limonene is used as the sole carbon source and is readily available at low cost (de Carvalho and da Fonseca 2003). Limonene can be extracted from the essential oils of orange and lemon peel as well as caraway seeds (Duetz et al. 2003). When grown on limonene as the sole carbon source, *R. erythropolis* DCL14 exhibits carveol dehydrogenase (CDH) activity (de Carvalho and da Fonseca 2002a). It has been reported by van der Werf et al. (1999a), that this CDH has a unique substrate specificity in comparison with other alcohol dehydrogenases. The microbial biotransformation substrate is a mixture of cis- and trans-carveol isomers. The CDH activity allows the stereoselective conversion of the trans-isomer to *R*-(-)-carvone, resulting in a second product of isomerically resolved cis-carveol (de Carvalho et al.

2005a) (see reaction scheme in Figure 2.3). The cis-isomer can also be transformed, but only when the concentration of trans-carveol reaches very low levels (de Carvalho and da Fonseca 2002a).

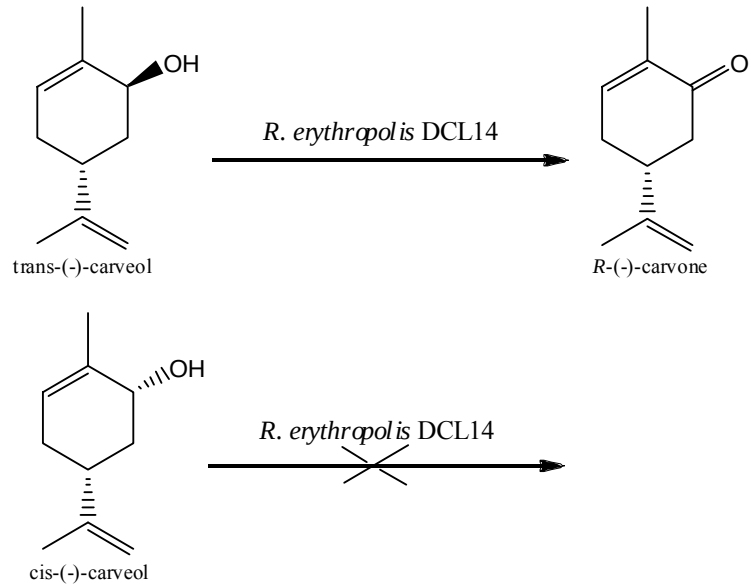


Figure 2.3: Reaction scheme of the carvone microbial biotransformation.

Since monooxygenases are dependent on cofactors, the successful production of carvone by microbial biotransformation involves the regeneration of the required cofactor (Mihovilovic et al. 2002). When *R. erythropolis* DCL14 is grown on limonene, the enzyme activity is NAD-dependent (de Carvalho and da Fonseca 2002a). The NAD-cofactor can be regenerated through cell growth and as such, the carbon source (limonene) and oxygen must be supplied throughout the biotransformation period. In order to obtain prolonged productivity, the cells must remain viable to maintain cofactor regeneration (de Carvalho and da Fonseca 2002a). Promising carvone yields have been obtained by microbial biotransformations which suggest that large scale production may one day be feasible (de Carvalho and da Fonseca 2006b).

In order to justify microbial biotransformation as an alternative production method for carvone, the advantages and disadvantages of the agricultural and

fermentative processes must be evaluated. From an environmental standpoint, it may seem that the agricultural production method would be favored; however this is not the case. A study conducted by Moll and Uiterkamp (1997) determined that the main disadvantages of fermentative production include the high energy requirement for moving parts in a reactor (ie. agitation), the low volumetric productivities and the need for expensive catalysts. The study determined that though the energy requirement for the fermentative production of carvone is high, the energy requirement for the production of its agriculturally derived counterpart is much greater. In addition to its high energy requirement, agricultural production also uses non-renewable resources such as fuel and fertilizers and toxic substances like pesticides (Moll and Uiterkamp 1997). Another disadvantage of the agricultural production method is that since the oils occur naturally in such low concentrations, a large amount of plant material waste is generated to obtain small amounts of products (Moll and Uiterkamp 1997).

2.4 Overview of Microbial Biotransformations

A microbial biotransformation involves the use of a biological catalyst, which can be either whole cells or isolated enzymes, to catalyze a chemical reaction (Burton 2001). There are several characteristics by which the biocatalyst can be evaluated which include: high stereoselectivity, high activity, flexible substrate selectivity, minimal side reaction productivity, stability under reaction conditions (Burton 2001) as will be discussed in Section 2.4.1. Microbial biotransformations can be used to convert pollutants to less toxic forms and also to make products with a high economic value. Recently, microbial biotransformation technology has become increasingly important in the production of

medium-priced, oxidized aromatic and aliphatic compounds for the chemical industry (Miller and Peretti 2002). The application of microbial biotransformation technology in the chemical industry is quite varied as production processes for more than 130 chemicals by have been commercialized (Straathof et al. 2002).

The production of desired chemicals by microbial biotransformations can offer several advantages over chemical production methods. The most significant advantage of the microbial biotransformation is the ability of the biocatalyst to be regio-, stereo- or enantioselective (de Carvalho et al. 2000). This selectivity proves to be very useful as many bioconversions involve the synthesis of products with one or more chiral centres (Zambianchi et al. 2004). Biocatalysts can modify a molecule at chemically inert carbons and modify a specific functional group in a multifunctional molecule (de Carvalho and da Fonseca 2006a). In chemical synthesis, multi-step procedures are used to obtain enantiomerically pure products, whereas biocatalysts have the ability to produce pure products in a single step (Zambianchi et al. 2004). It is important that food additives have very high purity such that they can be used commercially. Flavors, such as carvone, are bioactive compounds, meaning that their absolute configurations (chirality) affect odor perception (Krings and Berger 1998, de Carvalho et al. 2000). Finally, microbial biotransformations are useful because they operate under mild reaction conditions and can involve the use of inexpensive reagents (Schmid et al. 2001).

In order for microbial biotransformations to compete with chemical processes, a few limitations must be overcome. Microbial transformations often suffer from inhibition or toxicity due to the high substrate and product concentrations required at the industrial level (Straathof 2003). The presence of inhibitory or toxic substrates and/or

products can reduce the activity of the biocatalyst, which lowers its potential, leading to lower volumetric productivities (Lye and Woodley 1999). Other microbial biotransformation limitations include the chemical instability, low water solubility and high volatility of the substrate and product (Krings and Berger 1998) (which can also affect chemical transformations). Unfortunately, due to these limitations, the final product concentrations that can be reached can be far below those required for a commercially feasible process (Straathof 2003). Many of these limitations can be overcome by optimizing reactor configuration as will be discussed in Section 2.6.

2.4.1 Isolated Enzyme Versus Whole Cell Biotransformations

It is important to choose a biocatalyst that is tailored to the desired production system to achieve optimal reactor performance (de Carvalho et al. 2004). As stated in Section 2.4, proper biocatalyst selection will ensure high stereoselectivity, high activity, minimal side reaction productivity and stability under reaction conditions. Before about the 1960s, most bioprocesses were whole-cell based because efficient enzyme isolation techniques were not yet available (Woodley 2006). Currently, due to technological advances, it is possible to develop efficient processes using either whole cells or isolated enzymes. In general, isolated enzyme systems require investment upstream (to isolate the enzyme) and whole-cell processes require investment downstream (for product purification) (Woodley 2006). Isolation and purification of a specific enzyme can be challenging and also expensive whereas whole-cell catalysts, which are easier to obtain can add contaminants to the process (de Carvalho and da Fonseca 2006b). Krings and

Berger (1998) have suggested that whole cells should be used for complex systems and isolated enzymes should be used for simpler, single-step reactions.

In the case of the microbial biotransformation of carveol to carvone, the most important consideration is the cofactor requirement. In general, a cofactor is a compound that, together with a specific enzyme, facilitates a biological transformation. It is for this reason that the cofactor must be supplied in equimolar amounts to the enzyme to aid the microbial biotransformation (Mihovilovic et al. 2002). For certain processes, cofactors can be very costly which makes their use in microbial biotransformations unsustainable unless recycle is undertaken (van der Donk and Zhao 2003; Zhao and van der Donk 2003). The high cost of the cofactor necessitates *in situ* cofactor regeneration (Zhao and van der Donk 2003). Since living organisms provide natural recycling systems for cofactors through cell growth (Mihovilovic et al. 2002), whole cells should be used instead of isolated enzymes for applications requiring costly cofactors.

The application of whole cells can be advantageous because the cell walls and membranes protect the enzymes from factors such as shear forces in the bioreactor (de Carvalho and da Fonseca 2006b) except in the infrequent case of shear sensitive cells like mammalian cells. Whole cells can also be easily produced and stored, and as such, they can be used as recyclable catalysts (Mihovilovic et al. 2002). A significant disadvantage of using whole cells is the potential for unwanted side-reactions (Mihovilovic et al. 2002). These competitive processes can influence productivity and decrease the yield of the desired microbial biotransformation (Mihovilovic et al. 2002). In addition, the products of any unwanted side reactions can complicate downstream processing (Zambianchi et al. 2004). Other challenges associated with using whole cell biocatalysts are related to the

need to supply the cells with required medium components and oxygen through aeration. Often times, supplying sufficient aeration induces foaming in the reactor which needs to be controlled.

2.5 *Rhodococcus erythropolis*

The organism used to carry out the carvone biotransformation, *Rhodococcus erythropolis*, is part of the Actinomycetes phylum and the Nocardiaceae family. The Actinomycetes are bacteria that can occur in numerous natural and man-made environments (Goodfellow and Williams 1983). This family contains bacteria known for producing antibiotics, performing useful chemical modifications and degrading diverse pollutants (Goodfellow and Williams 1983) (see Table 2.2 for examples).

Table 2.2: A sample of the metabolic diversity exhibited by the members of the Nocardiaceae family.

Organism	Function	Reference
Rhodococcus sp. DSM 44126	naphthalene degrader	Grund et al. 1992
Nocardia asteroides	human pathogen that can cause bacterial meningitis	Prescott et al. 2002
Rhodococcus sp. strain RHA1	PCB and biphenyl degrader	Nandhagopal et al. 2001
Rhodococcus erythropolis TA57	degrades common herbicide component, triazine amine	Andersen et al. 2001
Rhodococcus IGTS8	desulfurization of petroleum products	Kaufman et al. 1999
Rhodococcus equi	human and animal pathogen that causes pulmonary infections	Weinstock and Brown 2002

Rhodococci are aerobic, gram-positive, non-motile bacteria that range from cocci to short rod shapes (Finnerty 1992). The *Rhodococcus* genus is well known for its broad

metabolic diversity (Finnerty 1992). *Rhodococcus erythropolis* is capable of performing oxidations, dehydrogenations, epoxidations, hydroxylations, hydrolysis, dehalogenations and desulfurisations (de Carvalho and da Fonseca 2005). A few commercially interesting bioprocesses catalyzed by *Rhodococcus erythropolis* include the production of acrylic acid and acrylamide, steroid conversions and bioremediation of chlorinated hydrocarbons and phenolics (Finnerty 1992). *Rhodococcus* IGTS8 expresses the complete '4S' desulfurization pathway in oil recovery which removes the sulfur from recalcitrant organic sulfur compounds while conserving the hydrocarbon value (Borole et al. 2002, Kaufman et al. 1999). This pathway is very useful for enhanced oil recovery as it can reduce air pollution by sulfur oxide emissions (Prescott et al. 2002). Figure 2.4 presents the '4S' desulfurization pathway for dibenzothiophene (DBT), the primary sulfur containing compound in diesel oil (Kaufman et al. 1999).

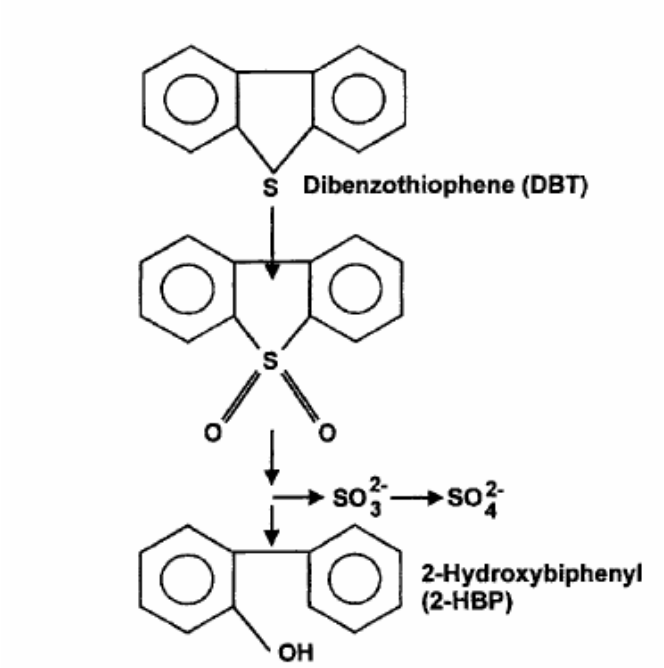


Figure 2.4: Desulfurization of DBT by *Rhodococcus* IGTS8 (Kaufman et al. 1999).

Since *Rhodococcus erythropolis* is a gram-positive bacterium, it possesses a cell wall that consists of a thick layer of peptidoglycan. The outer layer of the cell wall determines the hydrophilic or hydrophobic character of the cell (Borole et al. 2002). *Rhodococcus* organisms are very hydrophobic due to the presence of mycolic acids in the peptidoglycan layer (Borole et al. 2002). These mycolic acids include 34 – 52 carbon atoms and significant amounts of straight chain and branched fatty acids (Borole et al. 2002). *Rhodococcus* biocatalysts are so hydrophobic that they have been known to partition into an organic phase from an aqueous phase (Borole et al. 2002).

Rhodococci are also capable of producing biosurfactants when contacted with hydrophobic liquids (Borole et al. 2002), which have the potential to be valuable products in their own right (Goodfellow and Williams 1983). Neu and Poralla (1988) reported the discovery of a hydrophobic polysaccharide that has been characterized from a hexadecane-grown species that appears to contribute to the hydrophobicity of the cell surface. It is possible that these biosurfactants play an important role in the formation of very stable emulsions in the presence of hydrophobic liquids (Borole et al. 2002). The specific function(s) of these biosurfactants is not known, but Bredholt and Eimhjellen (1999) observed biosurfactant production during normal cell growth, under growth-limiting conditions and by resting cells, which suggests that the biosurfactants may serve numerous physiological purposes. It is certain however that the production of biosurfactants can be related to the development of emulsifying activity when organic solvents are present. An investigation by Bredholt and Eimhjellen (1999) which included the use of a variety of hydrophobic compounds, determined that no surfactants were found in the aqueous phase, however, a tightly bound polymeric compound with

emulsifying activity was found in the resulting emulsification. It was also determined that when the organisms are in the aqueous system, no emulsifying activity is present, but when they were exposed to a hydrophobic solvent, they developed emulsifying abilities (Bredholt and Eimhjellen 1999). It has been suggested that the development of the emulsifying activity in the presence of a hydrophobic liquid is coordinated with new cell wall synthesis since the emulsifying activity was inducible only under conditions of cell growth (Bredholt and Eimhjellen 1999). This emulsion formation reveals that there is potential for oil-emulsifying bacteria to be applied *in situ* to remediate oil contaminated sites (Bredholt et al. 1998).

2.5.1 DCL14

Rhodococcus erythropolis DCL14 is known to have several carveol dehydrogenase (CDH) activities (de Carvalho and da Fonseca 2006b). When grown on limonene or cyclohexanol, the cells produce CDHs capable of carrying out the cofactor dependent stereoselective oxidation of carveol to carvone (de Carvalho et al. 2005b). The major activity is NAD dependent, and the whole cells are capable of *in situ* cofactor regeneration (de Carvalho and da Fonseca 2006b).

This strain was isolated from a sediment sample from a ditch in Reeuwijk, The Netherlands (van der Werf et al. 1999b). *Rhodococcus erythropolis* DCL14 produces whitish to pink colonies on glucose-yeast extract agar (van der Werf et al. 1999b). As expected, the cell wall contains mycolic acids which attribute to the hydrophobic nature of *Rhodococcus erythropolis* DCL14 (van der Werf et al. 1999b). It has been shown that in the presence of organic solvents, this organism will migrate towards the

organic/aqueous interface in two-phase systems which is directly related to its hydrophobic nature (de Carvalho et al. 2004).

Rhodococcus erythropolis DCL14 has been known to produce interesting morphological changes under stress conditions. For example, when *Rhodococcus erythropolis* is subjected to nutrient shortage, slight morphological changes occur in cell shape (Goodfellow and Williams 1983). Under stress conditions, the cells aggregate to protect the population from the deleterious effects of the presence of a solvent or high concentrations of substrate and/or product (de Carvalho and da Fonseca 2005). This aggregation primarily affects the cell membrane and the ability of the cells to replicate (de Carvalho and da Fonseca 2005). Straathof (2003) reports that a hydrophobic substance can disturb the integrity of the cell membrane by dissolving in it which leads to cell permeabilization. This permeabilization affects not only the cell's ability to replicate, but also its biotransformation activity (Straathof 2003). In a two-phase reactor where the cells are exposed to a hydrophobic solvent, the cells develop the ability to change their morphology and their position in the organic/aqueous system (de Carvalho and da Fonseca 2004). The carbon source used may also determine the properties of the cell membrane, meaning that under stress conditions, cells grown on different carbon sources may exhibit different morphological changes (de Carvalho and da Fonseca 2004). Despite such physical changes, the cells are also able to adapt to the presence of a certain solvent or substrate through gradual acclimatization (de Carvalho and da Fonseca 2005). The cells can adapt their membrane composition to increase their resistance. de Carvalho and da Fonseca (2004) discovered that the resistance of the organism increased with

increasing inhibitory conditions which enables the cells to maintain viability under harsh conditions (de Carvalho and da Fonseca 2004).

2.6 Partitioning Bioreactor Configurations

A wide variety of bulk and fine chemicals can be produced by fermentation (Schulze and Wubbolts 1999). It is profitable to produce natural products via fermentation and since the products can be considered natural, the public may feel reassured that the product is safe for consumption as well as for the environment. Biotechnological processes can also be applied to the production of natural aroma compounds that are present in low concentrations in nature (Schrader et al. 2004).

Due to the increase in demand for fermentative products, there have been many attempts to increase productivity by improving reactor design In the case of cell-based processes, the reactors must be designed such that they can properly deliver nutrients, oxygen and substrate to the cells; as well, they are required to maintain appropriate temperature, pH and mixing. Despite the careful design of these reactors, certain bioprocesses can still lead to operational challenges which involve the biocatalyst. One major concern that arises in the production of microbiological products is cytotoxicity due to high concentrations of inhibitory substrates and/or products. Substrate inhibition can result in the need to work with small loadings which leads to more frequent substrate additions and higher labor requirement to maintain the process. Since high product concentrations within the reactor are desirable, often times the product will, at a certain concentration become inhibitory to the cell. This inhibition can result in no growth, decreased production rates and even cell death. The inhibition as a result of the

inhibitory product and/or substrate lowers fermentor productivity so that large fermentors are required to obtain suitable productivities (Roffler et al. 1988).

Partitioning bioreactors can enhance the performance of many bioprocesses by removing the inhibitory product(s) from the aqueous, cell-containing phase. A partitioning bioreactor is any reactor configuration that involves the delivery and/or removal of target compound(s) from the aqueous cell-containing phase with the aim of improving cell function and as a result, productivity. By removing an inhibitory product, the cell function is maintained which increases productivity. The removal of the target compound may involve the use of an extractant (i.e. adsorbent or absorbent). When an extractant is used, the product becomes concentrated in the extractant phase which provides benefits for subsequent downstream processing (Lye and Woodley 1999). In the case where the product has the potential to be degraded, *in situ* product removal can aid in the prevention of product degradation, since its residence time in the vicinity of the biocatalyst is limited (Zijilstra et al.1998). Partitioning bioreactors can also alleviate concerns dealing with substrates that have low water solubilities in that concentrated feedstocks can be applied (Roffler et al. 1984). An advantage to using these extractant methods for enzymatic reactions is that unfavorable reaction equilibria can be shifted to maximize productivity (Lye and Woodley 1999).

The majority of the fermentation products of interest in the pharmaceutical and food industries exhibit low water solubilities and are cytotoxic (to the production organism) to some degree (de Carvalho and da Fonseca 2005). Several bioreactor designs have been implemented in the past to overcome the issues of product inhibition and low substrate solubility in the aqueous phase. An overview of partitioning

bioreactors in terms of the various extractants or reactor configurations that can be applied can be found in Sections 2.6.1 to 2.6 3.

2.6.1 Adsorption

Adsorption occurs when the any molecule adheres to the surface of a solid structure called an adsorbent. There are several types of adsorbents that can be applied based on the nature of the target molecule. For example, silica gel can be used to adsorb hydrophilic/polar compounds and activated carbon can be used for hydrophobic/non-polar products. Adsorption is an effective and inexpensive way to remove inhibitory products. However, this adsorption system is restricted by the limited surface area and selectivity of the adsorbent.

Adsorbent materials can be packed into external (to the bioreactor) extraction columns. In this case, the adsorption occurs as the fermentation broth is cycled through the column and returns to the reactor at a product concentration that is sub-inhibitory to the organism (Roffler et al. 1988). This system is advantageous because the production and extraction occur in different vessels so the vessels can each be operated at their optimal conditions to maximize productivity (Roffler et al. 1998).

Ma et al. (2006) studied the removal of high concentrations of H_2S from waste gases using granulated activated carbon (GAC) as the adsorbent. GAC was chosen as the adsorbent because it has a uniform surface area and good resistance to crushing which allows better operational control of the process (Ma et al. 2006). The main limitation to this method of target compound removal is that once the compound has sorbed to the surface of the adsorbent, it must be removed which complicates downstream processing.

2.6.2 Membrane Reactors

Several membrane reactor configurations have been used in the past to partition inhibitory products in biochemical processes. This section outlines a few applications of these various membrane reactors.

Reverse osmosis is used to push a solution containing the desired product through a semi-permeable membrane such that the desired product passes through the semi-permeable membrane (Figure 2.5).

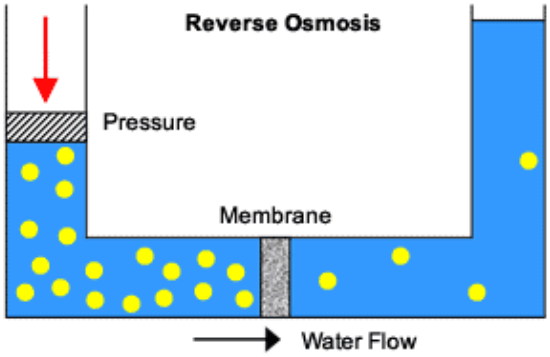


Figure 2.5: A membrane-aided separation technique: reverse osmosis.

Richter and Nottelmann (2004) studied lactate production in continuous fermentation using reverse osmosis to remove lactate, the inhibitory product. Reverse osmosis has certain limitations involving the membrane. An appropriate membrane must be chosen such that only the target molecule passes through the membrane, as well there is a high probability of membrane fouling, which will reduce the effectiveness of the process. Another significant disadvantage with reverse osmosis is the high pressure that is required to force the solution through the membrane.

Pervaporation is the membrane aided separation of a volatile product between a liquid and a vapor. In this case, the product-containing aqueous phase is passed over a selective membrane and an extracting vapour phase is passed in the opposite direction on

the other side of the membrane. The volatile product partitions through the membrane from the aqueous phase into the vapour phase. This system is depicted in Figure 2.6 where the liquid and vapor streams are flowing in opposite directions.

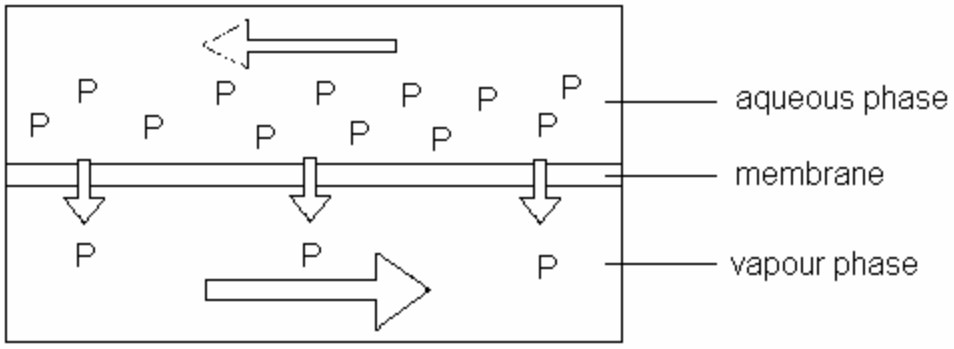


Figure 2.6: A membrane-aided separation technique: pervaporation.

Qureshi et al. (1992) used pervaporation with a silicone membrane to selectively remove acetone, butanol and ethanol from the ABE fermentation. Pervaporation is a difficult process to run successfully as the selectivity of the membrane must be well defined and the membrane is subject to fouling which reduces the effectiveness of the extraction process.

Perstraction is very similar to pervaporation because it is also characterized as membrane aided separation, but between two liquids, not a liquid and a gas (Figure 2.7). In this case, the fermentation broth containing the product (which does not have to be volatile) passes over a selective membrane in one direction and an extracting liquid phase passes in the opposite direction on the other side of the membrane such that the product partitions through the membrane into the extracting phase. Qureshi et al. (1992) also studied the effectiveness of acetone, butanol and ethanol removal from the fermentation medium of the ABE fermentation. In this case, they used a polypropylene membrane to

facilitate the extraction. It was found that perstraction was a more effective method of removal than pervaporation for the ABE fermentation.

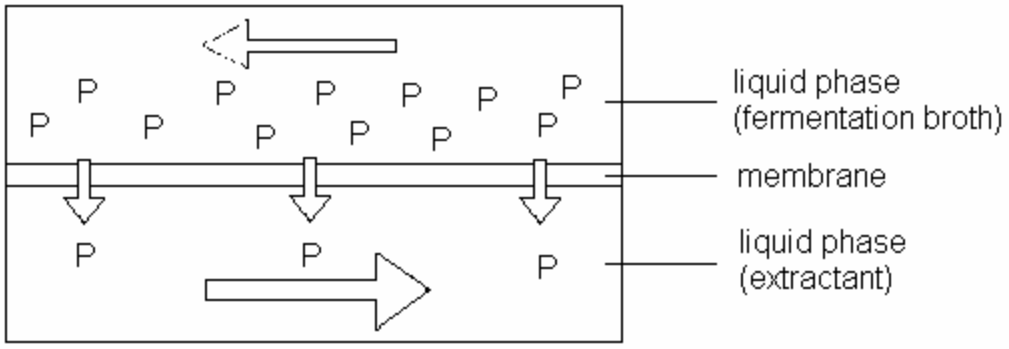


Figure 2.7: A membrane-aided separation technique: perstraction.

Perstraction faces the same limitations with respect to membrane selectivity and fouling as do reverse osmosis and pervaporation.

2.6.3 Two Phase Partitioning Bioreactors

A two phase partitioning bioreactor (TPPB) is comprised of an aqueous phase containing the biomass and a second, immiscible phase designed to partition inhibitory compounds (Malinowski 2001). Two phase partitioning bioreactors were developed to overcome product inhibition by the removal of inhibitory product(s) from the aqueous phase, which in turn increases product yield and simplifies downstream processing. Other advantages of the TPPB include the ability of the second phase to control the delivery of inhibitory substrate(s) and the second phase can also aid with low substrate solubility such that it can be added in higher concentrations. TPPBs are advantageous as they allow for *in situ* product removal. The most challenging and important part of setting up a

TPPB is the appropriate selection of the second phase. Two TPPB configurations are outlined in Sections 2.6.3.1 and 2.6.3.2.

2.6.3.1 Two Phase: Solvent

In situ product removal is required in order to obtain economically feasible product concentrations in biotechnological processes (Etschmann and Schrader 2006). Traditionally in a TPPB, a pure organic solvent has been used as the second phase (Prpich and Daugulis 2005). The organic solvent acts as a delivery and extraction phase for high concentrations of substrate and product to sustain high volumetric productivities (Lye and Woodley 1999). The partitioning of substrate and product between the organic and aqueous phases relies on thermodynamic equilibrium and the rate of metabolism of the cells (Daugulis 2001). Since the partitioning depends on the metabolic rate of the cells, the inhibitory or poorly soluble substrate can be added in very high concentrations and is transferred to the cells in the aqueous phase at sub-inhibitory levels (Daugulis 1997). This technology is very convenient as the system can be regarded as being self-regulating (Prpich and Daugulis 2004). Also, the immediate partitioning of the product into the solvent phase prevents the further conversion of product in the case of a biodegradable product (Schmid et al. 1998). Product recovery by solvent extraction is an attractive option because of the relatively low cost of implementation in terms of infrastructure and solvent cost (it can be recycled) (Mathys et al. 1999).

Due to poor solvent selection, the use of the solvent phase can have negative effects on the overall performance of the fermentation. The organic solvent can affect the performance of the biocatalyst as cell membranes are often a target for the deleterious

action of organic solvents (de Carvalho et al. 2004). The use of an organic solvent can lead to physical, morphological and biochemical changes in the microorganism (Serrano-Carreón et al. 2002) as discussed in Section 2.5.1. It is also possible, with highly hydrophobic organisms, that the cells are associated with the organic phase (MacLeod and Daugulis 2005). Of course this situation is not ideal since the cells in the solvent phase will be exposed to the high product and substrate concentrations within the solvent phase. In order to develop an efficient TPPB system, the solvent selection must be considered carefully, as described in Section 2.6.3.1.1.

2.6.3.1.1 Solvent Selection

In order to optimize the reactor performance, it is important to follow a rational solvent selection regime as developed by Bruce and Daugulis (1991). An appropriate solvent is chosen based on several factors including biocompatibility, bioavailability, partitioning capacity and selectivity, low volatility, low cost and safety. Possible solvents include organic solvents, ionic liquids and silicone oil (Prpich and Daugulis 2006). Many organic solvents are prone to being bioavailable, especially when a microbial consortium is used, which leads to the consideration of solvents such as silicone oil. Silicone oil (a "liquid polymer") is biocompatible and non-bioavailable, but it is limited in its partitioning capability due to its fixed molecular structure (Prpich and Daugulis 2006). Sometimes the solvent choice is limited due to bioavailability (degradation by cells) despite the fact that it exhibits desirable partitioning characteristics (Vrionis et al. 2002). The use of solvents as the second phase can also involve operating difficulties (i.e. the separation of phases when taking samples), as well as the disposal or recycling of the

solvent. An attractive alternative to the use of a liquid solvent in TPPBs is the use of solid polymer beads which are discussed in the next section.

2.6.3.2 Two Phase: Polymer Beads

Polymer beads are an excellent candidate for use as the second phase in a TPPB. It is well known that polymers are able to absorb small molecular weight compounds (Amsden et al. 2003). Polymer beads can be used in a TPPB because they are capable of partitioning substrates to the aqueous phase in response to the metabolic demand of the organisms (Amsden et al. 2003). Unlike most organic solvents, polymers are low-cost, non-volatile, biocompatible, non-biodegradable, reusable, easy to use and allow for complete recovery from the bioreactor. Since polymers are non-bioavailable, it is possible to use them in conjunction with a microbial consortium without degradation of the delivery phase (Amsden et al. 2003). The most important advantage is that the polymer structure can be tailored to enhance selective absorption of the desired product (Prpich and Daugulis 2006). The target molecules are absorbed into the polymer, unlike surface adsorption, and as such, there is no competition among target molecules (Prpich and Daugulis 2006).

2.6.3.2.1 Polymer Selection

As with solvent selection, it is important to choose the most suitable polymer in order to optimize the reactor configuration and maximize productivity. In order to choose an ideal polymer, there are several desirable characteristics that should be satisfied. The polymer should be commercially availability at low cost, nonhazardous,

biocompatible, non-biodegradable, resistant to biofilm formation, have an affinity for target molecule(s) and exhibit thermal stability for sterilization purposes (Rehmann et al. 2007). Since polymer beads are invariably biocompatible and non-bioavailable, the most important selection criterion is partitioning capability. As previously mentioned, it is possible to tailor the polymer such that it has a high affinity for the target molecule(s). Polymer beads can be modified by monomer selection, functionalization, co-polymerization, cross-linking, and polymer processing (Prpich and Daugulis 2005). Selection of a suitable polymer involves an understanding of the molecular structure of the polymer and its possible functional groups such that they can be modified to increase their affinity for the target molecule(s) (Prpich and Daugulis 2004). Depending on the structure of the target molecule, important factors for polymer selection can include the polarity and hydrogen bonding capabilities of the polymer. The polymer must also be amorphous (to allow the unrestrained transfer of substrate and product into and out of the polymer), inexpensive and commercially mass-produced to ensure availability and consistent quality (Prpich and Daugulis 2004). Finally, when dealing with a single organism, it is important that the polymer beads be resistant to sterilization via autoclaving to minimize the chance of contamination.

2.7 Discussion of Previous Work

The research group from the Division of Industrial Microbiology from Wageningen University and Research Centre in Wageningen, The Netherlands that isolated *Rhodococcus erythropolis* DCL14 also studied the various carveol dehydrogenases (CDHs) that were induced when *Rhodococcus erythropolis* DCL14 was

grown on various monoterpenes (van der Werf et al. 1999a). The stereoselective CDH activities of the various cell extracts were calculated and it was determined that the highest activity for trans-carveol resulted after growth on limonene and cyclohexanol (van der Werf et al. 1999a).

After the CDH activities had been characterized, a research group from the Center for Biological and Chemical Engineering at the Technical University of Lisbon in Portugal, set out to develop a reaction system for the bioconversion of trans-carveol to carvone using *R. erythropolis* DCL14 (Tecelão et al. 2001). Tecelão et al. (2001) completed the microbial biotransformation in both single phase and two phase organic solvent experiments in shake flasks with an aqueous volume of 20 mL using cells grown on limonene. Limonene was the carbon source (CDH inducer) of choice because the cells exhibited a higher growth rate than when grown on cyclohexanol (another CDH inducer). In the single phase fed-batch system, a total of 16.14 mg carvone was obtained after a total of four additions of 9.96 mg carveol. After each substrate addition, the reaction rate decreased until the fourth addition at which point, no further trans-carveol was converted. The reduction in reaction rate was attributed to substrate inhibition (build up of cis-isomer) and lack of cofactor regeneration (no carbon source was supplied for cell growth).

The second reaction system that was developed by Tecelão et al. (2001) was biphasic with 4 mL iso-octane as the second phase. The microbial biotransformation substrate was provided by the addition of the loaded iso-octane phase (23.9 g carveol/L). After the trans-isomer had been consumed, the organic solvent was removed and replaced with fresh solvent loaded with substrate (23.9 g carveol/L). The total carvone produced

in this system after 5 substrate additions was 255 mg. In this case, the reaction rates also decreased after each substrate addition suggesting substrate inhibition and cofactor depletion. The authors did recognize that the organism formed an emulsion with the second phase and by removing the organic phase, they were in turn removing some of the biomass which could have contributed to the decrease in reaction rate after each addition. In order to avoid the loss of biomass, the second phase was replaced with 20 mL of n-dodecane instead of 4 mL iso-octane (it is important to note that no systematic reason for the change in organic solvent was provided). In this manner, most of the organic solvent was removed after the depletion of the trans-isomer without disturbing the emulsion formed at the interface of the organic and aqueous phases. In this case, after three substrate additions (through replacement of the organic phase), the total carvone produced was 781 mg. Again, the reaction rates decreased with each substrate addition. In an attempt to overcome the decrease in reaction rate, co-substrates were added to try to relieve the potential lack of co-factor regeneration. The addition of propanone, glutamic acid, propanal and butanal did not alleviate the problem of reaction rate decrease.

After Tecelão et al. (2001) had characterized the single and two-phase reaction systems in shake flasks, another research team from the Technical University of Lisbon attempted to determine the effect of reactor configuration (using an extractant phase) on the microbial biotransformation of carveol to carvone by *Rhodococcus erythropolis* DCL14 (de Carvalho and da Fonseca 2002b). de Carvalho and da Fonseca (2002b) tested various reactor configurations including a mechanically stirred direct contact reactor (MSDCR) and a silicone tube membrane reactor. The MSDCR is simply another name for a TPPB since the system involves the direct contact between the aqueous phase and

the organic phase. The strategy behind selecting these reactor configurations was to overcome the low substrate solubility, and substrate and product inhibition in this microbial biotransformation system. The microbial biotransformation in the MSDCR was operated at three different sets of conditions. The first microbial biotransformation was operated as a two phase system with n-dodecane as the second phase at ambient temperature with a regenerative mineral medium as the aqueous phase. In this case, cell growth was observed, which led the authors to believe that there should be an increase in the rate of transformation of carveol, however since this was not the case, it was assumed that the new cells generated from glucose as the carbon source did not possess CDH activity. In this case, the final product concentration (referring to the organic phase of 60 mL) was 24.34 g/L. The second MSDCR configuration was operated at ambient temperature with a phosphate buffer acting as the aqueous phase to determine if a non-regenerative medium (i.e. phosphate buffer) would have an effect on the transformation rate. That is, the authors expected a lower transformation rate due to the lack of biomass growth. It was determined that the presence of a non-regenerative medium had no significant effect on the reaction rate after each carveol addition suggesting that the biomass that was produced in the first biotransformation did not have a significant effect on the reaction rate. The final reactor was operated similarly to the first but under a controlled temperature (28°C) to determine the effect of temperature control with mineral medium as the aqueous phase. However, it is difficult to compare these two reactors because part way through the transformation, the majority of the organic phase (n-dodecane) was removed and replaced with fresh organic phase to avoid product inhibition. In this case, the transformation rates after the first and second additions increased,

however the final product concentration referring to the organic phase (60 mL) was only 18.78 g/L. In conclusion, the set of operating conditions that achieved the best product yield were ambient temperature with mineral medium. It should be noted however, that the "controlled temperature, mineral medium" configuration may have produced a higher product yield had the majority of the second phase not been removed half way through the transformation. It is difficult to compare the productivities of the ambient and controlled temperature reactors with mineral medium as they did not follow the same methods.

The second reactor configuration that was tested by de Carvalho and da Fonseca (2002b) was the membrane reactor. The strategy behind this reactor configuration was to avoid the production of an emulsion since the cell-containing aqueous phase and the solvent phase were not in direct contact. In this case, a silicone tube that circulated the cell-containing aqueous phase (300 mL) was immersed in 250 mL n-dodecane that was loaded with 12.33 g/L carveol. The final carvone concentration (referring to the organic phase 250 mL) was 7.62 g/L. The major advantage of this reactor configuration was that no emulsion formation was observed because the hydrophobic cells were never in direct contact with the organic solvent. However, operating difficulties were encountered with this reactor configuration as a biofilm formation was observed on the surface of the tube which had the potential to limit the transfer of substrate and product between phases. It was determined that the membrane reactor is not an appropriate configuration for hydrophobic, slime producing strains.

In another article by de Carvalho and da Fonseca (2002a), an attempt was made to improve upon the microbial biotransformation yield by maintaining cell viability in a two

phase system throughout the transformation. The solvent selection was based upon cell viability and microbial biotransformation activity retention in the presence of several candidate solvents for use in a biphasic system. It was determined that n-dodecane (log P = 6.10) maintained the best activity and cell viability. It is interesting to note however that every other solvent that was tested had a log P lower than that of the critical log P of the organism and as expected, resulted in low activities and cell viabilities. This paper also focused on the inhibition effects of the substrate and product in a biphasic system. The effect of an increasing initial carveol concentration on the carvone production rate in a biphasic system was studied. The highest production rate was obtained at an initial carveol concentration of 19.03 g/L in the organic phase. The results also show the corresponding initial aqueous concentrations of carveol; however each sample point has a concentration higher than the aqueous solubility limit. For this reason, it is impossible to determine the proper inhibition limit for the aqueous phase with respect to initial carveol concentration from this work. Another study was completed by de Carvalho and da Fonseca (2002a) to determine the effect of initial carvone concentration on the production rate. The results show that the production rate decreases with increasing initial carvone concentrations, as expected. However, the initial concentrations only refer to the organic phase, which does not give the aqueous inhibition limit since no partition coefficient was provided. It is important to recognize the aqueous inhibition limits of the substrate and product since this is the concentration that will affect the cells.

The final paper of interest published by de Carvalho et al. (2005a) reports on solvent, substrate and product inhibition in aqueous systems as determined by cell viability. The results for substrate and product inhibition indicate that there is a certain

concentration at which cell viability is the lowest, however higher concentrations seem to have higher cell viability percentages. The explanation given for this inconsistency is that since the terpene concentrations were above the aqueous solubility limits, there was a decrease in the area of contact due to coalescence of terpene droplets. Again, it is important to determine these inhibitory concentrations in the aqueous phase within the aqueous solubility limits in order to determine their effects on the organisms.

The previous work conducted on this system (van der Werf et al. 1999a, Tecelão et al. 2001, de Carvalho and da Fonseca 2002a, de Carvalho and da Fonseca 2002b, de Carvalho et al. 2005a) was useful in identifying the favorable reaction conditions for the microbial biotransformation. It was determined that limonene was the best carbon substrate to be used to induce CDH activity, it was also recognized that the substrate and product (at certain concentrations) became inhibitory to the cells. The previous work has shown the successful production of carvone in single and two phase reaction systems, however, the results were limited by improper quantification of the inhibition limits of substrate and product and poor solvent selection for two phase reactor configurations. It is also important to note that though the emulsion formation in the biphasic system was mentioned, there was no report of any challenges, or measures undertaken to alleviate the difficulties in biomass quantification. As well, the majority of the work was completed in small scale (20 mL) shake flasks which do not have ideal aeration and agitation conditions. Most of the data presented is incomplete such that volumetric productivities (a common measure of reactor performance) cannot be calculated. For these reasons, the current work presented in this thesis was undertaken in an effort to properly characterize this system.

2.8 Summary and Scope of Current Work

The focus of this thesis is to enhance the microbial biotransformation of trans-carveol to (*R*)-(-)-carvone by *Rhodococcus erythropolis* DCL14 by developing an effective reactor configuration

The first part of the work in this thesis characterizes the single aqueous phase reaction system. The production capacity of the single phase system is maximized and the inhibitory effects of the substrate and product on *Rhodococcus erythropolis* DCL14 are studied. Also, some important properties of the organism, *Rhodococcus erythropolis* DCL14, are identified and methods to lessen the burden of working with such an organism are developed.

The next part of the work centers on the development of a TPPB reaction system in which the second phase is an organic solvent to overcome the low aqueous solubility and inhibition effects of the substrate and product. A rational solvent selection regime is applied to obtain a suitable second phase. Due to the hydrophobic nature of the organism, a "liquid polymer", silicone oil is applied as the second phase to try to overcome some operating difficulties.

The final part of the thesis is the development of a TPPB reaction system in which the second phase is comprised of solid polymer beads. Again, a rational selection regime was followed to obtain the best available polymer beads for use as a second phase. Also, a system where two different polymer beads were applied as the second phase was used to determine the effects of the combined partitioning activities. Finally a system that contained polymer beads in the reactor as well as in an external extraction column was employed to improve upon reactor performance.

Chapter 3.0 Materials and Methods

3.1 Chemicals and Polymers

(*R*)-(-)-carvone (CAS 6485-40-1) and (-)-carveol (CAS 99-48-9) were purchased from Sigma-Aldrich Canada Ltd. (+)-limonene (CAS 5989-27-5), methylene chloride (CAS 75-09-2), alcohol reagent (89 – 91% ethanol) (CAS 64-17-5), ethyl acetate (CAS 141-78-6) and methanol (CAS 67-56-1) were purchased from Fisher Scientific.

All organic solvents that were tested for use as the second phase in a TPPB are summarized in Table 3.1 and were purchased from Fisher Scientific with the exceptions of silicone oil (viscosity = 5 cSt), undecane and octane which were purchased from Sigma-Aldrich Canada Ltd. and bis(2-ethylhexyl)phthalate (BEHP) which was purchased from Alfa Aesar.

Table 3.1: Chemical and physical properties of the various organic solvents used.

Solvent	CAS	log P	Boiling Point (°C)	Cost[a] ($/L)	Identified Hazards[b]
1-dodecene	112-41-4	6.10	213	230	flash point is 77°C
1-octanol	111-87-5	3.00	195	178	flash point is 81°C
decane	124-18-5	5.01	174	409	flash point is 46°C
n-dodecane	112-40-3	6.10	217	438	none
1-tetradecene	1120-36-1	7.08	251	36	none
hexadecane	544-76-3	8.25	287	774	none
1-octadecene	112-88-9	9.04	179	28	none
silicone oil	63148-62-9	- [c]	>140	308	none
undecane	1120-21-4	5.74	196	543	flash point is 62°C
octane	111-65-9	4.27	124 - 127	103	flash point is 13°C
BEHP	117-81-7	8.39	230	34	none

[a] Approximate costs in Canadian dollars from company websites
[b] All solvents have the potential to cause eye and skin irritation, digestive and respiratory irritation if exposed
[c] There is currently no reported log P for silicone oil

Polymers were acquired from various companies: KRATON® D4150K (Kraton Polymers LLC), styrene/butadiene, ABA block copolymer (Scientific Polymer Products, Inc.), TONE™ P787 (Dow Chemical Canada Inc.), Desmopan 453 (Bayer MaterialScience), Elvax® 770 (Dupont), Nucrel® 925 (Dupont), ethylene/vinyl alcohol copolymer (Scientific Polymer Products, Inc.) and Hytrel®8206 (Dupont) and were used as received. For polymer data sheets, see Appendix E.

CelLytic™ B Cell Lysis Reagent (standard strength) and the Total Protein Kit, Micro Lowry, Peterson's Modification were purchased from Sigma-Aldrich Canada Ltd.

3.2 Microorganism and Medium Composition

3.2.1 Microorganism

Rhodococcus erythropolis DCL14 was originally obtained by the Division of Industrial Microbiology of the Wageningen Agricultural University, Wageningen , The Netherlands. The strain was isolated from a sediment sample from a ditch in Reeuwijk, The Netherlands (van der Werf et al. 1999b).

Rhodococcus erythropolis DCL14 was received as a donation from the Center for Biological and Chemical Engineering of the Technical University of Lisbon.

The stock culture was stored at -77°C in 5% dimethyl sulfoxide solution.

3.2.2 Medium Composition

The medium formulation is a modified version of that used by Wiegant and de Bont (1980). The growth medium formulation is as follows (added to 1 L tap water):

glucose (10 g), yeast extract (0.1 g), K_2HPO_4 (1.55 g), $NaH_2PO_4 \cdot H_2O$ (0.85 g), NH_4Cl (3 g), $MgCl_2 \cdot 6H_2O$ (0.075 g), $(NH_4)_2SO_4$ (1 g) and 0.2 mL trace element as described by Vishniac and Santer (1957). The medium used for fermentations contained no glucose and solid culture medium was prepared as above with glucose and 15g/L agar. The medium was autoclaved at 121°C for 45 minutes.

3.2.3 Inoculum Preparation

For all shake flask experiments and fermentations, 100 µL of *R. erythropolis* DCL14 stock culture was added to 50 mL medium containing glucose. The flasks were incubated at 30°C for 48 hours at 180 rpm. For fermentations, two 50 mL inoculum flasks were added to the reactor, which contained 3 L sterile glucose-free medium.

3.3 Analytics

3.3.1 Gas Chromatography

Calibration samples (5 mL) for carvone and carveol were prepared over a range of concentrations from 0 mg/L to the solubility limit (1350 mg/L for carvone and 2890 mg/L for carveol). The 5 mL samples were added to 5 mL ethyl acetate (used as the extractant). The samples were then vortexed for 10 seconds, twice. When the phases had separated, a 1 µL sample of the ethyl acetate layer was then injected into the gas chromatograph. The calibration curves can be seen in Appendix A.

A second set of calibration curves were completed as described above, but using methanol as the extractant. This second set of calibration curves was generated because

methanol was used as the extractant to remove the product from the polymer beads. Ethyl acetate could not be used as the extractant because when the beads were submerged in ethyl acetate, they dissolved. These calibration curves can also be seen in Appendix A.

The Varian Gas Chromatograph was equipped with an Agilent/J & W DB-WAX column (Part Number: 125 7032) with internal diameter of 0.53 mm and length of 30 m. The carrier gas was helium and had a flowrate of 30 mL/min. The hydrogen and air flowrates were 45 and 450 mL/min, respectively. The method used is as follows: injector temperature – 250°C, detector temperature – 270°C, oven temperature – 100°C, hold – 0.5 minutes, ramp to 160°C at 50°C/min, hold for 1 minute and finally ramp to 175°C at a rate of 50°C/min. The run time for this method is 3 minutes.

3.4 Quantification of Biomass

The hydrophobic nature of *R. erythropolis* DCL14 provided challenges with respect to the quantification of biomass. In the presence of an organic solvent, the centrifuged samples did not form a biomass pellet as expected. Instead, the centrifuged sample would result in the aggregation of biomass at the solvent/aqueous interface and also the formation of a small pellet at the bottom of the centrifuge tube.

Another challenge was discovered when the organism was subjected to high concentrations of the inhibitory substrate and product ((-)-carveol and (*R*)-(-)-carvone, respectively). As a stress response to the inhibitory conditions, the biomass would aggregate to form small particles in solution resulting in a non-uniform suspension.

In both of these cases, optical density was not an appropriate measure of biomass concentration and as a result, other methods were developed, as described in Sections 3.4.1 to 3.4.3.

Several other methods that were tested for biomass quantification but that were not used to obtain any results included the DNS glucose assay and the Lowry Protein Kit for protein quantification and are described in Appendix B.

3.4.1 Biomass Estimation: Absence of Organic Solvent, Substrate or Product

In aqueous media, the organism behaved as expected and produced a uniform suspension where the biomass could be quantified using optical density. In this case, the sample was centrifuged for 5 minutes at 3500 rpm and 20°C. The supernatant was removed and replaced with an equal volume of distilled water. The biomass pellet was resuspended and the optical density was measured at a wavelength of 650 nm. Though biomass quantification under these conditions was generally easier, there was still minor scatter in the data potentially due to the presence of wall growth, polymer particulates and minor morphology changes.

The calibration curve which can be found in Appendix C, was created by adding 100 µL *R. erythropolis* DCL14 stock to a 125 mL flask containing 50 mL glucose-containing medium. The flask was incubated at 30°C and 180 rpm for 48 hours. The 50 mL sample was then centrifuged for 5 minutes at 3500 rpm and 20 °C. The supernatant was removed and replaced with an equal volume of distilled water into which the pellet was resuspended. Serial dilutions of the cell suspension resulted in optical density

readings between 0 and 0.8 at a wavelength of 650 nm using an Ultraspec 3000 UV – vis spectrophotometer (Biochrom, UK). The rest of the cell suspension was air-dried under vacuum overnight using a 0.2 μm filter paper. The original cell concentration was determined based on the final cell dry weight and remaining volume of the cell suspension.

3.4.2 Biomass Estimation: Organic Solvent Present

In the presence of most organic solvents *R. erythropolis* DCL14 forms an emulsion. In order to remove the biomass from the emulsion and the remaining solvent, a washing method was developed using methylene chloride and ethanol.

To a 50 mL aqueous/organic sample containing biomass, 10 mL methylene chloride was added and vortexed for 10 seconds. The sample was then centrifuged for 5 minutes at 3500 rpm and 20°C. This cycle resulted in the aggregation of the biomass at the aqueous/methylene chloride and solvent interface. Then, most of the aqueous phase was aspirated off being careful not to disturb the biomass at the interface. Next, 30 mL ethanol was added and the sample was vortexed for 10 seconds. The sample was centrifuged again for 10 minutes at 3500 rpm and 20°C. This cycle results in the formation of a pellet. The supernatant can be decanted and the pellet can be resuspended in distilled water.

In certain cases, the emulsion was very thick and the above amounts of methylene chloride and ethanol were not sufficient to cause the biomass to separate. If either cycle did not perform the desired function, that cycle was repeated with fresh methylene chloride and/or ethanol until the desired result was achieved.

When the pellet from this treatment is resuspended in distilled water, it does not form a uniform suspension and the cell dry weight must be determined in order to obtain an accurate biomass concentration. For this final step, the 50 mL sample was dried in a pre-weighed metal dish in a 90°C oven overnight.

3.4.3 Biomass Estimation: Inhibitory Substrate and/or Product Present

In this case, when there was no solvent present, it was not necessary to wash the sample with methylene chloride and ethanol. However, the pellet would not resuspend uniformly in distilled water and the cell dry weight was measured instead. A known volume was dried in a pre-weighed metal dish in a 90°C oven overnight.

3.5 Substrate and Product Toxicity

In order to assess the relative aqueous toxicities of carveol (substrate) and carvone (product), assays were carried out in shake flasks to determine their effect on the biotransformation capacity, cell growth and cell morphology of *Rhodococcus erythropolis* DCL14. A 3 L reactor with inoculum as described above and gas-phase limonene as the carbon source was used to produce the biomass required for the tests. After 72 hours, there was sufficient biomass present in the inoculum bioreactor to start the toxicity tests. Two separate sets of 125 mL flasks sealed with rubber stoppers were prepared; one for carvone toxicity and another for carveol toxicity. A portion of the inoculum bioreactor contents was divided into 14 sterile 125 mL flasks (50 mL in each). For the carvone set, varying amounts of carvone were added to 5 flasks in the range of

100 – 1000 mg/L and to initiate the biotransformation, 500 mg/L carveol was also added. As for the carveol set, increasing concentrations of carveol were added in the amount of 500 - 1200 mg/L. The carveol and carvone concentration ranges were chosen such that they were below the substrate and product water solubilities, which are 2890 mg/L and 1350 mg/L, respectively. A control flask was also prepared with no carvone or carveol for comparison to the test flasks.

The flasks were incubated for 2 hours at 30°C and 180 rpm, after which the samples were centrifuged to remove the biomass and the aqueous substrate and product concentrations were determined using gas chromatography.

In order to test the effect of the inhibitory substrate and product on cell growth, the biomass pellet from each sample was resuspended in distilled water and a small amount was plated and incubated at 30°C for 48 hours. After 48 h, the cell growth on the surface of the plate was assessed by visual observation to determine if the majority of the cells were still viable after exposure to high concentrations of substrate and product.

3.6 Solvent Selection

3.6.1 Biocompatibility: Determination of Critical log P

Several organic solvents with a broad range of log Ps (1.25 to 9.04) and silicone oil were tested for their biocompatibility with *R. erythropolis* DCL14. To separate 125 mL flasks, 50 mL of sterile glucose-containing medium, 5 mL of the solvent being tested and 1 mL inoculum were added. To ensure sterility, the solvents were passed through a syringe with a sterile 0.2 μm nylon filter attached prior to use. A positive control flask

which contained no solvent was also prepared. The flasks were incubated at 30°C for 96 hours at 180 rpm. After incubation, the extent of biomass growth was measured relative to the control as described in Section 3.4.2.

3.6.2 Bioavailability

The solvents tested for bioavailability were those that had a log P above the critical log P of *R. erythropolis* DCL14, and were therefore biocompatible. To separate 125 mL flasks, 50 mL of sterile medium (no carbon source), 5 mL of the solvent being tested and 1 mL inoculum were added. To ensure sterility, the solvents were passed through a syringe with a sterile 0.2 μm nylon filter attached prior to use. A positive control flask with canola oil and a negative control with no second phase were also prepared in a similar manner. The flasks were incubated at 30°C for 96 hours at 180 rpm. After incubation, the biomass was measured as described in Section 3.4.2, above, and compared to the control flasks.

3.6.3 Determination of Solvent Partition Coefficients

The partition coefficients of carvone and carveol were determined for those solvents that were both biocompatible and non-bioavailable. Increasing concentrations of carvone and carveol that were below the aqueous solubility limits (0 – 1340 mg/L and 0 – 2400 mg/L, respectively) were added to 10 mL distilled water in a sealed 20 mL vial. Once the samples were well mixed, 5 mL of the solvent being tested was added and the samples were mixed at 180 rpm for 24 hours. After 24 hours, the solvent was aspirated

off and 5 mL of the aqueous phase was removed to be tested using the gas chromatograph as outlined in Section 3.3.1.

3.7 Polymer Selection

3.7.1 Bioavailability

Several polymers were tested for their bioavailability to *R. erythropolis* DCL14. To separate 125 mL flasks, 50 mL of sterile medium (no carbon source), 5 g of the polymer being tested and 1 mL inoculum were added. Prior to being added to the test flasks, the polymers were rinsed with water in an effort to remove any processing particles (i.e. powders) that were adhered to the polymer bead surface. Then, to reduce the chance of contamination due to the addition of the polymer beads, they were placed under a UV lamp for 5 hours before being added to the flasks. A positive control with glucose as the carbon source and a negative control with no polymer beads were also prepared. The flasks were incubated at 30°C for 96 hours at 180 rpm. After incubation, the aqueous phase was centrifuged, washed with distilled water and the optical density was measured at 650 nm and compared to the controls.

3.7.2 Determination of Polymer Bead Partition Coefficients

The partition coefficients of carvone and carveol were determined for eight different polymers. Increasing concentrations of carvone and carveol that were below the aqueous solubility limits (0 – 1340 mg/L and 0 – 2400 mg/L, respectively) were added to 10 mL distilled water in a sealed 20 mL vial. Once the samples were well mixed, 5 g of

the polymer being tested was added and the samples were mixed at 180 rpm for 24 hours. After 24 hours, 5 mL of the aqueous phase was removed to be tested using the gas chromatograph as outlined in Section 3.3.1.

3.7.3 Thermal Stability of Polymers

In order to determine whether or not the candidate polymers would be resistant to sterilization via autoclaving, 5 g of each type of polymer bead was added to separate 125 mL flasks and covered with water. The flasks were autoclaved at 121°C for 65 minutes (same conditions as for the reactors). After the autoclave cycle had finished, the resistance of the polymer beads were determined based on visual observation of the sample.

3.7.4 Biofilm Formation on Beads

Due to complications involving the hydrophobic nature of the organism, it was important to determine whether *R. erythropolis* DCL14 would produce a biofilm on the surface of the polymer beads. There was a concern that if a biofilm formed on the polymer, it could inhibit the partitioning of the substrate and product between the solid polymer and aqueous phases. The experiment was performed in 125 mL flasks containing 50 mL sterile growth medium, 2 g of the bead being tested and 1 mL inoculum. There were also negative control flasks for each polymer type containing 50 mL sterile growth medium and 2 g of beads. To reduce the chance of contamination due to the addition of the polymer beads, they were placed under a UV lamp for 5 hours before being added to the flasks. The optical density (650 nm) of the flasks was

measured every 24 hours for 96 hours. Biofilm formation was measured by comparison of the biomass concentration in each polymer-containing flask in the bulk suspension relative to a control flask containing no polymers.

3.8 Biotransformations

The reactor used to run the fermentations was a 5 L New Brunswick Scientific BioFlo III. The temperature and agitation were maintained at 28°C and 350 rpm, respectively. The pH was maintained automatically at 7.0 using 6M KOH and a Broadley James FermProbe. A Broadley James D100 Series Oxyprobe was used to track the dissolved oxygen level to ensure the system was not oxygen transfer limited. The air flow rate into the reactor was 1 L/min and was passed through a sterile 0.2 μm hydrophobic fluoropore PTFE filter. The carbon source, which was also used as the induction source, (+)-limonene, was supplied by passing an air stream through a sparger contained in a flask of liquid (+)-limonene. The limonene-saturated air flow rate into the reactor was equipped with a sterile 0.2 μm hydrophobic fluoropore PTFE filter and was 200 mL/min. The reactor containing the carbon-free medium was sterilized at 121°C for 65 minutes prior to use. The inoculum was prepared as outlined above in Section 3.2.3. The transformation began when the biomass concentration had reached approximately 500 mg/L. The air and (+)-limonene flows were continuously supplied throughout the duration of the transformation. The transformation substrate, (-)-carveol, was supplied in liquid form through a port on the reactor lid using sterile pipet tips to reduce the chance of contamination. The product and substrate concentrations of the microbial biotransformation were monitored frequently using the gas chromatograph and when the

trans-carveol isomer was depleted, more substrate was added such that its concentration in the reactor was below the aqueous solubility and so as not to unduly inhibit the cells present in the aqueous system. These substrate additions were added once the trans-carveol isomer was near depletion until the transformation ceased due to the accumulation of the toxic substrate and product.

3.8.1 Single Phase Biotransformation

The single phase reactor was prepared as outlined above, had a 3 L working volume and was operated at described in Section 3.8.

3.8.2 Two Phase Biotransformations: Liquid-liquid

These fermentations were undertaken as outlined in Section 3.8 and had a 3.5 L working volume (3 L medium and 0.5 L solvent). For the reactor with 1-dodecene (Section 4.4) as the second phase, the solvent could not be autoclaved due to its low flash point (Table 3.1). Instead of autoclaving, the solvent was passed through a sterile 0.2μm nylon filter prior to its use in the reactor. In the reactor that used silicone oil as the second phase (Section 4.5), the reactor and the solvent were autoclaved separately. In both cases, the solvent was added to the reactor just before the substrate, carveol (mixture of isomers), was added to initiate the transformation. Adding the second phase in this manner ensures the biomass concentration was able to reach the same level in the same time as in the single phase reactor, and that the metabolic state of the cells was similar. It also minimized the migration of cells into the second phase.

3.8.3 Two Phase Biotransformations: Solid-liquid

These fermentations were undertaken as outlined in Section 3.8. The first reactor had a working volume of 3.566 L (3 L medium and 0.566 L styrene/butadiene copolymer beads) and the second reactor had a working volume of 3.5 L (3 L medium, 0.25 L Hytrel® 8206 and 0.25 L styrene/butadiene copolymer beads). In each case, the reactor and the beads were autoclaved separately (the beads were covered with tap water) and the water was decanted just before the beads were added and the biotransformation was started. This method ensured consistency with the other two phase method.

3.8.4 Two Phase Continuous Biotransformation: Solid-liquid with Recirculation of Medium

This fermentation was undertaken as outlined in Section 3.8 and had a working volume of 3.822 L (3 L medium, 0.566 L styrene/butadiene copolymer beads in the reactor and 0.256 L Hytrel® 8206 beads in an external column). These bead volumes correspond to 235 g styrene/butadiene copolymer in the reactor and 300 g Hytrel® 8206 in the column. The styrene/butadiene copolymer beads were autoclaved separately from the reactor in a flask and were covered with water. The Hytrel® 8206 beads were autoclaved inside the glass column that contained approximately 10 mL water (to allow for steam formation). The ends of the glass column were fitted with a short length of rubber tubing that was loosely clamped to allow for pressure to escape. Once the biomass had reached the desired optical density, the styrene/butadiene copolymer beads were decanted and added aseptically to the reactor and the biotransformation was initiated by the addition of carveol (mixture of isomers). Once the carveol had been

added, the recirculation of the medium was started. The medium was circulated through sterile tubing, the glass column packed with Hytrel® 8206 and back into the reactor using a peristaltic pump operating at a rate of 30 mL/min. A schematic of the reactor setup is shown in Figure 3.1.

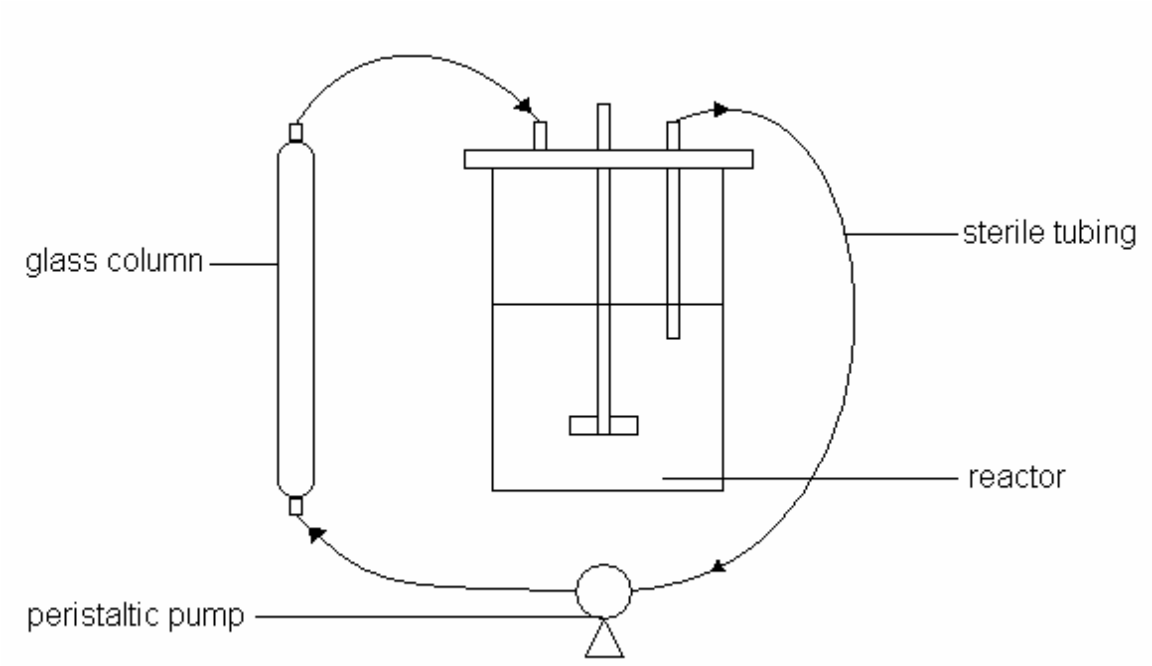


Figure 3.1: Schematic of reactor set-up with the external extraction loop.

3.8.5 Product Recovery From Polymer Beads

In order to demonstrate that it is possible to recover carvone from the polymer beads, methanol was used as an extractant. Once the biotransformation was complete, 3 beads were dried and then added to 10 mL methanol in a sealed 20 mL vial and placed in a rotary shaker for 24 h at 180 rpm and 30°C. After 24 hours, the beads were immersed in another (fresh) 10 mL of methanol. The product concentration in the methanol was tested using the gas chromatograph (and the calibration curve in Appendix A). This procedure was repeated every 24 hours until the amount of carvone recovered became

negligible and the total amount of carvone recovered was calculated by adding the masses of product recovered from each 24 h sample period.

Chapter 4.0 Results and Discussion

4.1 Substrate and Product Toxicity

In order to assess the relative toxicities of carveol (substrate) and carvone (product), assays were carried out in shake flasks to determine their effect on the biotransformation capacity, cell growth and cell morphology of *Rhodococcus erythropolis* DCL14. The most obvious means by which toxicity is quantified is by evaluating cell growth post-exposure to inhibitory concentrations; the concentration at which the cells' reproduction ceases is identified as the toxic threshold. However, in this case it is more important to consider the inhibitory effects of the substrate and product on the biotransformation capacity of the cells, since the end goal is to maintain a high biotransformation rate and not to produce biomass. Unfortunately, it is often very difficult to identify a critical concentration at which the microbial biotransformation capability completely stops. Consequently, the toxicity of the substrate and product in this case have been evaluated based on the increasing inhibition effect on the biotransformation rate.

To test the biotransformation capacity of the cells under potentially inhibitory conditions, the cells were subjected to increasing concentrations of carveol and carvone over a period of two hours. For carvone toxicity, five flasks containing 50 mL of cell suspension were subjected to increasing concentrations of carvone. In order to assess the biotransformation capacity of the cells in each of these flasks, 500 mg/L carveol (mixture of trans- and cis-isomers) was added to each flask to induce the biotransformation. After two hours, the final trans-carveol concentration was determined. Figure 4.1 presents the

results of the carvone toxicity experiment in terms of trans-carveol isomer that was not consumed during the two hour test period.

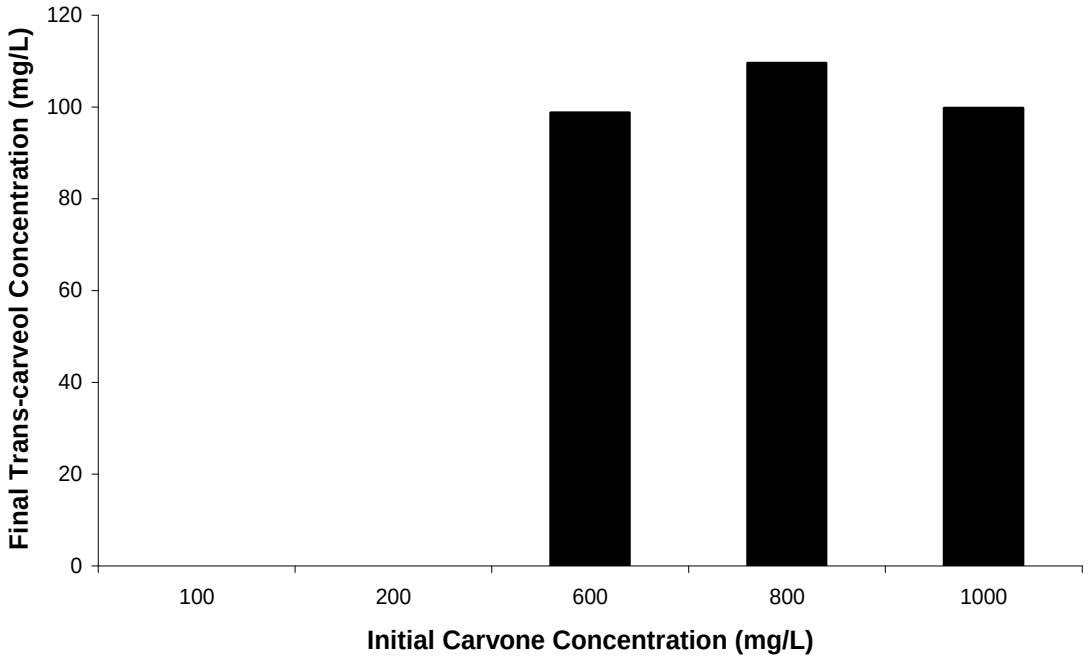


Figure 4.1: Inhibition effects of initial carvone concentration on the biotransformation capacity of *Rhodococcus erythropolis* DCL14 in terms of trans-carveol remaining.

It is evident from Figure 4.1 that between 200-600 mg carvone/L, there is an effect on the biotransformation rate. The five flasks which contained different and increasing concentrations of carvone contained the same initial amount of trans-carveol (approximately 275 mg/L). Over the two hour test period, the cells in the flasks containing lower initial carvone concentrations (100 and 200 mg/L) completely transformed the available trans-carveol isomer into carvone. As for the flasks containing higher initial carvone concentrations (600, 800, 1000 mg/L), approximately half of the available trans-isomer remained unconverted. These results imply that the biotransformation rate had decreased between a carvone concentration of 200-600 mg/L

since the available trans-carveol was not completely transformed in the two hour time period.

To determine the substrate toxicity, five flasks containing 50 mL of cell suspension were subjected to increasing concentrations of carveol (mixture of trans- and cis-isomers). The biotransformation began immediately after the addition of the substrate and after two hours, the final carvone concentration was determined. Figure 4.2 displays the result of the carveol toxicity analysis. These results are displayed in terms of carvone produced after the two hour incubation period as compared to the amount of carvone that could theoretically be produced based on the initial amount of trans-carveol present.

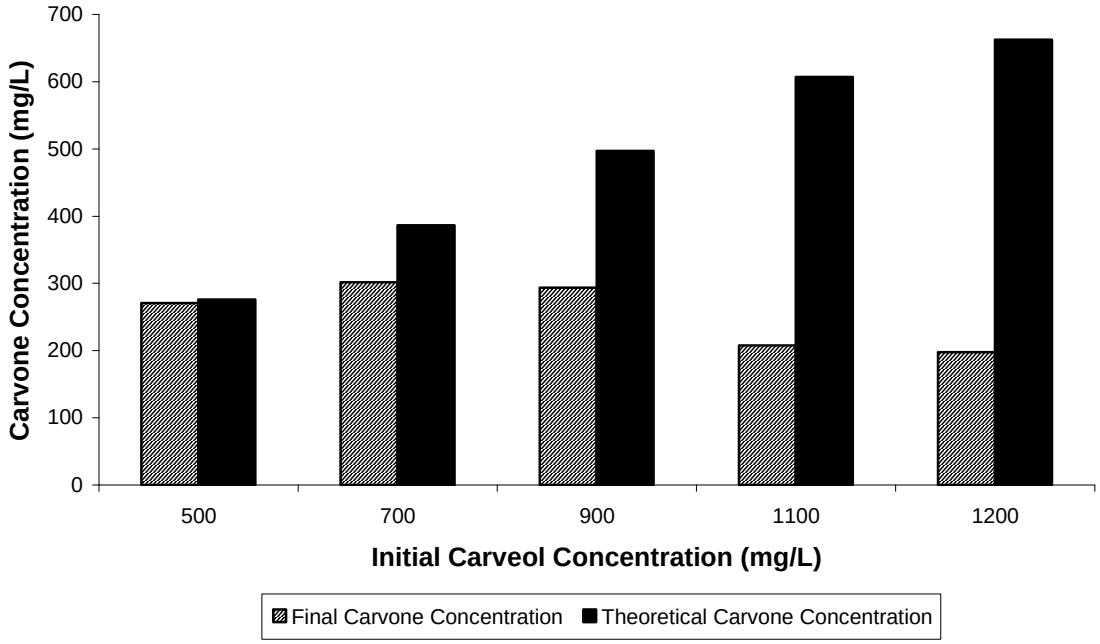


Figure 4.2: Inhibition effects of initial carveol concentration on the biotransformation capacity of *Rhodococus erythropolis* DCL14 in terms of carvone produced.

Standing alone, the resulting final carvone concentrations do not provide conclusive evidence of the inhibitory threshold of the initial carveol concentration because the amount of carvone produced is dependent on the initial amount of carveol present. For

this reason, the final carvone concentrations in Figure 4.2 were compared to the theoretical amount of carvone that could have been produced based on the amount of trans-carveol present (55.2% of total carveol added). For the lowest initial carveol concentration (500 mg/L), it is evident that all of the available trans-carveol isomer was consumed since the final carvone concentration in the flask appears to be almost equal to the expected theoretical amount of carvone. Above this initial carveol concentration (500 mg/L) it appears that the biotransformation capacity was inhibited since less carvone was produced in the two hour incubation period despite the fact that there was a higher concentration of substrate (trans-carveol) present in those flasks.

It should be noted that though the final procedure used to determine the toxicity effects of increasing initial carvone and carveol concentrations seems very straight forward, it was not easy to obtain consistent and reliable results. Several procedures were undertaken in an effort to quantify these very important thresholds. An effort was made to determine the interactive inhibition effects (i.e. a test with both carvone and carveol in increasing concentrations in the same flasks) however the results were inconclusive. It was very difficult to quantify the toxicity effect when both the initial and final carvone and carveol concentrations were changing. There were too many variables present to produce reliable results.

Previous studies were undertaken by de Carvalho and da Fonseca (2002a) in an effort to quantify the relative toxicities of carvone and carveol. The results obtained in their study cannot be compared to the current results because their toxicity experiments were carried out in biphasic systems. In each test flask, the substrate or product was added in a concentration greater than the aqueous solubility. As a result, all of the data

are represented in terms of the concentrations in the organic phase. In my opinion, these results are not very useful because they do not provide an aqueous phase toxicity for either compound which is the phase in which the cells are located, and where the toxicity limit should matter. Moreover, de Carvalho and da Fonseca (2002a) did not report estimated partition coefficients for carvone and carveol between water and their solvent phase. Since the toxicity results are reported in terms of concentrations in n-dodecane, and no partition coefficients were provided, these toxicity thresholds cannot be applied to any other system (with a different organic solvent). The results obtained by de Carvalho and da Fonseca (2002a) were equivocal and confirm the difficulty of determining these toxicity thresholds. It should be noted that de Carvalho and da Fonseca (2002a) did not perform any tests to quantify the outcome of interaction effects either.

In an effort to further investigate the inhibitory effects of substrate and product on the cells, the potential for cell growth after the two hour incubation period during which the cells were exposed to inhibitory concentrations was investigated. After the two hour test period, samples from each shake flask (both carveol and carvone toxicity flasks) were plated on agar medium and incubated for 48 h. After 48 h, every plate displayed substantial cell growth which indicates that in the cases where biotransformation capacity may be inhibited, the majority of the cells are still viable and able to replicate. This qualitative result is confirmed by an observation made by de Carvalho et al. (2005a) which states that increasing carvone concentrations affect primarily the enzymatic system of the cells and only at higher concentrations will carvone concentration have an effect sufficient enough to cause cell death.

Rhodococcus erythropolis DCL14 is known to exhibit morphological changes when exposed to high concentrations of inhibitory compounds (de Carvalho and da Fonseca 2005). In a different toxicity experiment during which the cells were exposed to higher concentrations of carveol and carvone than in Figures 4.1 and 4.2 above, morphological changes were observed. The cells aggregated to form a cellular structure as depicted in Figure 4.3.

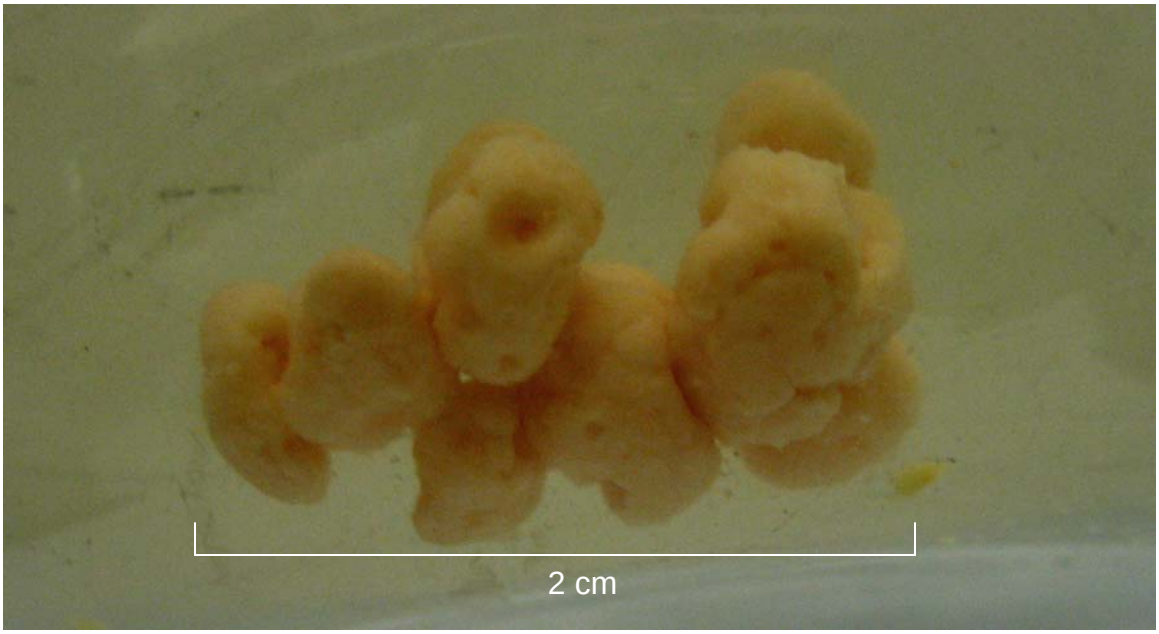


Figure 4.3: Aggregation of *Rhodococcus erythropolis* DCL14 when exposed to a high concentration of carveol.

The cells that formed the aggregation depicted above were originally part of a uniform cell suspension. After two hours of exposure to a high concentration of carveol (2400 mg/L), the cells aggregated into these particles and the surrounding medium was completely clear (free of cells). It has been suggested that these cells aggregate in an effort to protect the bulk of the population (on the inside of the sphere) from the inhibitory concentrations of substrate and product in the aqueous phase (de Carvalho and da Fonseca 2005).

Based on the data provided in Figures 4.1 and 4.2, the highest aqueous concentrations that would be attainable in a single phase system without inhibiting the biotransformation rate for carvone and carveol would be approximately 200-600 mg/L and 500 mg/L respectively. These results justify the use of a second phase in the bioreactor to obtain an overall higher product concentration and hence a higher productivity. In order to provide a base-case benchmark reactor performance, the biotransformation was tested in a single phase aqueous reactor as described in Section 4.2.

4.2 Single Phase Biotransformation

The biotransformation of trans-carveol to (*R*)-(-)-carvone was first carried out in a single phase reactor to determine a benchmark for the biotransformation system to which the results of all other reactor configurations would be compared. The measure of reactor performance would be quantified using several factors: total amount of substrate added, duration of biotransformation (before reaction rates declined significantly) and overall volumetric productivity.

Tecelão et al. (2001) previously developed a single phase reactor to carry out the biotransformation using a 20 mL aqueous working volume in shake flasks that were perforated with plastic tubing for aeration. In this case, carveol (mixture of isomers) was added in a fed-batch manner in 4 aliquots of 10.4 μL (a total of 39.85 mg). The total carvone produced was 16.14 mg. It was reported that after each substrate addition, the biotransformation rate decreased. The decrease in biotransformation rate was attributed to the build-up of the unconverted cis-isomer and the lack of cofactor regeneration due to the fact that no carbon source was provided throughout the transformation. From the

reported information, it is not possible to calculate a volumetric productivity since the duration of the biotransformation was not given and there is no figure similar to Figure 4.4 below from which to determine the time period. The time course data that are reported in Figure 4.4 below are the first ever reported for a single phase system of any size.

To begin the biotransformation, 3 mL of the substrate (mixture of isomers) was added to the 3 L aqueous phase reactor. This small amount of substrate was added initially to ensure that the substrate concentration was below the aqueous solubility limit and also to ensure the system was not overloaded with inhibitory substrate. The second and third substrate additions, which were provided after the trans-carveol present in the system was depleted, were only 1 mL in an effort not to overwhelm the system with the build-up of the unconverted cis-carveol isomer and the product, carvone. Figure 4.4 represents the changing substrate and product concentrations throughout the biotransformation period (43.75 – 59h).

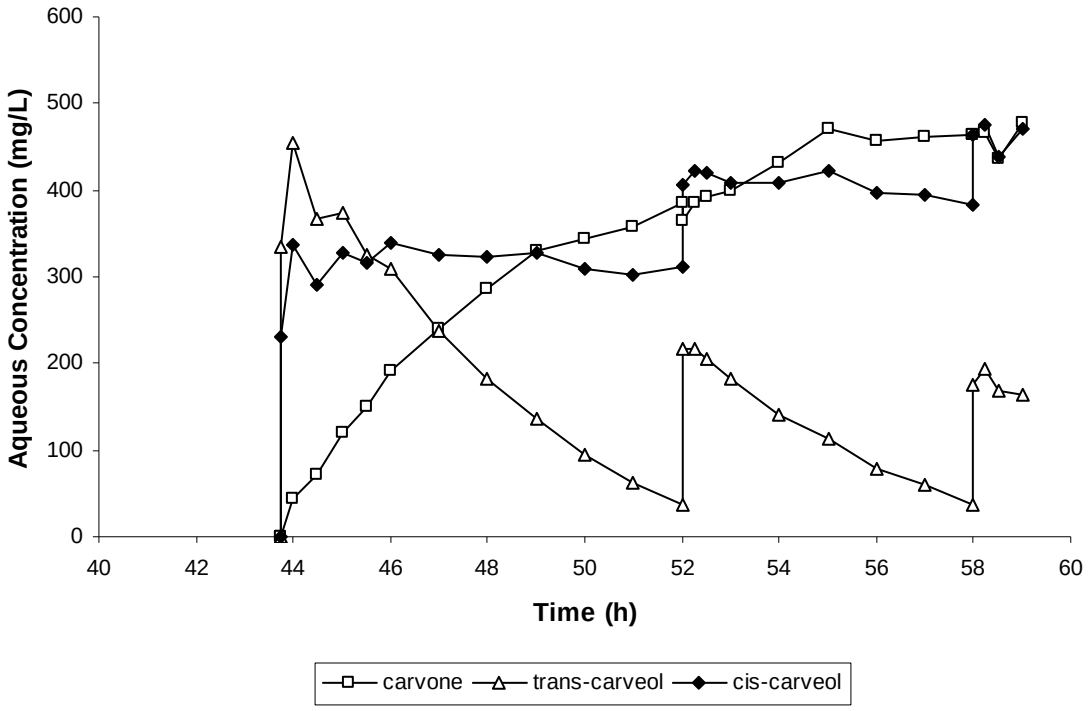


Figure 4.4: The time course of the substrate and product concentrations throughout the duration of the single phase biotransformation.

As expected, Figure 4.4 indicates that the trans-carveol isomer is transformed into carvone, and the cis-isomer is isomerically resolved and remains as a second product. The substrate additions were added in a fed-batch manner as follows: 3 mL added at t = 43.75 h, 1 mL added at t = 52 h and 1 mL added at t = 58 h. It is also important to note that at the end of the biotransformation, the remaining cis-isomer and the product are present in approximately the same concentration. This result is expected since the two isomers that make up the substrate are present in approximately equal amounts. The volumetric productivity of the single phase system was 31 mg/L·h, which was determined by dividing the overall product concentration (478 mg/L) by the total time of the biotransformation (15.25 h). After each substrate addition, the biotransformation rate decreased as depicted by the decreasing slope of the carvone curve. The decrease in

reaction rate can be explained by the inhibition effects that are present due to the accumulation of the inhibitory substrate and product. The final aqueous concentrations of carvone and cis-carveol were 478 mg/L and 470 mg/L respectively which agree with the predicted toxicity limits from Section 4.1. Previous work by Tecelão et al. (2001) suggested that the decrease in biotransformation rates could be due to cofactor depletion or biomass loss. In this case, to maintain conditions favorable for cofactor regeneration, the carbon substrate and biotransformation inducer (limonene) and air were supplied throughout the duration of the fermentation. For this reason, it is evident that cofactor depletion is not the reason for the decrease in reaction rate. As well, Figure 4.5 shows that the biomass concentration throughout the biotransformation (43.75 – 59 h) does not decrease substantially which means that the reduction in biotransformation rate is not due to loss of biomass.

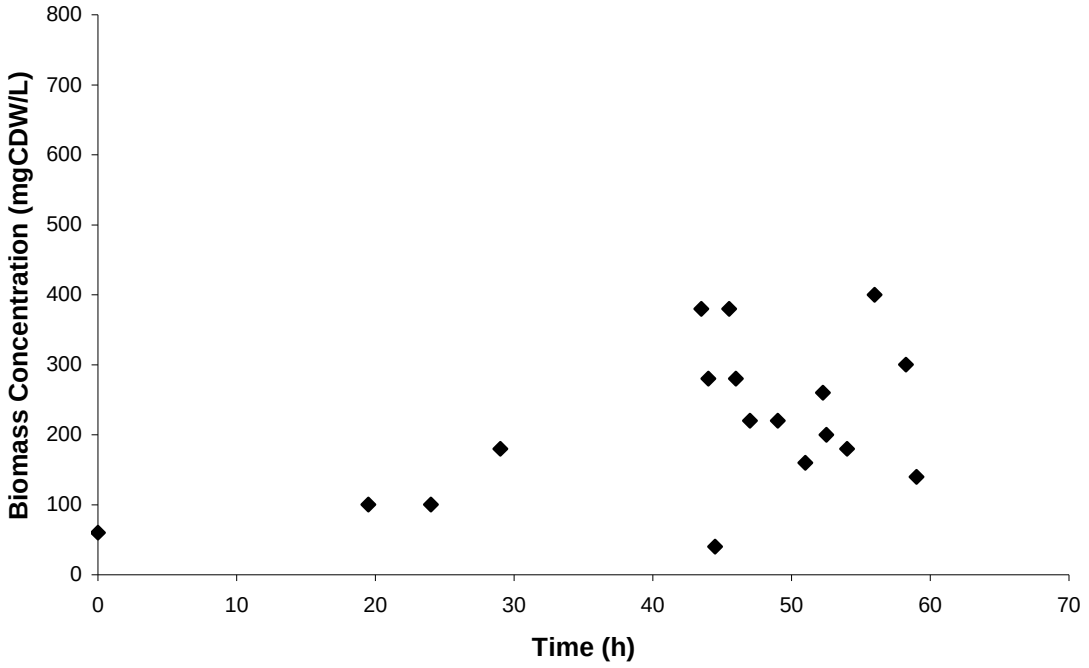


Figure 4.5: Time course of the biomass concentration throughout the single phase biotransformation.

The variability in the biomass data can be attributed to the difficulties associated with the quantification of this very hydrophobic organism (see Section 3.4). As the cis-isomer and carvone accumulate in the aqueous phase, the cells aggregate to form small particles (Figure 4.3). It has been suggested that the reason for this formation is to protect the inner cell population from the deleterious effects to the cell membrane by the accumulated substrate and product (de Carvalho and da Fonseca 2005). Therefore it would appear that the biomass was affected by the increasing cis-carveol and carvone concentrations in the aqueous phase, but the biotransformation continued, albeit at a lower rate.

It is apparent that the inhibitory effects of the substrate and product on the biotransformation rate are significant. In total, 5 mL of substrate (mixture of isomers) were added to the reaction system over a period of 15.25 h while the biotransformation was still occurring at a reasonable rate. For this system, the overall volumetric productivity was 31 mg/L·h. In order to improve upon the amount of substrate added, the productive time period of the biotransformation and the volumetric productivity, a second phase was applied to the reactor to alleviate the inhibitory concentrations of product and substrate in the aqueous phase.

4.3 Solvent Selection

There are three major factors to be considered when using rational solvent selection strategy to identify a solvent that is appropriate for use in a TPPB: biocompatibility, bioavailability and partitioning capacity.

4.3.1 Biocompatibility: Determination of Critical log P

The first major requirement for a solvent to be used in a TPPB is that it must be biocompatible. A biocompatible solvent ensures that its presence will not be inhibitory to the organism or affect its growth. Biocompatibility is usually evaluated based on the log P of the solvent and the critical log P of the organism. The log P of a solvent is defined as the logarithm of the partition coefficient of the solvent in a standard octanol-water two phase system (Bruce and Daugulis 1991). The critical log P of an organism is such that the organism will grow in the presence of solvents with log Ps greater than its critical log P and will not grow in solvents with log Ps lower than its critical log P.

The critical log P of *R. erythropolis* DCL14 was determined by exposing the cells to a selection of twelve organic solvents (see Table 3.1) with log Ps ranging from 1.25 – 9.04 as well as a "liquid polymer", silicone oil. Figure 4.6 clearly demonstrates that in the presence of solvents with a log P below 5, the growth of the organism was inhibited and above 5, it was not. For these reasons, the critical log P of *Rhodococcus erythropolis* DCL14 was estimated to be 5. This estimation roughly agrees with the reported critical log P of *Rhodococcus erythropolis* of 6 (Inoue and Horikoshi 1991). The results reported by Inoue and Horikoshi (1991) can be used for comparison because solvent tolerance is strain specific. Figure 4.6 does not include a data point for silicone oil since there is currently no reported log P for silicone oil. The final biomass concentration for the silicone oil flask was 1276 mg/L and since this concentration is in the range of the control sample (916 mg/L), silicone oil is also considered to be biocompatible.

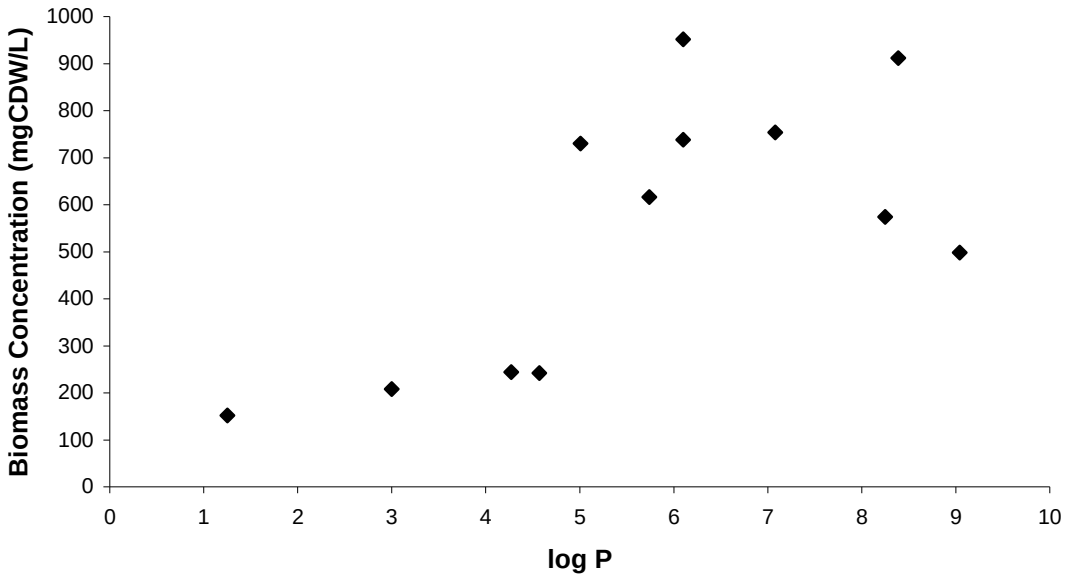


Figure 4.6: Effect of solvents with varying log Ps on the growth of *R. erythropolis* DCL14.

There is some variability in the biomass concentrations for those solvents above the critical log P. This variability is due to the difficulty of separating the biomass from the resulting emulsion (in those flasks with solvents that has a log P greater than 5) (see Section 3.4.2). The formation of the emulsion can be attributed to the ability of some organisms to secrete biosurfactants to increase the availability of a hydrophobic solvent (Madigan et al. 2000). To further validate this point, it has been suggested that emulsion formation in the presence of solvents is linked to cell growth (Bredholt and Eimhjellen 1999). Therefore, since the flasks containing solvents with log Ps greater than 5 all formed emulsions, it can be said with confidence that each of these flasks contained substantial biomass.

The biocompatible solvents (1-dodecene, n-dodecane, 1-tetradecene, hexadecane, BEHP, 1-octadecene and silicone oil) were further tested for bioavailability. Two biocompatible solvents, decane (log P = 5.01) and undecane (log P = 5.74), were not

included in further testing because their log Ps are too close to the critical log P and could potentially affect cell growth.

4.3.2 Bioavailability

Another very important factor in solvent selection is bioavailability of the solvent. It is important that the organism is not able to metabolize the solvent because this can cause loss of solvent volume (with increased cost for replacement), production of undesirable by-products and can also lead to substrate competition where the solvent is preferentially metabolized relative to the desired biotransformation substrate.

The seven biocompatible solvents from the previous section (4.3.1) were evaluated based on bioavailability. Figure 4.7 represents the relative bioavailabilities of the selected solvents with respect to a positive control sample containing corn oil.

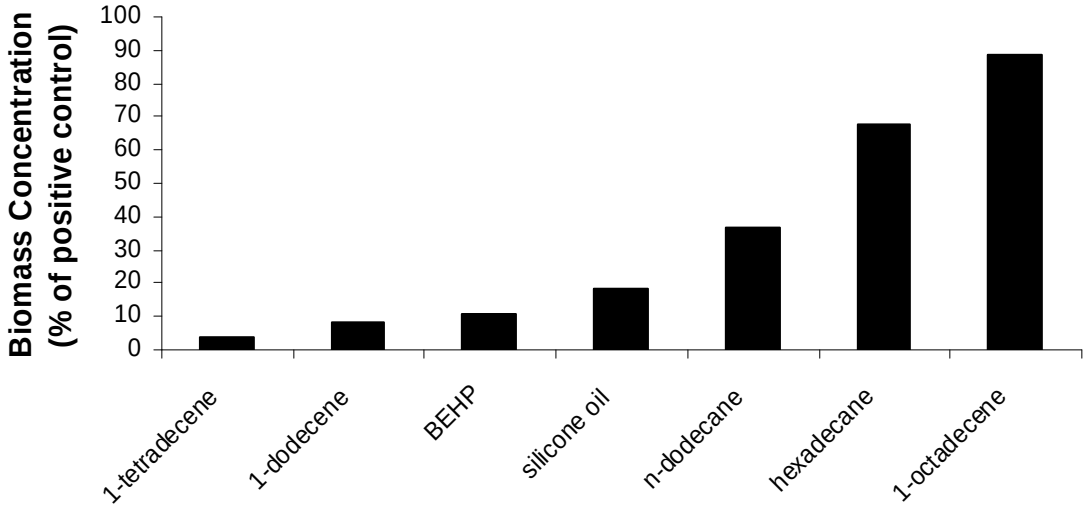


Figure 4.7: The relative bioavailabilities of seven biocompatible solvents.

It is not clearly evident whether or not a particular chemical class is bioavailable to *R. erythropolis* DCL14, as has been seen elsewhere (MacLeod and Daugulis 2005),

although it appears that alkanes are inherently bioavailable. The bioavailability of the even-chained alkenes is not consistent through the test samples. As expected, the molecularly complex silicone oil is relatively non-bioavailable and BEHP, a large branched-chain ester is also non-bioavailable. It is interesting to note that in Figure 4.7 bioavailability is not determined as "yes" or "no", but rather a sliding scale of relative bioavailabilities. It is hypothesized that if the solvents are bioavailable (as appears to be the case for n-dodecane, hexadecane and 1-octadecene) the organism's specific growth rates may be substrate (solvent) specific resulting in different biomass concentrations within the same incubation time.

The non-bioavailable solvents that were considered further include 1-tetradecene, 1-dodecene, BEHP and silicone oil.

4.3.3 Determination of Solvent Partition Coefficients

The biocompatible and non-bioavailable solvents were further evaluated based on their partitioning capacity of the target substrate and product molecules. Even though n-dodecane is slightly bioavailable (Figure 4.7) it was tested for partitioning capacity because this was the solvent used in the previous work by Tecelão et al. (2001), de Carvalho and da Fonseca (2002a, 2002b) and de Carvalho et al. (2005a). Also, though BEHP and 1-tetradecene were found to be non-bioavailable, their partitioning capacities were not determined because they formed an emulsion with the aqueous phase in the abiotic partitioning experiment and were eliminated to avoid further emulsion-related complications. The partition coefficients for carvone and carveol in the chosen solvents

are summarized in Table 4.1. The full plots for each of these partition coefficients can be found in Appendix D.

Table 4.1: Partition coefficients for carvone and carveol in various solvents.

Solvent	PC for Carvone	PC for Carveol
1-dodecene	38	8
n-dodecane	31	6
silicone oil	12	1

It is clear that 1-dodecene has the highest affinity for the target compounds and was therefore selected for use as the second phase in the TPPB. According to Table 3.1, 1-dodecene is also fairly low cost (relative to other organic solvents), has a low volatility and presents no major working hazards.

The selection of an organic phase for use in a two phase reactor was previously completed by de Carvalho and da Fonseca (2002a). In this case, the solvent selection criteria included the maintenance of cell viability and biotransformation activity in the presence of the solvent. It was determined that n-dodecane (log P = 6.01) satisfied both of these criteria, however, it is important to note that all of the other solvents that were tested by de Carvalho and da Fonseca (2002a) had a log P lower than the critical log P of the organism, and as expected, resulted in low activity and low cell viability.

4.4 Two Phase Biotransformation: Organic Solvent

Considering the above solvent selection criteria (i.e. biocompatibility, non-bioavailability and affinity for target molecules) 1-dodecene was chosen as an appropriate solvent for use as the second phase in the TPPB. The work in this section was completed jointly with a fourth year thesis project.

Despite the rational solvent selection that was applied (Section 4.3), the hydrophobic nature of *R. erythropolis* DCL14 resulted in several operational difficulties when attempting to complete the biotransformation in a two-phase system in which 1-dodecene was the second phase which included: challenging biomass quantification, formation of a third phase (emulsion formation) and difficulty in quantifying the amount of substrate and product in the system.

In an attempt to improve the reactor performance (from that in Section 4.2), 1-dodecene was used as the second phase in a TPPB. As a first attempt, to minimize the potential for contamination to the reactor, the solvent passed through a sterile 0.2 μm hydrophobic fluoropore PTFE filter (could not be autoclaved due to low flash point) into the reactor before it was inoculated. When the agitation was started and the reactor was inoculated, an emulsion was formed immediately between the solvent and aqueous phases due to the presence of *R. erythropolis* DCL14 and the vigorous mixing. It is possible that this extreme emulsion formation was not observed in the shake flask experiments used through the course of the rational solvent selection because the agitation was much less aggressive. After approximately 48 hours of growth in the reactor (when the biotransformation is usually started) there was insufficient biomass present in the aqueous phase. The presence of the solvent (and the resulting emulsion) slowed the growth rate such that the biomass concentration was not comparable with that used for the single phase system.

In order to begin the biotransformation with the same amount of biomass in the same metabolic state, another reactor, containing 3 L medium was inoculated and allowed to grow for 48 hours. In this time, sufficient biomass had accumulated and just

before the biotransformation was started, the solvent phase (which was again passed through a sterile 0.2 μm hydrophobic fluoropore PTFE filter) was added to the reactor. In this case, as soon as the solvent was added, an emulsion began to form and the biomass started to partition into the second phase. Nonetheless, the biotransformation was carried out but the results were inconclusive. Throughout the biotransformation, a third phase was produced possibly due to the formation of polysaccharides secreted by the organism in an effort to emulsify the solvent (Madigan et al. 2000) and is depicted in Figure 4.8.

Figure 4.8: Formation of the third phase in the presence of 1-dodecene.

Samples of the aqueous and organic fractions were assessed to determine the concentrations of substrate and product in the system, but the resulting values did not produce a properly closed mass balance. It is likely that the presence of the third phase

(which could not be tested by the gas chromatograph) contained the missing fractions of the mass balance.

Another major operational concern arose from the fact that the biomass is able to partition into the organic phase. Since the second phase is meant to act as a reservoir for inhibitory concentrations of the cytotoxic molecules, the biomass that is contained within the organic phase is unable to carry out the biotransformation. For this reason, the productivity of the system is greatly decreased.

The data from this biotransformation are not included since they did not produce any useful quantification of the substrate and product concentrations.

4.5 Two Phase Biotransformation: Silicone Oil

In order to overcome the operational difficulties encountered in the two-phase biotransformation with 1-dodecene as the second phase, a biotransformation was carried out with silicone oil as the second phase. The rationale behind choosing a solvent with less desirable partitioning capabilities such as silicone oil included the fact that the solvent would not be bioavailable to the organism and that the cells would likely not be able to partition from the aqueous phase into the silicone oil phase through emulsion formation. Silicone oil has been used in the past as the second phase in a liquid-liquid reactor because it is neither cytotoxic nor bioavailable (Guieysse et al. 2001). However, silicone oil is not necessarily an ideal candidate due to its fixed molecular structure which does no allow any opportunity to enhance substrate or product solubility (Prpich and Daugulis 2006).

The total working volume of this reactor was 3.5 L. The silicone oil (0.5 L) was autoclaved separately from the reactor containing 3 L of medium and was added to the reactor just before the biotransformation was initiated by the addition of carveol. As soon as the silicone oil was added to the reactor, the agitation was stopped and it was immediately apparent that the biomass had started to partition into the silicone oil phase as it changed from transparent to translucent and by the end of the biotransformation; the silicone oil phase was completely opaque as is depicted in Figure 4.9.

Figure 4.9: Evidence of biomass partitioning into silicone oil phase.

Despite this displacement of the biomass, the biotransformation was carried out and the time course data throughout the biotransformation period (43.5 to 72.5 h) is shown in Figure 4.10.

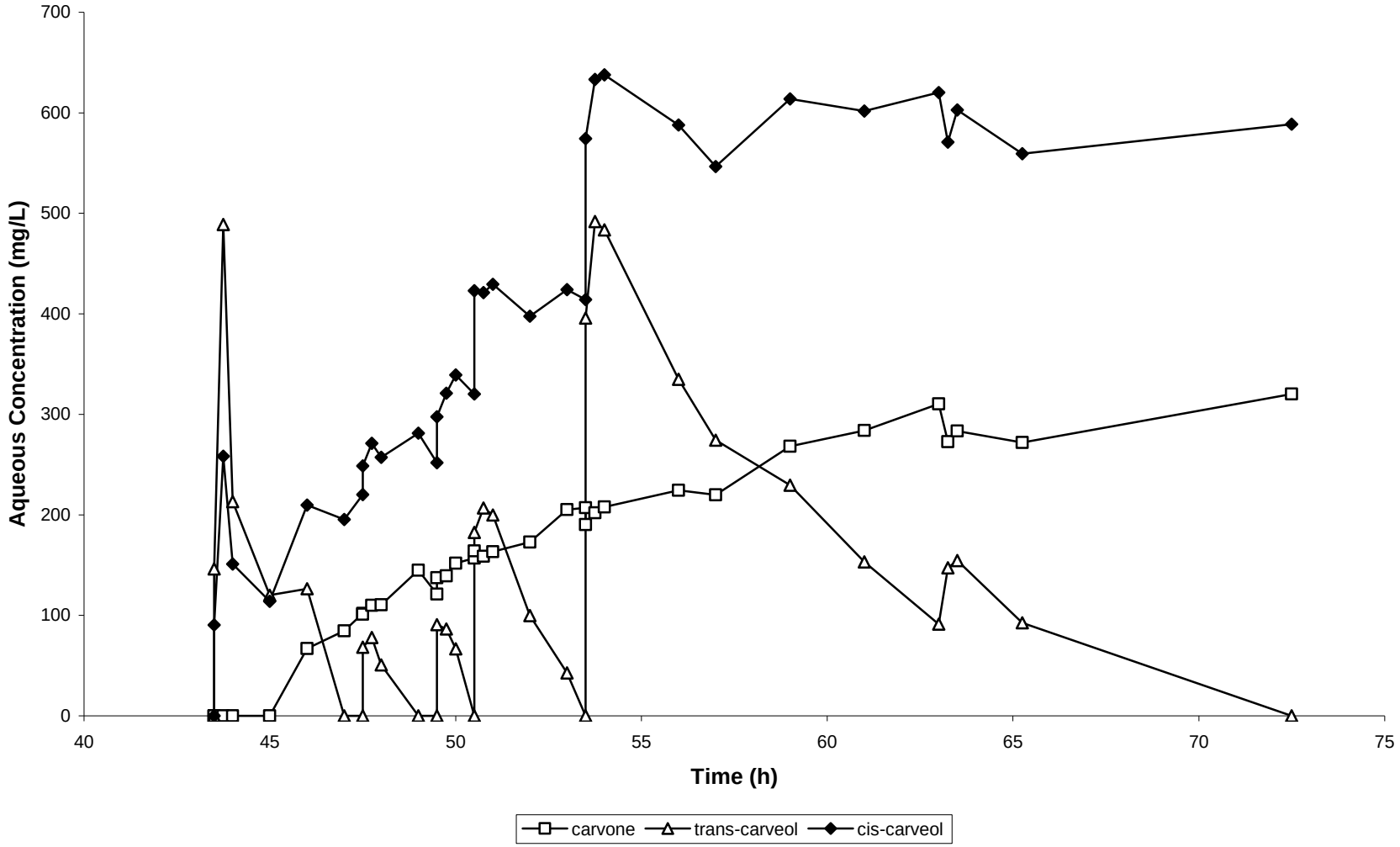


Figure 4.10: The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with silicone oil as the second phase.

To maintain consistency with the single phase biotransformation, the first substrate addition was 3 mL (at 43.5 h) and the second and third additions were both 1 mL (at 47.5 and 49.5 h respectively). After the first three additions, the system was still performing very well and further substrate additions (above those added in the single phase reactor) were employed. In an effort not to overwhelm the system, a fourth bolus of 2 mL of carveol was added at 50.5 h after which the system was still operating efficiently. A fifth aliquot of 5 mL of carveol was added to the system at 53.5 h, after which point the biotransformation rate decreased significantly. A final addition of 1 mL was added at 63.25 h and again, the biotransformation rate remained very low. Again, it is assumed that the biotransformation rates decreased with each addition due to the accumulation of the inhibitory product and cis-isomer. The final product concentration in the aqueous phase was 320 mg/L and the final cis-isomer concentration in the aqueous phase was 589 mg/L. Again, these final aqueous concentrations are consistent with the toxicity results from Section 4.1. The volumetric productivity achieved in this reactor configuration was 29 mg/L·h which was calculated from the total product concentration (823 mg/L) and the total time for the biotransformation (28.75 h), which is a slight decrease in productivity from that observed in the single phase (31 mg/L·h). The decrease in volumetric productivity can be attributed to the fact that the working volume of this reactor was larger and the biotransformation time was longer, resulting in a smaller volumetric productivity. Although these results seem unfavorable, when put in an industrial context, this reactor configuration would be favorable because the fermentation time is longer, meaning there is less down time (for setting up a new batch reactor) and in the long run

could improve volumetric productivity since the volumetric productivity would include turnaround time in an industrial application.

As in the single phase case, there was no cell growth observed throughout the biotransformation (Figure 4.11). Due to the presence of the solvent, however, there was a significant emulsion formed in the reactor. This emulsion caused difficulty in quantifying biomass since a centrifuged sample would result in the formation of a small pellet, as well as an accumulation of biomass at the solvent/aqueous interface. A procedure was developed to remove the biomass from the interface and is described in Section 3.4.2.

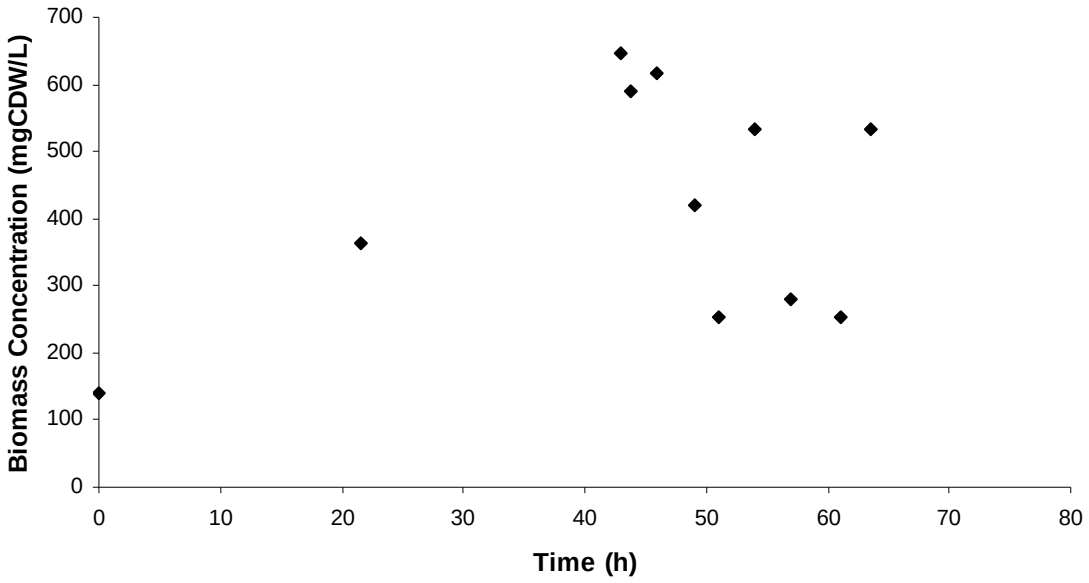


Figure 4.11: Time course of the biomass concentration throughout the two phase biotransformation with silicone oil as the second phase.

Though it appears in Figure 4.11 that the biomass concentration is decreasing during the biotransformation, it is believed that this trend is just a result of the difficulty in removing adequate biomass from the interface as the emulsion accumulated in the reactor.

In work previously published by Tecelão et al. (2001), the biotransformation was carried out in a biphasic system (in shake flasks) with a 20 mL aqueous phase and either a 4 mL iso-octane or a 20 mL n-dodecane as the second phase. It is important to note that a proper solvent selection regime was not applied in their work. This lack of strategy is evident since the first organic solvent to be used, iso-octane, had a log P lower than the critical log P of *R. erythropolis* DCL14 (4.07 versus 5.00). It is actually surprising that the non-biocompatible nature of the solvent did not hinder the biotransformation rate to a greater extent. To begin the biotransformation, the solvent phase was loaded with substrate (carveol, mixture of isomers at 157 mM). Once the trans-carveol isomer had been depleted, the solvent phase was removed and replaced with fresh solvent fully loaded with substrate. This strategy was meant to reduce the build-up of cis-carveol in the system. After each substrate addition, the biotransformation rate decreased. This decrease was attributed to the lack of co-factor regeneration (no carbon source was supplied) as well as a loss of biomass due to the removal of the solvent phase (and resulting emulsion). In an effort to conserve the biomass concentration, a larger solvent volume (20 mL) was applied and after the trans-carveol depletion, only a portion of the solvent was removed (and replaced with newly loaded solvent) such that the emulsion (containing cells) would remain intact. At this point the authors also switched the organic solvent from iso-octane, to a more biocompatible solvent, n-dodecane (log P = 6.10), however they did not provide a systematic explanation for the change in solvent choice. For the n-dodecane reaction system, a total of 1434 mg carveol (mixture of isomers) was added during the biotransformation and produced a total of 781 mg carvone.

Again, it is not possible to determine the length of biotransformation, and hence a volumetric productivity, because no time data were supplied.

4.6 Polymer Selection

As with choosing an appropriate solvent for use in a TPPB, a rational selection regime should be followed when choosing a suitable polymer. Candidate polymers may be chosen based on chemical structure which influences partitioning capacity (i.e. monomer selection, functional groups, copolymerization and crosslinking), bioavailability, thermal resistance to sterilization and resistance to biofilm formation. A comprehensive list of desirable polymer characteristics is detailed by Rehmann et al. (2007) which include the above characteristics among others. It is generally not necessary to verify biocompatibility of polymers because it is well known that polymers of the typed employed are biocompatible.

4.6.1 Bioavailability

Due to the complexity of their long chains, polymers are generally not susceptible to degradation by microorganisms. In order to confirm this for the tested polymers, the bioavailabilities of eight polymer beads were assessed and are summarized in Figure 4.12.

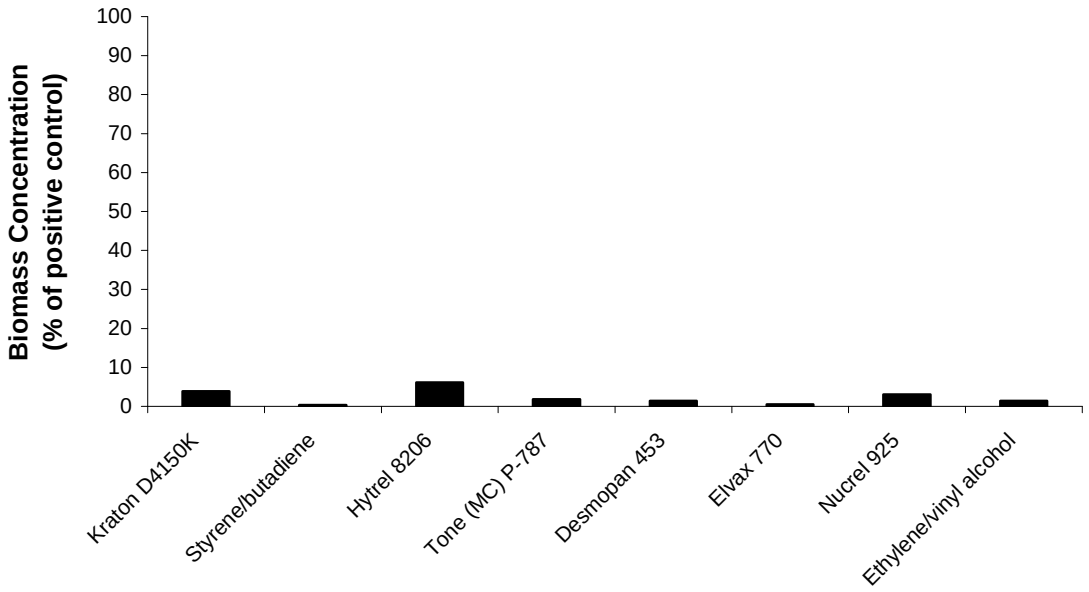


Figure 4.12: The relative bioavailabilities of eight polymer beads with respect to a positive control containing glucose as the carbon source.

Figure 4.12 clearly shows that none of the polymers are bioavailable since the relative biomass concentrations are very low compared to the positive control. Though these results indicate that the polymers are not bioavailable, the relative biomass concentrations are not zero. These non-zero results can be explained using two theories. First, the presence of residual processing materials (i.e. powder coating) on the surface of the polymers could have leached into solution during the 96 h incubation period and second, the hardness of the polymers. It is possible that small particulates were broken off the surface of the polymer beads after repeated friction with the glassware in the rotary shaker throughout the 96 h incubation period.

4.6.2 Determination of Polymer Bead Partition Coefficients

The partition coefficients of eight candidate polymers were determined and are summarized in Table 4.2. The polymers were purposefully chosen to include a "negative control" to demonstrate that not all polymers will have an affinity for the target molecules.

Table 4.2: Partition coefficients for carvone and carveol in various polymer beads.

Polymer	PC for Carvone	PC for Carveol
KRATON® D4150K	121	6
Styrene/butadiene	118	5
Hytrel® 8206	49	36
TONE™ P787	40	19
Desmopan 453	29	14
Elvax® 770	7	2
Nucrel® 925	2	1
Ethylene/vinyl alcohol	0	0

The figures used to determine the above partition coefficients can be found in Appendix D. Table 4.2 demonstrates that KRATON® D4150K and styrene/butadiene copolymer have superior partitioning capacity for carvone. Both of these polymers are styrene/butadiene copolymers where KRATON® D4150K has 31% styrene and styrene/butadiene copolymer has 28% styrene. The reason as to why these styrene/butadiene copolymers have an affinity for carvone is associated with their relative polarities. Since alcohols (carveol) are more polar than ketones (carvone) and the copolymers are non-polar this may explain why the polymer would have a higher affinity for the non-polar ketone. This explanation follows the classic case of "like dissolves like". It is interesting to note that most of the polymers that were tested have a much higher affinity for carvone than they do carveol. The exceptions include the Hytrel® 8206, TONE™ P787 and Desmopan 453 polymers all of which contain esters as a functional group. An explanation for their added affinity for carveol might be the

possibility of hydrogen bonding that the ester functional groups provide. There exists the potential for hydrogen bonding between the double-bonded oxygen atom in the ester functional group and the alcohol functional group on the carveol molecule. As expected, the polymer chosen to serve as a "negative control", ethylene/vinyl alcohol, did not exhibit any affinity for either carvone or carveol. Ethylene/vinyl alcohol is a polar molecule and as such would be expected to attract the polar carveol molecule. A suggestion as to why no uptake was observed is related to the glass transition temperature of the polymer (T_g). The T_g of this polymer (44% ethylene) is 55°C which means that the polymer configuration is rigid at 30°C (the temperature at which the experiments were run). Since carveol and carvone are relatively large molecules, their diffusion into the polymer would require the cooperative movement of the polymer chains. Since the experiment was run at a temperature below the T_g, it is expected that the polymer chains are very rigid and would not move to facilitate the absorption of the target molecules. Based on the partition coefficients listed in Table 4.2 above, the polymers with the highest affinity for carvone are KRATON® D4150K and styrene/butadiene copolymer where those with the highest affinity for cis-carveol (the secondary product) are Hytrel® 8206, TONE™ P787 and Desmopan 453.

4.6.3 Thermal Stability of Polymers

Since this system uses a pure culture of *Rhodococcus erythropolis* DCL14 to complete the biotransformation, it is essential that the polymer beads be sterilized before they are used in the reactor to reduce the chance of contamination. The simplest and most effective means of sterilization is via autoclaving. It is important that the polymer

beads maintain their shape and remain in separate pellets so that the surface area for absorption is consistent. Samples of each of the eight polymers used in the bioavailability and partitioning capacity experiments were autoclaved in shake flasks containing water to determine their resistance to sterilization in the autoclave. The results of this test are summarized in Table 4.3.

Table 4.3: Effect of heat on the polymer bead samples that were autoclaved to determine their ability to resist this method of sterilization

Polymer	Result
KRATON® D4150K	beads stuck together and did not easily separate
Styrene/butadiene	beads stuck together lightly, separated easily with agitation
Hytrel® 8206	unaffected
TONE™ P787	beads melted into one solid ball
Desmopan 453	unaffected
Elvax® 770	beads melted together but maintained their shape
Nucrel® 925	beads melted together into a disk
Ethylene/vinyl alcohol	beads melted together into a disk

The results indicate that the only polymer beads suitable to be sterilized by autoclave are Hytrel® 8206, Desmopan 453 and styrene/butadiene copolymer.

4.6.4 Biofilm Formation on Beads

Due to the previous problems that were encountered as a result of the extreme hydrophobicity of *Rhodococcus erythropolis* DCL14 in the presence of a hydrophobic solvent, it was considered necessary to determine whether or not a biofilm would form on the hydrophobic surface of the polymer beads. The formation of a biofilm could potentially inhibit the transfer of substrate and product between the aqueous and polymer phases. The results of this test are shown in Figure 4.13, which shows that there is no biofilm formation on the surface of the polymer beads.

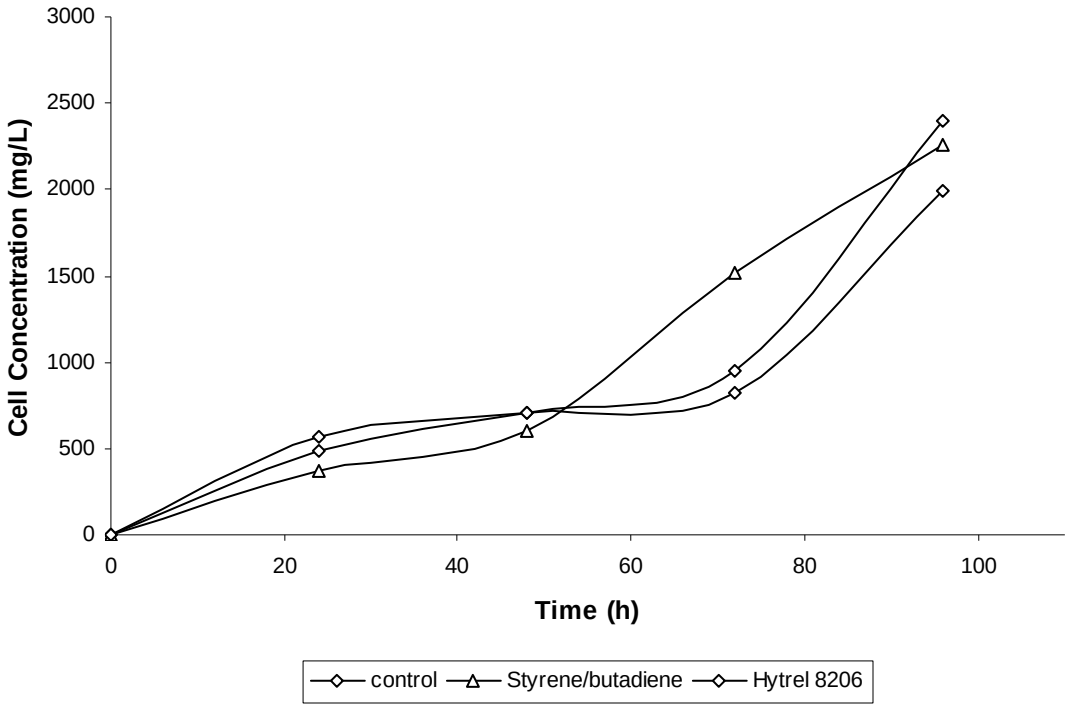


Figure 4.13: Assessment of the potential for biofilm formation on styrene/butadiene copolymer and Hytrel® 8206 with respect to a control flask containing no polymers.

The only two polymers tested were styrene/butadiene copolymer and Hytrel® 8206 because they met all the polymer selection criteria: non-bioavailable, good affinity for product and substrate (respectively) and suitable to be sterilized in the autoclave. Desmopan 453 was not considered for further testing because it's affinity for the target molecules did not compare to those of styrene/butadiene copolymer and Hytrel® 8206 for carvone and carveol, respectively. From Figure 4.13 it is apparent that there is no biofilm formation on the polymer beads since the suspended cell concentrations of the two samples containing polymer beads are very close to that of the control sample which did not contain polymer beads.

After completing a rational polymer bead selection, it was determined that the styrene/butadiene copolymer would be the appropriate choice for use as the second phase

in the two phase biotransformation with a solid second phase. Styrene/butadiene copolymer was chosen based on its high affinity for carvone, which is the target product molecule. Another candidate polymer, Hytrel® 8206 has a high affinity for the remaining substrate isomer (cis-carveol) and as such, could be used for the efficient removal of the substrate molecule.

4.7 Two Phase Biotransformation: Single Polymer

In order to maintain consistency between the various biotransformation reactor configurations, the working volume consisted of 3 L of medium that was autoclaved in the reactor and 566 mL (470 g) styrene/butadiene copolymer that was autoclaved separately and added aseptically to the reactor. The biotransformation was started shortly after the polymer beads were added with a bolus of 3 mL of substrate. In order to remain consistent with previous biotransformations, the second and third substrate additions were also 1 mL (at 46.5 and 48.5 h, respectively). The fourth, fifth, sixth and seventh substrate additions were each 5 mL and were added at 50 h, 54.25 h, 59.50 h and finally at 67 h. The biotransformation profile (43.75 to 78 h) is shown in Figure 4.14.

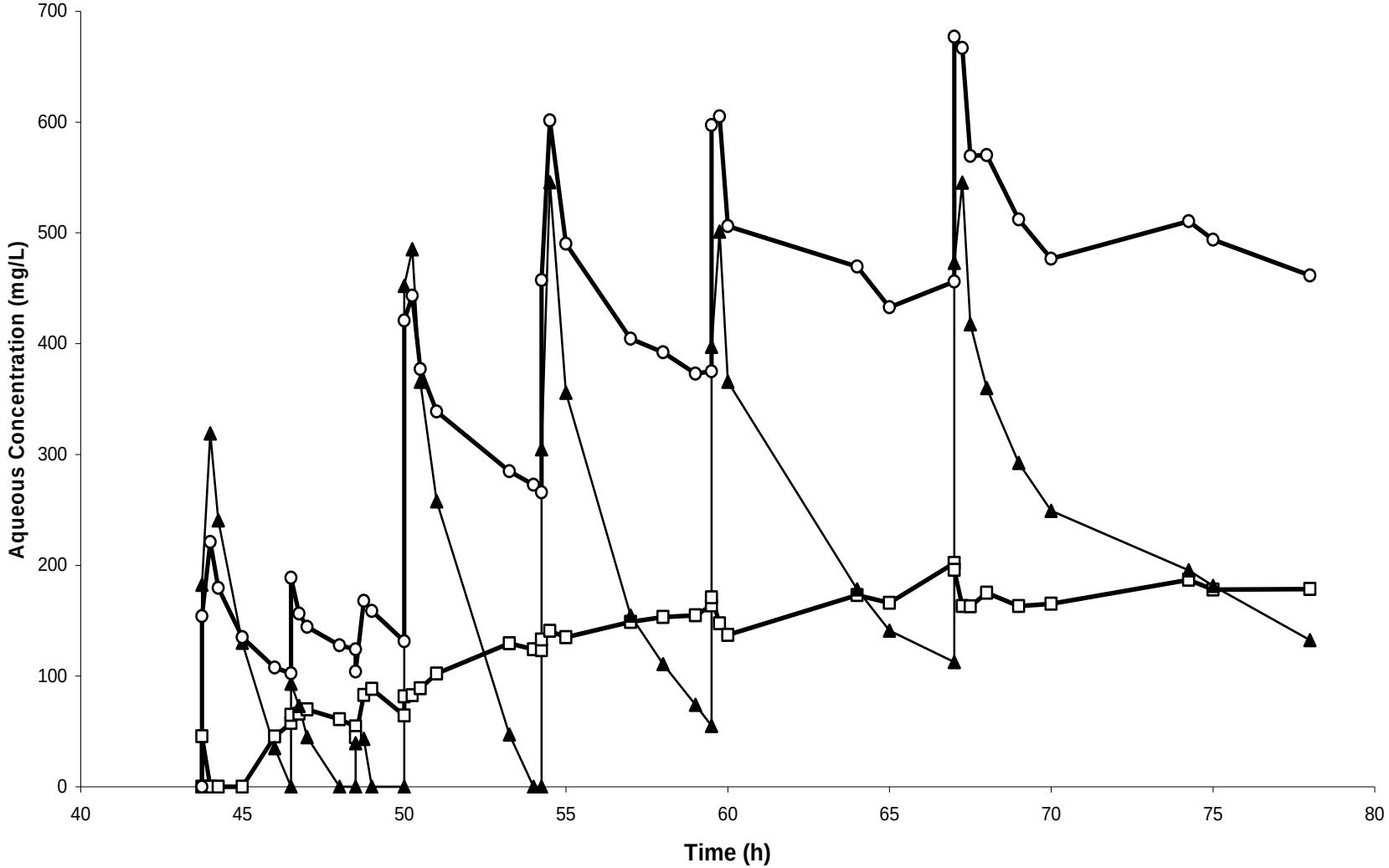


Figure 4.14: The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with styrene/butadiene copolymer as the second phase.

Again, it is quite obvious from the decreasing slope of the carvone curve that after each addition (Figure 4.14) the biotransformation rate decreases. Similar to the case of the single phase and two phase reactors, it is assumed that the biotransformation rates decrease with each substrate addition due to the accumulation of carvone and cis-carveol in the reaction system. The final aqueous concentrations of carvone and cis-carveol were 179 mg/L and 462 mg/L, respectively. These concentrations are slightly lower than what was expected based on the results from Section 4.1; however, the aqueous phase was temporarily exposed to higher concentrations of cis-carveol (around 600 mg/L) after the addition of each feed aliquot before the substrate was absorbed by the polymer beads. It is quite possible that these short exposure times to inhibitory concentrations were sufficient to inhibit the biotransformation capacity. The overall volumetric productivity of this reaction system is 102 mg/L·h, which was calculated using the final system carvone concentration of 3503 mg/L and the total biotransformation time of 34.25 h which is a significant improvement to the results obtained in both the single phase and two phase with silicone oil reactor configurations.

Upon further investigation of the cis-carveol curve in Figure 4.14, it does not appear to be consistent with that of the single phase reactor (Figure 4.4). It was expected that the cis-carveol curve would be made up of a series of steps corresponding to the substrate addition as in the single phase reactor. However, instead of a plateau appearing after each addition, there is a peak followed by a gradual decrease in aqueous concentration. It is assumed that this gradual decrease is the slow diffusion of cis-carveol into the polymer beads. It is well known that equilibrium in a liquid-liquid system is established almost instantaneously whereas equilibrium between a solid and liquid phase

can be delayed due to diffusion into the solid polymer matrix. Diffusivity quantifies the rate at which a molecule can diffuse into and out of a polymer matrix (Prpich and Daugulis 2004). The diffusion rates of carvone and carveol into styrene/butadiene copolymer have not been determined, however the diffusion rates of similar molecules (benzene and toluene) into styrene/butadiene copolymer, Hytrel® 8206 and ethylene/vinyl acetate (EVA) have been determined (Prpich and Daugulis 2004; Daugulis et al. 2003). The diffusion rates of benzene into EVA and styrene/butadiene copolymer were determined by Daugulis et al. (2003) to be 4.3×10^{-6} cm^2/s and 2.23×10^{-6} cm^2/s, respectively. The diffusion rates of phenol into Hytrel® 8206 and EVA are similar and are reported by Prpich and Daugulis (2004) to be 1.54×10^{-7} cm^2/s and 3.73×10^{-9} cm^2/s, respectively. It is also important to note that the surface area available for transfer in the solid-liquid system is less than that for the liquid-liquid system because the polymer beads are bigger than the solvent droplets formed as a result of vigorous agitation of the system allowing for more rapid transfer in a liquid-liquid system.

Aside from the improved volumetric productivity, increase in substrate addition (25 mL total) and increase in biotransformation time, this reactor configuration offers several operational advantages. First and foremost, there was no emulsion formation in the presence of the polymer beads. The lack of emulsion made it much easier to quantify biomass by cell dry weight as depicted in Figure 4.15. Also, the cells did not appear to produce any sort of polysaccharide which could cause the formation of a third phase and disturb the mass balance. It is also interesting to note that there were no morphological changes to the cells observed when the aqueous phase was saturated with inhibitory concentrations of substrate and product as were observed in the single phase bioreactor

configuration. It is hypothesized that any cell aggregation formations that were present would have been broken down by abrasion due to the solid polymer beads in the reactor.

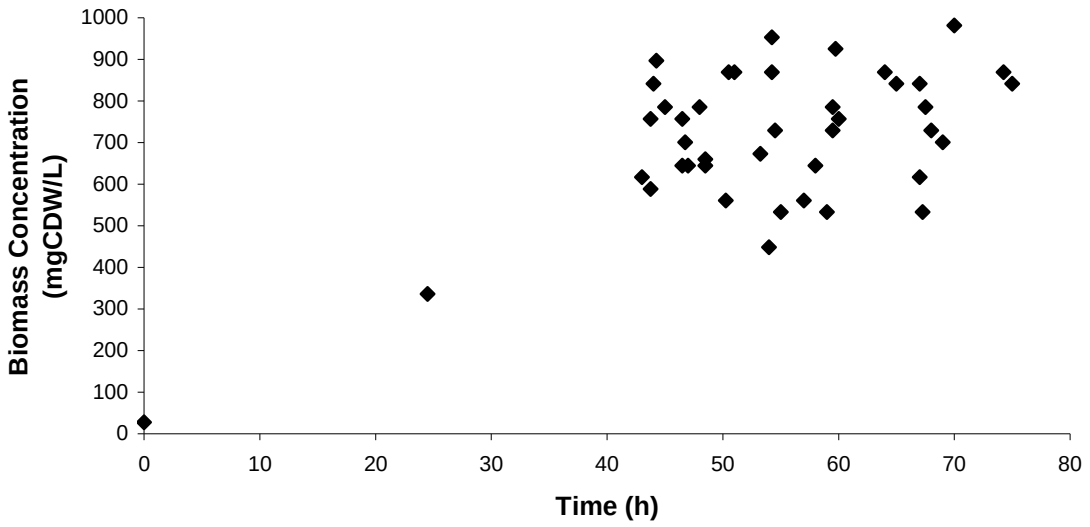


Figure 4.15: Time course of the biomass concentration throughout the two phase biotransformation with styrene/butadiene copolymer.

Figure 4.15 indicates that there is still some scatter in the biomass quantification throughout the duration of the biotransformation. It is however apparent that there is no significant decrease or increase in average cell concentration throughout the biotransformation period (43.75 to 78 h).

In an effort to demonstrate that it is possible to extract the product from the polymers at the end of the biotransformation, extractions from the polymer beads were performed into methanol. After 24 hours, it was evident that all of the carvone had partitioned out of the polymer phase, because the second extraction yielded no carvone. The total mass of carvone that was extracted from the 3 beads was approximately 4 mg which corresponds to a concentration in the polymers of 19890 mg/L (using the polymer density of 0.94 g/mL). Considering the final aqueous concentration, the partition

coefficient during operation was 111 which is in agreement with the previously determined abiotic partition coefficient of 118. These results demonstrate that in a relatively short time period (24 h) and using a small volume of methanol, it is possible to extract the product from the beads if an appropriate extractant is used.

4.8 Two Phase Biotransformation: Mixture of Two Polymers

In the previous two phase biotransformation with styrene/butadiene copolymer beads as the second phase, the ability for the organism to carry out the biotransformation was affected by inhibitory concentrations of substrate and product. Since the partition coefficient for the substrate, carveol, is much lower than that for the product, carvone, it accumulated more quickly in the aqueous phase causing acute toxicity. In an effort to balance out the partition coefficients of carvone and carveol in the reactor, a two phase biotransformation was carried out where two types of polymer beads were employed. The choice for the second polymer was Hytrel® 8206 because of its affinity for carveol. The goal of this biotransformation reactor configuration was to demonstrate the ability to tailor the mixture of polymer beads in the reactor to affect the ratio of inhibitory substances in the aqueous phase. This simple adjustment is an effect that is unique to the case where polymer beads are used as the second phase in a TPPB. Previous work by Bruce and Daugulis (1991) has demonstrated the ability to use mixtures of organic solvents as the extractant phase in a TPPB, however, the selection process for the co-solvents is quite involved and is not always reliable. Generally, a co-extractant is chosen to have superior partitioning capabilities; however these solvents are often non-biocompatible. The co-solvent can only be used in very small amounts such that the

aqueous solubility of the inhibitory solvent does not affect the integrity of the cell membrane in the aqueous phase. Another disadvantage is that it is difficult to predict the biocompatibility of mixtures of solvents as the log P strategy (Section 3.6.1) appears to be a poor means of predicting biocompatibility (Bruce and Daugulis 1991). Using mixtures of polymer beads is quite attractive because biocompatibility is not an issue (since the majority of polymers are inherently biocompatible) and as such the only selection criterion for choosing the polymer mixtures would be their partitioning affinity for the target molecule(s).

The total working volume of 3.5 L consisted of 3 L medium which was autoclaved in the reactor and 250 mL (235 g) styrene/butadiene copolymer and 250 mL (293 g) Hytrel® 8206 which were autoclaved separately in water which was decanted before the polymers were added to the reactor at which point the biotransformation was started.

The biotransformation was started with the addition of 3 mL of substrate at 43.5 h, followed by the second and third additions of 1 mL (at 47.75 and 49 h, respectively). The fourth and final substrate additions were each 5 mL (added at 50 and 56 h, respectively) at which point the biotransformation rate had decreased sufficiently that it was not feasible to continue the biotransformation. The final carvone and cis-carveol concentrations were 198 mg/L and 294 mg/L respectively (Figure 4.16). As expected, by comparison to the results of the toxicity experiment (Section 4.1), it is obvious that the biotransformation rate decreased due to the presence of inhibitory product and substrate concentrations. Again, the final cis-carveol concentration is lower than that found through the toxicity test, but it is apparent that immediately after the substrate addition

(before it is absorbed into the polymers) the aqueous phase (and hence cells) is exposed to concentrations of cis-carveol around 600 mg/L.

Figure 4.16 displays the time course data for the substrate and product throughout the biotransformation (43.5 to 67.5 h). In this case, as compared to the previous result in a TPPB with polymer beads used as the second phase (Section 4.7), it is clear that the combined partitioning capacity of styrene/butadiene copolymer and Hytrel® 8206 is inferior. After only 5 substrate additions (a total of 15 mL carveol), as compared to the 7 substrate additions from Section 4.7 (totaling 25 mL), the reaction rate had decreased significantly since the carvone accumulated in the aqueous phase at a much faster rate.

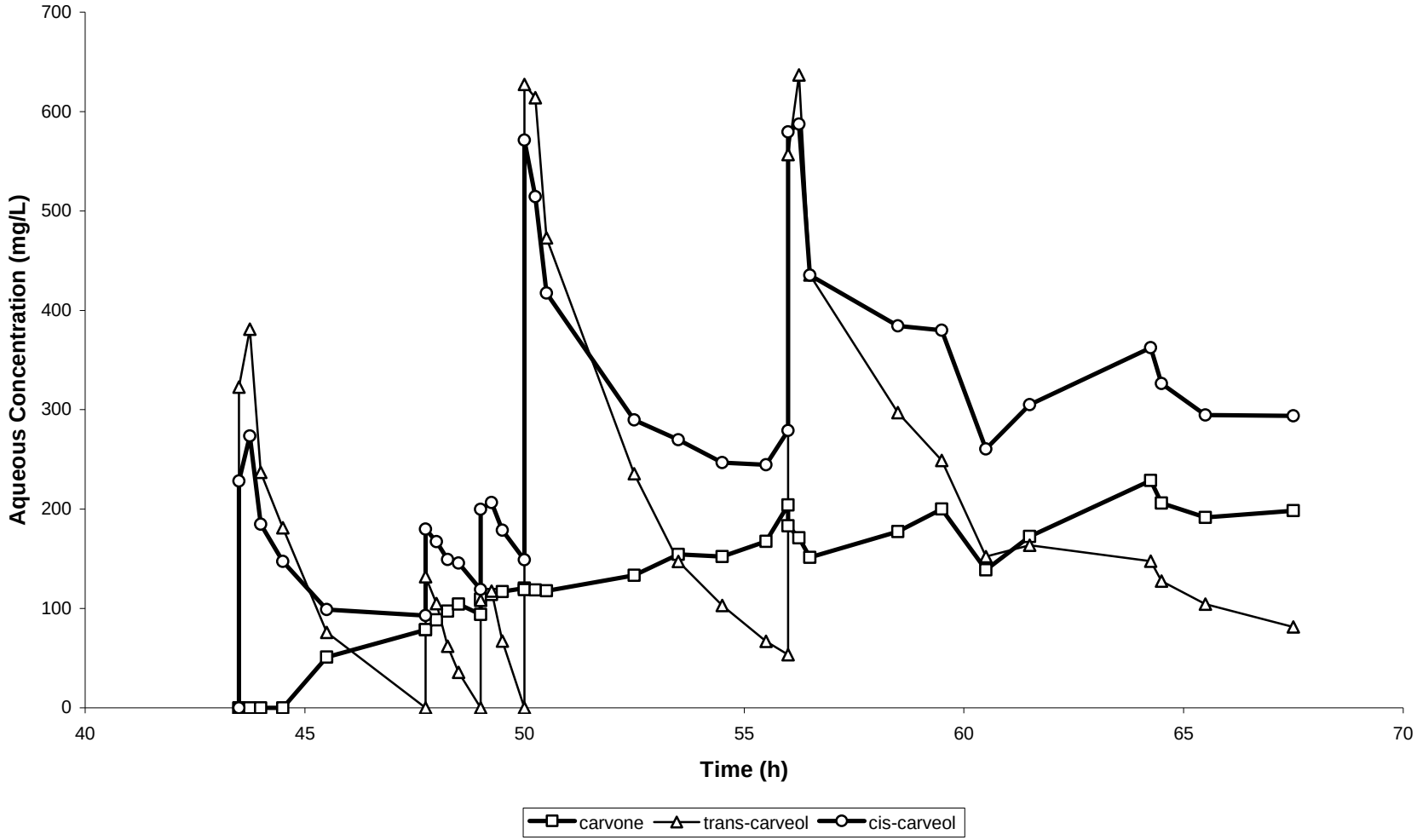


Figure 4.16: The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with a mixture of styrene/butadiene copolymer and Hytrel® 8206 as the second phase.

The styrene/butadiene copolymer beads have a very strong affinity for carvone (partition coefficient of 118); thus when half of the volume of these beads were replaced with Hytrel® 8206, with a much lower affinity for carvone (partition coefficient of 49), there was more carvone present in the aqueous phase. The volumetric productivity for this reactor was 106 mg/L·h (which was calculated using the final carvone concentration of 2537 mg/L in the system and the total time for the biotransformation 24 h) and as such, the data are successful in demonstrating that by changing the fractions of polymer in the reactor, the concentration of the target compound(s) in the aqueous phase can be manipulated. It is quite obvious that the aqueous concentration of the resulting cis-carveol isomer is lower due to the presence of Hytrel® 8206 which has a higher affinity for carveol than does styrene/butadiene copolymer (36 versus 5). Through further polymer selection, it may be possible to obtain a polymer that has a comparable affinity for carveol as styrene/butadiene copolymer has for carvone which would maintain low concentrations of both substrate and product in the aqueous phase. Again, it is interesting to note the slow diffusion of cis-carveol into the polymer beads as seen by the gradual decrease from its initial concentration after each addition. This affect is again due to a slower diffusion rate into the polymer phase than was experienced with the liquid-liquid two phase system.

The biomass in this reactor configuration behaved in a similar manner as the first TPPB with polymer beads as the second phase. Unexpectedly, there were no observable morphological effects to the cells in the presence of inhibitory substrate and product concentrations similar to those that were observed in the single phase reactor (Section 4.2). It is hypothesized that the biomass may in fact have formed small particles that

were broken apart by the abrasive action of the polymer beads in the reactor. The time course data for the biomass concentration throughout the fermentation are displayed in Figure 4.17.

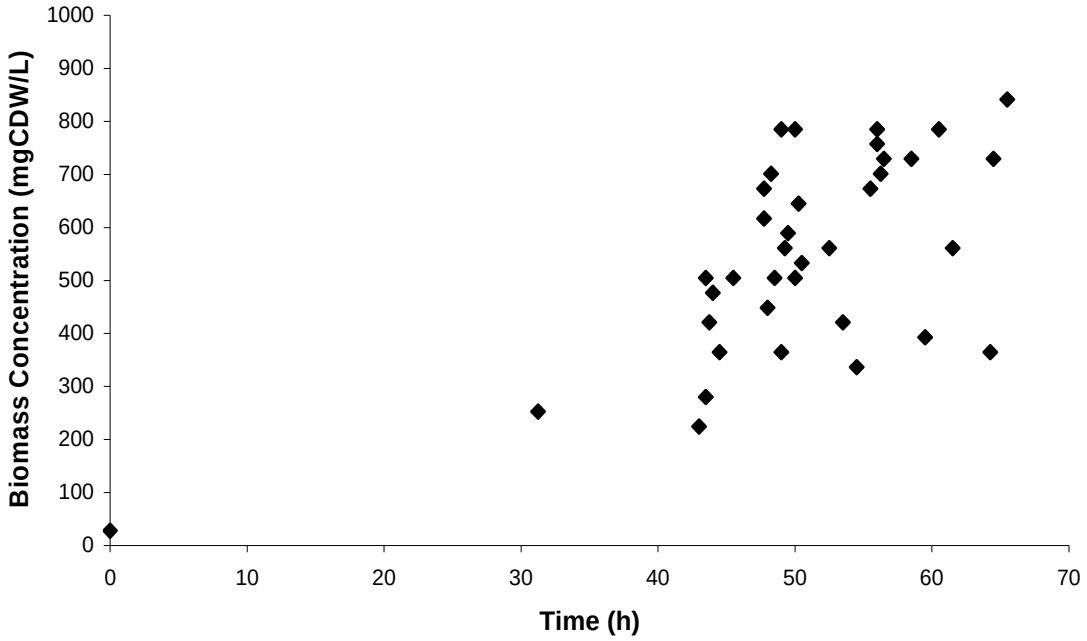


Figure 4.17: Time course of the biomass concentration throughout the two phase biotransformation with styrene/butadiene copolymer and Hytrel® 8206.

Again it is clear that there is some variability in the biomass data during the biotransformation, however it is not apparent that there is any trend for a steadily increasing or decreasing biomass concentration in the aqueous phase throughout the biotransformation period (43.5 to 67.5 h).

Again, once the biotransformation was completed, the product was extracted from both the styrene/butadiene copolymer and Hytrel® 8206 polymer beads into methanol. The final mass of carvone extracted from three styrene/butadiene copolymer and three Hytrel® 8206 polymer beads were 2 mg and 1 mg, respectively. These masses result in estimated partitioning coefficients of 81 for styrene/butadiene copolymer and 35 for

Hytrel® 8206 during the biotransformation. These partition coefficients do not correlate exactly with those achieved in the abiotic partitioning experiments; however they are within a suitable range.

4.9 Two Phase Continuous Biotransformation: Single Polymer with Recirculation of Medium

In a final attempt to improve the performance of this reaction system, an external extraction column was utilized which consisted of 300 g Hytrel® 8206. The strategy in this experiment was to minimize the accumulation of the cis-isomer in the aqueous phase. In the two-phase reactor with styrene/butadiene copolymer as the second phase, the biotransformation rate was inhibited by the accumulation of the cis-carveol isomer. In an attempt to reduce this accumulation, an external column containing Hytrel® 8206 polymer beads was added due to Hytrel® 8206's affinity for carveol. The working volume of the reactor included 3 L of medium in the reactor, 566 mL (470 g) styrene/butadiene copolymer (added to the reactor after being autoclaved separately) and 256 mL (300 g) Hytrel® 8206 contained within an external glass column through which the aqueous volume circulated (Figure 3.1). The medium was pumped through the extraction column at 30 mL/min using a peristaltic pump. The rationale for including Hytrel® 8206 in an extraction loop was to decrease the cis-carveol accumulation in the aqueous phase since Hytrel® 8206 has a higher affinity for carveol than does the styrene/butadiene copolymer (36 versus 5). This reactor configuration ensures that the polymer phase in the reactor (styrene/butadiene copolymer) will have the full extraction potential for carvone (as did the reactor configuration in Section 4.7) and the Hytrel®

8206 containing-column will act to reduce the accumulating cis-carveol isomer present in the aqueous phase.

From Figure 4.18, it is clear that this processing strategy was highly successful in improving reactor performance. A total of 35 mL carveol was added to the reactor before the reaction rate decreased significantly as compared to the biotransformation in Section 4.7 to which a total of 25 mL carveol was added throughout the biotransformation. The carveol additions in this experiment were 3 mL (at 43.5 h), 1 mL (at 46.5 h), 1 mL (at 47.5 h), followed by six 5 mL aliquots added at 48.5, 52.5, 56.5, 64.75, 70.5 and 78 h.

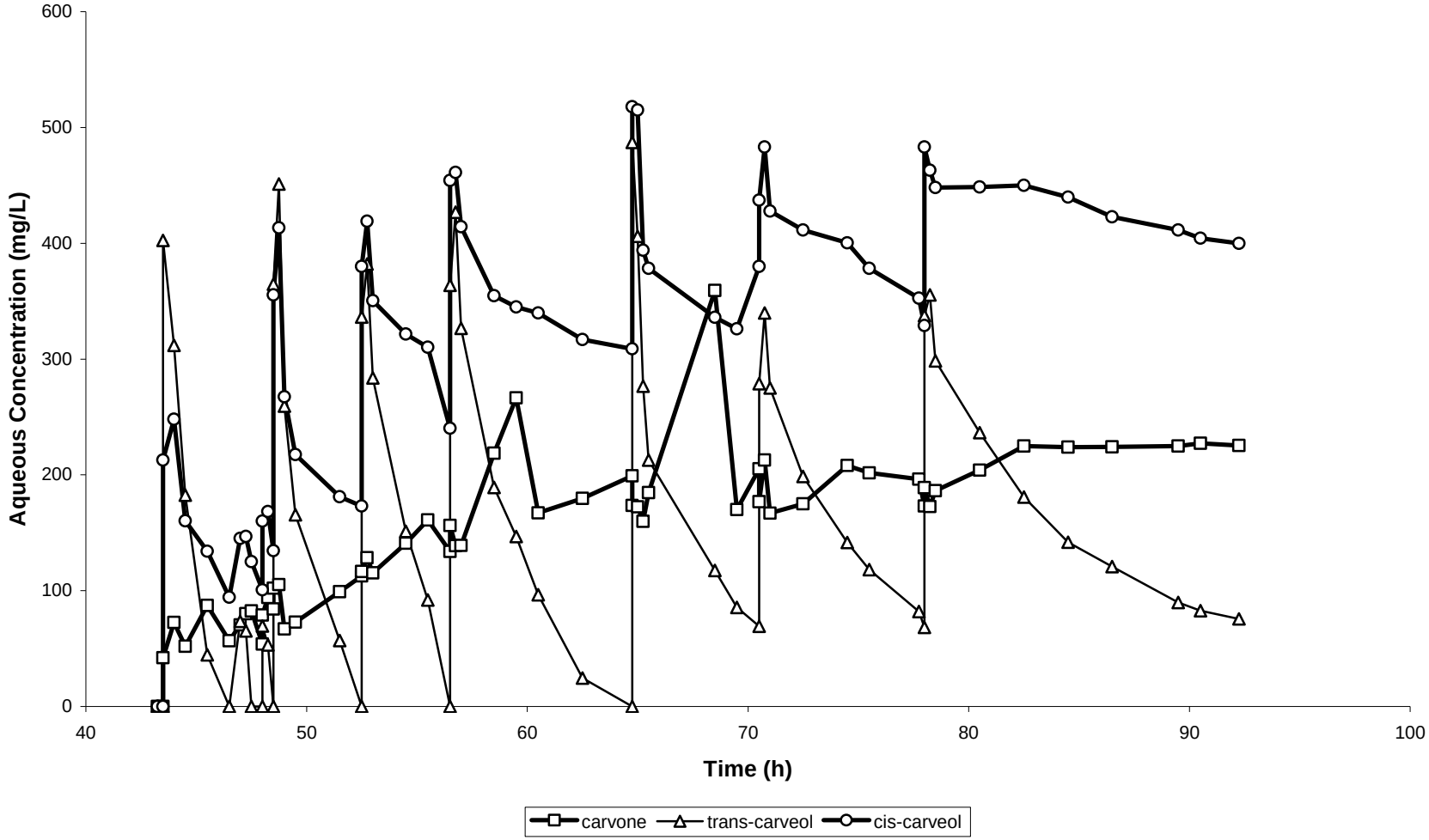


Figure 4.18 The time course of the substrate and product concentrations throughout the duration of the two phase biotransformation with styrene/butadiene copolymer as the second phase and Hytrel® 8206 contained within an external column.

The final carvone and cis-carveol concentrations in the aqueous phase were 225 mg/L and 400 mg/L respectively (Figure 4.18). These inhibitory concentrations (as confirmed by the toxicity experiment completed in Section 4.1) were responsible for the decrease in reaction rate with each substrate addition. The volumetric productivity for this reactor configuration was 99 mg/L·h, and was calculated using the system carvone concentration (4847 mg/L) and the total time of the biotransformation (48.75 h). The biomass concentration throughout the fermentation is displayed in Figure 4.19. The biomass concentration during the biotransformation is relatively constant again. The slight decrease in biomass concentration may be a result of minor biomass accumulation in the external column.

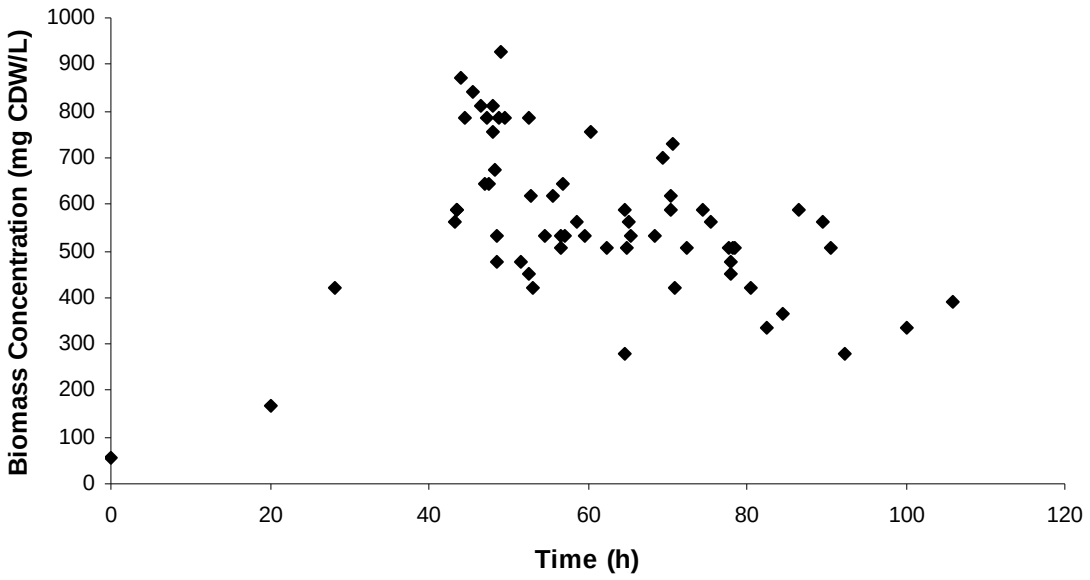


Figure 4.19: Time course of the biomass concentration throughout the continuous two phase biotransformation.

After the biotransformation was complete, the product was extracted from both the styrene/butadiene copolymer and the Hytrel® 8206 beads. The final mass of carvone extracted (after 24 hours) from the styrene/butadiene copolymer and Hytrel® 8206

polymer were 1 mg and 4 mg, respectively. These masses result in partitioning coefficients of 104 for styrene/butadiene copolymer and 35 for Hytrel® 8206 during the operation of the biotransformation which correlate well with the pre-determined abiotic partitioning coefficients.

Chapter 5.0 Conclusions

Tests confirmed that both the substrate, carveol, and product, carvone, are inhibitory to the cells of *Rhodococcus erythropolis* DCL14 and that the examined microbial biotransformation would benefit from the application of a two phase partitioning bioreactor (TPPB). The microbial biotransformation of trans-carveol to (*R*)-(-)-carvone by *Rhodococcus erythropolis* DCL14 was successfully completed in various TPPB configurations. The performance of the reactor was evaluated based on three criteria: volumetric productivity, duration of biotransformation and total volume of substrate added to the reactor. The single aqueous phase reactor which was performed as a benchmark reactor resulted in a volumetric productivity of 31 mg/L·h. Throughout the biotransformation, which lasted 15.25 h, 5 mL of carveol (mixture of isomers) was added to the reactor. In an attempt to improve reactor performance a rational solvent selection was carried out for application in a TPPB. The solvent that possessed all of the required characteristics (i.e. biocompatibility, non-bioavailability, high affinity for target compounds) was 1-dodecene. However, upon introduction of this solvent to the reactor, the hydrophobic nature of the organism led to the formation of a significant emulsion and a third phase which made quantifying substrate and product in the reactor very difficult. In order to overcome these operational difficulties, silicone oil (a liquid polymer) was used as the second phase. Again, in this case, the organism was able to partition into the second phase and form an emulsion. The volumetric productivity for this reactor configuration was 29 mg/ L·h and 13 mL of substrate was added over a 28.75 h biotransformation period. Though the volumetric productivity is lower than that of the

single phase reactor (due to the increase in volume and biotransformation time), the overall reactor performance was improved as confirmed by the addition of 13 mL of substrate (versus only 5 mL). Finally, three TPPB configurations in which the second phase consisted of solid polymer beads were examined. In the first reactor, styrene/butadiene copolymer was chosen to be the second phase after a rational polymer selection strategy had been employed, which included consideration of bioavailability, partitioning capacity, thermal stability and resistance to biofilm formation. This reactor achieved a volumetric productivity of 102 mg/L·h following a total substrate addition of 25 mL over a 34.25 h biotransformation time. The second reactor that contained a mixture of styrene/butadiene copolymer and Hytrel® 8206 (chosen for its affinity for the substrate) as the second phase achieved a volumetric productivity of 106 mg/L·h after the addition of 15 mL carveol over 24 hours. This reactor was successful in demonstrating the ability of mixtures of polymers to be applied to the TPPB to cause an effect on the aqueous concentrations of target molecules. The final (and most successful) reactor employed styrene/butadiene copolymer as the second phase in the reactor and also included an external extraction column containing Hytrel® 8206 beads through which the fermentation medium was circulated in an effort to reduce the accumulation of cis-carveol in the aqueous phase. The volumetric productivity of this reactor was 99 mg/L·h over a 48.75 h biotransformation time. The most obvious demonstration of the improved performance, however, was the addition of 35 mL of substrate throughout the transformation. It is important to note that the longer biotransformation time would in fact be advantageous in an industrial setting because of the reduction of turnaround time required, which would improve the volumetric productivity over repeated cycles of

operation and turnaround time. It was shown that it is possible, and simple, to remove the product from the beads once the transformation had ceased.

Chapter 6.0 Future Work

The work completed in this thesis leaves a few potential areas for future work. Most importantly, a better method of biomass quantification should be developed. Several challenges were encountered regarding biomass quantification in the presence of high substrate and product concentrations (due to morphological changes) and in the presence of a hydrophobic liquid (due to the formation of emulsions and the partitioning of the biocatalyst into the second phase). The morphological changes and emulsion formations may be avoided by the use of other organisms selected for their non-hydrophobic characteristics, including genetically modified cells that have been engineered to contain the appropriate biotransformation pathway.

The extent of work completed with the two phase systems in which polymer beads were used as the second phase opens up two new areas of research. The first of course is to complete further polymer selection, by tailoring the chemical composition for increased affinity for the target compounds. It would be advantageous to find a polymer that has a high affinity for the substrate as well as the product. The application of polymer beads to a reactor also opens up the possibility for the use of different reactor configurations. For instance, the continuous transformation used in this study with the external extraction column could be improved upon by exchanging a saturated column for a new column packed with fresh beads. This system has the potential for greatly increased productivity, as described by Prpich and Daugulis (2007) who used a similar reactor configuration. It is quite possible that this system could operate continuously if the saturated columns were continually replaced and if the organism was supplied with

the necessary nutrients, carbon source and air to carry out the biotransformation indefintely. Prpich and Daugulis (2007) have also explored the use of alternative polymer geometries as reactor internals. For these applications, the polymer beads can be melted into sheets and made to fit inside the reactor.

The present work also created a premise for the production of enantiomeric flavor and fragrance compounds in a TPPB. Since these compounds must be of very high quality for use in food applications, the use of an organic solvent (which would most likely affect the organoleptic properties of the product) is not an ideal system. However, the application of polymer beads as the second phase (which impart no scent or flavor to the product) should be explored further with other flavor and fragrance compounds.

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Appendix A:

Carvone and Carveol Gas Chromatograph Calibrations in Ethyl Acetate and Methanol

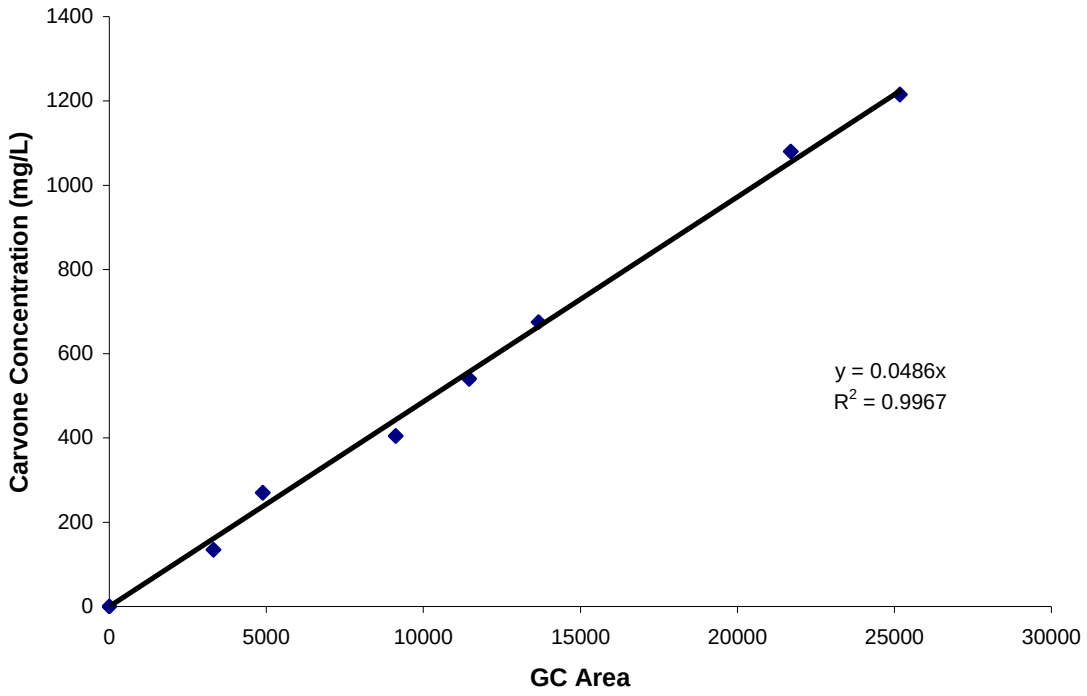

Figure A1: Carvone calibration curve using ethyl acetate as the extractant.

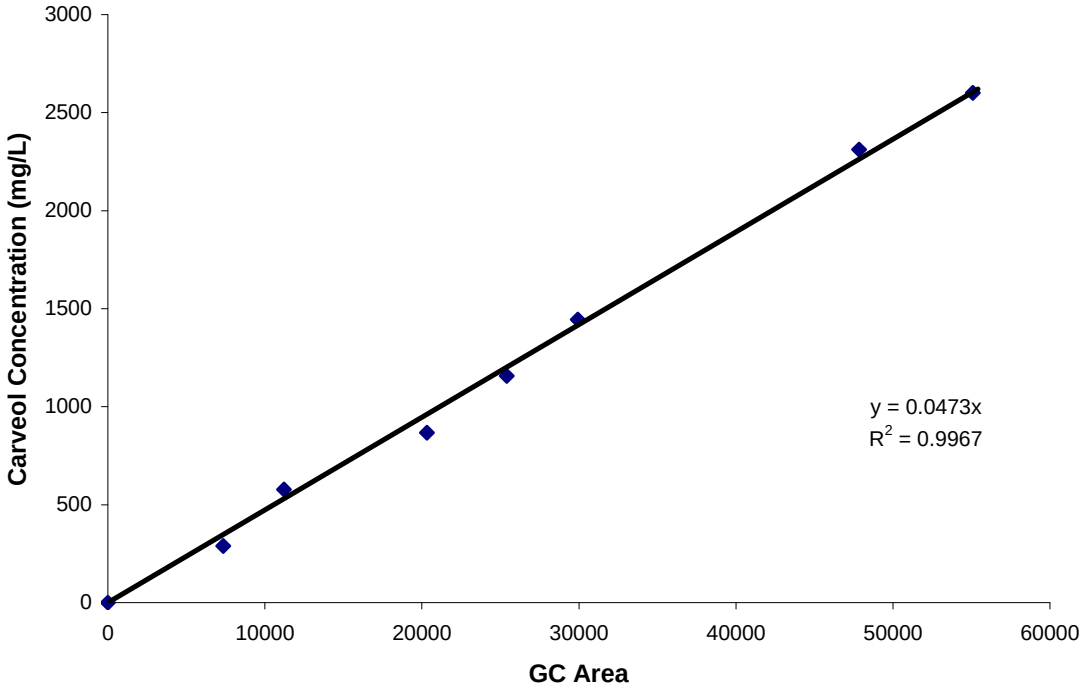

Figure A2: Carveol calibration curve using ethyl acetate as the extractant.

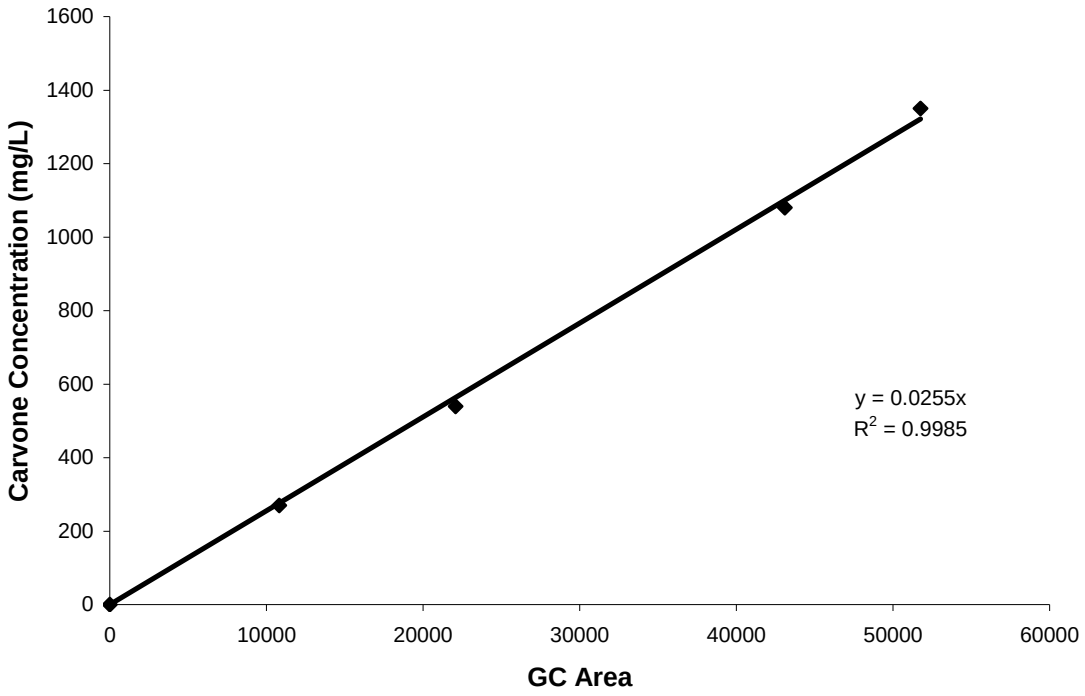

Figure A3: Carvone calibration curve using methanol as the extractant.

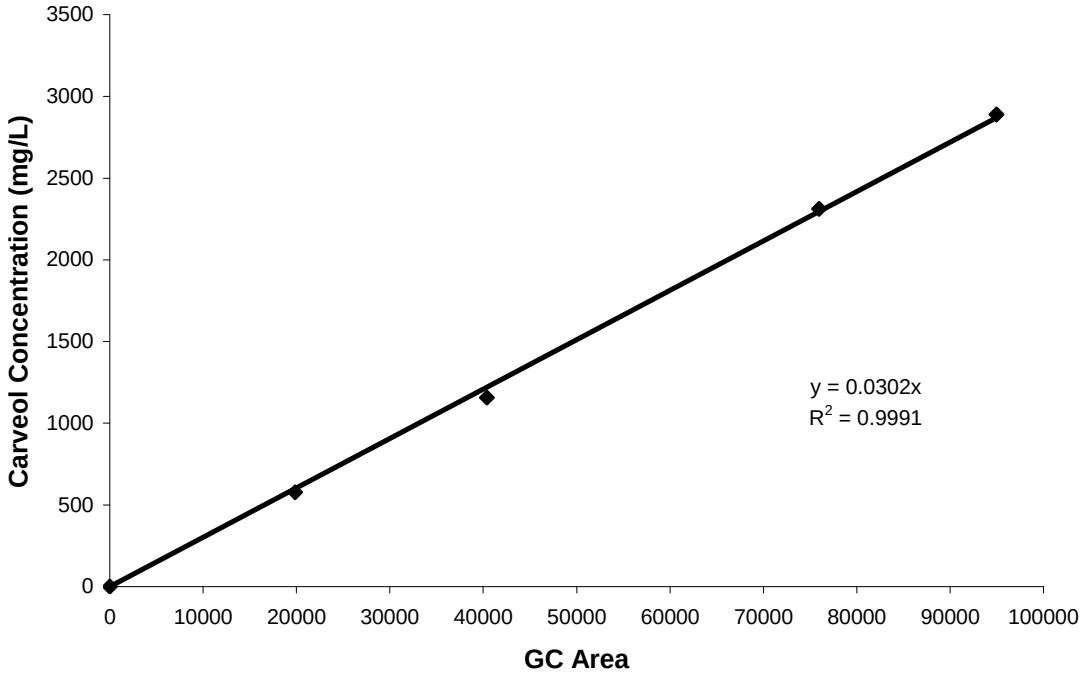

Figure A4: Carveol calibration curve using methanol as the extractant.

Appendix B:

Additional Attempts to Quantify Biomass in the Presence of a Hydrophobic Liquid Phase

Several attempts were made to obtain a reliable measure of biomass in the presence of a second liquid phase. *Rhodococcus erythropolis* DCL14 is a very hydrophobic organism and in the presence of a second liquid phase would form an emulsion from which it was very difficult to separate the biomass.

For the solvent biocompatibility tests (Section 3.6.1), the medium contained glucose as a carbon source and for this reason, an attempt to quantify biomass using a dinitrosalicylic acid (DNS) assay was performed (as outlined by Miller 1959). It was hypothesized that biomass growth could be quantified based on glucose substrate consumed instead of biomass concentration. A calibration curve for the colorimetric DNS assay is shown in Figure B1.

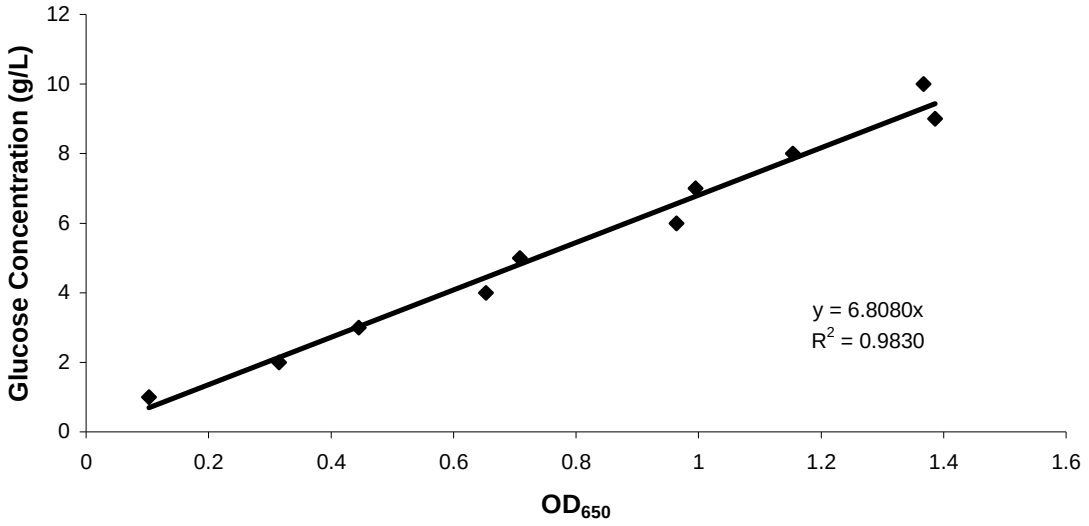

Figure B1: DNS assay calibration curve.

The resulting glucose concentrations did not provide conclusive data. It was determined that this test was unsuccessful because some of the solvents were bioavailable and were consumed preferentially (or in combination) with the glucose substrate. The DNS assay was not an effective means of biomass quantification.

Another strategy for the determination of biomass concentration was the Lowry Protein Assay. In order to measure protein concentration, the proteins must first be extracted from the cells. The simplest and most economical method of cell disruption was to use CelLytic^{TM}B cell lysis agent. In order to extract the proteins, a cell suspension was added to a pre-weighed test tube and centrifuged for 10 minutes at 3500 rpm and 20°C. The supernatant was then poured off and the sample was centrifuged for another 2 minutes, and the remaining supernatant was aspirated off of the biomass pellet. The tube was then weighed to determine the weight of wet cell paste. As described in the CelLytic^{TM}B instruction manual, ten times the weight in grams of CelLytic^{TM} B Cell Lysis Agent in mL was added to the wet cell paste. The pellet was then resuspended and mixed for 15 minutes at 180 rpm. Finally, the sample was centrifuged for 5 minutes to remove any remaining cell debris from the supernatant. The supernatant was then tested using the Lowry Assay. The calibration curve for the Lowry Assay is shown in Figure B2.

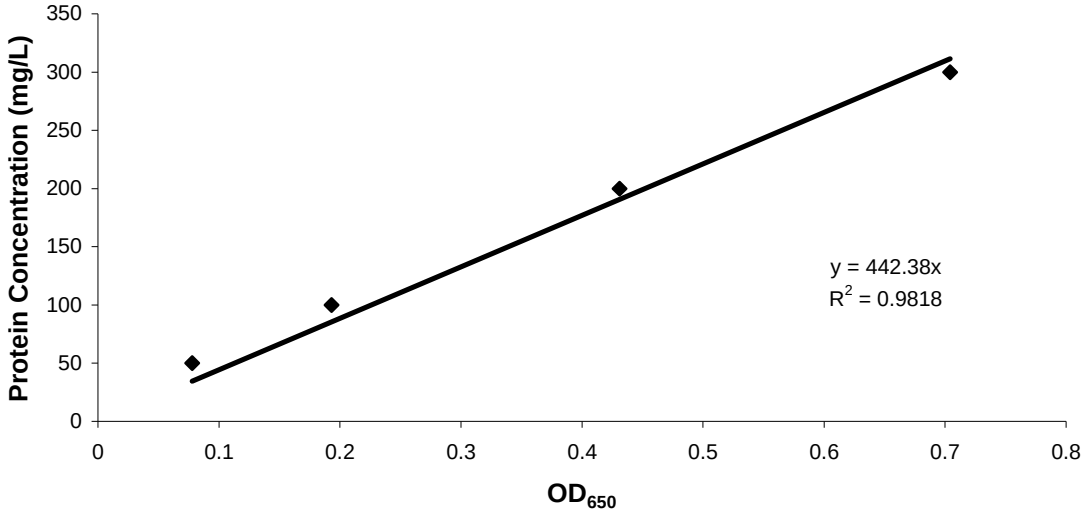

Figure B2: Lowry assay calibration curve for determination of protein concentration.

Once the proteins had been extracted from the cells, their concentration was determined using the Total Protein Kit, Micro Lowry, Peterson's Modification from Sigma-Aldrich. The procedure that was followed to obtain the protein concentration was taken directly from the information pamphlet provided with the kit as described below. The only variation is that the standards made for the calibration curve were made with CelLytic^{TM}B as the buffer instead of water as the pamphlet suggested such that they would be consistent with the samples to be tested.

Once the proteins had been extracted using CelLytic^{TM}B, 1 mL of Lowry Reagent Solution was added to 1 mL of supernatant and mixed. The samples were allowed to sit at room temperature for 20 minutes, followed by the addition of 0.5 mL Folin's and Ciocalteu's Phenol Reagent and mixing. The samples remained at room temperature for another 30 minutes while the color developed. The optical density was then measured at 650 nm to determine the protein concentration using the calibration curve (Figure B2). This method of biomass quantification was also unsuccessful because when a sample containing a second liquid phase was centrifuged, a good amount of biomass was retained at the organic/aqueous interface. Since the full amount of biomass in the sample was not available for protein extraction a true measure of biomass concentration could not be obtained by this method.

Appendix C:

Optical Density versus Cell Dry Weight Calibration Curve for Determination of Biomass Concentration

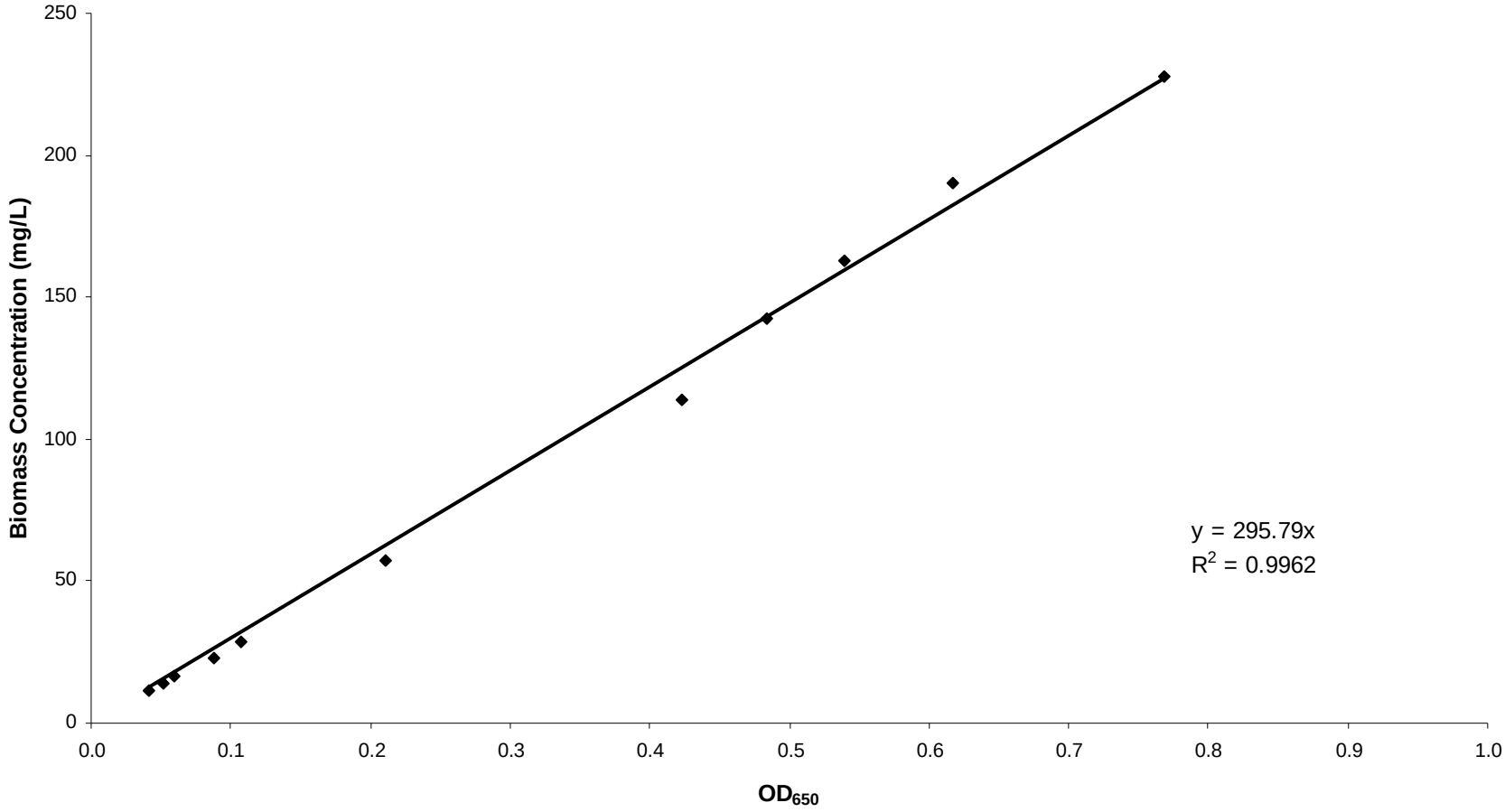

Figure C1: Calibration curve for the determination of biomass concentration in the absence of a second liquid phase.

Appendix D:

Partition Coefficient Plots for the Tested Solvents and Polymers

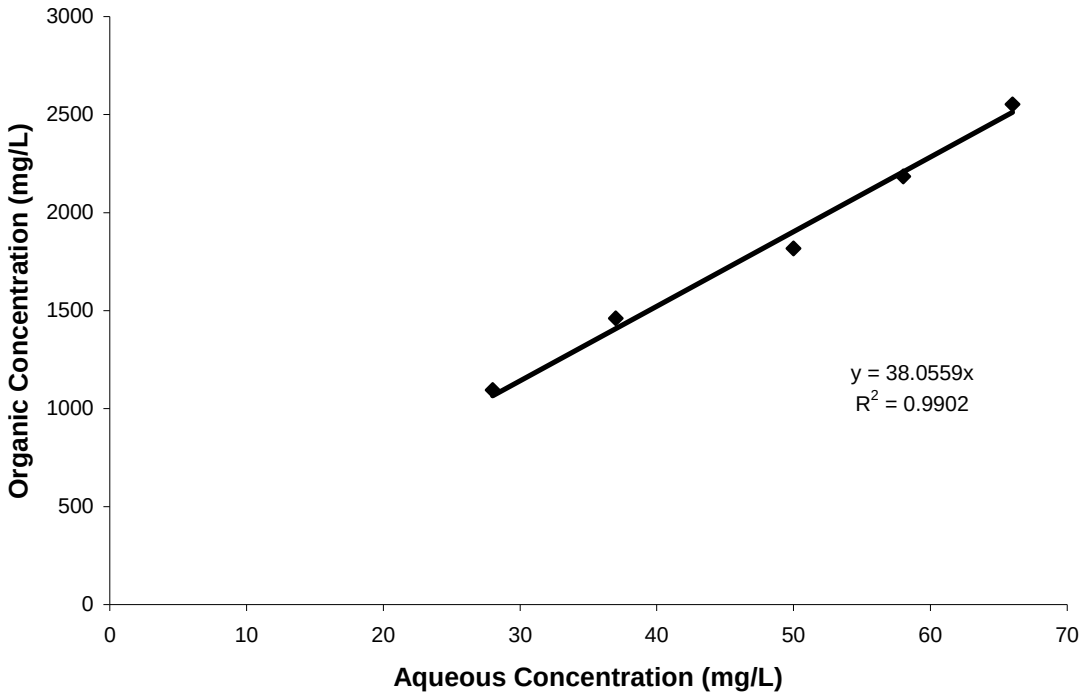

Figure D1: Carvone in 1-dodecene.

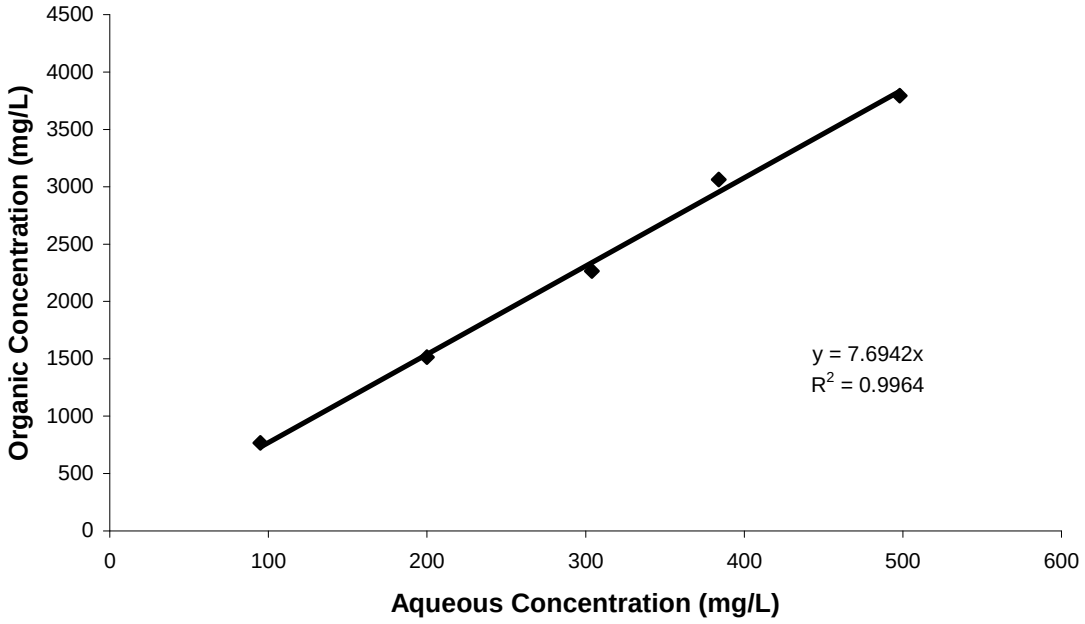

Figure D2: Carveol in 1-dodecene.

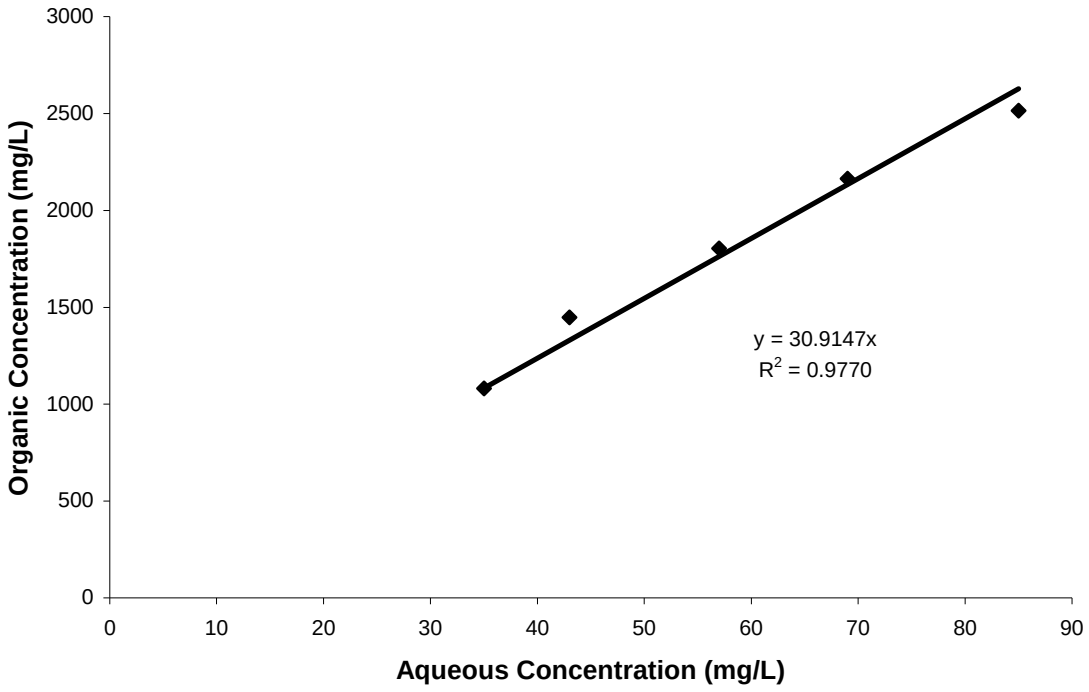

Figure D3: Carvone in n-dodecane.

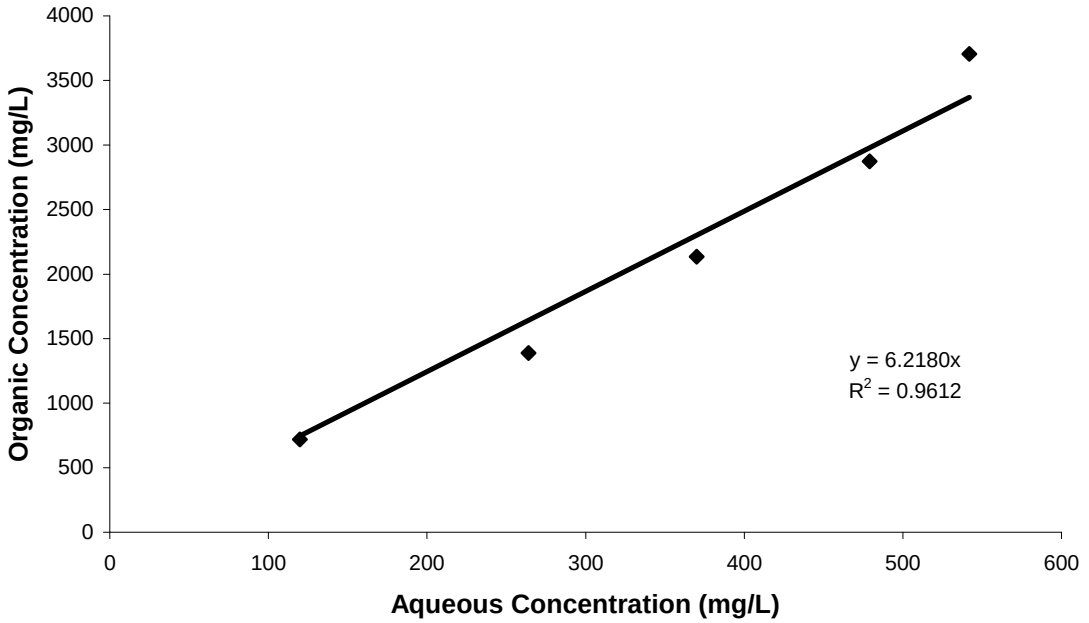

Figure D4: Carveol in n-dodecane.

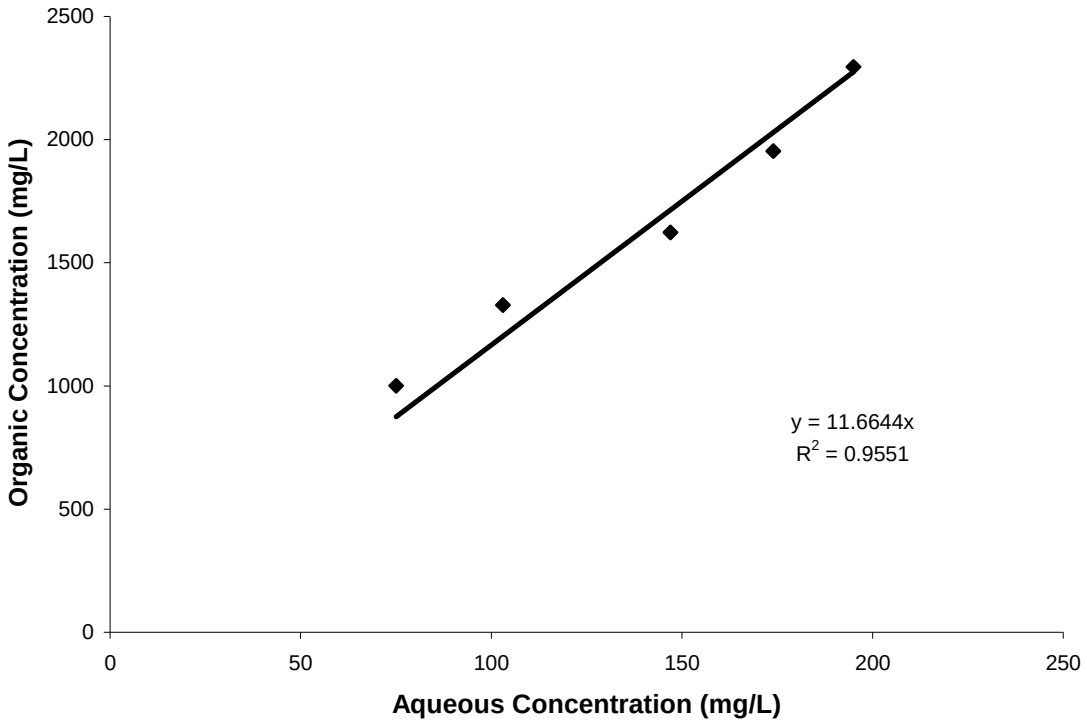

Figure D5: Carvone in silicone oil.

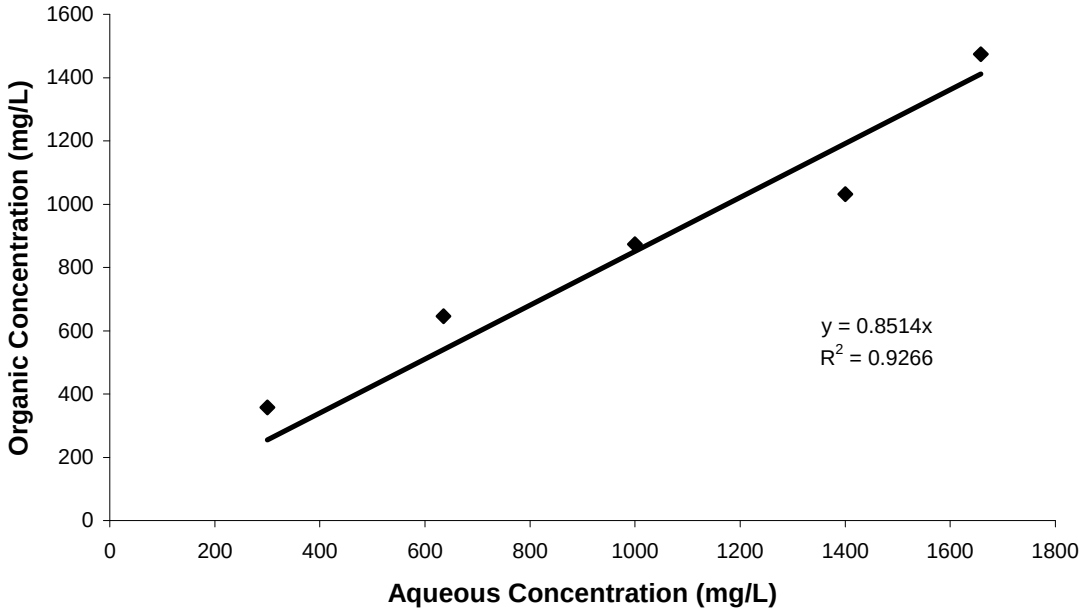

Figure D6: Carveol in silicone oil.

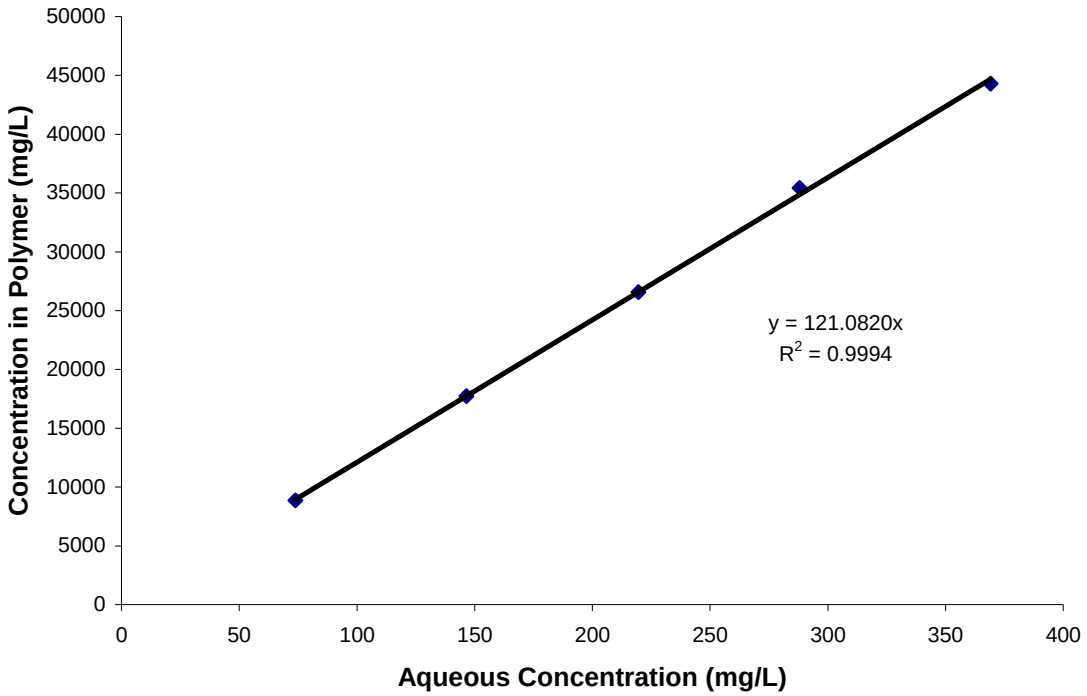

Figure D7: Carvone in KRATON® D1504K.

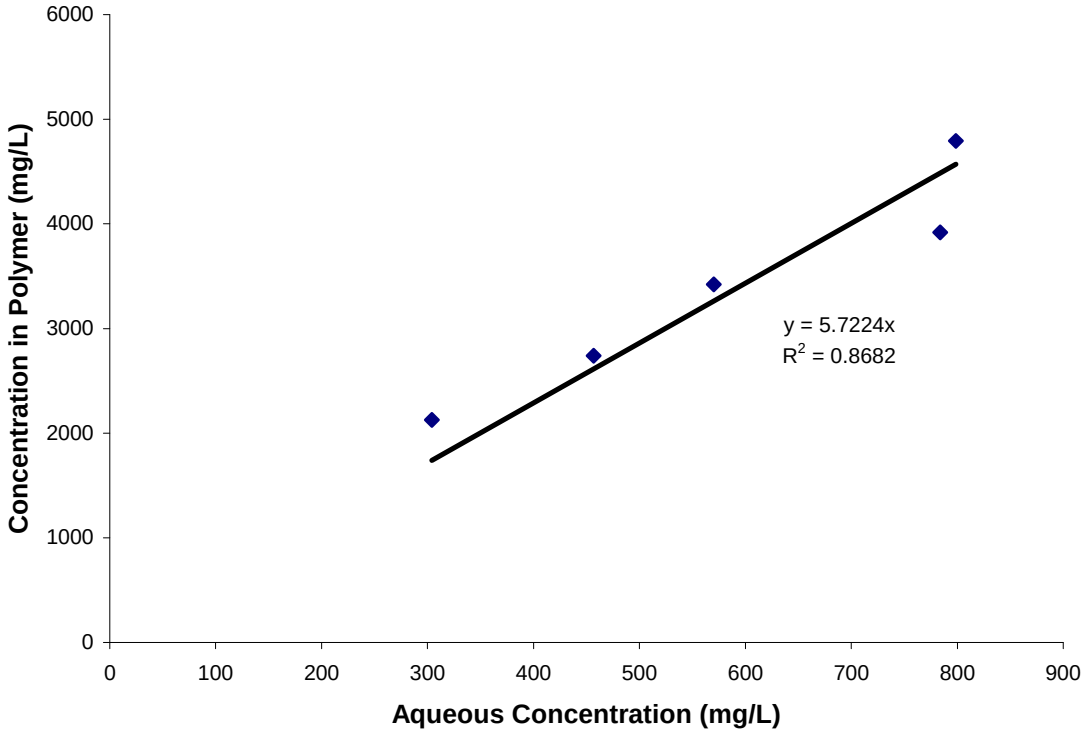

Figure D8: Carveol in KRATON® D1504K.

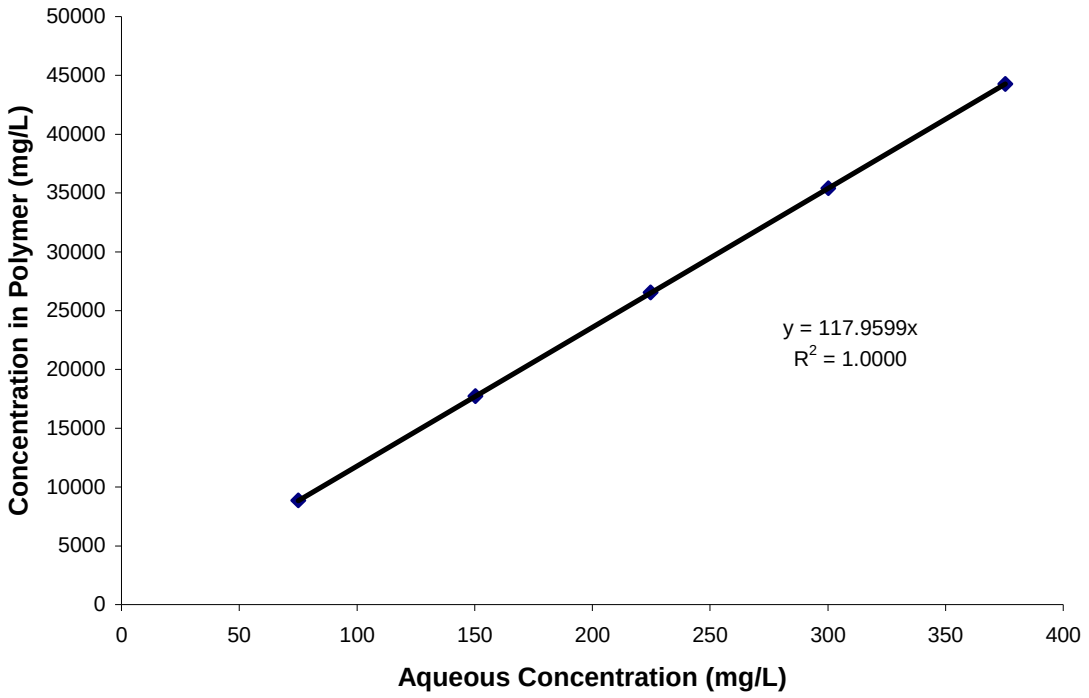

Figure D9: Carvone in styrene/butadiene copolymer.

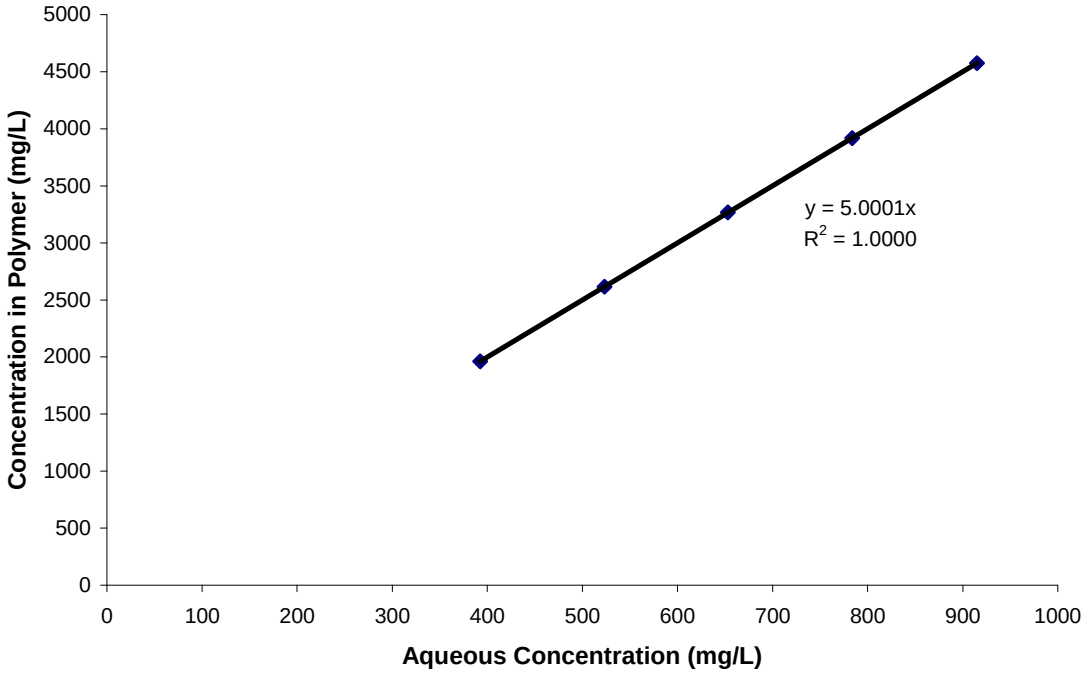

Figure D10: Carveol in styrene/butadiene copolymer.

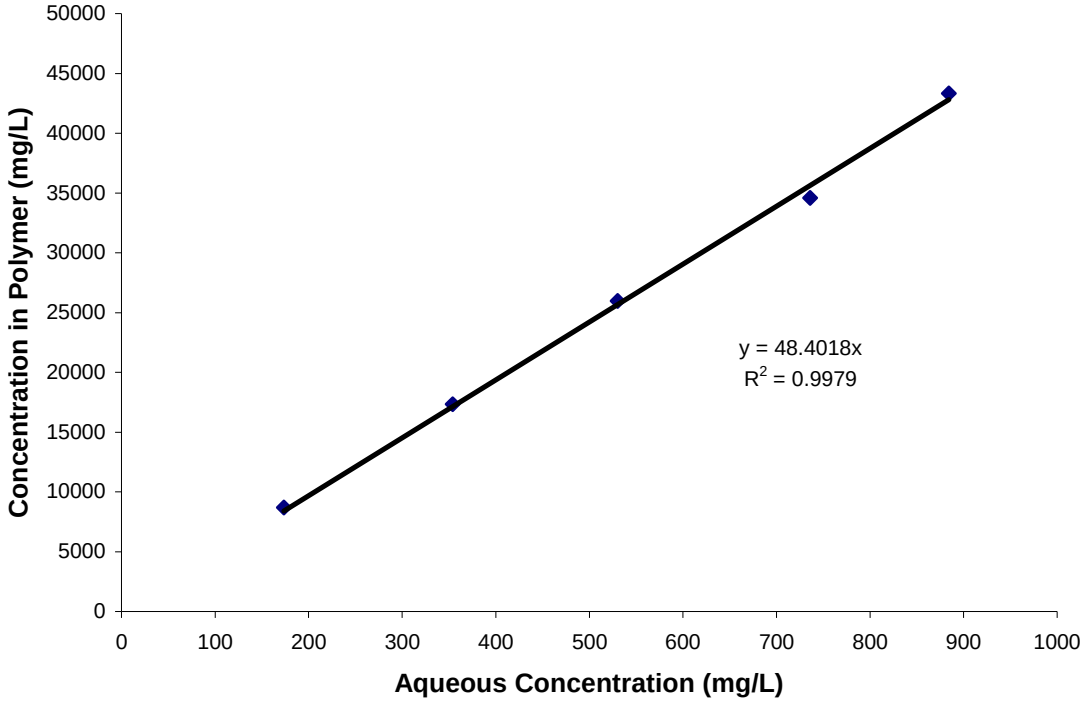

Figure D11: Carvone in Hytrel® 8206.

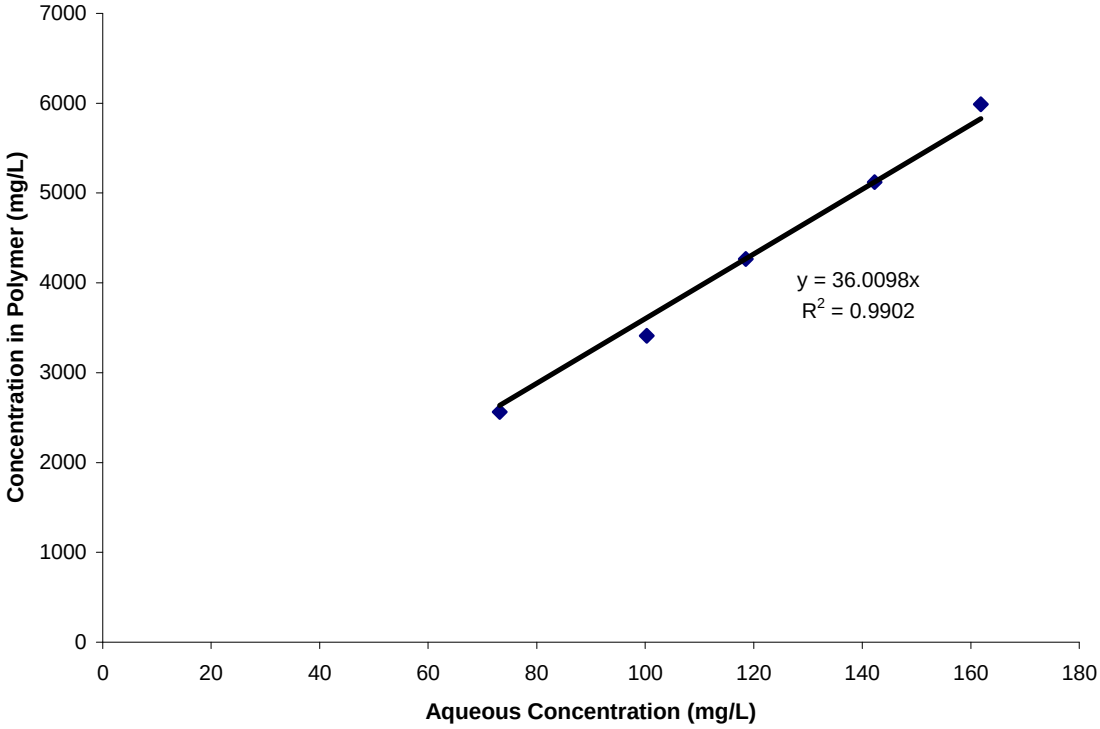

Figure D12: Carveol in Hytrel® 8206.

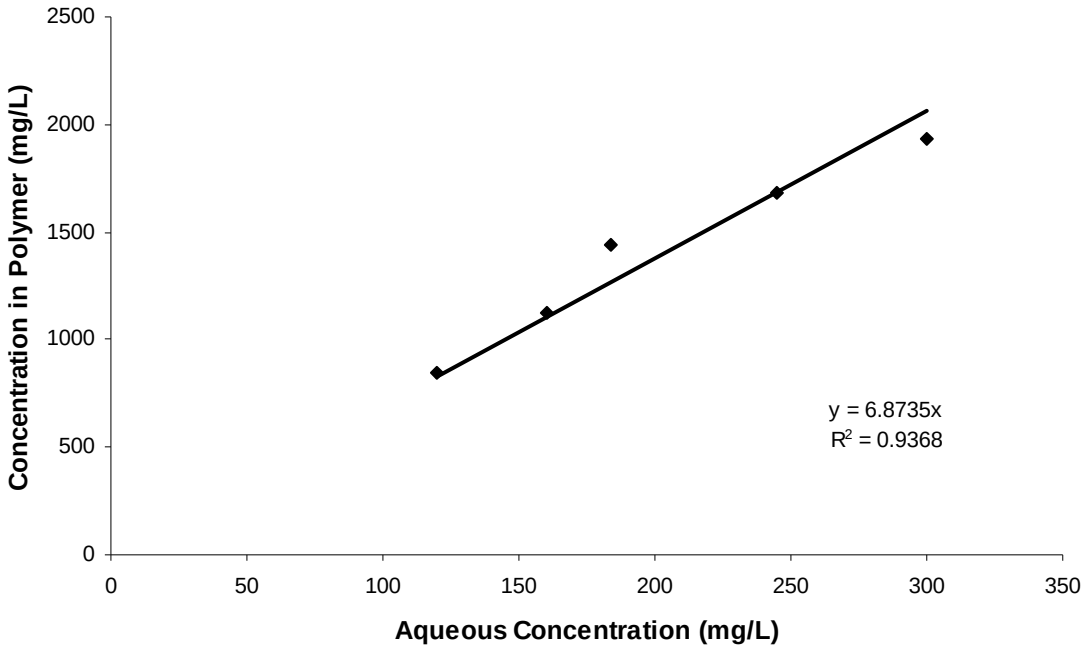

Figure D13: Carvone in Elvax® 770.

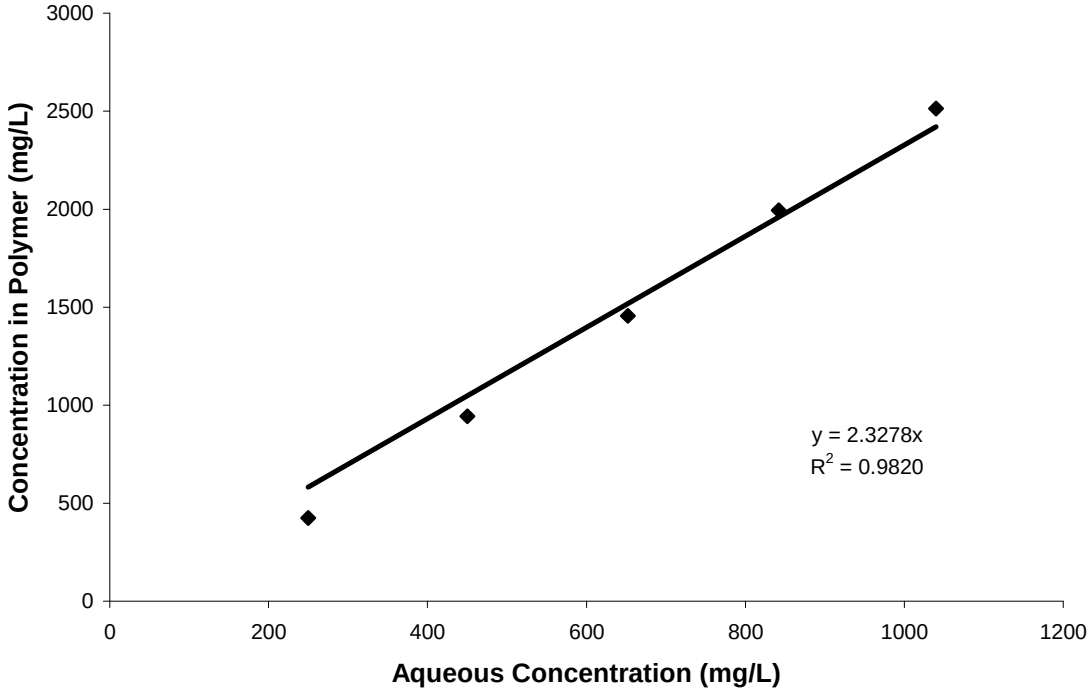

Figure D14: Carveol in Elvax® 770.

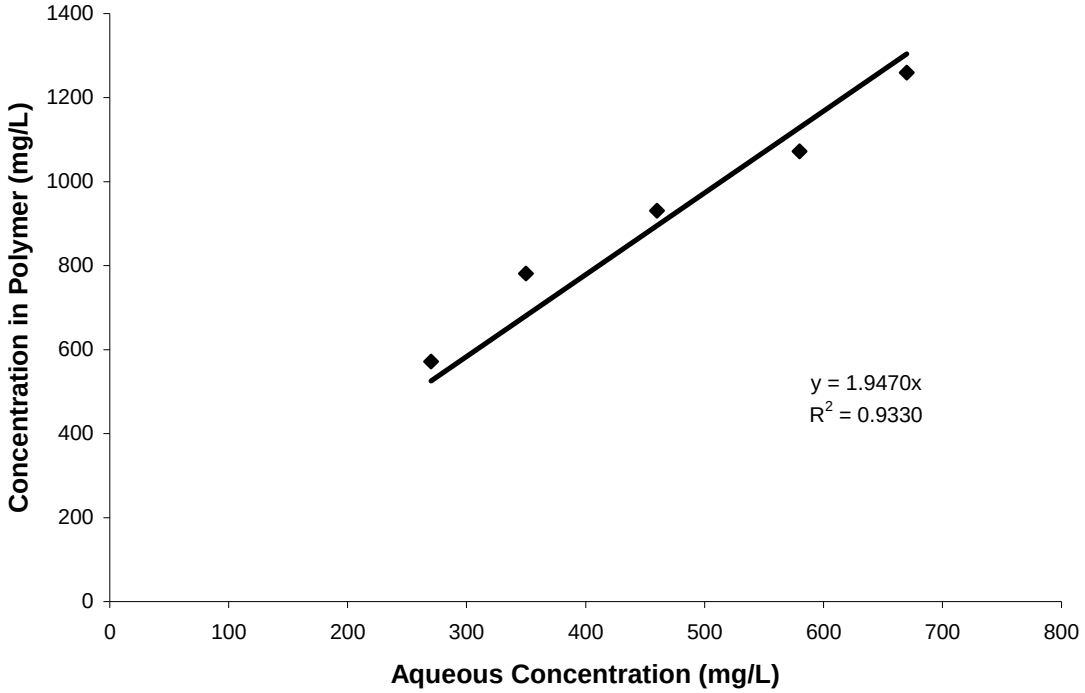

Figure D15: Carvone in Nucrel® 925.

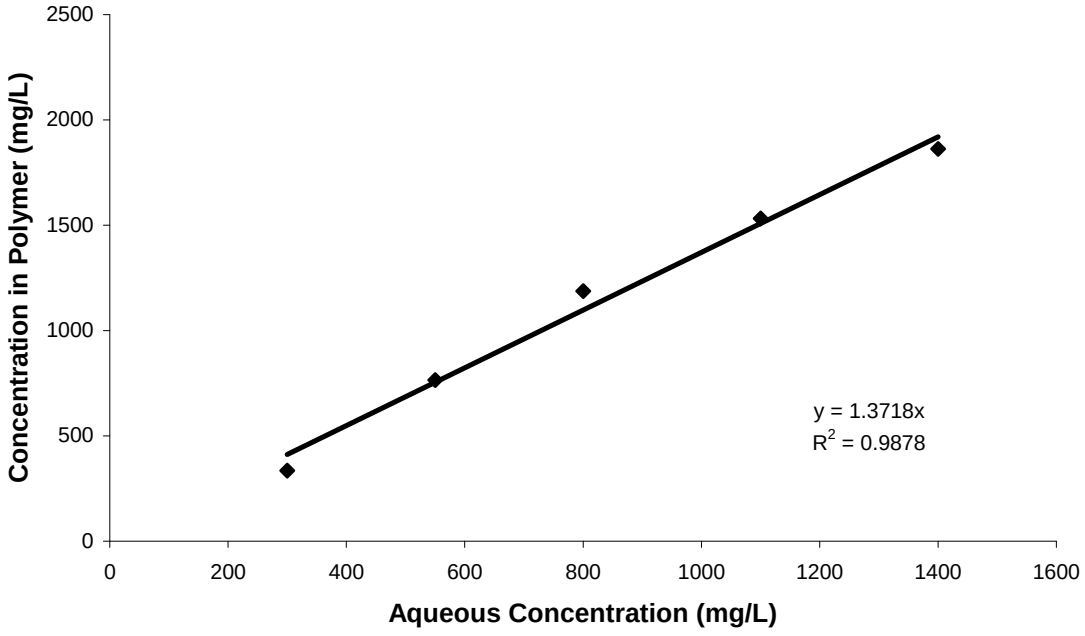

Figure D16: Carveol in Nucrel® 925.

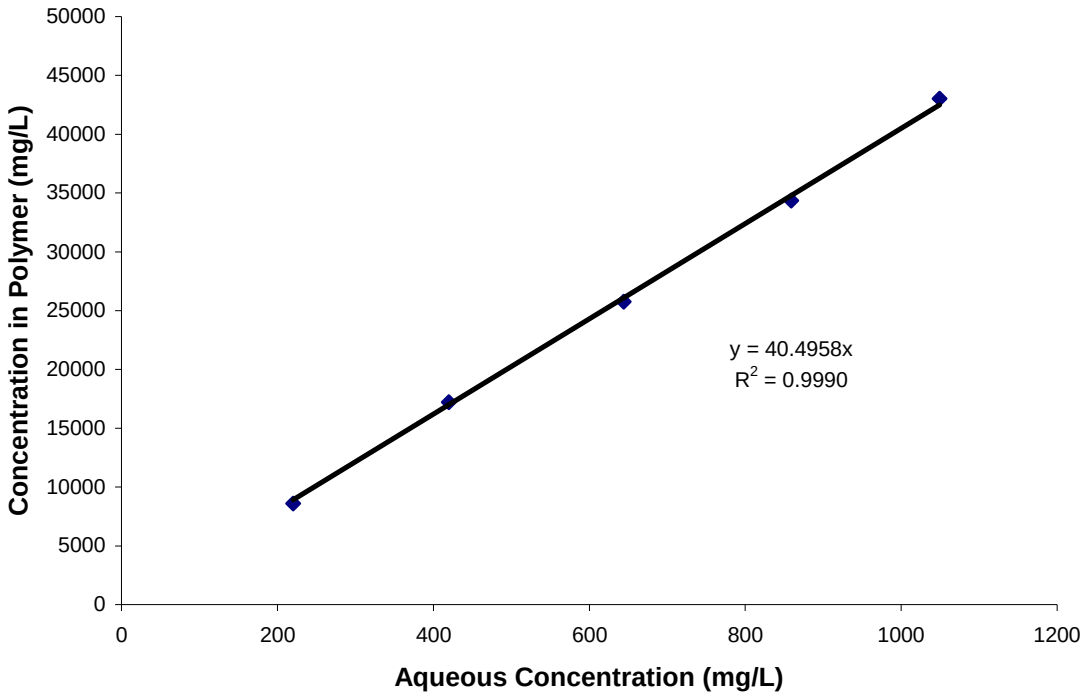

Figure D17: Carvone in TONE™ P787.

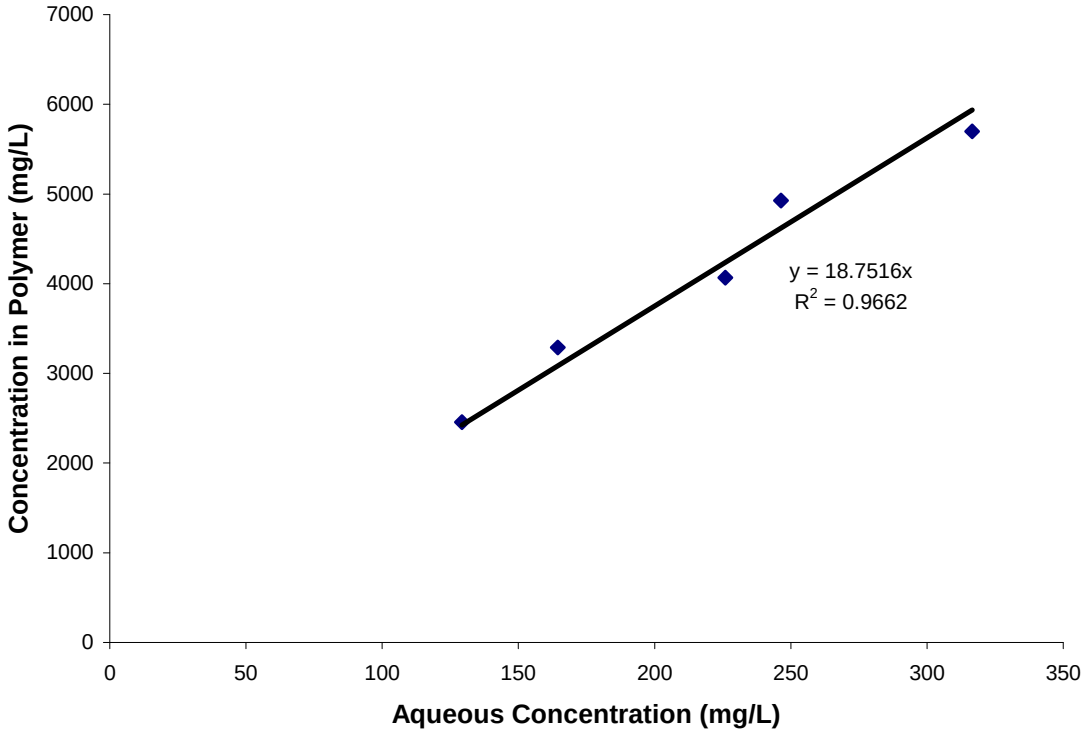

Figure D18: Carveol in TONE™ P787.

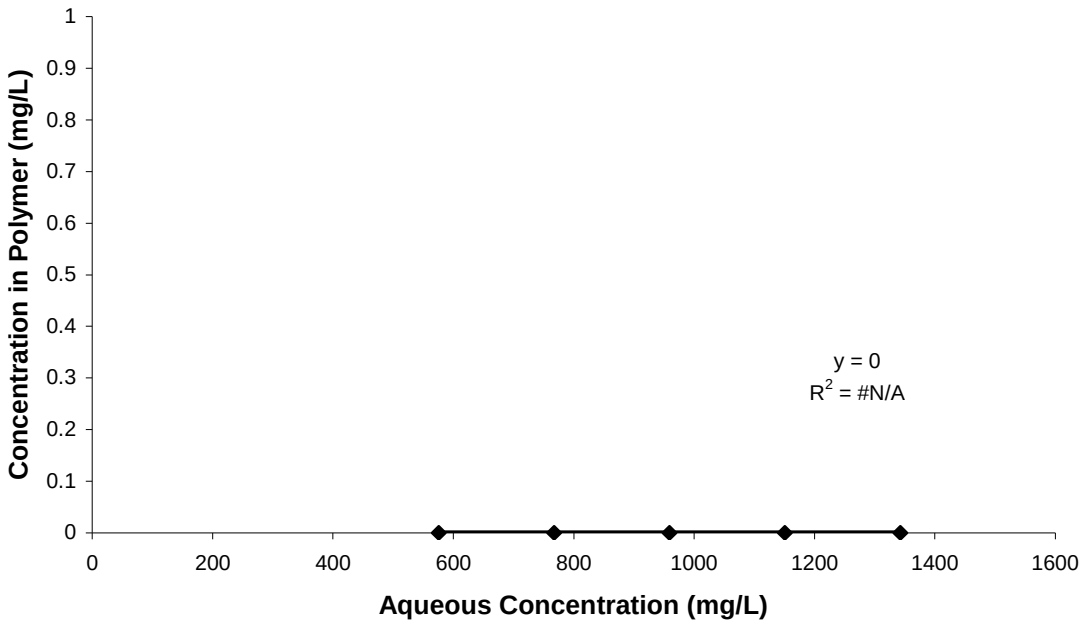

Figure D19: Carvone in ethylene/vinyl acetate.

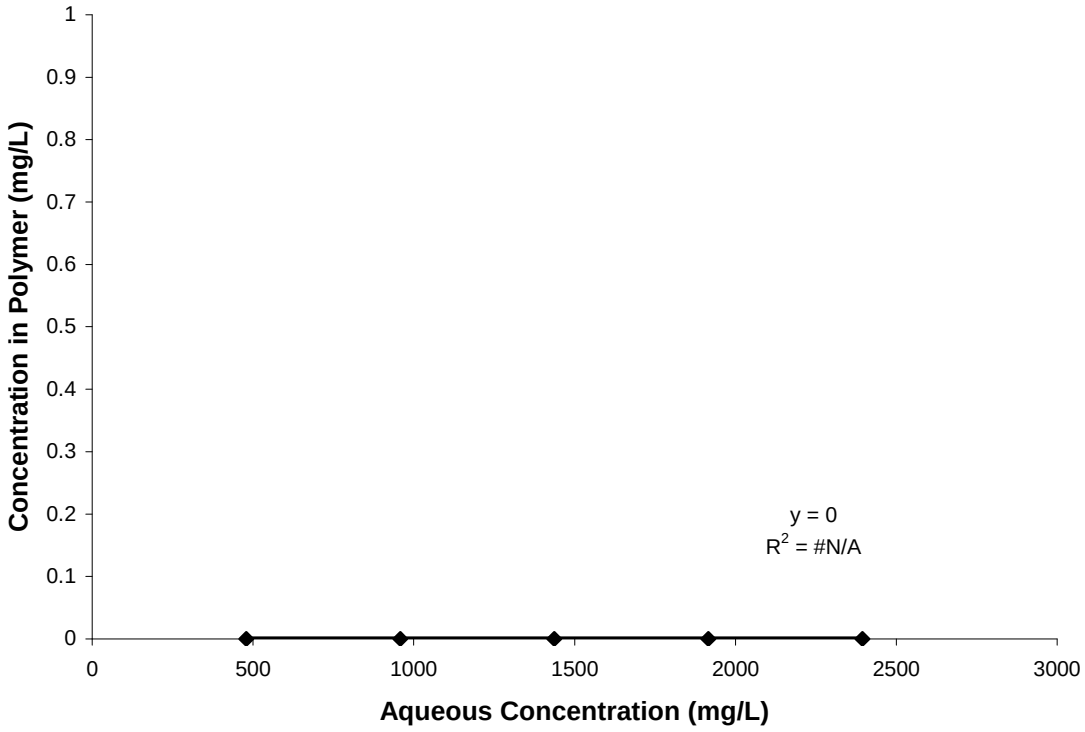

Figure D20: Carveol in ethylene/vinyl acetate.

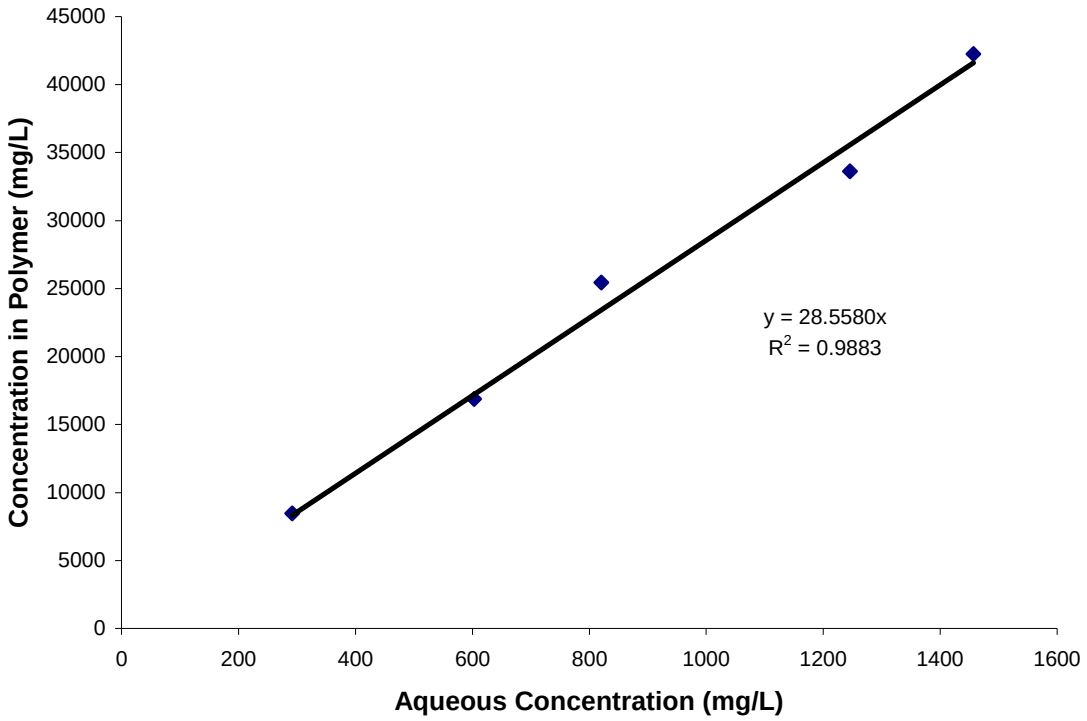

Figure D21: Carvone in Desmopan 453.

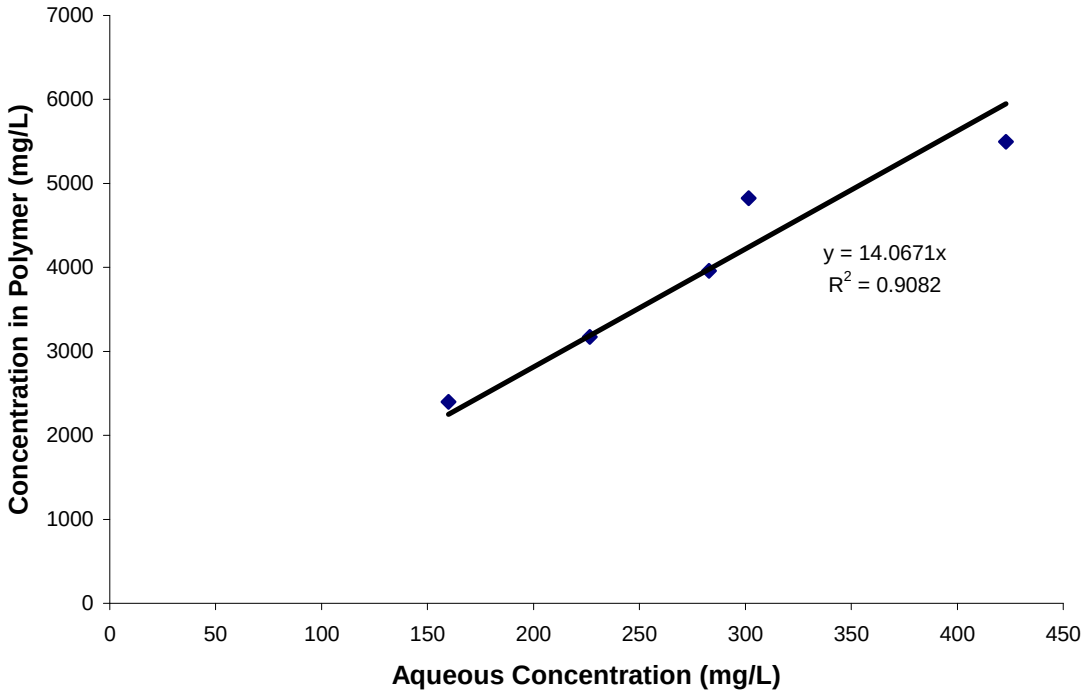

Figure D22: Carveol in Desmopan 453.

Appendix E:

Polymer Data Sheets

KRATON ® D4150K

K0249 North America 1/11/2007	KRATON® D4150K	Data Document

Identifier : K249DDd07U

Description

Kraton D4150K is a linear triblock copolymer based on styrene and butadiene with a polystyrene content of 31%. It is supplied from North America in the physical form identified below.

- Kraton D4150K -16 supplied as a dusted porous pellet.

Kraton D4150K is used as a modifier of bitumen and polymers. It is also suitable as an ingredient in formulating compounds for footwear applications, molded and extruded goods applications and may be used in formulating adhesives, sealants, and coatings. This product is also available as an undusted porous pellet (-00).

Sales Specifications

Property	Test Method	Units	Sales Specification Range	Notes
Polystyrene Content	BAM 919	%w	29 TO 33	
Oil	BAM 905	%w	32.3 TO 34.3	
Volatile Matter	BAM 907	%w	<= 0.7	
Dust, Talc	BAM 908	%w	0.25 TO 0.45	
Primary Antioxidant Content	BAM 929	%w	0.13 TO 0.35	

Typical Properties (These are typical values and may not routinely be measured on finished product)

Property	Test Method	Units	Typical Value	Notes
Styrene / Rubber ratio	n/a		31/69	
Diblock content	n/a		17	
Solution Viscosity	BAM 922	cps	850	a
Melt Index 200°C, 5kg	n/a	gms/10 Min.	9.5	
Specific gravity	ASTM D4025	gm/cc	0.92	
Hardness	ASTM 2240	Shore A (10s)	45	b
Tensile strength	ASTM D-412	psi	2,800	c
Elongation at break	ASTM D-412	%	1,400	c
300% Modulus	ASTM D-412	psi	160	c

a 25%w toluene solution at 25C
b Typical values on polymer compression molded at 300F.
c Measured on films cast from a solution in toluene.

Packaging

End Use Requirements

If the finished article is intended for use in food contact applications, toys, or human contact areas, manufacturers of the final product should observe all relevant regulations. Detailed information is available from Kraton Polymers.
For food packaging, manufacturers of the final product should ensure that all ingredients used comply with applicable regulations. Some of these regulations require tests to be carried out on the final product, e.g. migration. These are the responsibility of the final product manufacturer.

Restrictions on Medical/Healthcare Applications

Products or compounds made from Kraton Polymers' products shall not be used in any of the following applications: (a) cosmetics, (b) drugs and other pharmaceuticals, and (c) Class II and Class III Medical Devices, as defined in 21 CFR 860.3 (hereinafter collectively referred to as "Medical/Healthcare Applications"). Kraton Polymers requires that it give its prior written approval before its products are used in such Medical/Healthcare Applications. Please contact your Kraton Polymers Sales Representative for more details before using our products in these specific applications.
KRATON POLYMERS HAS NO SPECIFIC EXPERTISE IN THE MEDICAL/HEALTHCARE MARKET OR MEDICAL/HEALTHCARE APPLICATIONS AND DOES NOT INTEND TO PERFORM TESTING, CLINICAL STUDIES OR OTHER INVESTIGATIONS OF THE SUITABILITY OF ITS PRODUCTS FOR THESE SPECIFIC APPLICATIONS. KRATON POLYMERS MAKES NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE (INCLUDING MEDICAL/HEALTHCARE APPLICATIONS) FOR ITS PRODUCTS.
EACH CUSTOMER OR USER OF KRATON POLYMERS' PRODUCTS IS SOLELY RESPONSIBLE FOR DETERMINING THE SUITABILITY OF THE MATERIALS IT SELECTS FOR THE INTENDED PURPOSE. FOR MEDICAL/HEALTHCARE APPLICATIONS, EACH CUSTOMER OR USER MUST CONDUCT ITS OWN STUDIES, REGISTRATIONS, AND OTHER RELATED ACTIVITIES TO ESTABLISH THE SAFETY AND EFFICACY OF ITS PRODUCTS.
Do not use Kraton Polymers' tradenames, trademarks, logos or other similar identifying characteristics for the manufacture, sale or promotion of products intended for Medical/Healthcare Applications.

Safety and Handling Precautions

Read the Material Safety Data Sheet for Kraton Polymers' products carefully and thoroughly before beginning any work with such products. Additional information relating to the health, safety, storage, handling and processing of Kraton Polymers' products can be found in the Kraton Polymer HSE Fact Sheet (K0155), available from your local Kraton Polymers Sales Representative. Kraton Polymers also recommends that customers or users consult other sources of safety information, for example, the current edition of the "Code of Practice on the Toxicity and Safe Handling of Rubber Chemicals," British Rubber Manufacturers Association Limited (www.brma.co.uk).
Kraton Polymers' products and compounds can accumulate electrostatic charges when rubbed, chafed or abraded. Processing and storage equipment for use with Kraton Polymers' products should provide a means of dissipating any charges that may develop.

When processing Kraton Polymers' products, maintain a fire watch if the material reaches 225°C (437°F) for Kraton IR and Kraton D (polymers and compounds), and 280°C (536°F) for Kraton G (polymers and compounds). The temperatures listed above are indicated only for safety reasons (risk of fire and product degradation) and are not necessarily recommended for processing. Degradation of the polymer (polymer breakdown) will start at lower temperatures depending on the specific processing conditions. Therefore, operating below these temperatures does not guarantee the absence of product degradation.

Kraton Polymers' products (the neat resin or the base product) are high molecular weight polymers which by all accounts are non-toxic and biologically inactive.

Warranty

The information contained in this publication is, to the best of Kraton Polymers' knowledge, true and accurate, but any recommendations or suggestions that may be made are without guarantee, since the conditions of use and storage are beyond Kraton Polymers' control. The customer understands that it shall make its own assessment to determine the suitability of a Kraton Polymers' product for a particular purpose. Further, nothing contained herein shall be construed as a recommendation to use any Kraton Polymers product in conflict with existing patents. All products purchased from or supplied by Kraton Polymers are subject to terms and conditions set out in the applicable contract, order acknowledgement and/or bill of lading. Kraton Polymers warrants only that its products will meet those specifications designated therein.
KRATON POLYMERS MAKES NO OTHER WARRANTY, EITHER EXPRESS OR IMPLIED INCLUDING MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE; OR THAT THE USE OF SUCH INFORMATION OR KRATON POLYMERS PRODUCT WILL NOT INFRINGE ANY PATENT.

For further information:		**In Asia Pacific**	
USA Tel (toll free):	+1-800-4-Kraton (+1 800-457-2866)	Tel Japan	+81-3-3769-5990
USA Tel:	+1-281-504-4950	Hong Kong	+852-250-80-657
USA Fax:	+1-281-504-4953	Taiwan	+886-2-2545-1538
In Europe / Africa		China	+86-21-5109-5212
Europe Fax:	+44-(0)1829-773-130	South East Asia / India	+91-11-628-4324
In South America		Australia	+61-41-937-5055
Fax:	+55-(0)19-3874-7275	Or contact your local KRATON Polymers Representitive **Visit us at www.kraton.com** **Email info@kraton.com**	

Styrene/butadiene, ABA Block Copolymer

Table E1: Properties of Styrene/butadiene, ABA Block Copolymer.

Property	**Value**	**Unit**
Approximate MW	100 000	g/mol
Viscosity	1200	cp
Melt Flow Index	6	g/10min
Physical Form	rubbery pellets	-
Size	2 - 4	mm
Density	0.94	g/cc

SCIENTIFIC POLYMER PRODUCTS, INC.

MATERIAL SAFETY DATA SHEET

Effective Date:	06/96	**Catalog Number:**	451
Revision Date:	04/05	**Page Number:**	1

Scientific Polymer urges each customer or recipient of this MSDS to study it carefully to become aware of and understand the hazards associated with the product. The reader should consider consulting reference works or individuals who are experts in ventilation, toxicology, and fire prevention, as necessary or appropriate to use and understand the data contained in this MSDS.

To promote safe handling, each customer or recipient should: 1) Notify its employees, agents, contractors and others whom it knows or believes will use this material or the information in this MSDS and any other information regarding hazards or safety; 2) Furnish this same information to each of its customers for the product; 3) Request its customers to notify their employees, customers, and other users of the product of this information.

SECTION 1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Product Name Styrene/butadiene, ABA block copolymer

CAS Number: 9003-55-8 **Molecular Formula:** $(C_8H_8.C_4H_6)x$

Manufacturer Information:

Scientific Polymer Products
6265 Dean Parkway
Ontario, NY 14519

Non-Emergency Phone Number: 585/265-0413
Emergency Phone Number (24 hrs): 1-800-255-3924 (CHEM TEL)
Website: www.scientificpolymer.com

SECTION 2. COMPOSITION AND INFORMATION ON INGREDIENTS

Ingredient	CAS Number	%
Styrene/butadiene, ABA block copolymer	9003-55-8	~100
Antioxidant/stabilizer/may contain dusting agent		

SECTION 3. HAZARDS IDENTICATION

Emergency Overview:
Molten product adheres to the skin and causes burns. Electrostatic charges may be generated during handling. Risk of self-ignition of bulk product above certain temperatures. Dust explosion can occur.

Signs and Symptoms of Potential Overexposure:
This material is a rubber compound, which is essentially non-toxic. Material is not irritating. If polymer dusts are generated, they could scratch the eyes and cause minor irritation to the respiratory tract.

SECTION 4. FIRST AID MEASURES

INHALATION:
Remove to fresh air. If rapid recovery does not occur, obtain medical attention.

EYE CONTACT:
Flush eyes with water. Seek medical attention if necessary.

SKIN CONTACT:
If contact with hot material, cool the burn area by flushing with large amounts of water. DO NOT attempt to remove anything from the burn area or apply burn creams or ointments. Cover the burn area loosely with a sterile dressing, if available and seek medical attention.

INGESTION:
No specific measures.

SECTION 5. FIRE FIGHTING MEASURES

Flash Point/Method: N/A **Autoigition Temperature:** N/A

Flammable Limits: **UFL:** N/A **LFL:** N/A

Fire Fighting Instructions:
Full protective clothing and self-contained breathing apparatus.

Extinguishing Media:
Foam, water spray or fog, dry chemical powder, CO2, sand or earth may be used for small fires only. Do not use water in a jet – may disperse fire.

Fire and Explosion Hazards:
None known

Hazardous Combustion Products:
May include CO, CO2.

SECTION 6. ACCIDENTAL RELEASE MEASURES

Avoid raising dusts. Shovel up and place in to suitable containers for disposal.

SECTION 7. HANDLING AND STORAGE

HANDLING:
Avoid generation or accumulation of dusts. Take precautionary measures against static discharges, earth/ground all equipment. Avoid contact with heated or molten product. Do not breathe dust. Do not breathe fumes or vapors from heated product. Use local exhaust ventilation over processing area.

When processing this product, maintain a fire watch if the material reaches 225ºC. The temperature listed are indicated only for safety reasons (risk of fire and product degradation) and are not necessarily recommended for processing. Degradation of the polymer (polymer breakdown) will start at lower temperatures depending on the specific processing conditions. Therefore, operating below these temperatures does not guarantee the absence of product degradation. Static charge build up can be a potential fire hazard when used in the presence of volatile or flammable vapors or in high airborne dust concentrations.

STORAGE:
Keep container dry. Keep in a cool, well ventilated area. Keep away from direct sunlight and other sources of heat or ignition. Avoid storage of bulk product at temperatures above ambient to minimize risk of exothermic degradation, self heating and possible self ignition. Avoid storage under pressure or at elevated temperatures to minimize particulate clustering.

SECTION 8. EXPOSURE CONTROLS AND PERSONAL PROTECTION

EXPOSURE LIMITS:
Rubber Fume
TLV (EH40)
Rubber Manufacturing and Processing Giving Rise to Rubber Dust and Rubber Fume

Rubber Fume:	MEL/TWA (8 h) = 0.6 mg/m3
Rubber Process Dust:	MEL/TWA (8 h) = 6 mg/m3

MEL = Maximum Exposure Limit

Dust, Respirable Dust
TLV (EH40)

Total Inhalable Dust:	TWA (8 h) = 10 mg/m3
Respirable Dust:	TWA (8h) = 4 mg/m3

Personal Protective Equipment:
RESPIRATORY: Where local exhaust ventilation is not practicable and odors are detected use a negative pressure half face respirator equipped with a cartridge designed to protect against organic vapors and if dust is also present a particulate pre-filter should also be used. For high airborne dust concentrations use a cartridge designed to be used against nuisance dust. SKIN: Cloth gloves if desired. EYES: Dust-tight mono goggles.

Engineering Controls:
Use local exhaust ventilation

SECTION 9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance: White rubbery pellets **Odor:** Essentially odorless

Specific Gravity: <1 Solubility in Water: Insoluble

SECTION 10. STABILITY AND REACTIVITY

Stability:
Stable under ambient conditions. Oxidizes exothermically above ambient temperatures

Hazardous Polymerization: Will not occur

Conditions to Avoid:
Accumulation of product in areas exposed to elevated temperatures for extended periods in air may result in self-heating and auto ignition. Avoid elevated temperatures in storage for prolonged periods of time (example: 5 days at 200ºF).

Incompatibility: Strong oxidizing agents

Hazardous Decomposition Products:
Hazardous vapors from heated products are not expected to be generated under normal processing temperatures and conditions. Although highly dependent on temperature and environmental conditions, a variety of thermal decomposition products may be present if the product is over heated, is smoldering or catches fire. These range from simply hydrocarbons (such as methane and propane) to toxic/irritating vapors (such as CO, CO2, acrolein, aldehydes and ketones).

SECTION 11. TOXICOLOGICAL INFORMATION

Basis for Assessment:
Toxicological data have not been determined specifically for this product. Information given is based on a knowledge of the toxicology of similar products.

Acute Oral Toxicity:	Expected to be of low toxicity, LD50 > 2,000 mg/kg
Acute Dermal Toxicity:	Expected to be of low toxicity, LD50 > 2,000 mg/kg
Skin Irritation:	Not expected to be irritating
Eye Irritation:	Not expected to be irritating
Skin Sensitization:	Not expected to be a skin sensitizer
Repeated Dose Toxicity:	Repeated exposure does not cause significant toxic effects.
Mutagenicity:	Not considered to be a mutagenic hazard.

SECTION 12. ECOLOGICAL INFORMATION

Basis for Assessment:
Ecotoxicological data have not been determined specifically for this product. The information given below is based on a knowledge of the components and the ecotoxicology of similar products.

Mobility:	Floats on water. Remains on surface of soil.
Persistence/Degradability:	Expected to be not biodegradable. Persists under anaerobic conditions.
Bioaccumulation:	Not expected to bioaccumulate

Acute Toxicity (Fish, Invertabrates, Algae, Bacteria) and Sewage Treatment:
Expected to be practically non-toxic, LC/EC/IC 50 > 1,000 mg/l

SECTION 13. DISPOSAL CONSIDERATIONS

Waste Disposal:
Recover or recycle if possible, otherwise incineration or licensed landfill. However, local, state, federal, international, or country specific regulations take precedents. They may vary, and they may be more stringent but they must be strictly enforced and complied with.

If this material becomes a waste and has not been chemically altered, it is not considered a hazardous waste defined by RCRA (40 CFR 261).

SECTION 14. TRANSPORT INFORMATION

US Department of Transportation Classification:
The material is not subject to DOT regulations under 49 CFR Parts 171-180.

International Air Transportation Association Classification:
This material is not classified as hazardous under IATA regulations

International Maritime Organization – IMDG:
This material is not classified as hazardous under IMDG regulations

UN, IMO, ADR/RID, ICAO Code:
This material is not dangerous for conveyance under these codes.

SECTION 15. REGULATORY INFORMATION

The regulatory information provided is not intended to be comprehensive. Other local, state, federal, international or country specific regulations may apply to this material.

EUROPE – EC Classification: Not classified as dangerous under EC criteria

US Legislation:
US Federal – Superfund Amendment & Reauthorization Act (SARA) Title III:
This material is not regulated under SARA Title III

US Federal – Toxic Substances Control Act (TSCA) Inventory Status:
Component(s) of this material is (are) listed on the EPA TSCA Inventory of Chemical Substances.

US State – California Safe Drinking Water:
This material is not regulated by the California Safe Drinking Act.

US State – Toxic Enforcement Act (Proposition 65):
This material is not regulated by the Toxic Enforcement Act (Proposition 65)

US State – New Jersey Right to Know List:
This material is not regulated by the New Jersey Right to Know Act.

US State – Pennsylvania Right to Know List:
This material is not regulated by Pennsylvania Right to Know Act

SECTION: 16. OTHER INFORMATION

HMIS HAZARD RATING

HEALTH:	0
FLAMMABILITY:	1
REACTIVITY:	0

PERSONAL PROTECTION: B

FIRE:
Material that must be preheated before ignition can occur.
HEALTH:
Materials which on exposure under fire conditions would offer no hazard beyond that of ordinary combustible material.
REACTIVITY:
Materials which in themselves are normally stable, even under fire exposure conditions, and which are not reactive with water.

This material is intended for laboratory use only. It is not sold or intended for drug, household or other uses. The information represents the most accurate and complete data currently available to us. However, we make no warranty, express or implied, with respect to such information, and we assume no liability resulting from its use.

TONE™ P787

TONE P787 Polymer

CAS Reg. No. 24980-41-4

Description

TONE™ P787 polymer is a linear polycaprolactone polyester with high crystallinity and a low melting point, which can be injection molded, extruded or blended with other polymers. TONE P787 polymer was specially formulated for use in high melt strength applications.

Applications

- Hot Melt Adhesives
- Shoe Counters
- Biodegradable Applications
- Molding and Thermal Transfer
- Polymer Modification
- Pigment Dispersant
- Extrusion Aid

Advantages

- Low-Melting
- Low Toxicity
- Biodegradable (Compostable)
- Good Adhesion
- Good Blend Compatibility
- High Crystallinity (Green Strength)

Physical Properties(1)	
Physical Form	pellets
Melt Flow at 80 °C/44 psi, g/10 min	0.5
Molecular Weight, Number Average (approx.)	80000
Tensile Strength, 2 in/min, psi (MPa)	6000 (41.4)
Tensile Modulus, psi (MPa)	56,000 (386)
Elongation to Break, 2 in/min, %	900
DSC Glass Transition Temp. (T_g), °C	-69
Melting Temperature, °F (°C)	140 (60)
Viscosity at 200 °C, Poise at 55 °C	1500
Density, g/cc at 23 °C	1.145

(1) These data are typical values and cannot be construed as specifications. For product handling and safety aspects, please refer to the Material Safety Data Sheet.

Storage and Handling

Store below 120 °F in a well-sealed container. Product will absorb water moisture if exposed to the atmosphere for prolonged periods, resulting in accelerated degradation.

Melt process at a 93 - 120 °C zone temperature (For detailed conditions, see full product brochure). Do not heat above 90 °C for prolonged periods. Polycaprolactone in the melt will solubilize other resins present in the equipment. Avoid contact with zinc, galvanized iron, copper and its alloys.

For further information contact: Customer Information Group

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e-mail: dicinfo@euronet.nl

United States and Canada: Tel: 1-800-447-4369
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Fax: 1-989-832-1465 (U.S.A.)

Published December 2001

Form No. 321-00052

Desmopan 453

Bayer MaterialScience

DESMOPAN 453

Thermoplastic Polyurethane

Polyester-Based Grade

Description

Desmopan 453 resin is a polyester-based thermoplastic polyurethane with a Shore hardness of approximately 53D.* It can be processed by injection molding; extrusion processes are not recommended.

Applications

Desmopan 453 resin offers outstanding abrasion resistance, impact strength, toughness, and flexibility. It also exhibits excellent low-temperature and compression properties as well as excellent fuel and oil resistance. Applications include casters, couplings, toplifts, sleeves, gears, and other injection molded items. However, as with any product, use of Desmopan 453 resin in a given application must be tested (including field testing, etc.) in advance by the user to determine suitability.

Storage

Desmopan thermoplastic polyurethane resins are hygroscopic and will absorb ambient moisture. The presence of moisture can adversely affect processing characteristics and the quality of parts. Therefore, the resins should remain in their sealed containers and be stored under cool and dry conditions until used. Storage temperature should not exceed 86°F (30°C). Unused resin from opened containers, or reground material that is not to be used immediately, should be stored in sealed containers.

Drying

Prior to processing, Desmopan 453 resin must be thoroughly dried in a desiccant dehumidifying hopper dryer. Hopper inlet air temperature should be 210–230°F (99–110°C). To achieve the recommended moisture content of less than 0.03%, the inlet air dew point should be 0°F (-18°C) or lower. The hopper capacity should be sufficient to provide a minimum residence time of 2 hours.

Injection Molding

General-purpose screws are satisfactory for use with Desmopan 453 resin. The recommended screw length-to-diameter (L/D) ratio is 20:1 with a compression ratio of 2.5–3:1. Screws with a compression ratio greater than 4:1 should be avoided.

Typical Injection Molding Conditions

Barrel Temperature:	
Rear	380–410°F (190–210°C)
Middle	380–420°F (190–220°C)
Front	390–430°F (195–225°C)
Nozzle	400–440°F (200–230°C)
Ideal Melt Temperature	410°F (210°C)
Mold Temperature:	
Stationary Part	60–110°F (15–45°C)
Moving Part	60–110°F (15–45°C)
Injection Pressure:	
1st Stage	7,000–13,000 psi
2nd Stage	6,000–10,000 psi
Clamp Pressure	3–5 ton/in^2 of projected part area
Shot Weight	40–80% of rated barrel capacity
Timers (per 0.125-in cross section):	
Boost	5–10 sec
2nd Stage	10–20 sec
Cool	20–30 sec

Typical values for mold shrinkage to be taken into account are as follows:

Cross Section	Mold Shrinkage
Less than 1⁄8 inch	7–10 mils per inch
1⁄8 to 1⁄4 inch	10–15 mils per inch
Over 1⁄4 inch	15–20 mils per inch

For treatments such as postcuring, an additional 1 to 1.5 mil per inch should be added.

**These items are provided as general information only. They are approximate values and are not part of the product specifications.*

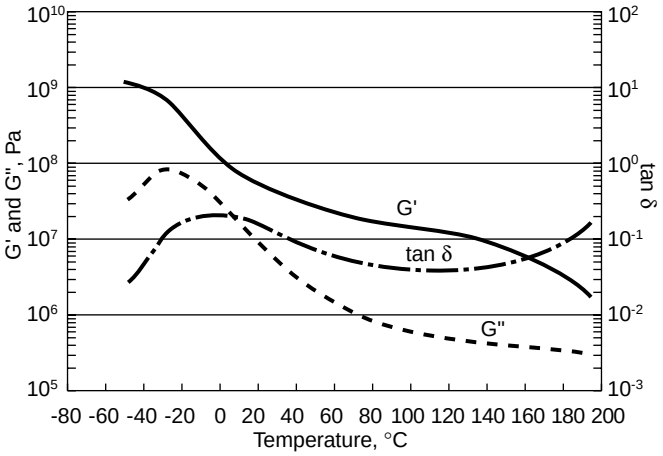
Regrind Usage

For Desmopan resin, up to 20% regrind may be used with virgin material, depending upon the end-use requirements of the molded part and provided that the material is kept free of contamination and is properly dried (see section on Drying). Any regrind used must be generated from properly molded parts, sprues, and/or runners. All regrind used must be clean, uncontaminated, and thoroughly blended with virgin resin prior to drying and processing. Under no circumstances should degraded, discolored, or contaminated material be used for regrind. Materials of this type should be discarded.

Improperly mixed and/or dried resin may diminish the desired properties of Desmopan resin. You must conduct testing on finished parts produced with any amount of regrind to ensure that your end-use performance requirements are fully met. Regulatory organizations (e.g., UL) may have specific requirements limiting the allowable amount of regrind. Because third party regrind generally does not have a traceable heat history, nor offer any assurance that proper temperatures, conditions, and/or materials were used in processing, extreme caution must be exercised in buying and using regrind from third parties.

The use of regrind material should be avoided entirely in those applications where resin properties equivalent to virgin material are required, including but not limited to color quality, impact strength, resin purity, and/or load-bearing performance.

Dynamic Mechanical Analysis of Desmopan 453 Resin



Health and Safety Information

Appropriate literature has been assembled which provides information concerning the health and safety precautions that must be observed when handling Desmopan 453 resin. Before working with this product, you must read and become familiar with the available information on its hazards, proper use and handling. This cannot be overemphasized. Information is available in several forms, e.g., material safety data sheets and product labels. Consult your local Bayer MaterialScience resentative or contact Bayer's Product Safety and Regulatory Affairs Department in Pittsburgh, PA.

Note: The information contained in this bulletin is current as of July 1995. Please contact Bayer MaterialScience to determine whether this publication has been revised.

Typical Physical Properties* of Natural Resin	ASTM Test Method (Other)	Units U.S. Conventional	Units SI Metric	Desmopan 453 Resin U.S. Conventional	Desmopan 453 Resin SI Metric
General					
Specific Gravity	D 792 (ISO 1183)			1.22	
Shore Hardness	D 2240 (ISO 868)	D Scale		53	
Taber Abrasion:	D 3489 (ISO 4649)				
H-18, 1000-g Load, 1,000 Cycles		mg Loss		50	
Bayshore Resilience	D 2632	%		40	
Mold Shrinkage at 100-mil Thickness:	D 955 (ISO 2577)				
Flow Direction		in/in (mm/mm)		0.008	
Cross-Flow Direction		in/in (mm/mm)		0.008	
Mechanical					
Tensile Strength	D 412 (ISO 37)	lb/in^2	MPa	5,000	34.5
Tensile Stress at 50% Elongation	D 412 (ISO 37)	lb/in^2	MPa	1,800	12.4
Tensile Stress at 100% Elongation	D 412 (ISO 37)	lb/in^2	MPa	2,000	13.8
Tensile Stress at 300% Elongation	D 412 (ISO 37)	lb/in^2	MPa	3,100	21.4
Ultimate Elongation	D 412 (ISO 37)	%		500	
Tear Strength, Die "C"	D 624 (ISO 34)	lbf/in	kN/m	900	157.6
Flexural Modulus:	D 790 (ISO 178)				
158°F (70°C)		lb/in^2	MPa	7,390	51.0
73°F (23°C)		lb/in^2	MPa	15,000	103.4
-22°F (-30°C)		lb/in^2	MPa	110,000	758.4
Compression Set:	D 395-B (ISO 815)				
As Molded (Post-Cured)**					
22 Hours at 158°F (70°C)		%		62 (35)	
22 Hours at 73°F (23°C)		%		17 (15)	
Compressive Load:	D 575				
2% Deflection		lb/in^2	MPa	100	0.7
5% Deflection		lb/in^2	MPa	350	2.4
10% Deflection		lb/in^2	MPa	650	4.5
15% Deflection		lb/in^2	MPa	950	6.6
20% Deflection		lb/in^2	MPa	1,250	8.6
25% Deflection		lb/in^2	MPa	1,550	10.7
50% Deflection		lb/in^2	MPa	4,550	31.4
Thermal					
Low-Temperature Brittle Point	D 746 (ISO 974)	°F	°C	<-90	<-68
Glass Transition Temperature (Tg)	(DMA)#	°F	°C	-29	-34
Vicat Softening Temperature (Rate A)	D 1525 (ISO 306)	°F	°C	293	145

** These items are provided as general information only. They are approximate values and are not part of the product specifications.*
*** Postcured 16 hrs at 230°F (110°C).*
 # DMA—Dynamic Mechanical Analysis.

Desmopan 453 Resin Property Changes after Aging	ASTM Test Method (Other)	Unit of Change	70 Hrs	7 Days	14 Days	21 Days
Hot Air at 212°F (100°C)	D 573 (ISO 216)					
Tensile Strength		%	+5	-10	-3	-3
Tensile Stress at 100% Elongation		%	+4	0	+9	+8
Tensile Stress at 300% Elongation		%	+10	+7	+10	+10
Ultimate Elongation		%	+4	0	+5	+4
Hardness		Shore D	-3	-2	0	-2
ASTM Oil #1 at 212°F (100°C)	D 471 (ISO 175)					
Tensile Strength		%	—	-6	-5	-15
Tensile Stress at 100% Elongation		%	—	+10	+6	+3
Tensile Stress at 300% Elongation		%	—	+13	+3	-10
Ultimate Elongation		%	—	0	-4	-11
Hardness		Shore D	—	-2	0	0
Volume		%	—	0	0	0
ASTM Oil #3 at 212°F (100°C)	D 471 (ISO 175)					
Tensile Strength		%	—	+11	-8	-14
Tensile Stress at 100% Elongation		%	—	+4	+3	+2
Tensile Stress at 300% Elongation		%	—	+7	+2	-2
Ultimate Elongation		%	—	+8	+9	+11
Hardness		Shore D	—	0	0	0
Volume		%	—	+5	+5	+6
Fuel A at 73°F (23°C)	D 471 (ISO 175)					
Tensile Strength		%	0	+3	+1	+8
Tensile Stress at 100% Elongation		%	+4	+3	+4	+8
Tensile Stress at 300% Elongation		%	+5	+5	+5	+10
Ultimate Elongation		%	-6	-3	-6	+2
Hardness		Shore D	+2	-2	0	0
Volume		%	+2	0	+1	+1
Fuel C at 73°F (23°C)	D 471 (ISO 175)					
Tensile Strength		%	—	-15	-27	-31
Tensile Stress at 100% Elongation		%	—	-26	-26	-25
Tensile Stress at 300% Elongation		%	—	-30	-29	-29
Ultimate Elongation		%	—	+8	+6	+2
Hardness		Shore D	—	-10	-10	-9
Volume		%	—	+18	+18	+18

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Elvax® 770

DuPont™ Elvax® 770

Elvax® resins Product Data Sheet

Description

Product Description	DuPont™ Elvax® 770 is an ethylene-vinyl acetate copolymer resin for use in industrial applications.

Restrictions

Material Status	• Commercial: Active
Availability	• Globally

Typical Characteristics

Composition	9.5% By Weight Vinyl Acetate Thermal Stabilizer: BHT antioxidant
Applications	Elvax® resins can be used in a variety of applications involving molding, compounding, extrusion, adhesives, sealants, and wax blends. For additional information and properties associated with specific applications, please refer to the Grade Selector Guides found on the Elvax® website for industrial applications. http://www.dupont.com/industrial-polymers/elvax/index.html.

Typical Properties

Physical	Nominal Values	Test Method (s)	
Density ()	0.93 g/cm³	ASTM D792	ISO 1183
Melt Index (190°C/2.16kg)	0.8 g/10 min	ASTM D1238	ISO 1133

Thermal	Nominal Values	Test Method (s)	
Melting Point (DSC)	96°C (205°F)	ASTM D3418	ISO 3146
Vicat Softening Point ()	80°C (176°F)	ASTM D1525	ISO 306

Processing Information

General

Maximum Processing Temperature	230°C (446°F)
General Processing Information	Elvax® resins can be processed by conventional thermoplastic processing techniques, including injection molding, structural foam molding, sheet and shape extrusion, blow molding and wire coating. They can also be processed using conventional rubber processing techniques such as Banbury, two-roll milling and compression molding. Elvax can be used in conventional extrusion equipment designed to process polyethylene resins. However, corrosion-protected barrels, screws, adapters, and dies are recommended, since, at sustained melt temperatures above 446°F (230°C), ethylene vinyl acetate (EVA) resins may thermally degrade and release corrosive by-products.

FDA Status Information	ELVAX® 770 EVA Resin complies with Food and Drug Administration Regulation 21 CFR 177.1350(a)(1) - - Ethylene-vinyl acetate copolymers, subject to the limitations and requirements therein. This Regulation describes polymers that may be used in contact with food, subject to the finished food-contact article meeting the extractive limitations under the intended conditions of use, as shown in paragraph (b)(1) of the Regulation.
Safety & Handling	A Product Safety Bulletin, Material Safety Data Sheet, and more detailed information on compounding and processing Elvax® resins for specific applications are available from your DuPont Packaging and Industrial Polymers representative.

Read and Understand the Material Safety Data Sheet (MSDS) before using this product

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guarantees of results and assumes no obligations or liability in connection with its advice.

This data sheet is effective as of 01/15/2007 04:32:12 PM and supersedes all previous versions.

Nucrel® 925

DuPont™ Nucrel® 925

Nucrel® resins Product Data Sheet

Description

Product Description	Nucrel® 925 is a copolymer of ethylene and methacrylic acid, made with nominally 15 wt% methacrylic acid. It is inherently flexible without the need for plasticizers. The resin can be pigmented, UV-stabilized for exterior applications and painted or plated for special decorative effects. Nucrel® 925 has excellent tensile strength and low temperature impact properties.

Restrictions

Material Status	• Commercial: Active

Typical Characteristics

Composition	15% By Weight Methacrylic Acid

Typical Properties

Physical	Nominal Values	Test Method(s)	
Density ()	0.94 g/cm³	ASTM D792	ISO 1183
Melt Flow Rate (190°C/2.16kg)	25 g/10 min	ASTM D1238	ISO 1133

Thermal	Nominal Values	Test Method(s)	
Melting Point (DSC)	92°C (198°F)	ASTM D3418	ISO 3146
Freezing Point (DSC)	74°C (165°F)	ASTM D3418	
Vicat Softening Point ()	67°C (153°F)	ASTM D1525	ISO 306

Processing Information

General

Maximum Processing Temperature	235°C (455°F)
General Processing Information	Nucrel® 925 is readily processed in conventional extrusion equipment. Melt temperature may be varied over the range of 135-235°C (275-455°F). Materials of construction used in the processing of this resin should be corrosion resistant. Stainless steels of the types 316, 15-5PH, and 17-4PH are excellent, as is quality chrome or nickel plating, and in particular duplex chrome plating. Type 410 stainless steel is satisfactory, but needs to be tempered at a minimum temperature of 600°C (1112°F) to avoid hydrogen-assisted stress corrosion cracking. Alloy steels such as 4140 are borderline in performance. Carbon steels are not satisfactory. While stainless steels can provide adequate corrosion protection, in some cases severe purging difficulties have been encountered. Nickel plating has been satisfactory, but experiments have shown that chrome surfaces have the least adhesion to acid based polymers. In recent years, the quality of chrome plating has been deteriorating due to environmental pressures, and the corrosion protection has not always been adequate. Chrome over top of stainless

steel seems to provide the best combination for corrosion protection and ease of purging.

If surface properties of the extruded resin require modification (such as, lower C.o.F. for packaging machine processing), refer to the Conpol™ Processing Additive Resins product information guide.

After processing Nucrel, purge the material out using a polyethylene resin, preferably with a lower melt flow rate than the Nucrel resin in use.
The "Disco Purge Method" is suggested as the preferred purging method, as this method usually results in a more effective purging process. Information on the Disco Purge Method can be obtained via your DuPont Sales Representative.

Never shut down the extrusion system with Nucrel in the extruder and die. Properly purge out the Nucrel with a polyethylene, and shut down the line with polyethylene or polypropylene in the system.

FDA Status Information

Nucrel® 925 resin conforms to the U.S. Code of Federal Regulations, Title 21, paragraph 177.1330, covering their use as a food contact surface subject to the extractives limitations on the finished food contact articles as described in the regulation.

Safety & Handling

Nucrel® methacrylic acid copolymer resins as supplied by DuPont are not considered hazardous materials. As with any hot material, care should be taken to protect the hands and other exposed parts of the body when handling molten polymer. At recommended processing temperatures, small amounts of fumes may evolve from the resins. When resins are overheated, more extensive decomposition may occur. Adequate ventilation should be provided to remove fumes from the work area. Disposal of scrap presents no special problems and can be by landfill or incineration in a properly operated incinerator. Disposal should comply with local, state, and federal regulations. Resin pellets can be a slipping hazard. Loose pellets should be swept up promptly to prevent falls. For more detailed information on the safe handling and disposal of DuPont resins, a Material Safety Data Sheet can be obtained from the DuPont Packaging and Industrial Polymers website or by contacting your sales representative.

Read and Understand the Material Safety Data Sheet (MSDS) before using this product

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This data sheet is effective as of 05/15/2007 02:07:36 PM and supersedes all previous versions.

Ethylene/vinyl Alcohol Copolymer

Table E2: Properties of Ethylene/vinyl Alcohol Copolymer.

Property	**Value**	**Unit**
Approximate MW	-	g/mol
Viscosity	-	cp
Melt Flow Index	3.5	g/10min
Physical Form	pellets	-
Size	2	mm
Density	1.14	g/cc

SCIENTIFIC POLYMER PRODUCTS, INC.

MATERIAL SAFETY DATA SHEET

Effective Date:	06/96	**Catalog Number:**	960
Revision Date:	05/03	**Page Number:**	1

Scientific Polymer urges each customer or recipient of this MSDS to study it carefully to become aware of and understand the hazards associated with the product. The reader should consider consulting reference works or individuals who are experts in ventilation, toxicology, and fire prevention, as necessary or appropriate to use and understand the data contained in this MSDS.

To promote safe handling, each customer or recipient should: 1) Notify its employees, agents, contractors and others whom it knows or believes will use this material or the information in this MSDS and any other information regarding hazards or safety; 2) Furnish this same information to each of its customers for the product; 3) Request its customers to notify their employees, customers, and other users of the product of this information.

1. CHEMICAL PRODUCT AND COMPANY INFORMATION

IDENTIFICATION

Product Name Ethylene/vinyl alcohol copolymer

Molecular Formula $(C_4H_6O_2.C_2H_4O.C_2H_4)x$

COMPANY INFORMATION

Scientific Polymer Products
6265 Dean Parkway
Ontario, NY 14519

Telephone: 585/265-0413
Fax: 585/265-1390
website: www.scientificpolymer.com

EMERGENCY INFORMATION

24 Hours a day: CHEM-TEL 1-800-255-3924

2. COMPOSITION INFORMATION

Component	CAS#	%
Ethylene/vinyl alcohol copolymer	26221-27-2	>99

3. HAZARDS INFORMATION

EMERGENCY OVERVIEW

Appearance: Off-white pellets **Odor:** Odorless

Hazards of Product: None known

Potential Health Effects:
Powdered or finely ground dust cloud could cause eye irritation. Molten or heated material can cause serious burns to unprotected skin.

4. FIRST AID PROCEDURES

INHALATION:
If overcome by finely ground or heated fumes, immediately remove victim to fresh air. If victim has stopped breathing, give artificial respiration, preferably mouth to mouth. Get medical attention immediately.

EYE CONTACT:
If finely ground dusts gets in the eyes, immediately flush eyes with plenty of cool water to remove particles from eyes.

SKIN CONTACT:
Wash affected area with soap and water.

INGESTION:
Call a physician or the Poison Control Center immediately.

NOTES TO PHYSICIAN: None known

5. FIREFIGHTING MEASURES

Flash Point: 550ºF (COC) **Autoignition Temperature:** N/A
Flammable Limits: N/A

EXTINGUSIHING MEDIA:

SPECIAL FIREFIGHTING PROCEDURES:
Fire fighters should wear self contained breathing apparatus in the positive pressure mode with a full face piece when there is a possibility of exposure to smoke, fumes or hazardous decomposition products.

UNUSUAL FIRE AND EXPLOSION HAZARDS:
The application of high velocity water will spread the burning surface layer. Finely ground powder particles in the atmosphere are combustible and may be explosive. This material may generate static electrical charges when conveyed or poured in an extremely dry environment.

HAZARDOUS COMBUSTION PRODUCTS:
CO, olefinic and paraffinic compounds, trace amounts of organic acids, ketones, aldehydes and alcohols may be formed.

6. ACCIDENTAL RELEASE MEASURES

Steps to be taken if material is released or spilled:
Pellets spilled on walking surfaces constitute a slipping hazard. Work areas should be kept clean and free of pellets. Normal procedures for clean up. Use good housekeeping practices.

7. HANDLING AND STORAGE

HANDLING:
When handling finely ground material, ground transfer, blending and dust collecting equipment to prevent static sparks. Remove all ignition sources from handling area. Do not use near open flame or areas where smoking is permitted.

STORAGE:
Keep container dry. Keep container closed to prevent contamination.

8. EXPOSURE CONTROLS AND PERSONAL PROTECTION

EXPOSURE LIMITS:
PEL (OSHA Permissible Exposure Limit): No OSHA PEL for this compound. For nuisance dust: 15 mg/m3 (respirable) – 8 hour TRWA.

TLV (ACGIH Threshold Limit Value): No ACGIH TLV for this compound. For nuisance dust: 10 mg/m3 – 8 hour TWA.

PERSONAL PROTECTION:

Respiratory:
Where exposure is likely to exceed acceptable criteria, use NIOSH/MSHA approved respiratory protection equipment. Respirators should be selected based on the form and concentration of contaminant in air in accordance with OSHA 29 CFR 1910.134.

Eye Protection:

Wear safety glasses meeting the specifications of ANSI standard Z87.1 where no contact with eye is anticipated. Chemical safety goggles meeting ANSI standard Z87.1 should be worn if there is a possibility of eye contact.

Skin Protection:
Wear heat protective gloves and clothing if there is a potential contact with heated material.

ENGINEERING CONTROLS:
Provide adequate local exhaust ventilation to maintain air concentration of dust, fumes and vapors below acceptable exposure criteria. Provide adequate mechanical ventilation at the points of extrusion where polymer is at elevated temperatures coming from the extruder.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance:	Off-white pellets	**Odor:**	Odorless
Melting Point:	157-191ºC	Solubility in Water:	Essentially none

10. STABILITY AND REACTIVITY

Stability:	Stable	Hazardous Polymerization:	Will not occur
Conditions to Avoid:	Extreme heat above 460ºF		
Materials to Avoid:	None known		

10. TOXICOLOGICAL INFORMATION

No information available

12. ECOLOGICAL INFORMATION

No information available

13. DISPOSAL CONSIDERATIONS

All recovered material should be packaged, labeled, transported, and disposed or reclaimed in conformance with applicable laws and regulations and in conformance with Good Engineering Practices.

14. TRANSPORT INFORMATION

Shipping Name:	Not regulated	Hazard Class:	N/A

Packing Group: N/A UN#: N/A

15. REGULATORY INFORMATION

TSCA: This product is listed in the TSCA Inventory of Chemical Substances

Canadian Domestic Substances List (DSL): Is listed in the Canadian Domestic Substances List.

16. OTHER INFORMATION

HMIS HAZARD RATING

HEALTH: 1
FLAMMABILITY: 1
REACTIVITY: 0

PERSONAL PROTECTION: B

FIRE:
Material that must be preheated before ignition can occur.
HEALTH:
Materials which on exposure under fire conditions would offer no hazard beyond that of ordinary combustible material.
REACTIVITY:
Materials which in themselves are normally stable, even under fire exposure conditions, and which are not reactive with water.

This material is intended for laboratory use only. It is not sold or intended for drug, household or other uses. The information represents the most accurate and complete data currently available to us. However, we make no warranty, express or implied, with respect to such information, and we assume no liability resulting from its use.

Hytrel® 8206

Table E3: Properties of Hytrel® 8206 Polymer.

Property	**Value**	**Unit**
Approximate MW	-	g/mol
Viscosity	-	cp
Melt Flow Index	-	g/10min
Physical Form	oval shaped beads	-
Size	4 – 5	mm
Density	1.17	g/cc