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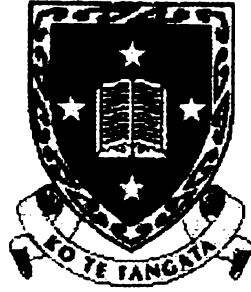
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Anaerobic Transformation of Halocarboxylic
Acids and Phenolic Compounds by
Photosynthetic Bacteria

*A thesis submitted in partial fulfilment of the requirements for
the degree of Doctor of Philosophy at The University of Waikato*

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ABSTRACT

The ability of photosynthetic bacteria to transform phenolic compounds and halocarboxylic acids under photoheterotrophic growth conditions was assessed and studied.

Attempts to isolate strains of phototrophic bacteria capable of degrading phenolic compounds yielded a strain of the purple nonsulfur bacteria species *Rhodopseudomonas palustris*. The isolated strain showed growth to a high cell density on phenol, although this may have been in conjunction with a sulfate reducing bacteria. Certain type strains of purple nonsulfur bacteria were able to grow on phenol and a small number of substituted phenols. *Rhodospirillum rubrum* DSM 467 showed growth to a high cell density on the pesticide 2,4-dinitrophenol at a concentration of 1.5 mM. The initial step in the degradation was shown to be a reduction of the *ortho*-nitro group to an amino group, transforming the compound to 2-amino-4-nitrophenol.

A number of purple nonsulfur bacteria type strains were reported for the first time to utilise halocarboxylic acids for photoheterotrophic growth. Growth rates and optimum growth concentrations increased when cells were previously adapted for growth on the halocarboxylic acid substrates. Collectively, the purple nonsulfur bacteria can grow at the expense of brominated, chlorinated, and fluorinated acetic and propionic acids. The mechanism of degradation was shown to be via reductive dehalogenation. The results suggest that the purple nonsulfur bacteria may play a significant role in the transformation of phenolic and halogenated compounds in anaerobic sediments, particularly in cases of heavy contamination where the ability of these bacteria to grow on high concentrations of these compounds could allow them to proliferate.

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ABBREVIATIONS

PNSB	Purple Nonsulfur Bacteria		
<i>Blc.</i>	<i>Blastochloris</i>	<i>Rfx.</i>	<i>Rhodoferax</i>
<i>Phs.</i>	<i>Phaeospirillum</i>	<i>Rmi.</i>	<i>Rhodomicrobium</i>
<i>Rba.</i>	<i>Rhodobacter</i>	<i>Rpi.</i>	<i>Rhodopila</i>
<i>Rbi.</i>	<i>Rhodobium</i>	<i>Rpl.</i>	<i>Rhodoplanes</i>
<i>Rcy.</i>	<i>Rhodocyclus</i>	<i>Rps.</i>	<i>Rhodopseudomonas</i>
<i>Rdt.</i>	<i>Rhodothalassium</i>	<i>Rsi.</i>	<i>Rhodospira</i>
<i>Rdv.</i>	<i>Rhodovulum</i>	<i>Rsp.</i>	<i>Rhodospirillum</i>
<i>Rss.</i>	<i>Roseospira</i>	<i>Rvb.</i>	<i>Rhodovibrio</i>
<i>Rvi.</i>	<i>Rubrivivax</i>		
2-CP	2-chlorophenol	2-NP	2-nitrophenol
3-CP	3-chlorophenol	3-NP	3-nitrophenol
2,4-DCP	2,4-dichlorophenol	4-CP	4-chlorophenol
2,4-DNP	2,4-dinitrophenol	4-NP	4-nitrophenol
BPA	2-bromopropionic acid	FAA	Fluoroacetic acid
2-CPA	2-chloropropionic acid	CAA	Chloroacetic acid
CPA	3-chloropropionic acid	BAA	Bromoacetic acid
ATCC	American Type Culture Collection		
DSM	Deutsche Sammlung von Mikroorganismen		
ESMS	Electrospray Mass Spectroscopy		
GC	Gas Chromatography		
HPLC	High Performance Liquid Chromatography		
SDS	sodium dodecyl sulfate		
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis		
ATP	adenosine triphosphate		
NAD	nicotinamide adenine dinucleotide		
NADH	nicotinamide adenine dinucleotide (reduced)		
CoA	coenzyme A		
bchl	bacteriochlorophyll		

CHAPTER 1

INTRODUCTION

The purple nonsulfur bacteria are the most metabolically diverse of all groups of bacteria. The preferred mode of growth is photoheterotrophy, although certain members are capable of photoautotrophic, chemoautotrophic, chemoheterotrophic or fermentative growth.

The purple nonsulfur bacteria occur predominantly in aquatic habitats in or near the aerobic/anaerobic transition zone. Diffusion of substrates occurs from aerobic and anaerobic environments, providing a variety of potential substrates. The purple nonsulfur bacteria have the ability to metabolise a wide range of organic compounds.

Metabolism of a number of aromatic compounds by the purple nonsulfur bacteria has been reported (Harwood and Gibson 1988); there are however no reports of phenolic compound degradation by these organisms. Pollution of freshwater masses such as lakes and estuaries by phenolic compounds derived primarily from industry waste and pesticide usage is of global concern. With their diversity of metabolism, the purple nonsulfur bacteria could potentially play an important role in the removal of such contaminants from these environments.

Straight-chain carboxylic acids have been tested for use by many species under anaerobic phototrophic conditions (Janssen 1985). However, there are no references to utilisation of halogenated carboxylic acids under these conditions. The aim of this study was to investigate metabolism of these compounds by the purple nonsulfur bacteria.

The work was carried out as follows:

- Attempts were made to isolate anaerobic photosynthetic organisms capable of growing at the expense of phenolic compounds. Over a 12 month period, many enrichments were performed on a number of anaerobic sediment samples taken from a variety of sites that were potentially subject to aromatic pollutant loading. Both batch and continuous culture enrichment techniques were employed. One strain obtained from the isolation work along with a large number of purple nonsulfur bacteria type strains, were screened for their ability to utilise phenol and a range of substituted phenolic compounds for photoheterotrophic growth. This work is described in Chapter 4.
- A large number of purple nonsulfur bacteria type strains were screened for their ability to utilise a range of mono-halogenated carboxylic acids for photoheterotrophic growth. From the positive results, degradation pathway elucidation studies and dehalogenase identification work was performed. This work is described in Chapter 5.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review comprises three main sections. The first section is a general overview of the purple nonsulfur bacteria (PNSB) with an emphasis on metabolic capabilities. The second section discusses the various processes of the anaerobic microbial degradation of aromatic compounds, detailing those carried out by the purple nonsulfur bacteria. The third section is a general discussion on microbial dehalogenation and dehalogenases.

Part A: The Purple Nonsulfur Bacteria

Bacteria similar to presently recognised purple nonsulfur bacteria were described in some detail by Molisch in 1907 (Imhoff and Trüper 1989). He proposed that all purple bacteria be incorporated into the order Rhodobacteria and into two families, the Thiorhodaceae and Athiorhodaceae. In the 8th edition of Bergey's Manual of Determinative Bacteriology, Pfennig and Trüper (1974) renamed these families *Rhodospirillaceae* and *Chromatiaceae*. The genus *Ectothiorhodospira* has now been separated from the *Chromatiaceae* to form the new family *Ectothiorhodospiraceae* and the *Rhodospirillaceae* have been renamed the purple nonsulfur bacteria (PNSB).

2.2 Characteristics of the Purple Nonsulfur Bacteria

The purple nonsulfur bacteria are by far the most diverse group of phototrophic bacteria as can be seen in their wide range of morphologies, internal membrane structure, carotenoid composition, and utilisation of carbon sources and electron donors (Imhoff and Trüper 1989). Table 2.1 shows the major differences between the purple nonsulfur bacteria and the purple sulfur bacteria.

Characteristics distinguishing the two groups	<i>Chromatiaceae</i> and <i>Ectothiorhodospiraceae</i>	Purple nonsulfur bacteria
Principal mode of photosynthesis	photoautotrophic	photoheterotrophic
Range of photoassimilable organic substrates	narrow	broad
Aerobic growth	no [#]	yes
Ability to oxidise sulfide	yes	yes [*]
Sulfide toxicity	low	high
Ability to use intermediates in the oxidation of sulfide to sulfate	yes	no [#]
Mole %G+C	45-70	61-72

a few exceptions

* normally only at low concentrations

Table 2.1 The major differences between the purple nonsulfur bacteria and the purple sulfur bacteria.

2.3 Photosynthetic pigments

The photosynthetic pigments present in anoxygenic phototrophs represent a major cytological property that has been used in the classification of these organisms. Present in these organisms are bacteriochlorophylls and carotenoid pigments. The types of pigment and the type of photosynthetic membranes in which they are contained vary markedly between the groups of bacteria. The photosynthetic

pigments of the purple nonsulfur bacteria are comprised of bacteriochlorophyll *a* or bacteriochlorophyll *b* and various carotenoids depending on the species. These are located in the cytoplasmic membrane and internal membrane systems that may be in the form of lamellae, tubes or vesicles. The carotenoids present in species within the purple nonsulfur bacteria are shown in Table 2.2.

Carotenoid Series	Major Pigments	Colour of Mass Cell Suspension
1. Normal spirilloxanthin	Lycopene, rhodopin and spirilloxanthin.	Red, brown, orange
2. Rhodopinal	Lycopenal and rhodopinal.	Purple, purple-violet
3. Alternative spirilloxanthin	Spheroidene, spheroidenone, neurosporene and derivatives.	Brown, yellow-brown, yellow-green
4. Okenone	Okenone, methoxylated ketocarotenoids	Purple-red

Madigan 1988

Table 2.2 Carotenoids of the purple nonsulfur bacteria.

2.4 Genera of the Purple Nonsulfur Bacteria

The following is a discussion of the presently recognised genera of purple nonsulfur bacteria, with an emphasis on the diversity between species in each genus.

2.4.1 *Blastochloris*

The species *Rps. viridis* (Drews and Giesbricht 1966) and *Rps. sulfoviridis* (Keppen and Gorlenko 1975) are now accommodated in the genus *Blastochloris* (Hiraishi 1997). These species are unique amongst the purple nonsulfur bacteria in their possession of bacteriochlorophyll *b* as their major pigment, instead of

bacteriochlorophyll *a*. Anaerobically grown cultures are therefore green. *Blc. sulfoviridis* can utilise sulfide and thiosulfate as electron donors; *Blc. viridis* cannot.

2.4.2 *Phaeospirillum*

The species *Rsp. fulvum* (van Niel 1944) and *Rsp. molischianum* (Giesberger 1947) are now accommodated in the genus *Phaeospirillum* (Imhoff *et al.* 1998). Both species contain lycopene and rhodopin as the major carotenoids, giving cell suspensions a brown colour. In addition, both species are more sensitive to oxygen than the red spirilla of the genus *Rhodospirillum*.

2.4.3 *Rhodobacter*

There are currently five species in the genus *Rhodobacter*. Cells are ovoid to rod-shaped with many species forming chains of cells. All species divide by binary fission and possess internal photosynthetic membranes of the vesicular type with the exception of *Rba. blasticus* (Eckersley and Dow 1980) which undergoes sessile budding, and has lamellar membranes. Until recently, *Rba. blasticus* was classified as a member of *Rhodopseudomonas* until molecular evidence proved otherwise (Kawasaki *et al.* 1993).

Physiologically, *Rhodobacter* species are among the most versatile of the purple nonsulfur bacteria. The type species of the genus, *Rba. capsulatus* (van Niel 1944), can perform all possible modes of growth; photoautotrophy, photoheterotrophy, chemoheterotrophy, chemolithotrophy, and fermentative metabolism. Differentiation of this species with *Rba. sphaeroides* (van Niel 1944) is often difficult. The fact that *Rba. capsulatus* is noted for a tendency to produce zigzag chains of cells, is up to 10⁵ times more sensitive to penicillin and is more restricted in carbon source utilisation than *Rba. sphaeroides* (*Rba. capsulatus*

cannot use mannitol, sorbitol, ethanol or citrate), may be of use (Weaver, Wall and Gest 1975).

Rba. azotoformans is the newest member of this genus (Hiraishi, Muramatsu and Ueda 1996), and is closely related to *Rba. sphaeroides*. *Rba. azotoformans* is mainly characterised by a high level of NO_3^- reduction, producing hydrogen gas, although some strains of *Rba. sphaeroides* also show this property (Sato et al. 1976).

Rba. veldkampii (Hansen and Imhoff 1985) has a marked tolerance to sulfide, about 5mM, and is dependant on a reduced sulfur compound for growth. Its lack of a sodium ion requirement sets it apart from the marine species of genus *Rhodovulum*.

2.4.4 *Rhodobium*

This genus contains two marine species with lamellar photosynthetic membranes and budding morphology. *Rbi. orientis* (Hiraishi et al. 1995a) and *Rbi. marinum*, which was previously classified as *Rps. marina* (Imhoff 1983), are both moderately halophilic. They are readily distinguishable in a number of characteristics, notably the low level of absorption at 803nm of *Rbi. marinum* (a property shared with *Rhodospirillum rubrum*). *Rbi. orientis* is capable of denitrification and is able to photoassimilate ethanol or propionate.

Molecular work performed in this lab has shown evidence for the placement of the *Rhodopseudomonas* species *Rps. julia* (Kompantseva 1989) into *Rhodobium* to reflect its true phylogenetic affiliation (Charlton and McGrath unpublished data).

2.4.5 *Rhodocista*

Kawasaki, Hoshino, Kuraishi and Yamasoto (1992) isolated two strains of a cyst-forming purple nonsulfur bacterium which they named *Rhodocista centenaria*. These are now recognised to be of the same species as an organism isolated by Favinger *et al.* (1989). To this point the prior name *Rhodospirillum centenum* has been retained.

2.4.6 *Rhodocyclus*

There are currently two recognised *Rhodocyclus* species. The type species *Rcy. purpureus* (Pfennig 1978a) was isolated from a swine waste lagoon in Ames, Iowa, and is probably a rare species in nature. Cells are half-circle to ring shaped and non-motile. It is unable to fix dinitrogen and has a relatively limited ability to utilise varied carbon sources, although it does possess the enzyme pathways for degradation of aromatic compounds.

Rcy. tenuis (Pfennig 1969b) cells are thin spirals, motile by polar flagella, and capable of nitrogen fixation. Strains are known which vary in their carotenoid composition, with brownish-red strains containing spirilloxanthin and purple-violet strains containing rhodopinal series carotenoids.

Phylogenetic studies by Hiraishi (1994) have shown that *Rcy. tenuis* DSM 110, a purple strain, must be regarded as a new species of the genus, since it is located outside the cluster encompassing *Rcy. purpureus* and other *Rcy. tenuis* strains. A brownish-pink organism isolated in this laboratory from a winery drainage ditch could also be described as a new *Rhodocyclus* species, since it exhibits differences in carbon source utilisation patterns and shows a 16S rDNA similarity of 94.1% to 96.2% to other members of the genus (Charlton and McGrath unpublished data).

2.4.7 *Rhodoferax*

The only species in this genus is *Rfx. fermentans* (Hiraishi, Hoshino and Satoh 1991). The most characteristic feature of this species is its ability for rapid fermentative growth under anaerobic and dark conditions.

2.4.8 *Rhodomicrobium*

The sole member of this genus, *Rmi. vannielii* (Duchow and Douglas 1949), possesses morphological characteristics unique amongst the purple nonsulfur bacteria. Swarmer cells are motile by peritrichous flagella. Non-motile mother cells undergo division by budding from filaments, branching of which may form dendroid aggregates. In addition, several smaller cells called exospores may be formed at the end of a filament (Gorlenko 1969). These exospores are more resistant to heat and dryness than normal cells. *Rmi. vannielii* has a high sulfide tolerance relative to most members of the purple nonsulfur bacteria, about 2-3mM.

2.4.9 *Rhodopila*

Rpi. globiformis (Pfennig 1974) is the only species of the genus *Rhodopila*. It is relatively restricted in the range of carbon sources it can utilise. Cells are oxygen sensitive and grow in the dark only at low oxygen tensions. *Rpi. globiformis* has a lower pH optimum than most purple nonsulfur bacteria and is closely related to other bacteria with acidic optima, including those of the genus *Acidiphilum* which contains photosynthetic members (Sievers *et al.* 1994).

2.4.10 *Rhodoplanes*

The genus *Rhodoplanes* contains the species *Rpl. roseus*, formerly known as *Rhodopseudomonas rosea* (Janssen and Harfoot 1987), and *Rpl. elegans*, a recent isolate from activated sewage (Hiraishi and Ueda 1994b). Their inability to utilise medium or long-chain fatty acids is characteristic. *Rpl. roseus* is unable to grow on valerate or higher fatty acids, while *Rpl. elegans* shows growth on caproate. Both species are capable of photoautotrophic growth with thiosulfate as electron donor, in the presence of low quantities of yeast extract. Dark growth is possible aerobically, or anaerobically using nitrate respiration. Complete denitrification to N₂ is possible.

Strains IL-245 and ANT-P-1 isolated by Kawasaki *et al.* (1993), are likely to be from a third species of *Rhodoplanes* as indicated by 16S rDNA sequences.

2.4.11 *Rhodopseudomonas*

Cells of the genus *Rhodopseudomonas* are rod-shaped and motile. The internal photosynthetic membranes of the cells are present as lamellae.

Hiraishi and Ueda (1994b) have proposed the gradual reclassification of all members of *Rhodopseudomonas* other than the type species *Rps. palustris*, due to the genus being polyphyletic as shown by 16S rDNA analyses of the members. Hence the genus has been progressively reduced in size. Currently, in addition to the type species, only *Rps. acidophila*, *Rps. julia* and *Rps. cryptolactis* remain. *Rps. viridis* and *Rps. sulfoviridis* have been transferred to the genus *Blastochloris* (Hiraishi 1997).

Rps. palustris (van Niel 1944) is the most commonly isolated of all the purple nonsulfur bacteria (Biebl and Drews 1969; Lee 1992) and is capable of photoheterotrophic growth on the widest range of carbon sources of all the purple

nonsulfur bacteria. Cells of *Rps. palustris* commonly form characteristic rosettes in older cultures.

Rps. rutila (Akiba, Usama and Horikoshi 1983), has an identical 16s rDNA sequence to *Rps. palustris*. It has hence been suggested that the two organisms are synonymous (Hiraishi et al. 1992), although there are some marked dissimilarities in carbon utilisation patterns and vitamin requirements.

The recent discovery of 26 strains morphologically and physiologically similar to *Rps. palustris*, falling into 7 phylogenetically distant clusters and containing a total of 13 genospecies (Kompantseva et al. 1996), have further complicated this picture, making the identification of an isolate such as *Rps. palustris* no longer straightforward.

Rps. acidophila (Pfennig 1969a) has a pH optimum for growth of 5.5-6.0, with cells larger than those of other *Rhodopseudomonas* species. *Rps. cryptolactis* (Stadtwald-Demchick, Turner and Gest 1989) is a thermotolerant species with a growth optimum of 40°C and a maximum growth temperature of 46°C. This degree of thermotolerance may be more widespread among *Rhodopseudomonas* with *Rps. palustris*-like isolates from work carried out in this lab showing similar temperature optima and ranges (Charlton, unpublished results).

Rps. julia (Kompantseva 1989) is a species with a preference for acidic growth conditions, but has unique metabolic capabilities. It is capable of photoautotrophic growth on elemental sulfur, and when growing on sulfide, deposits sulfur globules both extracellularly and intracellularly. 16S rDNA sequences have however shown this species to be more phylogenetically related to members of the genus *Rhodobium* than *Rhodopseudomonas*. (Charlton and McGrath, unpublished results).

Finally, *Rhodopseudomonas* strain G1 (Resnick and Madigan 1989) is a mildly thermophilic organism containing bacteriochlorophyll *b*, and may accordingly be

transferred to the genus *Blastochloris*. Its maximum temperature for growth is 47°C and it has an obligate requirement for reduced sulfur compounds.

2.4.12 *Rhodospira*

The only member of this recently formed genus is the newly described *Rsi. trueperi* (Pfennig *et al.* 1997). Cells are vibrioid- to spirilloid-shaped and possess internal membranes of the vesicular type. Sulfide is required for growth and is oxidised to elemental sulfur which is deposited extracellularly. *Rsi. trueperi* has a minimum NaCl requirement of 0.5% and an optimal concentration for growth of 2%.

2.4.13 *Rhodospirillum*

The cells of this genus are spiral in shape and possess internal photosynthetic membranes in the form of vesicles or lamellae. Pigments present are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin or lycopene and rhodopin series.

Rsp. rubrum is the type species of the genus and was the first anoxygenic phototroph isolated in pure culture. It is also the most studied of all the purple and green bacteria.

Rsp. centenum (Favinger, Stadtwald and Gest 1989) is unusual among the purple nonsulfur bacteria in its ability to form heat- and desiccation-resistant cysts. These cysts can survive heating at 75°C for 48 hours. Not surprisingly, most *Rsp. centenum* strains have been isolated from thermal regions (Nickens *et al.* 1996). The maximum growth temperature of this organism is 47°C. Some cells contain refractile bodies identical in appearance to the "R-bodies" observed in some non-photosynthetic bacteria, predominantly endosymbionts (Pond *et al.* 1989).

Both *Rsp. rubrum* and *Rsp. centenum* contain carotenoids of the spirilloxanthin series, and are red in colour.

The remaining species of this genus, *Rsp. photometricum* (Giesberger 1947), possesses lycopene and rhodopin as the major carotenoids, and hence cultures of this organism are brown.

Rhodospirillum sp. E-12 is an as yet undescribed isolate whose closest phylogenetic relative is *Rsp. photometricum* (Kawasaki et al. 1993).

2.4.14 *Rhodotalassium*

The species *Rsp. salexigens* (Drews 1981) is now accommodated in the genus *Rhodotalassium* (Imhoff et al. 1998). *Rdt. salexigens* has a NaCl requirement of 5-20% and cannot use sulfide or thiosulfate.

2.4.15 *Rhodovibrio*

The species *Rsp. salinarum* (Nissen and Dundas 1984) and *Rsp. sodomense* are now accommodated in the genus *Rhodovibrio* (Imhoff et al. 1998). *Rvb. salinarum* and *Rvb. sodomensis* are similar to *Rdt. salexigens* in their NaCl requirement and inability to utilise sulfide or thiosulfate.

2.4.16 *Rhodovulum*

The genus *Rhodovulum* was created to accommodate a group of marine species once classified as *Rhodobacter*. 16S rDNA studies have clearly shown these organisms to form a phylogenetically distinct cluster from the terrestrial and freshwater members of *Rhodobacter*. The species are *Rdv. adriaticum* (Neutzling et al. 1984), *Rdv. euryhalinum* (Kompantseva 1985) and *Rdv. sulfidophilum* (Hiraishi and Ueda 1994a). A further species, *Rdv. strictum* was described later

(Hiraishi and Ueda 1995b). The genera can be distinguished on the basis of salt requirement for optimal growth and sulfide tolerance.

Rdv. adriaticum is one of the few non-motile species of purple nonsulfur bacteria, and along with *Rdv. euryhalinum* is unable to assimilate sulfate. *Rdv. strictum* has both a narrow pH and salinity range for growth.

Two ferrous-iron-oxidising species *Rdv. iodosum* and *Rdv. robiginosum* have recently been added to this genus (Straub *et al.* 1999).

2.4.17 *Roseospira*

The species *Rsp. mediosalinum* (Kompantseva and Gorlenko 1985) is now accommodated in the genus *Roseospira* (Imhoff *et al.* 1998). *Rss. mediosalina* has a NaCl requirement of 4-7% and can use sulfide as an electron donor.

2.4.18 *Rubrivivax*

The only species of this genus is *Rvi. gelatinosus* (Willems, Gillis and deLey 1991). *Rvi. gelatinosus* has the ability to liquefy gelatin, a property unusual among the purple nonsulfur bacteria and only shared with *Rfx. fermentans* and some strains of *Rba. capsulatus*. Cells contain sphaeroidene carotenoids which give cultures their unusual peach or brown colour.

2.5 Diversity within the Purple Nonsulfur Bacteria

The diversity within the PNSB is summarised in Tables 2.3, 2.4 and 2.5. These tables summarise the important differences between the genera of the PNSB, with emphasis on those that are useful in identification. Table 2.5 can be used as a diagnostic table although the data is incomplete and the substrate concentrations used by the different workers have not been standardised.

Species	Cell shape	Motility	Conditions for dark growth	Mode of cell division
<i>Blc. sulfoviridis</i>	ovoid/rod	+	microaerophilic	sessile budding
<i>Blc. viridis</i>	ovoid/rod	+	microaerophilic	sessile budding
<i>Phs. fulvum</i>	spiral	+	microaerophilic	binary fission
<i>Phs. molischianum</i>	spiral	+	microaerophilic	binary fission
<i>Rba. azotoformans</i>	ovoid/rod	+	aerobic	binary fission
<i>Rba. blasticus</i>	ovoid/rod	-	aerobic	binary fission
<i>Rba. capsulatus</i>	ovoid/rod	+	aerobic	binary fission
<i>Rba. marinus</i>	ovoid/rod	+	aerobic	binary fission
<i>Rba. sphaeroides</i>	spherical/rod	+	aerobic	binary fission
<i>Rba. veldkampii</i>	ovoid/rod	-	aerobic	binary fission
<i>Rbi. marinum</i>	ovoid/rod	+	microaerophilic	sessile budding
<i>Rbi. orientis</i>	rod	+	aerobic	sessile budding
<i>Rcy. purpureus</i>	half circle/circle	-	microaerophilic	binary fission
<i>Rcy. tenuis</i>	spiral	+	microaerophilic	binary fission
<i>Rdt. salexigens</i>	rod/spiral	+	aerobic	binary fission
<i>Rdv. adriaticum</i>	ovoid/rod	-	microaerophilic	binary fission
<i>Rdv. euryhalinum</i>	coccoid/rod	+	microaerophilic	binary fission
<i>Rdv. strictum</i>	ovoid/rod	+	aerobic	binary fission
<i>Rdv. sulfidophilum</i>	ovoid/rod	+	aerobic	binary fission
<i>Rdv. iodosum</i>	ovoid/rod	-	aerobic	binary fission
<i>Rdv. robiginosum</i>	ovoid/rod	-	aerobic	binary fission
<i>Rfx. fermentans</i>	curved rods	+	aerobic/fermentation	binary fission
<i>Rmi. vanniellii</i>	ovoid	+	microaerophilic	budding with tube
<i>Rpi. globiformis</i>	spherical/ovoid	+	microaerophilic	binary fission
<i>Rpl. elegans</i>	rod	+	aerobic	budding
<i>Rpl. rosea</i>	flask to rod	+	microaerophilic	sessile budding
<i>Rps. acidophila</i>	ovoid/rod	+	aerobic	budding with tube
<i>Rps. cryptolactis</i>	rod	+	aerobic	budding
<i>Rps. julia</i>	variable rods	+	microaerophilic	sessile budding
<i>Rps. palustris</i>	ovoid/rod	+	aerobic	budding with tube
<i>Rsi. trueperi</i>	spiral	+	aerobic	binary fission
<i>Rsp. centenum</i>	comma/spiral	+	aerobic	binary fission
<i>Rsp. photometricum</i>	spiral	+	microaerophilic	binary fission
<i>Rsp. rubrum</i>	spiral	+	aerobic	binary fission
<i>Rss. mediosalina</i>	spiral	+	microaerophilic	binary fission
<i>Rvb. salinarum</i>	rod/spiral	+	aerobic	binary fission
<i>Rvb. sodomensis</i>	rod/spiral	+	aerobic	binary fission
<i>Rvi. gelatinosus</i>	rod/spiral	+	aerobic	binary fission

Table 2.3 Diversity within the purple nonsulfur bacteria.

Species	Colour of cell suspension	Bchl	Internal membrane structure
<i>Blc. sulfovirdis</i>	green	<i>b</i>	Lamellar
<i>Blc. viridis</i>	green	<i>b</i>	Lamellar
<i>Phs. fulvum</i>	brown	<i>a</i>	Lamellar
<i>Phs. molischianum</i>	brown	<i>a</i>	Lamellar stacks
<i>Rba. azotoformans</i>	yellow-green-brown	<i>a</i>	Vesicles
<i>Rba. blasticus</i>	orange-brown	<i>a</i>	Lamellar
<i>Rba. capsulatus</i>	yellow to brown	<i>a</i>	Vesicles
<i>Rba. marinus</i>	yellow-brown	<i>a</i>	Vesicles
<i>Rba. sphaeroides</i>	green-brown to brown	<i>a</i>	Vesicles
<i>Rba. veldkampii</i>	yellow-brown	<i>a</i>	Vesicles
<i>Rbi. marinum</i>	pink-red	<i>a</i>	Lamellar
<i>Rbi. orientis</i>	pink-red	<i>a</i>	Lamellar
<i>Rcy. purpureus</i>	purple-violet	<i>a</i>	Tubes
<i>Rcy. tenuis</i>	purple-violet/brown-orange	<i>a</i>	Tubes
<i>Rdt. salexigens</i>	red	<i>a</i>	Lamellar
<i>Rdv. adriaticum</i>	brown	<i>a</i>	Vesicles
<i>Rdv. euryhalinum</i>	green-yellow to brown	<i>a</i>	Vesicles
<i>Rdv. strictum</i>	yellow-green to brown	<i>a</i>	Vesicles
<i>Rdv. sulfidophilum</i>	yellow-brown to brown	<i>a</i>	Vesicles
<i>Rdv. iodosum</i>	yellow-brown	<i>a</i>	Vesicles
<i>Rdv. robiginosum</i>	yellow-brown	<i>a</i>	Vesicles
<i>Rfx. fermentans</i>	peach-brown	<i>a</i>	Tubes
<i>Rmi. vanniellii</i>	orange-brown	<i>a</i>	Lamellar
<i>Rpi. globiformis</i>	purple-red	<i>a</i>	Vesicles
<i>Rpl. elegans</i>	pink	<i>a</i>	Lamellar
<i>Rpl. rosea</i>	pink	<i>a</i>	Lamellar
<i>Rps. acidophila</i>	purple-red/orange-brown	<i>a</i>	Lamellar
<i>Rps. cryptolactis</i>	red	<i>a</i>	Lamellar
<i>Rps. julia</i>	pink to purple	<i>a</i>	Lamellar
<i>Rps. palustris</i>	red-brown	<i>a</i>	Lamellar stacks
<i>Rsi. trueperi</i>	red	<i>b</i>	Vesicles
<i>Rsp. centenum</i>	red	<i>a</i>	Lamellar
<i>Rsp. photometricum</i>	brown	<i>a</i>	Lamellar stacks
<i>Rsp. rubrum</i>	red	<i>a</i>	Vesicles
<i>Rss. mediosalina</i>	red	<i>a</i>	Vesicles
<i>Rvb. salinarum</i>	red	<i>a</i>	Vesicles
<i>Rvb. sodomensis</i>	red	<i>a</i>	Vesicles
<i>Rvi. gelatinosus</i>	yellow-brown to pink	<i>a</i>	Tubes

Table 2.4 Further diversity within the purple nonsulfur bacteria.

2.6 Metabolic Capabilities of the Purple Nonsulfur Bacteria

The purple nonsulfur bacteria are among the most metabolically diverse of all groups of bacteria. Outlined in this section are the metabolic capabilities of the group.

2.6.1 Phototrophic Metabolism

All members of the purple nonsulfur bacteria share the ability to photosynthesise. This is defined as the ability to transform light energy via a membrane-bound photosynthetic apparatus into an electrochemical proton gradient, which can be used for the generation of ATP and the reduction of NAD^+ .

2.6.1.1 Cyclic Photophosphorylation

During light growth, ATP is produced by a process known as cyclic photophosphorylation. This term describes a process during which electrons are cycled between reaction-centre bacteriochlorophylls, a primary acceptor, and several electron carriers such as quinones, nonheme iron-sulfur proteins, and cytochromes. All are associated with the photosynthetic membrane system which is continuous with the cytoplasmic membrane. (Drews 1978, Trüper 1989).

Light is harvested by antenna pigments which consist of bacteriochlorophyll and carotenoids bound to proteins. From the light harvesting pigments, the energy is transferred to reaction centres which consist of 4 molecules of bacteriochlorophyll, 2 molecules of bacteriopheophytin, and a single molecule of carotenoid. Closely associated with the reaction centre is an iron-ubiquinone complex. Stretching through the membrane is a complex containing a cytochrome b, a cytochrome c, and an iron-sulfur protein. When light energy is transferred from the antenna complex to the reaction centre, one electron per light quantum is translocated from

bacteriochlorophyll to bacteriopheophytin and further to the ubiquinone-iron complex. At the reaction centre bacteriochlorophyll, an electron from cytochrome c_2 immediately replaces the donated one. The reduced ubiquinone travels to the bc_1 complex where the electron is donated to cytochrome c_2 and two proteins are moved to the topological outside of the cell. Cytochrome c_c acts as a mobile electron carrier between the bc_1 complex and the reaction centre complex. The proton gradient thus created drives ATP synthase, forming ATP in the cytoplasm. The purple nonsulfur bacteria generate NADH by reverse electron transfer (Knaff 1978). The electrons required are derived from the oxidation of external electron donors such as H_2 , reduced sulfur compounds or organic compounds.

2.6.1.2 Photoautotrophic metabolism

Photoautotrophic metabolism requires light, carbon dioxide as sole carbon source, suitable photosynthetic electron donors (H_2 , reduced sulfur compounds, or organic compounds) for generation of NADH, and anaerobic conditions.

Use of H_2 as an electron donor for photoautotrophic growth by a member of the purple nonsulfur bacteria, *Rhodospirillum rubrum*, was first observed by Ormerod and Gest (1962). Uptake of H_2 and CO_2 in *Rsp. rubrum* was shown not to occur until all available organic substrate was depleted (Schick 1971).

Sulfide is used as an electron donor by many members of the purple nonsulfur bacteria provided the concentration is low enough to avoid toxicity. It is oxidised to sulfate or sulfur depending upon the species (Trüper and Pfennig 1978). Thiosulfate is another reduced sulfur compound that may be used by a more limited number of purple nonsulfur bacteria, including *Rba. veldkampii*, *Rdv. adriaticum*, *Rdv. sulfidophilum*, *Rps. palustris* and *Blc. sulfoviridis* (Trüper and Fischer 1982). Widdel *et al.* (1993) isolated strains resembling *Rps. palustris* and

Rmi. vannielii which were capable of photoautotrophic growth using Fe^{2+} as electron donor.

2.6.1.3 Photoheterotrophic metabolism

Photoheterotrophy in the light under anaerobic conditions is the preferred mode of growth of all the purple nonsulfur bacteria. Since the purple nonsulfur bacteria are able to use light energy for growth, organic carbon sources need not be diverted to ATP production, and thus a much higher molar cell yield can be obtained than is possible for chemotrophic anaerobes. Organic carbon sources serve primarily as a source of cellular carbon under phototrophic conditions, but may also function as electron donors for non-cyclic photosynthesis. In the presence of inorganic electron donors, they may be wholly photoassimilated (Imhoff and Trüper 1992).

A large number of organic compounds support growth. These include various alcohols (Foster 1944; Lee 1992; Charlton 1997), fatty acids (van Niel 1944; Janssen and Harfoot 1987; Charlton 1997), tricarboxylic acid intermediates, amino acids, carbohydrates, compounds containing sulfur moieties (McGrath 1995; Heising *et al.* 1996) and also some one-carbon compounds (Quayle and Pfennig 1975; Quadri and Hoare 1968). A few purple nonsulfur bacteria are capable of metabolising a range of aromatic compounds (Dutton and Evans 1978; Harwood and Gibson 1988).

Many substrates utilised by the purple nonsulfur bacteria are more reduced than cell material. This results in the net fixation of CO_2 which is used as an electron sink by the cells. If the oxidation state of a substrate is higher than that of cell material, there is a net production of CO_2 . Under optimal growth conditions, no products other than CO_2 may be produced.

The general theme for photoassimilation is that carbon sources are broken down to a common pool of metabolic intermediates which are then drawn off for cellular

synthesis. It is assumed that reactions of the tricarboxylic acid cycle lie at the centre of the assimilation process. Sugars may be degraded by either the Embden-Meyerhoff or Entner-Doudoroff pathways. *Rba. capsulatus* unusually possesses both these pathways (Sojka 1978).

The mechanisms of CO₂ fixation during photoheterotrophic growth are varied, depending on the specific substrates in many cases. The reductive pentose phosphate cycle is not involved in CO₂ fixation in the presence of malate or acetate. Instead, a number of carboxylating reactions are involved in photoheterotrophic CO₂ fixation (Kondratieva 1979; Anderson and Fuller 1967).

2.6.2 Other modes of metabolism

The purple nonsulfur bacteria can also metabolise in the absence of light, both with and without the presence of oxygen. This is of course required for energy generation by cells outside daylight hours.

2.6.2.1 Chemoheterotrophic metabolism

All the purple nonsulfur bacteria are capable of growth in the dark using oxygen as a terminal electron acceptor. Some are able to grow aerobically under full oxygen tension while others are more sensitive to oxygen and require microaerophilic conditions. (see Table 2.6). Organic compounds that can be utilised under phototrophic conditions can, in the main, be used in the dark. With the need to divert substrate utilisation into ATP production, the cell yield for dark growth is far lower.

Growth may also be supported under dark conditions by various forms of anaerobic respiration, using nitrate, nitrous oxide, DMSO or TMAO as electron acceptors (Ferguson *et al.* 1987). The ability to denitrify has been found in *Rba*.

azotoformans (Hiraishi et al. 1996), several strains of *Rba. sphaeroides* (Sato et al. 1976; Michalski and Nicholas 1988) and *Rps. palustris* (Klemme et al. 1980) and in the species of the genus *Rhodoplanes* (Hiraishi and Ueda 1994b).

Poor dark growth may also occur at the expense of cell storage products such as PHB (poly- β -hydroxybutyrate) or metabolic intermediates such as fumarate.

2.6.2.2 Chemolithotrophic metabolism

Lithotrophic growth using H₂ as electron donor in aerobic respiration has been described in *Rba. capsulatus* (Madigan and Gest 1979) and *Rvi. gelatinosus* (Siefert and Pfennig 1979). The ability to use H₂ in the dark may be widespread among the purple nonsulfur bacteria, as most species possess uptake hydrogenases. (Yoch 1978).

Rba. capsulatus may also grow aerobically in the dark using sulfide as electron donor (Kompantseva 1981).

2.6.2.3 Fermentative metabolism

In the absence of light and external electron acceptors, some purple nonsulfur bacteria are able to resort to a fermentative metabolism. *Rsp. rubrum* was the first species of purple nonsulfur bacteria shown to grow by fermentation, using pyruvate as substrate (Uffen and Wolfe 1970), and producing acetate, formate, CO₂ and H₂ (Uffen 1973). *Rsp. rubrum* ferments fructose to succinate, acetate, propionate, formate, CO₂ and H₂ (Schön and Biedermann 1973). Other products are produced by different species.

2.7 Utilisation of Organic Compounds by the Purple Nonsulfur Bacteria

The range of organic compounds used as substrates for growth by the purple nonsulfur bacteria is unrivalled among the photosynthetic bacteria. Discussed below is the utilisation by these organisms of some major groups of organic substrates.

2.7.1 Aromatic compounds

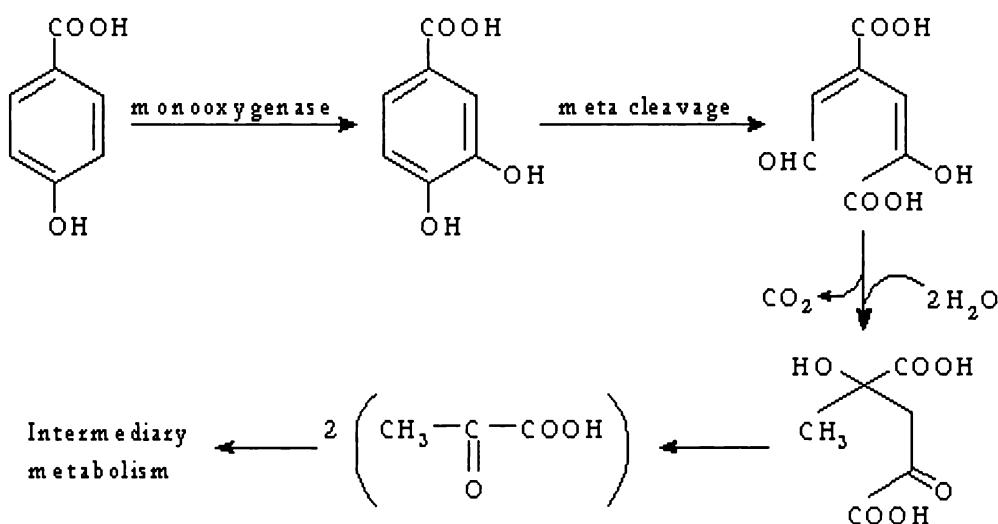


Figure 2.1 Pathway of aerobic *p*-hydroxybenzoate metabolism by *Rps. palustris*.

The purple nonsulfur bacteria are capable of utilising a number of aromatic compounds for growth, both aerobically and anaerobically. The anaerobic utilisation of aromatic compounds by these organisms will be discussed further in Section 2.8.5.

Rps. palustris has been the most widely studied of the purple nonsulfur bacteria for aromatic utilisation. Aromatic compounds used as substrates for aerobic growth include; *p*-hydroxybenzoate (Figure 2.1), caffeate, cinnamate and various phenyl-fatty acids (Harwood and Gibson 1988; Dutton and Evans 1978). *Rba. blasticus*, *Rmi. vanniellii*, *Rsp. rubrum* and *Rvi. gelatinosus* are other species that have been reported to utilise a range of aromatic compounds for growth, although in many cases, growth on these compounds aerobically only supports very poor growth (Wright and Madigan 1991; Rahalkar, Joshi and Shivaraman 1991; Shoreit and Shabeb 1994).

2.7.2 Fatty acids

Fatty acids have mainly been used in diagnostic tests to aid identification of purple nonsulfur bacteria isolates. Straight chain fatty acids from 1 to 9 carbons were commonly used. This can be seen in Table 2.5 (from Trüper and Pfennig 1992). Fatty acids have also been used as carbon sources for the enrichment of purple nonsulfur bacteria; caprylate and perlargonate have been said to be specific for the enrichment of brown spirilla (van Niel 1944).

Janssen (1985) and Janssen and Harfoot (1987) carried out a systematic study on the utilisation of straight-chain fatty acids by a wide selection of purple nonsulfur bacteria, and found that in most cases growth would occur if the concentration was suitable. Charlton (1997) performed a similar study using branched-chained fatty acids as substrates and showed that a large number of species were able to utilise methyl-branched fatty acids as carbon sources for growth. *Rcy. purpureus* was the only species that was able to utilise 2-ethylbutyrate, an ethyl-branched fatty acid. Utilisation of fatty acids is presumed to occur via the reactions of β -oxidation, which are widespread among Gram negative bacteria.

2.7.3 Alcohols

Alcohols have been considered as good substrates for the growth of purple nonsulfur bacteria (van Niel 1944). Only methanol and ethanol have been used generally for identification purposes.

2-propanol was used in early metabolic studies to show that organic compounds can act as hydrogen donors in photosynthesis (Foster 1940). Using *Rvi. gelatinosus*, Siegel and Kammen (1950) confirmed the conversion of 2-propanol to acetone and further catabolism of acetone, as shown by the proposed pathway in Figure 2.2.

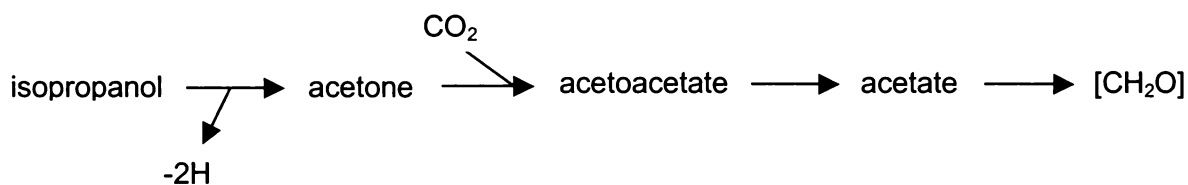


Figure 2.2 Utilisation of isopropanol by *Rvi. gelatinosus*.

Lee (1992) carried out a systematic study on the utilisation of straight-chain primary alcohols by a wide selection of purple nonsulfur bacteria. Some species were shown to use only short-chain alcohols, while others could use alcohols up to octadecanol. Several species lacked the ability to utilise any alcohol.

Charlton (1997) has suggested using growth on branched-chain alcohols for diagnostic and characterisation purposes of the purple nonsulfur bacteria. A smaller number of species are able to use branch-chain alcohols than can use fatty-acids, and within these species, many can only use a limited number. Secondary alcohols were shown to support growth only of *Rvi. gelatinosus*.

2.7.4 Glycerol and diols

Of the 26 species of purple nonsulfur bacteria that have been tested for utilisation of glycerol, as reported in the diagnostic Table 2.5, 13 gave positive growth. Glycerol is assimilated by *Rba. sphaeroides* by the pathway shown in Fig 2.3 (Pike and Sojka 1975).

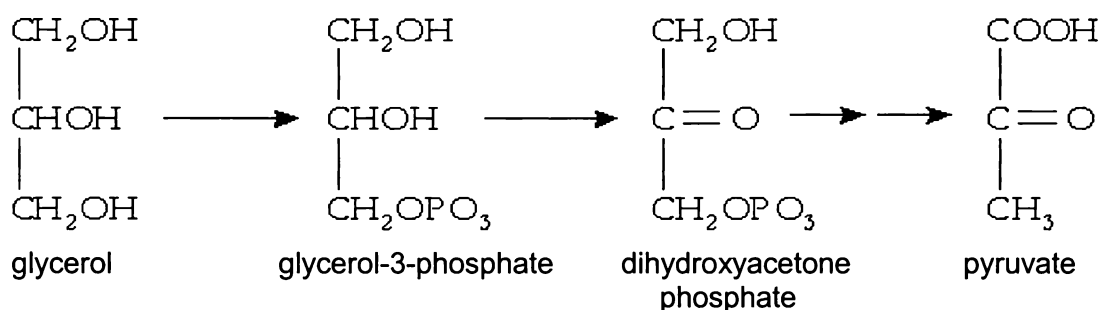


Figure 2.3 Assimilation of glycerol by *Rba. sphaeroides*.

Charlton (1997) reported that growth of the purple nonsulfur bacteria on diols is far less common than for growth on fatty acids or alcohols. Of the species tested, it was found that ethylene glycol is more widely used than glycerol. Propanediols are also readily used by the purple nonsulfur bacteria. Those species able to grow on propan-1,3-diol also showed good growth on malonate, suggesting a possible conversion of the former to the latter, or to malonyl-CoA. By contrast, 4-carbon diols were shown to be recalcitrant to degradation by the purple nonsulfur bacteria. *Rba. sphaeroides* was the only species to grow commonly on these compounds.

Part B: Anaerobic Degradation of Aromatic Compounds

A large variety of aromatic compounds exist in the environment, both as part of the natural carbon cycle and as a result of human activities. Many of these compounds are recalcitrant and require the action of microbes for their dissimilation.

Under aerobic conditions, ring fission of aromatic compounds is achieved by a variety of bacteria that possess mono- and dioxygenases. Molecular oxygen is incorporated into the degradation products and is hence essential for the function of these enzymes (Dagley 1971).

Under anaerobic conditions, the metabolic fate of organic compounds and their mineralisation to CO_2 or CH_4 depends on the availability of inorganic electron acceptors such as NO_3^- , SO_4^{2-} , or of CO_2 , or of light (Evans and Fuchs 1988).

2.8 Anaerobic processes

The anaerobic processes involved in the degradation of aromatic compounds are denitrification, sulfate reduction, fermentation, methanogenesis, and anaerobic photometabolism. Table 2.6 summarises the aromatic compounds degraded in association with these processes.

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Energy-yielding process	Organisms	Substrates
Denitrification	<i>Alcaligenes</i> <i>Paracoccus denitrificans</i> <i>Bacillus</i> <i>Pseudomonas</i>	Benzoate Hydroxybenzoate Protocatechuate Vanillate <i>o,m,p</i> -phthalate 2-aminobenzoate Phenol <i>o,m,p</i> -cresol
Sulfate reduction	<i>Desulfovibrio</i> <i>Desulfococcus</i> <i>Desulfonema</i> <i>Desulfosarcina</i>	Benzoate Hydroxybenzoates Phenylacetate Phenol Indole
Fermentation	<i>Coprococcus</i> <i>Streptococcus</i> <i>Pelobacter acidigallici</i> <i>Eubacterium oxidoreductans</i>	Phloroglucinol Resorcinol Gallate, pyrogallol Polyphenols Quercetin
Methanogenic fermentation	Microbial consortia: fermentative bacteria + acetogenic and methanogenic bacteria	Lignin, Benzoate Chlorobenzoate Tyrosine Cinnamate Phenylpropionate Phenylacetate Phenol, Chlorophenol Catechol Hydroquinone Ferulate, Vanillate Syringate Phenylalanine Tyrosine, Tryptophan Benzene Chlorobenzene Chlorophenoxyacetates Nitrophenols Chloroguaiacols
Photosynthetic phosphorylation	<i>Rhodopseudomonas</i> spp. <i>Rubrivivax gelatinosus</i> <i>Rhodospirillum</i> spp. <i>Rhodobacter</i> spp. <i>Rhodomicrobium vannielii</i>	Benzoate <i>m,p</i> -Hydroxybenzoate Phloroglucinol Cinnamate Ferulate Phenylbutyrate Phenylpropionate Phenylvalerate Chlorobenzoate

Modified from Evans and Fuchs (1988).

Additional data from Harwood and Gibson (1988), Shoreit and Shabeb (1994)

Table 2.6 Anaerobic metabolism of aromatic compounds.

2.8.1 Denitrification

Denitrifying bacteria chiefly derive energy from electron transport phosphorylation during nitrate respiration, obtaining cell carbon from breakdown products of an organic compound.

Initially it was thought that the ability of denitrifying strains to degrade aromatic compounds was due to them being photosynthetic. However, Taylor *et al.* (1970) isolated a *Pseudomonas*-like organism which anaerobically metabolised benzoate in the obligatory presence of nitrate as electron acceptor. The organism was later classified as *Alcaligenes xylosoxidans*. Williams and Evans (1975) isolated a strain of *Paracoccus denitrificans* that metabolised benzoate by way of ring reduction to cyclohexanecarboxylate, ultimately yielding adipate. The initial ring reduction is likely coupled to a low-potential reductant, possibly a flavodoxin or ferredoxin (Evans and Fuchs 1988).

Schennen *et al.* (1985) were able to show the anaerobic degradation of 2-fluorobenzoate by a benzoate-utilising denitrifying bacterium. The fluoride was eliminated reductively and the benzoate was thioesterified by an induced benzoyl-CoA synthetase before being metabolised.

Bakker (1977) demonstrated that an enrichment culture comprised predominantly of *Pseudomonas* and *Spirillum* species, degraded phenol and the cresols anaerobically in a nitrate-mineral salts medium. [*ring*-U-¹⁴C]phenol was converted to ¹⁴CO₂, radioactive cell material and labelled *n*-caproate and acetate were detected in the culture fluid. Using pure strains of *Pseudomonas*-type bacteria, Tschech and Fuchs (1987) showed that phenol was degraded in a nitrate-mineral salts medium by carboxylation of the phenol to 4-hydroxybenzoate. This reaction was shown to be catalysed by a phenol carboxylase, described as a Mn²⁺ enzyme. Figure 2.4 shows the two proposed pathways for phenol degradation by denitrifying bacteria.

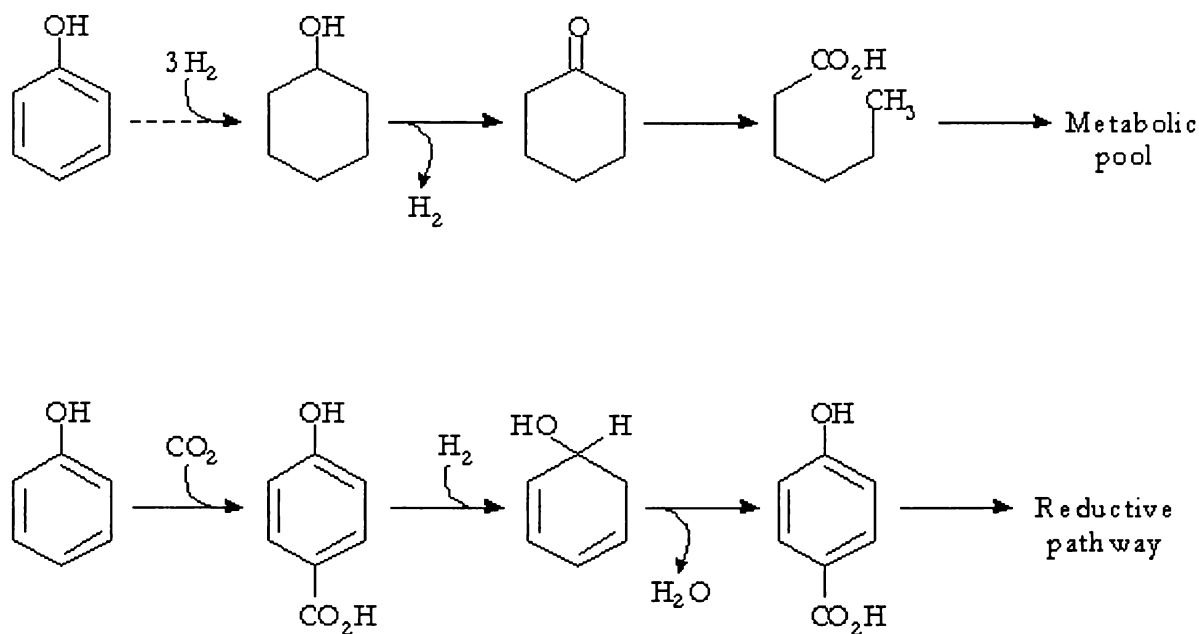


Figure 2.4 Proposed pathways for the anaerobic metabolism of phenol by denitrifying bacteria.

2.8.2 Sulfate Reduction

Sulfate-reducing bacteria couple the oxidation of organic compounds with water to the exergonic reduction of sulfate via sulfite to sulfide. Energy is derived predominantly from electron transport phosphorylation during sulfite reduction. Cell carbon is derived from breakdown products of the organic compound.

Although early studies did not show that sulfate reducers could degrade aromatic compounds, a number of bacteria capable of benzoate degradation were isolated in coculture with sulfate reducers (Balba and Evans 1980; Mountfort and Bryant 1982). Widdel (1980) isolated four genera of sulfate reducers from various environments using benzoate as the substrate; *Desulfovibrio*, *Desulfococcus*, *Desulfonema*, and *Desulfosarcina*. All strains isolated were capable of degrading a variety of aromatic compounds including; benzoate, cyclohexanecarboxylate, phenylacetate, 3-phenylpropionate, and hydroxybenzoates.

Bak and Widdel (1986) isolated a bacterium, *Desulfobacterium phenolicum*, that completely oxidised phenol with sulfate as terminal electron acceptor. The indole-oxidising *Desulfobacterium indolicum* was also isolated (Bak and Widdel 1986a). The degradation of *p*-cresol under sulfate reducing conditions has been reported by Häggblom *et al.* (1990). The proposed pathway for *p*-cresol degradation is via oxidation of the methyl substituent to *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate.

2.8.3 Fermentation

In fermentation, microorganisms derive their energy from substrate-level phosphorylation reactions. Organic compounds serve as electron donors and acceptors.

Species of *Coprococcus* and *Streptococcus* were isolated from the rumen and shown to ferment phloroglucinol via dihydrophloroglucinol to acetate (Tsai and Jones 1975; Patel *et al.* 1981). Schink and Pfennig (1982) isolated five strains of a novel, strictly anaerobic bacterium from marine sediment using gallic acid, pyrogallol, phloroglucinol, or 2,4,6-trihydroxybenzoate as substrate. All substrates were fermented to acetate and CO₂. The bacteria was named *Pelobacter acidigallici*. Coculture studies with *P. acidigallici*, *Acetobacterium woodii*, and methanogenic bacteria showed the transformation of methoxylated gallic acid derivatives into methane and CO₂.

The degradation of various aromatic compounds by fermentation has been shown to rely on syntrophic relationships in coculture. Tschech and Schink (1986) showed that degradation of various mono- and dihydroxybenzoates by fermenting isolates was conditional on the presence of *Desulfovibrio vulgaris* or *A. woodii* as hydrogen scavenger.

Krumholz and Bryant (1986) isolated an anaerobic chemoorganotroph, *Syntrophococcus sucromutans*, from the rumen that cleaved methyl-ether linkages of substituted monobenzenoids, e.g. syringate, caffeate, and vanillin.

2.8.4 Methanogenesis

In anoxic marine environments, methanogenesis is the process by which most organic matter is degraded. Pure cultures of methanogenic bacteria are able to use only a small number of simple substrates for growth. All methanogenic consortia must therefore rely on syntrophic associations with fermenters that degrade complex organic compounds into usable products for the methanogens.

Initial work performed by Boruff and Buswell (1934) and Tarvin and Buswell (1934) showed that organic acids, including aromatic acids, were decomposed into CO₂ and CH₄ in sewage consortia. Later, ¹⁴C-labelled benzoate was shown to be converted to ¹⁴CO₂ and ¹⁴CH₄ by an adapted methanogenic consortium (Clark and Fina 1952). Further work by different researchers involving isotopic trapping experiments showed that a number of fermentation products were formed from benzoate degradation. The variety of fermentation products were attributed to the different consortia, made up of multiple species, studied in different laboratories (Fina *et al.* 1978; Balba and Evans 1978; Shlomi *et al.* 1978). All the results did however support the view that benzoate is initially reduced, probably as the CoA ester. This is followed by a β-oxidation sequence leading to ring cleavage and aliphatic acids. Figure 2.5 summarises the probable pathways for benzoate and phenol degradation by adapted bacterial consortia from a variety of methanogenic ecosystems.

Much work has also been performed on the conversion of phenols and catechols to CH₄ and CO₂. Healy and Young (1978) showed that the fermentation gases were produced within days of adding the substrates to adapted sewage consortia.

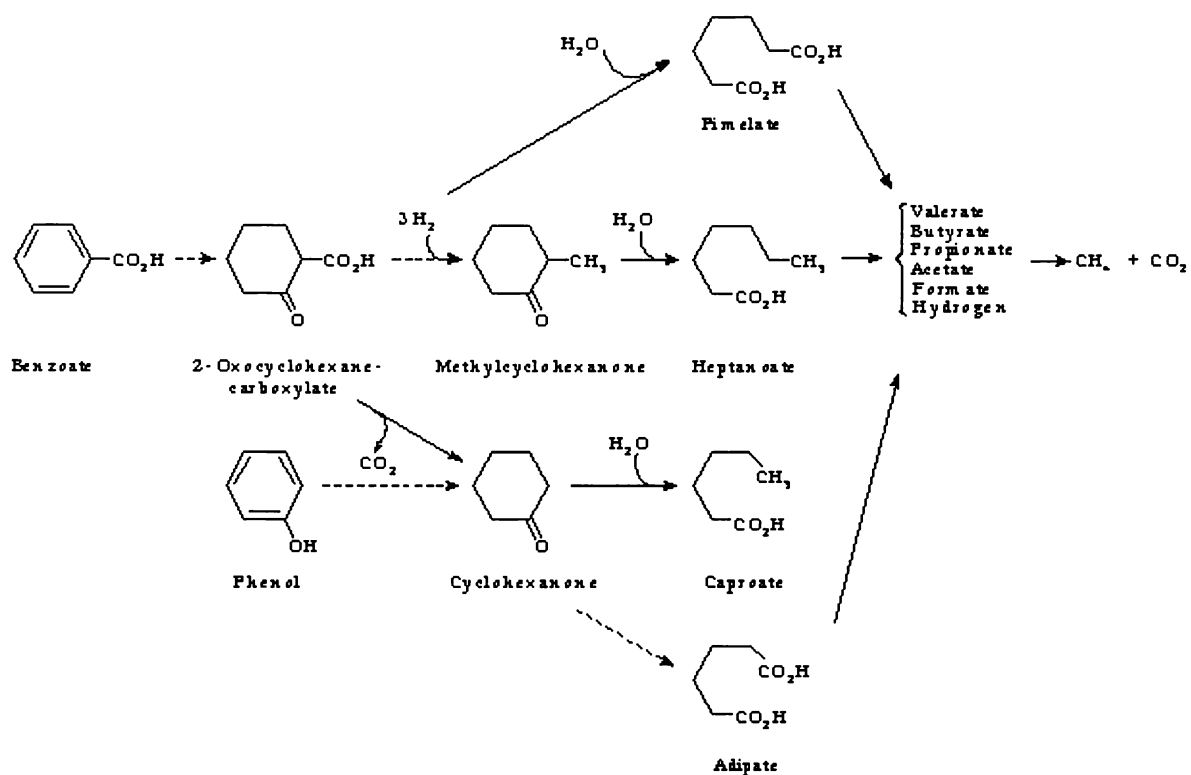


Figure 2.5 Probable pathways for the methanogenic fermentation of benzoate and phenol.

Knoll and Winter (1987) found that benzoate was formed from phenol in sewage sludge in the presence of H_2 and CO_2 ; the metabolism may have proceeded via 4-hydroxybenzoate. Other work has included the study of cresol metabolism in anoxic water (Smolinski and Suflita 1987; Häggblom *et al.* 1990), and the mineralisation of pentachlorophenol (Mikesell and Boyd 1986).

The metabolism of phenol and catechol by methanogenic consortia was studied in detail by Balba and Evans (1980). They observed that an enrichment culture actively metabolising catechol, converted *cis*-1,2-[U- ^{14}C]benzenediol into radioactive phenol, cyclohexanol, cyclohexanone, 2-hydroxycyclohexanone, adipate, succinate, propionate, acetate, CH_4 , and CO_2 .

Other aromatic compounds shown to be degraded were methoxybenzoates, protocatechuate, β -phenylpropionate, cinnamate, phenylacetate, phenylalanine, tyrosine, and tryptophan (Balba *et al.* 1979; Balba and Evans 1980).

From the results of the latter three mentioned above, tentative metabolic pathways for the methanogenesis of aromatic amino acids were proposed. The anaerobic transformations of the alanine side chains were shown to be similar to those observed in the rumen (Scott *et al.* 1964); a unique characteristic of the process is the dismemberment of the aromatic residues for cell growth and methanogenesis. Aromatic hydrocarbons such as toluene and benzene (Kuhn *et al.* 1985), and lignin monomers such as ferulate (Healy *et al.* 1980) have also been shown to be degraded under methanogenic conditions.

2.8.5 Anaerobic Photometabolism

Several species of the purple nonsulfur bacteria can grow anaerobically in the light at the expense of simple aromatic compounds as sole carbon source.

Initial attempts to elucidate the aromatic breakdown of aromatic acids such as benzoate were based on a literal interpretation of the general scheme for photosynthesis in bacteria and plants. One of the products of the light reaction was a strong oxidant which, in plants, was converted into molecular oxygen; in bacteria the proposedly light-induced "bound oxygen" was used to oxidise substrates. Proctor and Scher (1960a, 1960b) surmised that molecular oxygen and the light-oxidant were equivalent and the pathway for benzoate metabolism was similar to those of well-known aerobic processes.

Leadbetter and Hawk (1965) and Button and Evans (1967) grew cells of *Rhodospseudomonas palustris* photosynthetically on benzoate and 3-hydroxybenzoate. Neither substrate promoted growth under aerobic conditions,

hence molecular oxygen and any light-generated oxidant were not equivalent. Growth under anaerobic conditions in the light afforded benzoate utilisation.

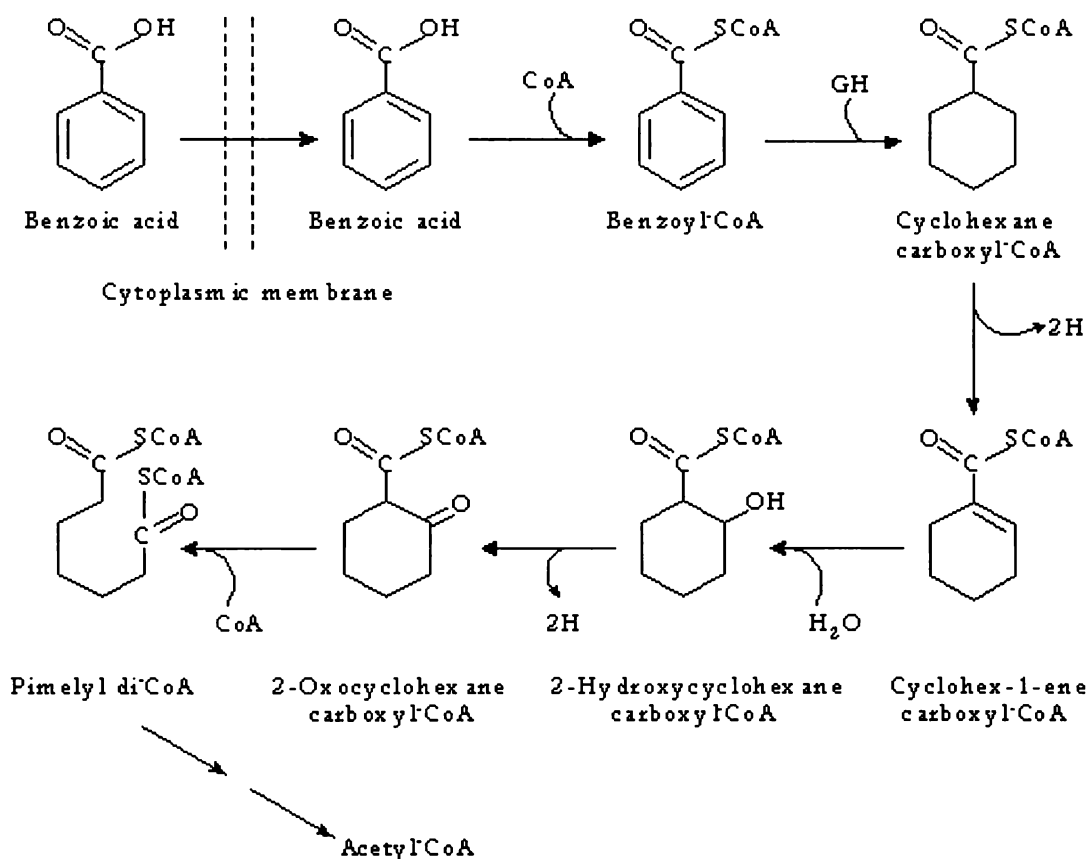
2.8.5.1 Anaerobic benzoate metabolism by *Rhodopseudomonas palustris*

By incubation of unlabeled test intermediates with cells actively photometabolising ¹⁴C-labelled benzoate, Dutton and Evans (1968) were able to determine the key intermediates in the anaerobic photosynthetic metabolism of benzoate by *Rps. palustris*. The five products that incorporated the isotope were: cyclohex-1-enecarboxylate, cyclohexanecarboxylate, 2-hydrocyclohexanecarboxylate, 2-oxocyclohexanecarboxylate, and pimelate. Guyer and Hegeman (1969) confirmed these results by growing isolated mutants of *Rps. palustris* on the proposed intermediates. Each strain was able to grow on all proposed intermediates later in the pathway than the compound for which it was selected, but not on the preceding intermediates.

Dutton and Evans (1970) found that monocarboxylic acids can inhibit the rate of benzoate utilisation by whole cells. After the acid was utilised the rate was recovered, suggesting that fatty acids compete as substrates for enzymes or cofactors, such as coenzyme A, involved in benzoate photoassimilation.

Whittle *et al.* (1976) verified this by showing that cell-free extracts from cells grown photoheterotrophically on benzoate were capable of catalysing the thioesterification of benzoate to benzoyl-CoA. Hutber and Ribbons (1983) followed this work with an identification of an acyl-CoA synthetase in addition to confirming the presence of all the enzymes required for the β -oxidation of cyclohexanecarboxyl-CoA by *Rps. palustris* cells grown anaerobically on benzoate. Further work has shown that an O₂-sensitive benzoyl-CoA ligase exists. It catalyses the formation of acyl-CoA from carboxylate and coenzyme A with high

specificity for benzoate (Geissler *et al* 1988). Intracellularly, it has been shown that benzoate is immediately converted to benzoyl-CoA and the kinetics of benzoate uptake match the kinetic properties of the acyl-CoA ligase, confirming its role in catalysing the first degradation step (Harwood and Gibson 1986). Figure 2.6 illustrates the proposed pathway for benzoate metabolism by *Rps. palustris*.



Evans and Fuchs (1988)

Figure 2.6 Proposed pathway of benzoate metabolism by *Rps. palustris*.

2.8.5.2 Further anaerobic aromatic metabolism by the purple nonsulfur bacteria

Pfennig *et al.* (1965) showed that the metabolism of benzoate by *Rcy. purpureus* and *Rsp. fulvum* occurred via the same mechanism as for *Rps. palustris*. Studies by Harwood and Gibson (1988) showed that *Rps. palustris* could utilise a range of aromatic compounds in addition to benzoate under phototrophic conditions. Such compounds include caffeate, cinnamate, ferulate, and 4-hydroxybenzoate. *Rps. palustris* has also been shown to grow phototrophically on halogenated benzoic acids by reductive dehalogenation and subsequent utilisation of the remaining acid (van der Woude *et al.* 1994).

Other purple nonsulfur species known to grow at the expense of aromatic compounds include *Rba. blasticus*, *Rmi. vanniellii*, *Rsp. rubrum* and *Rvi. gelatinosus*. (Wright and Madigan 1991; Rahalkar *et al.* 1991; Shoreit and Shabeb 1994). Whittle *et al.* (1976) showed that *Rvi. gelatinosus* grows anaerobically in the light on phloroglucinol, with intermediates such as dihydrophloroglucinol and 2-oxo-4-hydroxyadipate appearing in the culture fluid. However, little is known of the reactions involved, including the ring-cleavage mechanism. Blasco and Castillo (1992) reported that a strain of *Rba. capsulatus* was able to transform 2,4-dinitrophenol to 2-amino-4-nitrophenol without further metabolism. Growth in the presence of mono-nitrophenols with acetate as carbon source was also reported, although the degradation product remained unidentified. Zengler *et al.* (1999) isolated a strain of *Blc. sulfoviridis* capable of utilising toluene for phototrophic growth. The mechanism was shown to proceed via benzylsuccinate, the same activation mechanism reported for denitrifying and sulfate reducing bacteria.

Part C: Bacterial Dehalogenation and Dehalogenases

Halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use as herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticisers, and intermediates for chemical syntheses. Because of their toxicity, persistence, and ubiquitous distribution, halogenated compounds are of public concern (Fetzner and Lingens 1994).

Many natural abiotic and biotic processes contribute to halogenated substances in the environment, an example being the release into seawater of volatile halogenated organics by many species of marine macroalgae. In 1986, it was reported that more than 700 natural halogenated compounds have been identified (Niedleman and Geigert 1986). These naturally occurring halogenated compounds may have an important role to play in the adaptation of microorganisms to degrade xenobiotic halogenated compounds.

The recalcitrance of halogenated organic compounds is related to the number, type, and position of the halogen substituents. The carbon-halogen bond is regarded as increasingly recalcitrant with increased electronegativity of the substituent, and polyhalogenated compounds are more difficult to degrade than those compounds with only one or two substituents.

This section will discuss the different mechanisms utilised in the microbial dehalogenation of both haloaromatic and haloaliphatic compounds, and the dehalogenase enzymes involved.

2.9 Mechanisms of Microbial Dehalogenation

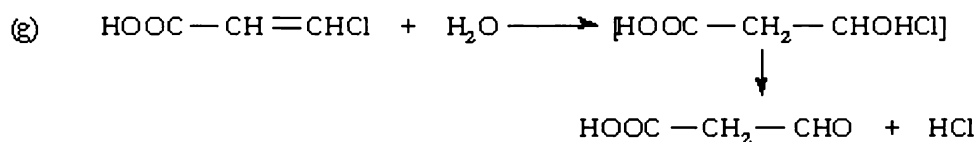
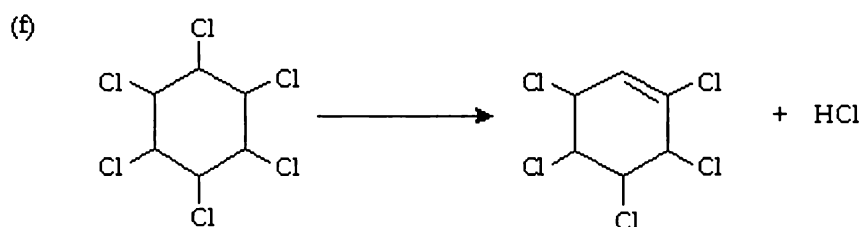
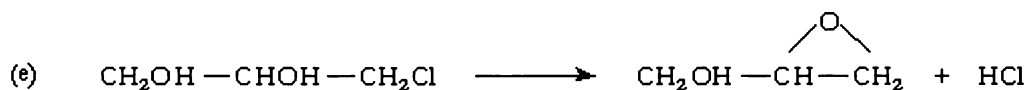
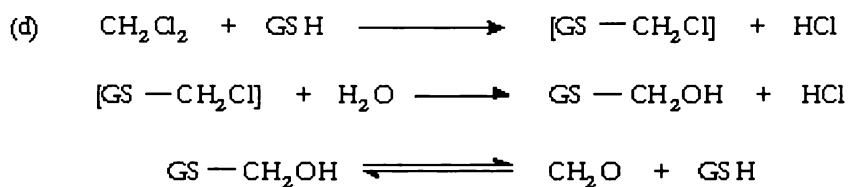
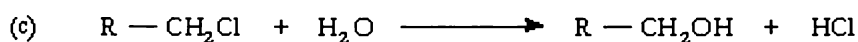
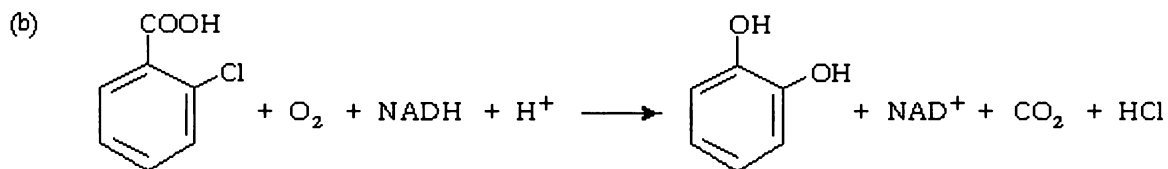
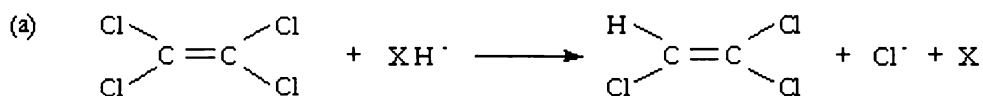
Microbial dehalogenation may involve the spontaneous chemical breakage of the carbon-halogen bond from unstable primary products of an unassociated enzyme

reaction, or a fortuitous removal of the halogen as a result of the action of broad-specificity enzymes. The process that will be discussed in depth is the cleavage of the carbon-halogen bond as catalysed by specific enzymes, the dehalogenases.

Seven mechanisms of microbial dehalogenation involving dehalogenases are known so far. Figure 2.7 illustrates each of these mechanisms.

- (a) Reductive dehalogenation involves the removal of the halogen substituent which is replaced by a hydrogen.
- (b) Oxygenolytic dehalogenation reactions are catalysed by mono- or dioxygenases and incorporate one or two atoms of molecular oxygen into the substrate.
- (c) Hydrolytic dehalogenation involves a nucleophilic substitution reaction during which the halogen is replaced with a hydroxy group derived from water.
- (d) Thiolytic dehalogenation occurs in some dichloromethane-utilising bacteria and involves the formation of an *S*-chloromethyl glutathione conjugate with release of the halogen.
- (e) Intramolecular substitution involves an intramolecular nucleophilic displacement, yielding epoxides. Involved in the dehalogenation of vicinal haloalcohols.
- (f) Dehydrohalogenation involves the elimination of HCl and the formation of a double bond.
- (g) Hydration reactions involve the addition of a water molecule to an unsaturated bond. Dehalogenation occurs by chemical decomposition of an unstable intermediate. Occurs in vinylic compounds such as 3-chloroacrylic acid.

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Fetzner and Lingens (1994)

Figure 2.7 Dehalogenation mechanisms. (a) Reductive dehalogenation; (b) oxygenolytic dehalogenation; (c) hydrolytic dehalogenation; (d) thiolytic dehalogenation; (e) intramolecular substitution; (f) dehydrohalogenation; (g) hydration.

2.9.1 Reductive Dehalogenation

Reductive dehalogenation is an important means of biodegradation of numerous compounds, including organochlorine pesticides, alkyl solvents, and aryl halides. It is the only mechanism known for the biodegradation of certain significant pollutants such as polychlorinated biphenyls, hexachlorobenzene, and pentachlorophenol. Reductive dehalogenation occurs mainly under anaerobic conditions and is the initial step in the anaerobic biodegradation of most aryl halides. In addition, reductive dehalogenation is involved in the aerobic degradation of certain highly halogenated compounds.

Reductive dehalogenation involves the removal of a halogen substituent from a molecule with the concurrent addition of an electron to the molecule. Two processes of reductive dehalogenation have been identified. The first process, hydrogenolysis, is the replacement of a halogen substituent of a molecule with a hydrogen atom. The second process involves the removal of two halogen substituents from adjacent carbon atoms with the formation of an additional bond between the carbon atoms, and is known as vicinal reduction or dihaloelimination. Hydrogenolysis can dehalogenate alkyl or aryl halides, whereas vicinal reduction can transform only alkyl halides. Both processes require an electron donor (Mohn and Tiedje 1992).

2.9.1.1 Reductive dehalogenation of haloaliphatic compounds

Halogenated aliphatic compounds are of major concern environmentally due to their recalcitrance and the quantities being produced. Many one- and two-carbon halogenated solvents including chloroform, tetrachloroethene, and chloromethane, have been widely used in industry for degreasing machinery and in the manufacturing of electrical components. Halogenated carboxylic acids, such as dichloropropionic acid (DPA), have been produced and applied as pesticides.

Among the many dehalogenating mechanisms identified as degrading these compounds, reductive dehalogenation is one of the most important, particularly in anaerobic environments. It is common to find partially dehalogenated compounds as the major contaminant in these environments. An example of this is that *cis*-dichloroethene is the major contaminant when trichloroethene has entered anaerobic aquifers. However, Freedman and Gossett (1989) showed that complete reductive dehalogenation of trichloroethene is possible under methanogenic conditions.

Single species of bacteria capable of reductive dehalogenation of aliphatic compounds are numerous, and appear to be widespread. Species with the ability to reductively dehalogenate, frequently have a wide substrate range. Examples of this include *Methanobacterium thermoautotrophicum* which dehalogenates methanes, ethanes and ethenes (Belay and Daniels 1987), and *Escherichia coli* which dehalogenates methanes and the alkyl section of the pesticide DDT (Criddle *et al.* 1990; Langlois 1967).

Haloalkanes and haloethenes are reductively dehalogenated by a wide range of bacteria, however halocarboxylic acid degradation by this means has not been commonly observed. Nakamura *et al.* (1991) showed that *Clostridium* species were able to degrade low molecular weight halocarboxylic acids such as chloroacetate and 3-chloropropionate by reductive dehalogenation.

Table 2.7 summarises the haloaliphatic compounds degraded by reductive dehalogenation and the consortia and pure cultures known to achieve this.

Substrate	Inoculum/Consortia	Culture
3-Chloropropionic acid		<i>Clostridium</i> spp.
1,2-Dibromo-3-chloro-propane	Soil	
2,3-Dibromobutane	Soil	
Haloethanes	Bioreactor Subsoil Marl, muck	<i>A. woodii</i> <i>M. thermoautotrophicum</i> <i>Methanococcus</i> spp. <i>D. autotrophicum</i>
Haloethenes	Bioreactor Enrichment culture Marl, muck Soil, subsoil	<i>A. woodii</i> <i>M. thermoautotrophicum</i> <i>Methanococcus</i> spp. <i>Methanosarcina</i> spp. <i>D. tiedjei</i>
Halomethanes	Soil Sewage sludge	<i>A. woodii</i> <i>Clostridium</i> spp. <i>D. autotrophicum</i> <i>E. coli</i> <i>M. thermoautotrophicum</i> <i>Methanosarcina</i> spp. <i>P. putida</i>
Trichloroethane		<i>Clostridium</i> spp.

From Mohn and Tiedje (1992)

Table 2.7 Microbial consortia and pure cultures that reductively dehalogenate haloaliphatic compounds.

2.9.1.2 Reductive dehalogenation of haloaromatic compounds

Haloaromatic compounds of environmental significance include pesticides, solvents, and products from numerous industrial processes. Examples of these compounds include halogenated anilines, benzenes, biphenyls, phenoxyacetates, and phenols. The degradation of pentachlorophenol (PCP) in paddy soil was the first recognised reductive dehalogenation of an aromatic compound (Ide *et al.* 1972). Since that time, aromatic reductive dehalogenation has been found and studied in a variety of undefined anaerobic communities, although this activity has not been reported for many pure cultures.

Halogenated benzoic acids have long been studied as compounds reductively dehalogenated by various microorganisms. Of particular interest to this study are the reports of strains of the purple nonsulfur bacterium *Rhodopseudomonas palustris* growing via reductive dehalogenation on 3,5-dichlorobenzoic acid, 3-bromobenzoic acid, and all three isomers of monochlorobenzoic acid (Kamal and Wyndham 1990; Woude *et al.* 1994). The reductive dechlorination of chlorinated phenols, particularly PCP, has been studied in depth. A number of undefined anaerobic consortia have been shown to reductively remove chlorine atoms from PCP. An example of this is the complete reductive dechlorination and mineralisation of PCP by monochlorophenol acclimated activated sewage sludge, as observed by Mikesell and Boyd 1986. Far less has been reported on pure culture reductive dehalogenation of PCP. The ability of the aerobes *Rhodococcus chlorophenolicus* and *Flavobacterium* to reductively remove chlorines from PCP metabolites has been reported (Apajalahti and Salkinoja-Salonen 1987; Steiert and Crawford 1986). Other haloaromatic compounds observed to be reductively dehalogenated either by undefined consortia or pure cultures include chlorobenzenes (Fathepure *et al.* 1988), chloroanilines (Kuhn and Suflita 1989), chlororesorcinols (Fathepure *et al.* 1987), chlorocatechols (Allard *et al.* 1991), and polychlorinated biphenyls (Nies and Vogel 1990).

The bacterium that has been most studied for its aromatic reductive dehalogenating ability is *Desulfomonile tiedjei* DCB-1. Dehalogenation activity within this organism is substrate specific, with activity preferentially directed toward the *meta* position of aromatic substrates. In addition, monochlorinated phenols are not dehalogenated, whereas higher chlorinated phenols are (Mohn and Kennedy 1992). Further studies of *D. tiedjei* have suggested some answers as to why aromatic reductive dehalogenation is most commonly found in complex communities. Dolfig and Tiedje 1986, found that initially, *D. tiedjei* could only be

cultured on 3-chlorobenzoate while in a stable, defined consortium with a benzoate-fermenting rod and an H₂-consuming methanogen (*Methanospirillum* sp.). In this consortium, *D. tiedjei* was dehalogenating the 3-chlorobenzoate, the fermentor was fermenting the benzoate intermediate, and both *D. tiedjei* and the *Methanospirillum* sp. were using the H₂ product from fermentation. Dolfig and Tiedje found that benzoate degradation was reliant on a low H₂ partial pressure, and this was maintained in part by the requirement of H₂ for reductive dehalogenation. The likelihood that the majority of reductively dehalogenating bacteria are involved in such syntrophic relationships in the environment was hence postulated as a reason for the difficulties in obtaining pure cultures of such organisms.

2.9.1.3 Reductive dechlorination of organochlorine pesticides

Reductive dehalogenation in anaerobic environments was found to be of great importance in the degradation of several organochlorine pesticides. With the exception of PCP, all reports have been of alkyl reductive dehalogenation.

The reductive dehalogenation of pesticides such as DDT, lindane, alachlor, dieldrin, heptachlor, and methoxychlor has been shown by pure cultures of bacteria as well as undefined consortia. Figure 2.8 illustrates two of these reactions; (a) the alkyl hydrogenolysis alachlor by anaerobic stream sediment, and (b) the vicinal reduction of lindane to γ -3,4,5,6-tetrachloro-1-cyclohexene by facultative anaerobes.

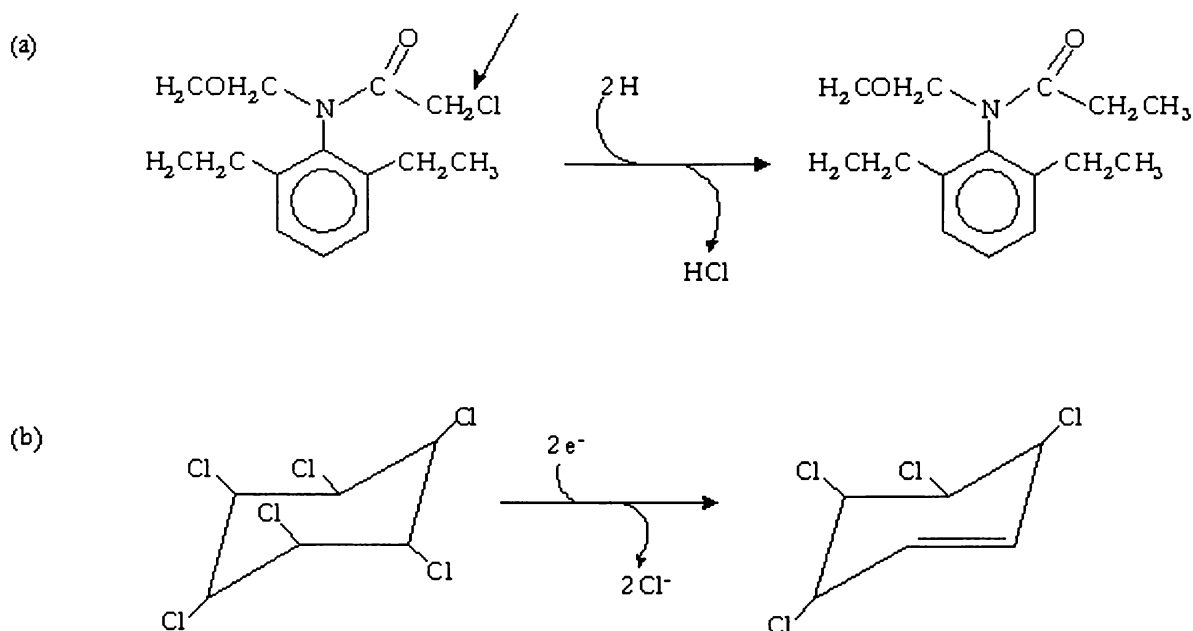


Figure 2.8 Reductive dechlorination mechanisms for alachlor and lindane.
 (a) Alkyl hydrogenolysis of alachlor by anaerobic stream sediment.
 (b) Vicinal reduction of lindane to γ -3,4,5,6-tetrachloro-1-cyclohexene.

2.9.2 Other Dehalogenation Mechanisms

A number of other mechanisms have been identified for the dehalogenation of aromatic and aliphatic compounds. These are summarised below with reference to the dehalogenases involved.

2.9.2.1 Oxygenolytic Dehalogenation

Oxygenolytic dehalogenation is the most common mechanism identified for the removal of halogen substituents from aromatic compounds. The mechanism is defined as the incorporation of one or two molecules of oxygen into a substrate with the associated removal of halogen substituents. The enzymes involved in oxygenolytic dehalogenation are the oxygenases. Two types of oxygenase exist, the monooxygenases and the dioxygenases.

The aerobic degradation of PCP has been reported for a number of bacteria including *Arthrobacter* and *Flavobacterium*. Schenk *et al.* (1990) showed that a PCP 4-hydroxylase from an *Arthrobacter* strain catalysed a NADPH- and oxygen-dependant dehalogenation, suggesting a monooxygenase-type reaction. Xun *et al.* (1992a) purified a PCP 4-hydroxylase from a *Flavobacterium* strain that catalysed the incorporation of ^{18}O from $^{18}\text{O}_2$ but not from H_2^{18}O into the reaction product tetrachloro-*p*-hydroquinone. This proved the monooxygenase mechanism of tetrachloro-*p*-hydroquinone formation as shown in Figure 2.9.

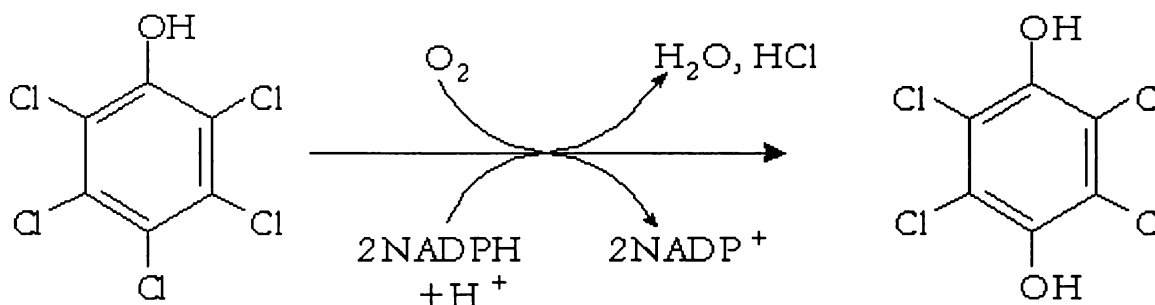


Figure 2.9 Proposed mechanism for the monooxygenase catalysed formation of tetrachloro-*p*-hydroquinone by a *Flavobacterium* strain.

Xun *et al.* (1992b) reported that the same PCP 4-monooxygenase was able to catalyse the *para*-hydroxylation of a broad range of substituted phenols. In addition to removing halogens, it could also catalyse the removal of nitro, amino and cyano groups. Other compounds known to be dehalogenated by monooxygenase catalysed reactions include less highly halogenated phenols, chlorinated guaiacols, and chlorinated syringols (Hägglom *et al.* 1988).

Examples of dehalogenation involving dioxygenase enzymes include the conversion of 2-chlorobenzoate to catechol and the conversion of 4-chlorophenylacetate to 3,4-hydroxyphenylacetate (Figure 2.10), both by species of *Pseudomonas* (Fetzner *et al.* 1992). Dioxygenases, like monooxygenases, are aspecific and can convert several substrates with other substituents at the position of the halogen.

Very little is known of the role of oxygenases in the dehalogenation of aliphatic halogenated compounds. It is thought that oxygenases may play a role in the utilisation of methyl chloride by a strain of *Hyphomicrobium*, and in the growth of a *Pseudomonas* strain on 1,2-dichloroethane (Hartmans *et al.* 1986; Stucki *et al.* 1983).

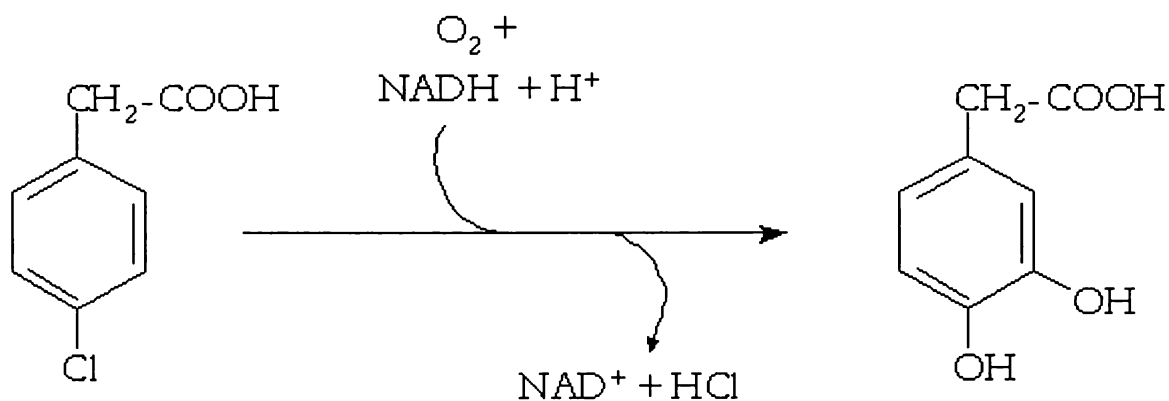


Figure 2.10 The dioxygenase mediated conversion of 4-chlorophenylacetate to 3,4-hydroxyphenylacetate by a species of *Pseudomonas*.

2.9.2.2 Hydrolytic Dehalogenation

Hydrolytic dehalogenation reactions are defined as the nucleophilic displacement of the halogen with a hydroxy group from water, and are catalysed by hydrolytic dehalogenases. The principal substrates for these enzymes are 2-halocarboxylic

acids and haloalkanes.

Enzymes that convert only 2-haloacetates have been termed haloacetate dehalogenases, while enzymes that convert both haloacetates and longer-chain halocarboxylic acids are termed 2-haloacid dehalogenases. Haloacetate dehalogenases are classified into two types; the enzymes which are active in the cleavage of the carbon-fluorine bond of fluoroacetate, and those enzymes which do not catalyse this reaction. These are named type 1 and type 2 haloacetate dehalogenases respectively. Au and Walsh (1984) suggested that a type 1 haloacetate dehalogenase from a *Pseudomonas* strain was encoded by a plasmid. Since this time, both type 1 and type 2 haloacetate dehalogenases have been shown to be plasmid-borne, with some organisms carrying a plasmid encoding for both types of enzyme (Kawasaki *et al.* 1981). 2-haloacid dehalogenases have been classified into five mechanistic groups as suggested by Weightman *et al.* (1982) and Hardman (1991). These groups along with representative enzymes and the organisms from which they were isolated are summarised in Table 2.8.

Group	Characteristics	Organism (Enzyme)
1.	Reaction results in inversion of configuration L-2MCPA is converted, D-2MCPA is not Reaction is unaffected by sulfhydryl-blocking agents (MCPA = monochloropropionic acid)	<i>Pseudomonas</i> (HI, HII) <i>Rhizobium</i> (HI) <i>P. cepacia</i> (HdIIVa) <i>X. autotrophicus</i> (DhIB) <i>P. putida</i> (HadL, DehH109)
2.	Reaction results in inversion of configuration Both L-2MCPA and D-2MCPA are converted Reaction is unaffected by sulfhydryl-blocking agents	<i>P. putida</i> (DehII) <i>Rhizobium</i> (HII)
3.	Catalysis with retention of configuration Both L-2MCPA and D-2MCPA are converted Reaction is inhibited by sulfhydryl-blocking agents	<i>P. putida</i> (DehI)
4.	Reaction results in inversion of configuration D-2MCPA is converted; L-2MCPA is not Reaction is unaffected by sulfhydryl-blocking agents	<i>Rhizobium</i> (HIII) <i>P. putida</i> (HadD)
5.	Enzymes with elevated activity toward Trichloroacetate	

Table 2.8 Mechanistic groups of 2-haloacid dehalogenases.

Group 1 2-haloacid dehalogenases are active toward the L-isomer of 2-monochloropropionic acid, yielding D-lactate as the product. Goldman *et al* (1968) and van der Ploeg *et al.* (1991) suggested two possible mechanisms to explain the observed inversion of configuration: (i) nucleophilic attack by activated water or (ii) attack by a carboxylate group of the enzyme, followed by ester hydrolysis (Fig 2.11). Both structural and genetic data obtained from isolated 2-haloacid dehalogenases support the latter mechanism for enzymes of this group.

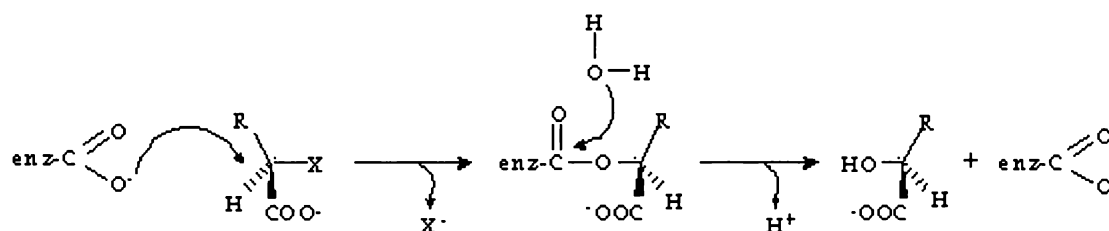


Figure 2.11 Suggested dehalogenation mechanism for group 1 2-haloacid dehalogenase.

2-haloacid dehalogenases of group 2 also catalyse the halide hydrolysis with inversion of product configuration. However, these enzymes are active toward both L- and D- isomers of 2-monochloropropionic acid.

Group 3 dehalogenases also use both L- and D- isomers of monochloropropionic acid, however the product of the reaction has the same optical configuration as the substrate. These enzymes are inhibited by sulfhydryl reagents. To account for this it has been suggested that a double inversion mechanism involving a thioether intermediate occurs, this would also result in the observed retention of configuration (Weightman *et al.* 1982).

Three 2-haloacid dehalogenases were isolated from a *Rhizobium* species and one from a *Pseudomonas putida* strain, each being active only toward the D-isomer of 2-monochloropropionic acid. The reaction results in the inversion of product configuration, and the enzymes are classified as group 4 2-haloacid dehalogenases. The suggested mechanism is that of a base catalysis, promoting nucleophilic attack with water as the nucleophile (Smith *et al.* 1990).

A fifth group of 2-haloacid dehalogenases may be represented by enzymes with elevated activity toward trichloroacetate, catalysing the complete hydrolytic dechlorination of this substrate.

Direct hydrolytic dehalogenation of haloalkanes was first observed with the haloalkane dehalogenase from the nitrogen-fixing hydrogen bacterium *Xanthobacter autotrophicus* GJ10. The strain is capable of the rapid utilisation of C-1 to C-4 -halogenated *n*-alkanes and α,ω -dihalogenated *n*-alkanes, but is inactive towards halogenated carboxylic acids (Janssen *et al.* 1985). The enzyme was purified and incubated with 1,2-dichloroethane in the presence of H_2^{18}O , which resulted in the incorporation of ^{18}O into 2-chloroethanol and onto the carboxylate group of a nucleophilic residue on the enzyme called Asp-124 (Franken *et al.* 1991). This result, in addition to mutagenesis and crystallographic studies, allowed for the proposal of a haloalkane dehalogenase mechanism (Pries *et al.* 1994; Verschueren *et al.* 1993). It was concluded that the substrate was attacked by the nucleophilic residue of Asp-124 and the subsequent covalent ester intermediate was cleaved by a water molecule, releasing the alcohol. Haloalkane-degrading organisms usually possess a single dehalogenating enzyme active toward a broad range of haloalkanes. However, Scholtz *et al.* (1988) showed that an *Arthrobacter* strain contains three haloalkane dehalogenases. This indicated that haloalkane utilisers may also possess multiple enzyme forms, just as the 2-haloacid-utilising bacteria do.

Both Gram negative and Gram positive bacteria have been shown to possess haloalkane dehalogenases and the list of substrates known to be dehalogenated includes a large number of mono- and di-, chloro-, bromo-, and iodoalkanes of chain length C1-C16.

2.9.2.3 Thiolytic Dehalogenation

Thiolytic dehalogenation can occur by either one of two reactions: (i) spontaneous decomposition of a glutathione adduct (in dihalomethane metabolism) and (ii) substitutive displacement of the organic group by a second glutathione molecule (in pentachlorophenol metabolism). Both reactions are preceded by the nucleophilic displacement of the halogen substituent with a glutathione conjugate, catalysed by a glutathione transferase. Bacteria that have shown the ability to dehalogenate using either of these mechanisms include *Methylobacterium* and *Flavobacterium* (Kohler-Staub and Leisinger 1985; Xun *et al.* 1992). Figure 2.12 shows an example of the use of a second glutathione molecule to remove a chlorine from the PCP-degradation product, tetrachloro-*p*-hydroquinone by a *Flavobacterium*.

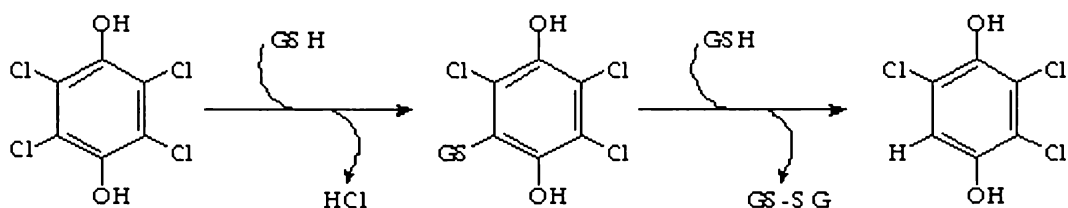


Figure 2.12 Thiolytic dehalogenation of tetrachloro-*p*-hydroquinone by a species of *Flavobacterium*.

2.9.2.4 Intramolecular Substitution

The intramolecular nucleophilic displacement of *vic*-haloalcohols to their corresponding epoxides is catalysed by haloalcohol lyases. Only two of these enzymes have been characterised in any detail; they were isolated from a *Corynebacterium* species and an *Arthrobacter* species (Nagasawa *et al.* 1992; van den Wijngaard *et al.* 1991). Both enzymes were very similar and were more active toward bromoalcohols than chloroalcohols. The enzymes use 2-propanols with the halogen substituents in the 1- or 3-position.

2.9.2.5 Dehydrohalogenation

Dehydrohalogenation is the first step in the metabolism of γ -hexachlorocyclohexane by a strain of *Pseudomonas paucimobilis* and is catalysed by the enzyme dehydrochlorinase. A molecule of HCl is eliminated and a double bond is formed to give the product γ -pentachlorocyclohexene, which is further converted by the same enzyme to 1,3,4,6-tetrachloro-1,4-cyclohexadiene. The enzyme is active with α -, γ -, and δ -hexachlorocyclohexane, but not with the β -isomer or several linear chloroalkanes (Nagata *et al.* 1993).

2.9.2.6 Hydration

A hydration type dehalogenation has been proposed for the bacterial conversion of a few compounds that carry a halogen substituent on an unsaturated carbon atom. Such compounds include aliphatic acrylic acids and aromatic compounds.

The best evidence for a hydratase-type reaction comes from studies on 4-chlorobenzoate degradation by a *Pseudomonas* strain. The hydratase system converts 4-chlorobenzoate to 4-hydroxybenzoate. Müller *et al.* (1984) showed by oxygen isotope-incorporation experiments that the hydroxyl oxygen is derived from

water. The dehalogenation reaction can be considered a concerted activity of various enzymes as the reaction can only occur after activation of the substrate to its coenzyme A derivative (Loeffler and Müller 1991).

Some coryneform bacteria are also capable of a hydration-type dehalogenation, showing activity toward *cis*- and *trans*-3-chloroacrylic acid. The addition of water across the double bond yields an unstable intermediate, which is spontaneously decomposed with release of the halide (Hartmans *et al.* 1991).

2.10 Applications of Dehalogenation

Technological applications of bacterial transformations of halogenated compounds can be considered with respect to two different objectives: (i) synthesis and (ii) degradation (Fetzner and Lingens 1994).

2.10.1 Synthesis

Microbial strains or purified dehalogenases may be used for the biosynthetic generation of halogenated synthetic intermediates or novel compounds. Currently, halogenated compounds are synthesised predominantly by chemical techniques. However, biotransformations could provide economic and versatile alternatives.

One such example of this is in the production of esters of 2-monochloropropionic acid, which are used as intermediates in the production of pharmaceuticals. When racemic 2-monochloropropionate is used for chemical syntheses, racemic products are generated. However, in most cases only one of the isomers is biologically active, and half of the racemic product would act as expensive 'chiral ballast' or could even

lead to toxic side effects. This is overcome by selective dehalogenation of the unwanted isomer of 2-monochloropropionate by the chiral specificity of 2-haloacid

dehalogenases (Motosugi *et al.* 1983). The commercial production of L-2-monochloropropionate from racemic 2-monochloropropionate for use in herbicide manufacture is achieved by a strain of *Pseudomonas putida* (Taylor 1987).

Other commercially important intermediates that are known to be produced via dehalogenase activity include (R)-3-chloro-1,2-propanediol, (S)-epichlorohydrin, and β -hydroxynitriles.

2.10.2 Degradation

The application of dehalogenating bacteria and enzymes in environmental and waste management systems is receiving increasing attention.

In the area of bioaugmentation, much work has been carried out with reference to dehalogenating bacteria. The stimulation of in situ organisms by optimisation of their nutrient supply or by addition of substrate analogues is one area of bioaugmentation. It was observed by Wyndham and Straus (1988) that low phosphate concentrations in the water of a chlorobenzoate-contaminated creek were responsible for limiting growth of a chlorobenzoate-degrading *Alcaligenes* species. Analogue enrichment with aniline was used successfully as a method for the removal of 3,4-dichloroaniline from contaminated soil. The effect of aniline was to induce pathways that cometabolised 3,4-dichloroaniline (You and Bartha 1982). Another method of bioaugmentation is the addition of wild-type or mutant microorganisms to contaminated sites. An example of this method is the treatment of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol contaminated effluents from 2,4-D manufacture by defined bacterial strains (Finn 1983). Other examples include: the bioremediation of PCP contaminated soil by PCP-degrading strains of a *Flavobacterium* species (Crawford and Mohn 1985), the mineralisation of polychlorinated biphenyls in soils by a *Pseudomonas* strain (Hickey *et al.* 1993),

and the removal of tetrachloromethane from groundwater by another *Pseudomonas* strain (Criddle *et al.* 1990).

Downstream use of biofilters in industrial processes is another method by which contaminants can be removed. Strains of *Hyphomicrobium* have been used as inocula in trickling filters to remove dichloromethane from industrial waste gases (Hartmans and Tramper 1991). Mixed culture biofilters have also been used, an example of which is the removal of 1,2-dichloroethane and vinyl chloride from waste gases with vinyl chloride-utilising strain of *Mycobacterium* and the dichloroethane utiliser *Xanthobacter autotrophicus* GJ10 (Hartmans *et al.* 1992).

At this stage, many companies prefer physiochemical halogenated-waste treatment methods, which, although expensive, guarantee high efficiency. However, the use of natural, adapted, or genetically engineered organisms is becoming a more viable option as the difficulties and costs of conventional cleanup methods increase.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This chapter provides an overview of the materials and methods used throughout this study. More detailed methodologies used in specific experiments are described in the relevant chapters.

3.2 List of organisms used

All culture collection strains of purple nonsulfur bacteria used in this study were obtained as gift cultures from previous workers. Table 3.1 lists the culture collection strains used.

<i>Blc. viridis</i> DSM 133	<i>Rmi. vannielii</i> DSM 162	<i>Phs. molischianum</i> DSM 120
<i>Rba. blasticus</i> DSM 2131	<i>Rpl. roseus</i> DSM 5909	<i>Rsp. photometricum</i> DSM 122
<i>Rba. capsulatus</i> DSM 1710	<i>Rps. acidophila</i> DSM 137	<i>Rsp. rubrum</i> DSM 467
<i>Rba. sphaeroides</i> DSM 158	<i>Rps. palustris</i> ATCC 33872	<i>Rvi. gelatinosus</i> DSM 1709
<i>Rcy. purpureus</i> DSM 168	<i>Rps. palustris</i> DSM 123	

Table 3.1 List of purple nonsulfur bacteria culture collection strains used in this study.

All other organisms used in this study were obtained from the environment via enrichment and isolation. Table 3.2 lists the isolates used in this study and the environment from which they came.

ISOLATES	SOURCE
<i>Rba. blasticus</i> TK 2	Te Koutu Lake
<i>Rps. palustris</i> CCFEP 1	Farm effluent pond
<i>Rps. palustris</i> CCLRSP 1	Lake Rotoroa subsidiary pond
<i>Rps. palustris</i> CCLRSP 2	Lake Rotoroa subsidiary pond
<i>Rps. palustris</i> CCLRSP 3	Lake Rotoroa subsidiary pond
<i>Rps. palustris</i> Eff 1	Farm effluent drain
<i>Rps. palustris</i> FEP 1	Farm effluent pond
<i>Rps. palustris</i> FEP 2	Farm effluent pond
<i>Rps. palustris</i> FP 1	Farm pond
<i>Rps. palustris</i> FP 2	Farm pond
<i>Rps. palustris</i> TK 1	Te Koutu Lake
<i>Rps. palustris</i> TK 2	Te Koutu Lake
<i>Rmi. vanniellii</i> FEP 2	Farm effluent pond

Table 3.2 List of purple nonsulfur bacteria isolates used in this study.

3.3 Growth media and associated additives

All chemicals used in the preparation of growth media were of analytical grade, and were supplied by Aldrich Chemical Company (Milwaukee, USA) or Sigma Chemical Company (St. Louis, USA). All gases used were obtained from New Zealand Industrial Gases (Wellington, New Zealand).

Water used for preparation of growth media and additives was supplied by a Millipore Milli-Q filtration system (Millipore Corporation, Massachusetts, USA).

3.3.1 Basal salts medium

The following were dissolved in distilled water to a total volume of 1L:

0.5g KH_2PO_4 , 0.2g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4g NaCl, 0.05g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.4g NH_4Cl , 0.02g yeast extract, 1mL trace element solution (Section 3.33), 1mL Vitamin B₁₂ (cyanocobalamin) solution at 10mg/mL, and 5mL ferric citrate solution at 1g/mL.

The medium was sterilised by autoclaving (Biebl and Pfennig 1981).

Yeast extract was added to the medium as a source of vitamins in place of a defined vitamin solution. In addition, yeast extract acts as a ready substrate for respiration during dark incubation, allowing the removal of trace amounts of dissolved oxygen which would affect phototrophic growth upon transfer to the light. At 0.002%, yeast extract supports a small amount of growth. This is useful in detecting toxicity of a test substrate. If no growth occurs on the yeast extract, the test substrate is inhibitory at the concentration tested.

3.3.2 Medium M-27

The following were dissolved in distilled water to a total volume of 1L:

0.5g KH_2PO_4 , 0.4g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4g NaCl , 0.05g CaCl_2 , 0.4g NH_4Cl , 1g yeast extract, 1mL trace element solution, 1mL Vitamin B_{12} (cyanocobalamin) solution at 10mg/mL. 5mL ferric citrate solution at 1g/mL, 0.5mL ethanol, and 1g disodium succinate. The medium was sterilised by autoclaving.

3.3.3 Trace element solution

The following salts were dissolved in 6.5mL of 25%(v/v) hydrochloric acid:

60mg H_3BO_4 , 100mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 120mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 70mg ZnCl_2 , 25mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 15mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and 25mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$. This solution was made up to 1L with distilled water, and sterilised by autoclaving.

3.3.4 Bicarbonate solution

A 7.5% bicarbonate solution was prepared by dissolving 7.5g NaHCO_3 to a total volume of 100mL in carbon dioxide saturated distilled water.

The water was saturated by alternately flushing the headspace of a 500mL flask with CO_2 , and shaking the stoppered vessel vigorously. this process was continued until no negative pressure was felt on removal of the stopper

(Lee 1992). The solution was then either autoclaved in sealed universal bottles, or filter sterilised.

Bicarbonate solution was generally prepared when required, and used immediately. In general, 0.2mL of sterile bicarbonate solution was added aseptically to each Hungate tube, bringing the final pH to the desired 6.8 (Charlton 1997).

3.3.5 Sulfide solution

5g $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ was dissolved in 90mL of distilled water which had been degassed under vacuum for 15 minutes. The solution was neutralised by slow addition with stirring of 5M HCl, and made up to 100mL with more degassed distilled water. The resulting 5% sulfide solution was autoclaved in a serum vial with the headspace flushed with oxygen-free nitrogen gas.

3.3.6 DCMU solution

0.0233g of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was dissolved in 100mL of distilled water which had been degassed by boiling in a microwave oven. The resulting 1mM solution was filter sterilised through a Supor Acrodisc 32 syringe-tip filter (Gelman Sciences, Ann Arbor, USA) into a sterile 150ml serum vial. The headspace was then flushed with oxygen-free nitrogen gas and the vial sealed.

3.3.7 Medium for *Rhodopseudomonas acidophila*

The medium for *Rhodopseudomonas acidophila* was the basal salts medium described in Section 3.3.1, with the pH adjusted to 5 prior to autoclaving.

3.3.8 Preparation of anaerobic media

All growth experiments were carried out under anaerobic conditions. Large quantities of growth media were dispensed anaerobically by the following method, which is similar to that described by Patel, Morgan and Daniel (1985).

1 litre of medium was degassed in an Oxoid dispenser by boiling vigorously for 1 minute in a microwave oven at high power. Porcelain chips were added to minimise bumping.

The headspace of the dispenser was flushed with a stream of oxygen-free nitrogen gas directed through a bent wide-bore (typically 18-gauge) syringe-needle. This flow was maintained to retain an oxygen-free headspace over the medium throughout the dispensing procedure. The medium was allowed to cool until it was safe to handle (typically 30 minutes).

A second bent syringe-needle carrying oxygen-free nitrogen was attached to the outlet pipe of the dispenser in such a fashion that both might be placed in the mouth of the recipient vessel upon dispensing. This allowed the headspace of the recipient vessel to be displaced with nitrogen, ensuring anaerobic transfer. The vessel was sealed with an s-butyl rubber stopper immediately after dispensing.

Hungate tubes were filled with 9.5mL of medium. Serum vials were filled with 20mL, 50mL or 100mL, depending on their size. Occasionally, the appropriate volume of medium was degassed directly in a serum vial by boiling in a microwave oven.

3.3.9 Sterilisation of media

All media were sterilised by autoclaving using a time-temperature automatic autoclave (Mercer and Sons Ltd, Christchurch, New Zealand) at 121°C and one atmosphere overpressure for 20 minutes.

3.3.10 Sterile manipulations

All manipulations involving sterile media and substrates were performed in a sterile laminar flow cabinet (Nuair Ltd, Maine, USA).

3.4 Growth substrates

All substrates for growth were prepared as 100mM stock solutions in degassed distilled water, and stored under oxygen-free nitrogen in serum vials or Bellco bottles sealed with a s-butyl rubber stopper. This procedure minimised the introduction of oxygen to anaerobic growth vessels when adding the substrate. All stock solutions were sterilised by filter sterilisation as described in Section 3.3.6.

Structural formulae and abbreviations of substrates used in this study are displayed below.

3.4.1 Halocarboxylic acids

Some of the halocarboxylic acids used were light sensitive, as a precaution all stock solutions of halocarboxylic acids were stored at room temperature in the dark.

HALOCARBOXYLIC ACID	ABBREVIATION	STRUCTURE
Bromoacetic acid	BAA	Br-CH ₂ COOH
Chloroacetic acid	CAA	Cl-CH ₂ COOH
Fluoroacetic acid	FAA	F-CH ₂ COOH
Iodoacetic acid	IAA	I-CH ₂ COOH
2-Bromopropionic acid	2-BPA	CH ₃ CH(Br)COOH
2-Chloropropionic acid	2-CPA	CH ₃ CH(Cl)COOH
3-Chloropropionic acid	3-CPA	Cl-CH ₂ CH ₂ COOH

Table 3.3 Abbreviations and structural formulae of halocarboxylic acids used.

3.4.2 Phenols

Due to the light sensitivity of all phenols used, the precaution of storing all stock solutions at room temperature in the dark was taken.

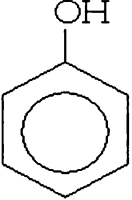
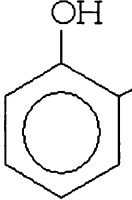
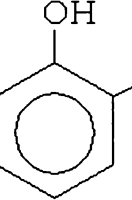
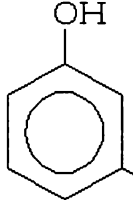
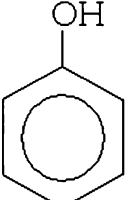
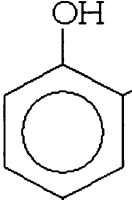
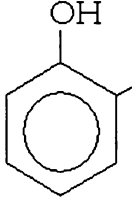
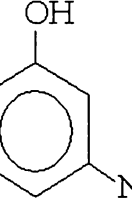
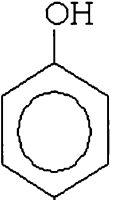
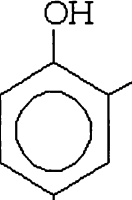
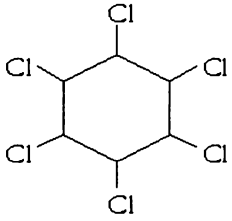
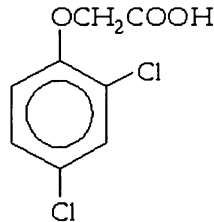
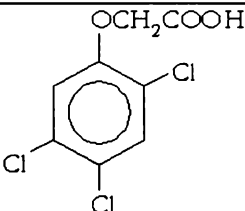
 <p>Phenol</p>	 <p><i>o</i>-cresol</p>	 <p><i>o</i>-chlorophenol (2-CP)</p>	 <p><i>m</i>-chlorophenol (3-CP)</p>	 <p><i>p</i>-chlorophenol (4-CP)</p>
 <p>2,4-dichloro-phenol (2,4-DCP)</p>	 <p><i>o</i>-nitrophenol (2-NP)</p>	 <p><i>m</i>-nitrophenol (3-NP)</p>	 <p><i>p</i>-nitrophenol (4-NP)</p>	 <p>2,4-dinitro-phenol (2,4-DNP)</p>

Table 3.4 Abbreviations and structural formulae of phenols used.

3.4.3 Miscellaneous substrates

The structural formulae of substrates used in this study that did not fall into a specific family of compounds are shown below.

COMPOUND	ABBREVIATION	STRUCTURE
Bromopropane	BP	$\text{CH}_3\text{CH}_2\text{CH}_2\text{-Br}$
Chloropropane	CP	$\text{CH}_3\text{CH}_2\text{CH}_2\text{-Cl}$
γ -hexachlorocyclohexane Prepared as a stable stock emulsion of 10mM by sonication using an Ultrasonic Processor Model W-380 (Heat-Systems-Ultrasonics, Inc.).	Lindane*	
2,4-dichlorophenoxyacetic acid Solubility in water achieved by dropwise addition of 0.1M NaOH until dissolved.	2,4-D*	
2,4,5-trichlorophenoxyacetic acid Solubility in water achieved by dropwise addition of 0.1M NaOH until dissolved.	2,4,5-T*	

* Common names

Table 3.5 Abbreviations and structures of miscellaneous compounds used.

3.4.4 Safety precautions

The majority of the compounds used as substrates in this study are volatile and considered highly toxic. All manipulations involving neat liquids or pure solids were carried out in a Captair Hazardous Chemical Cabinet, Model 5008NU (Captair Ltd, Boston, USA). Filter sterilisation of stock solutions was carried out in a Class II Flow Cabinet whilst wearing an Easi-Air breathing apparatus (3M Corporation, St. Paul, USA).

3.5 Incubation of cultures

After inoculation, cultures were incubated in the dark at 25°C for 24 hours to enable them to remove traces of oxygen through respiration. Upon transfer to the light, residual oxygen would cause photooxidative damage to the cells.

Cultures were incubated in the light at a constant temperature of 30°C. Continuous illumination of approximately 1200 lux was provided by lamps fitted with 100 W tungsten bulbs.

3.6 Methods related to enrichment, isolation and identification

Discussed below are the methods related to the enrichment, isolation and identification of the bacteria isolated through the course of this study.

3.6.1 Sampling

Samples of the upper 2-3cm of sediment from suitable sites were taken using a spatula or long-handled sampling ladle, depending on water depth. Sediment samples were placed in screw-cap universal bottles or 1 litre screw-cap bottles, depending on sample size. Bottles were filled to about 75% volume with sediment that was overlain to the brim with water from the sample site. Samples were stored in the dark at 25°C until required.

3.6.2 Enrichment

The three basic techniques employed in this study for the enrichment of anoxygenic phototrophs are outlined below. Further details are discussed in Chapter 4 of this study.

3.6.2.1 Liquid enrichment

All liquid enrichments were performed in Hungate tubes containing; basal salts medium (Section 3.3.1), a suitable carbon source, sample inoculum, and 0.1mL of DCMU solution (Section 3.3.6) to inhibit the growth of algae.

Sediment sample bottles were inverted several times to mix the sediment and overlaying water to form a slurry. Approximately 0.2mL of this slurry was injected aseptically via a hypodermic syringe and needle into a pre-dispensed Hungate tube (Section 3.3.8) containing the balance of the contents listed above. Upon addition of the inoculum, the enrichment tube was placed in the dark overnight before light incubation (Section 3.5).

3.6.2.2 Winogradsky columns

Winogradsky columns were prepared in glass columns measuring approximately 20cm in height and 5cm in diameter.

A sheet of filter paper of 10cm in diameter was torn into small pieces of 1cm diameter or less and placed at the base of the column as an additional source of carbon for cellulose degraders. Approximately 2g of CaSO_4 was added to the filter paper to encourage the proliferation of sulfate reducers at the base of the column, hence providing the column with a sulfide gradient.

A slurry of sample water and sediment was then added to the column, care was taken to avoid the introduction of air-pockets during packing. The slurry was added to approximately 75% the height of the column. A cotton wool ball of approximately 2cm in diameter was soaked in a 100mM solution of an appropriate carbon source and placed with forceps into the slurry about half way down the column on the inner wall. This created a diffusion gradient of the carbon source and allowed for the formation of colonies able to utilise the particular compound. The slurry was then overlain with 4cm of basal salts medium. Aluminium foil was then used to

seal the mouth of the column to prevent moisture loss by evaporation during the incubation.

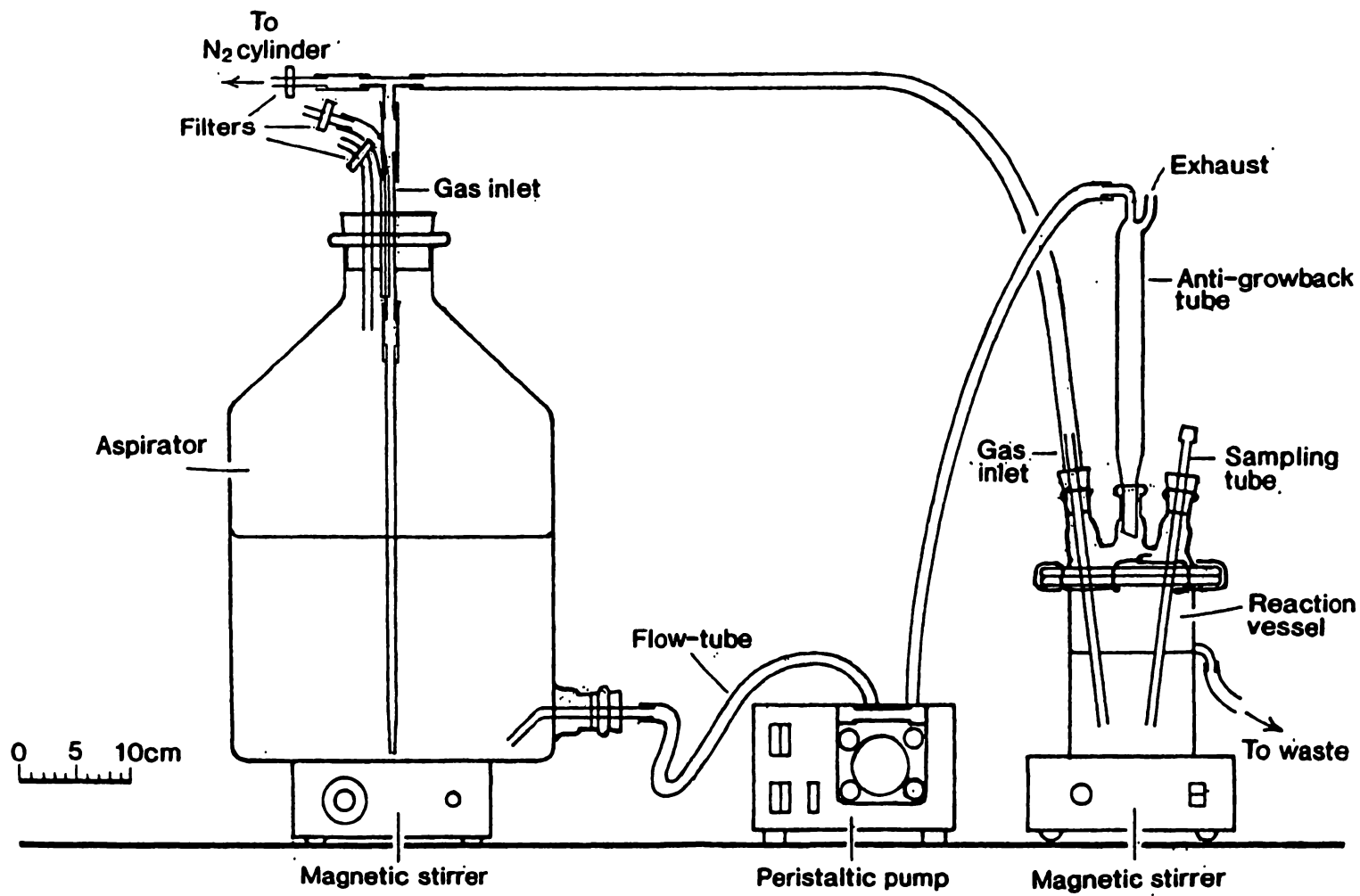
The column was then placed in the dark overnight to allow respirative removal of excess oxygen before being placed in the light (Section 3.5). The same portion of the column was illuminated throughout the incubation. Incubation times varied between three weeks and two months.

After a suitable incubation time, pigmented colonies growing between the mud and the glass were removed using a long Pasteur pipette or thin spatula and placed in serial dilution tubes (Section 3.6.3.1).

3.6.2.3 Continuous culture

Figure 3.1 represents the continuous culture system employed for the isolation of some strains in this study.

A 20 litre aspirator was approximately half filled with degassed basal salts medium with a suitable carbon source added to a concentration of 1mM. The aspirator was sealed with a stopper through which two glass tube were inserted. One of these tubes acted as a gas inlet and was immersed most of the way into the medium. The tubing from the gas cylinder was fitted with a 0.2 μm pore size in-line filter. Another tube was fitted with similar filter and acted as an exhaust. From the outlet, s-butyl rubber tubing was used to connect the aspirator to the reaction vessel via an anti-growback tube. The reaction vessel included a sampling port which was comprised of a thin piece of glass tubing with a rubber septum through which samples could be taken with a 10ml hypodermic syringe and needle. The sampling tube was held in place by a rubber stopper through which it was previously inserted. Another tube acted as a gas inlet to the reaction vessel. Attached to the overflow outlet of the vessel was a length of rubber tubing which connected it to a 5 litre stoppered waste vessel. The reaction vessel was filled to just below the



3.1 Schematic of the continuous culture system

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overflow outlet with degassed basal salts medium. Both the aspirator and reaction vessel were fitted with magnetic stirrers. The whole system was then flushed with oxygen-free nitrogen gas.

The gas inlet tube was then removed from the nitrogen source and sealed off. The whole system shown in Figure 3.1, excluding the peristaltic pump, was autoclaved at 121°C for 90 minutes at one atmosphere overpressure. To allow for gas volume changes during autoclaving, the aspirator stopper was rested on an angle and covered with aluminium foil.

Once the system had cooled, a peristaltic pump was added to the system in the position shown in Figure 3.1. The flow rate was maintained at a constant 20 ml/hr. The gas inlet tube was connected to an oxygen-free nitrogen cylinder and the system was continuously gassed throughout incubation.

During incubation, aluminium foil was placed around the aspirator to avoid any possible photooxidation of substrate. This was not deemed to be necessary for the reaction vessel, as the concentration of substrate at any one time is minimal and the smaller volume and the microorganisms present would allow for anaerobic conditions to be more effectively maintained. An inoculum of sample sediment slurry was added through the sampling tube with a hypodermic syringe and needle before the system was incubated at 30°C with constant illumination of the reaction vessel.

3.6.3 Isolation

Two basic methods for the isolation of individual strains of bacteria from enrichment cultures were used in this study. These are outlined below.

3.6.3.1 Agar shake tubes

The most commonly used method for the isolation of individual strains in this study was the agar shake method.

Approximately 200 tubes were prepared at the same time. 3mL of 2.2% molten purified agar was dispensed into each disposable glass tube. Each tube was capped with a loose-fitting plastic cap and all tubes were then autoclaved (Section 3.3.9). The tubes were then stored in the dark at 5°C until required.

Five tubes were then placed in a beaker of hot water and heated in a microwave oven until the agar had melted. 6mL of basal salts medium, 0.2mL of bicarbonate solution, and an appropriate concentration of carbon source were then added to each tube, upon which each tube was placed in a 45°C waterbath. An inoculum of 0.05mL from an enrichment culture was then added aseptically to a single tube and mixed thoroughly by rolling between the palms of the hands. From this tube, approximately two drops were poured into the second tube in the series and this was mixed as for the first tube. Two drops from tube two was then poured into a third tube, and the process repeated until the dilution series was completed.

To prevent diffusion of oxygen into the agar during incubation, a wax plug was used as a barrier at the agar-air interface. This consisted of a 3:1 (v/w) mixture of paraffin and paraffin wax that was previously sterilised in an oven at 160°C for six hours. Once the agar in the tubes had set, 0.2mL of the hot molten paraffin mixture was poured onto the agar surface. After overnight incubation in the dark at room temperature, the region of wax adjacent to the glass was melted slightly to ensure a seal between the glass and the wax plug. The tubes were then incubated

as described in Section 3.5.

After an appropriate incubation period, tubes with well-separated colonies were selected for isolation. Commonly, tubes four and five in the series were selected. A diamond pen was used to score the surface of the tube and just prior to breakage, the surface was swabbed with 70% ethanol and allowed to dry. A hammer was then used to deliver a light tap on the scored surface of the tube, causing it to crack. The broken glass was then removed with sterile forceps to expose the agar surface.

Using a syringe and needle containing 0.1mL of anaerobic medium, a single colony was removed from the agar and injected directly into a pre-dispensed Hungate tube. This was repeated for other colonies of interest in the agar.

3.6.3.2 Dilution series

When attempting to isolate organisms from liquid enrichments, it was sometimes necessary to employ a dilution series to further enrich for the organisms required prior to an agar shake series.

Commonly, the dilution series consisted of five Hungate tubes containing basal slats medium, bicarbonate solution, and a carbon source. An inoculum of 0.1mL of enrichment culture was added to the first tube, and 100-fold dilutions were performed along the dilution series.

All tubes were incubated as described in Section 3.5, and the inocula from the fourth and fifth tubes of the series were used for agar shake series.

3.6.4 Identification

As described in Chapter 4 of this study, all of the isolates obtained belonged to the purple nonsulfur bacteria. The positive identification of the isolates obtained required the analysis of a number of characteristics. These are outlined below.

3.6.4.1 Morphology

The purple nonsulfur bacteria are morphologically diverse; hence microscopic examination of a growing culture, in most cases, yielded enough information to place an isolate in a genus. The morphological properties of importance included; cell shape, cell size, mode of division, cell aggregation, motility, presence of sphaeroidene carotenoids, and colour of cell suspension.

Figure 3.2 represents a scheme for the morphological characterisation of the isolates into their genera.

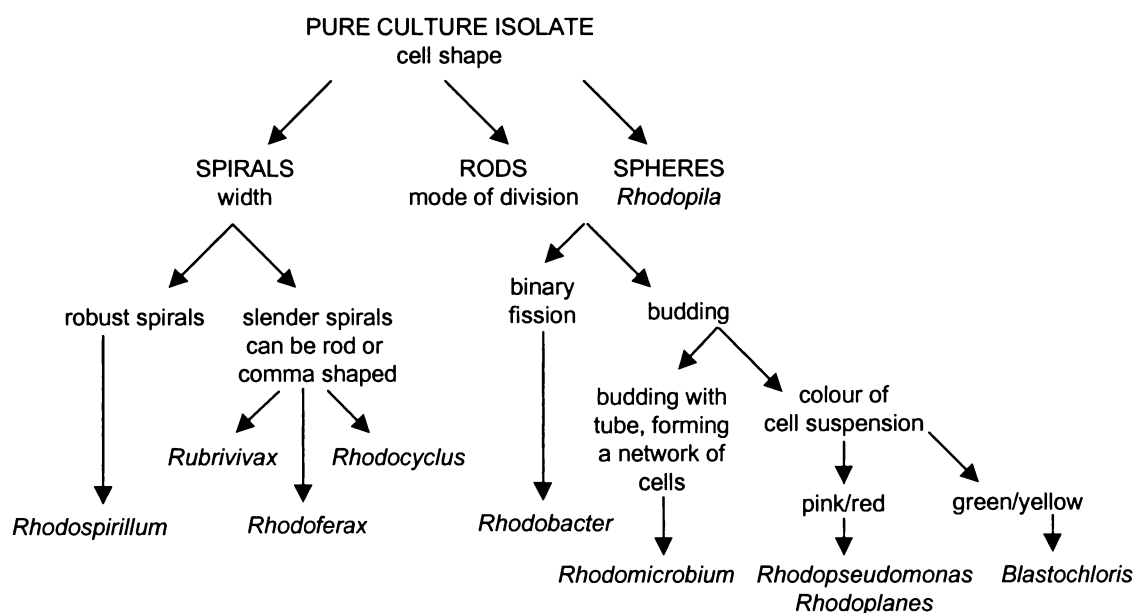


Figure 3.2 Scheme for the identification of genera of purple nonsulfur bacteria.

The presence of sphaeroidene carotenoids in *Rhodobacter* species allows these organisms to be differentiated from *Rhodofera* and *Rhodocyclus*, which are also rod-shaped and divide by binary fission. The species *Rubrivivax gelatinosus* also possesses sphaeroidene carotenoids but is easily distinguished from *Rhodobacter* species by the formation of macroscopic cell aggregates in cultures of the organism. Cells of *Rba. capsulatus* tend to form zigzag chains whereas cells of *Rba. sphaeroides* seldom form chains.

Rcy. tenuis is characteristically spiral shaped with the width of the spirals being much smaller than that of the smallest *Rhodospirillum* species, *Rsp. fulvum*.

Isolates of the genera *Rhodospirillum* or *Phaeospirillum* can be distinguished according to the colour of phototrophically grown cultures. *Phs. fulvum* and *Phs. molischianum* are coloured brown and are thus difficult to distinguish from *Rsp. photometricum*; *Rsp. rubrum* is coloured red. *Phaeospirillum* species can be distinguished morphologically on the basis of size whereas *Rsp. rubrum* can be distinguished from the other red spirilla by having no obligate NaCl requirement.

Rhodopseudomonas palustris is most easily distinguished from other species of *Rhodopseudomonas* by the tendency of cells to form rosettes. Phototrophically grown cultures of *Rhodopseudomonas* tend to grow as a deep-red colour, whereas the morphologically similar *Rhodoplanes* grow light-red to pink.

3.6.4.2 Substrate utilisation

The utilisation of certain substrates by the isolates obtained was useful in confirmation at the species level. Table 2.5 shows which substrates may be useful in determination.

Benzoate, ethanol and mannitol can be used as determinative carbon sources for distinguishing *Rps. palustris* from species of the genus *Rhodoplanes*.

Rps. palustris is capable of using these substrates as sole carbon source for growth, whereas *Rhodoplanes* species are not.

Ethanol is also used as a sole carbon source for growth by *Rba. sphaeroides*. This is useful in its determination over the very similar *Rba. capsulatus* which cannot utilise ethanol.

Of the brown spirillum species, only *Rsp. photometricum* can grow on fructose and glucose as sole carbon sources. *Phs. fulvum* is the only brown spirillum that can utilise benzoate, and the only one that cannot use lactate.

The carbon sources selected for diagnostic purposes in this study were; benzoate, citrate, ethanol, fructose, glucose, lactate and mannitol.

3.6.4.3 Light and electron microscopy

For the routine examination of isolates for morphological characteristics, an Olympus BH-2 phase contrast microscope was used (Olympus Optical, Tokyo, Japan). Transmission electron microscopy was used to determine membrane structure.

3.6.4.4 Colour of cell suspension

The colour of cell suspensions was determined visually for cells grown photoheterotrophically for seven days using pyruvate as sole carbon source.

3.6.4.5 Oxidation of sphaeroidene carotenoids

Isolates that were suspected of belonging to the genus *Rhodobacter* were tested for the presence of sphaeroidene carotenoids. Approximately 2mL of photoheterotrophically grown cells which appeared yellow-brown in colour were transferred into a 10mL glass tube and shaken vigorously to incorporate air into the sample. This was then allowed to stand for up to one hour before being

examined. Samples which had changed to a red colour indicated that sphaeroidene carotenoids present had been oxidised to sphaeroidenone.

3.6.5 16S rDNA sequence analysis

The final confirmation of the identification of an isolate was the sequencing of the 16S rDNA molecule of the organism.

3.6.5.1 DNA Extraction

The following protocol was used in this study for the extraction of total genomic DNA.

- 10mL of photoheterotrophically grown cells were centrifuged at 8000 x *g* for 10 minutes in a Beckman induction drive centrifuge, and then resuspended in 20mM Tris (pH 7.6), 100mM EDTA to a final volume of 400 μ L in a 1.5mL microcentrifuge tube.
- Lysozyme was added to the cells at a concentration of 15mg/mL, and the mixture incubated at 37°C for 60 minutes or until viscous.
- 45 μ L of 10% SDS and 45 μ L of 20mg/mL proteinase K were added, and the mixture was incubated at 55°C for 30 minutes with occasional mixing.
- An equal volume of a 24:23:1 solution of phenol:chloroform:*iso*-amyl alcohol was added to the tube, mixed gently, and centrifuged at 15900 x *g* for 2 minutes in an Eppendorf bench centrifuge (Model 5415). The top layer was removed and placed in a new tube. This step was repeated.
- To the resulting aqueous phase, enough chloroform to fill the tube was added and then centrifuged as above. The top layer was transferred to a new tube.
- 3M NaCl was added to give 0.2M final concentration, followed by 2.2 volumes of 100% ethanol. Contents were mixed and incubated at -20°C for 30 minutes.

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- The nucleic acid was then pelleted by centrifugation at 12700g for 10 minutes at 4°C. The resulting pellet was washed with 70% ethanol and allowed to air dry before being resuspended in 400µL of TE buffer (pH 8.0) containing 50µg/mL of RNase followed by incubation at 37°C for 30 minutes.
- The mixture was then extracted once with 24:23:1 phenol:chloroform:*iso*-amyl alcohol and then extracted once with chloroform (as above), before the DNA in the final aqueous phase was precipitated with NaCl and ethanol (as above).
- The final pellet was resuspended in TE buffer.
- DNA concentration was calculated from the OD_{260/280} ratio of a 1/100 dilution in Milli-Q water.

3.6.5.2 Polymerase Chain Reaction

The protocol for the amplification of the 16S rDNA gene by the polymerase chain reaction (PCR) is outlined below.

PCR Reaction Mixture

10µL 10x PCR buffer

5µL Taq polymerase (5 units total)

2.5µL dNTPs at 25mM

1µL pA primer at 50µM : Primer sequence: 5'-AGAGTTTGATCCTGGCTCAG-3'

1µL pH* primer at 50µM: Primer sequence: 5'-AAGGAGGTGATCCAGCCGCA-3'

5µL template DNA (50ng total DNA)

Milli-Q water to 100µL

The PCR buffer, Taq polymerase, and dNTPs were obtained from Boehringer Mannheim Ltd (Mannheim, Germany). The primers were synthesised by Life Technologies Ltd (Gaithersburg, USA).

PCR Reaction

3 minutes of denaturation at 94°C

30 cycles of: (a) 45 seconds of denaturation at 94°C

(b) 1 minute of annealing at 50°C

(c) 1.75 minutes of extension at 72°C

5 minute final extension at 72°C

4°C indefinite

The PCR reaction was performed in a Techne Thermocycler (Techne Ltd, Cambridge, UK). To check for the efficiency of the reaction, a sample of the amplified DNA was electrophoresed on a 1% agarose gel at 80volts for 25 minutes. If only one distinct PCR product was visible, then the remaining DNA was cleaned using the Wizard-Prep System (Promega Corp., Madison, USA). This procedure involved adding the 100µL of PCR product to 100µL of Purification Buffer and 1mL of Purification Resin and mixing. The mixture was then passed through the column provided, to which the DNA binded. The column was then washed with 2mL of 80% isopropanol and the DNA eluted with 50µL TE buffer into a new tube.

To check for purity, the cleaned PCR product was electrophoresed as above. The appearance of a single PCR product band with no dNTP or primer dimer contamination bands meant the DNA was pure enough for sequencing.

3.6.5.3 DNA Sequencing

The direct sequencing of the purified PCR product was achieved using the Prism Ready Reaction Dichlororhodamine Terminator Cycle Sequencing Kit (Perkin-Elmer Corp., Norwalk, USA). Sequences were analysed on the Applied Biosystems Model 377 automated DNA sequencer from Perkin-Elmer Corp.

Sequencing was performed by the Waikato DNA Sequencing Facility (The University of Waikato, Hamilton, New Zealand). Sequences were compared with those in the GenBank database using the Blastn programme for the MacIntosh.

The sequencing primer used was Sef 1*, and had the sequence:

5'-CTAACTACGTGCCAGCAGCCG-3'

3.7 Methods related to the measurement of growth

Cultures were tested for utilisation of substrates in Hungate tubes or serum vials. Growth was determined by visual assessment or spectrophotometric measurement.

3.7.1 Visual assessment

This method was used for the initial screening tests for substrate utilisation. It was also useful for assessing growth of cells which formed aggregated colonies on a particular substrate. Positive growth was taken to be a visibly more intense pigmentation in growth tubes than negative control tubes. The size of the undisturbed cell pellet which formed at the bottom of Hungate tubes was also compared.

3.7.2 Spectrophotometric measurement

For the spectrophotometric measurement of growth in Hungate tubes, absorbance was read using a Sequoia-Turner Model 340 spectrophotometer fitted with a light-tight cover. For the spectrophotometric measurement of growth in serum vials, 2mL samples were removed and absorbance was measured on a Shimadzu UV-250 spectrophotometer (Shimadzu Co., Tokyo, Japan). Growth was measured at 660nm on both machines and compared to a negative control.

3.7.3 Total protein determination

In many cases, assessment of growth was made by determination of total cell protein using the Lowry method (from Scopes 1987).

To 1mL of sample (containing up to 0.5mg of protein) was added 3mL of Reagent A. This was mixed thoroughly and placed in a boiling water bath for 15 minutes. After cooling to approximately 30°C, 0.5mL of Reagent B was added and upon mixing, was left to stand for 30 minutes to allow colour development. Upon standing, the optical density was read at 750nm using a Sequoia-Turner Model 340 spectrophotometer. Optical density values were converted to μg protein/mL from a bovine serum albumen standard curve.

Reagent A: 2% Na_2CO_3 in 50mL of 0.1M NaOH;
plus 0.05% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1mL of sodium tartrate

Reagent B: 1:1 mixture of Folin-Ciocalteu reagent and water

3.8 Methods related to the identification and quantitation of products and substrates

Several analytical techniques were used in order to obtain quantitative measurements of substrate and product concentrations throughout the course of growth experiments. The techniques are described below.

3.8.1 Capillary Gas Chromatography

Capillary gas chromatography was used for the quantitation of carboxylic acids, alkanes, and phenolic compounds. The protocols for the analysis of each are described below.

3.8.1.1 Analysis of carboxylic acids

All quantitation of carboxylic acids was performed by capillary gas chromatography. 1mL samples of culture fluid from bacterial cultures grown on halocarboxylic acids as substrate, were disrupted by ultrasonication for 5 minutes with an ultrasonic liquid processor (Model W-380, Heat Systems Ultrasonics, Farmingdale, USA). Samples were then centrifuged at 5000g for 15 minutes in an Eppendorf centrifuge. 2 μ L of the resulting supernatant was injected onto an SGE BP-21 fused-silica capillary column (SGE Inc., Austin, USA), fitted to a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionisation detector. The column was 25m in length and had a bonded FFAP phase with a 0.25 μ m film thickness. The column was run isothermally at 210°C for 20 minutes for each sample with an injector temperature of 210°C and a detector temperature of 240°C.

3.8.1.2 Analysis of phenolic compounds

Sample supernatants for the analysis of phenolic compounds were prepared as for carboxylic analysis above. 0.5mL of the supernatant was extracted twice with an equal volume chloroform. 2 μ L of the resulting sample was injected onto a Supelco PTE-5 QTM fused-silica capillary column (Supelco Inc., Bellefonte, USA), fitted to a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionisation detector. The column was 15m in length and had a 0.53mm internal diameter with a 0.50 μ m film thickness. The column was isothermally at 240°C with an injector temperature of 250°C and a detector temperature of 250°C.

3.8.2 Electrospray Mass Spectrometry

Electrospray mass spectrometry was used for the qualitative identification of products of metabolism.

1mL samples from cultures or cell free systems were sonicated and centrifuged as for Section 3.8.1.1. The resulting supernatant was then diluted 1:1 with an 50:50 (vol/vol) mixture of acetonitrile and water, and injected onto a VG Platform II electrospray mass spectrometer at a cone voltage of 20V. A 50:50 mixture of acetonitrile and water was used as the mobile phase.

Samples that contained phenolic compounds, were extracted twice in a 1:1 volume of liquid phosphine oxide.

3.8.3 Analysis of Cl⁻ and Br⁻ concentration

The measurement of chloride and bromide concentration was achieved by either of two methods in this study. Both of these methods are described below.

3.8.3.1 Ferric thiocyanate method

This method was used for long term growth studies where chloride or bromide concentration was measured.

- A 1mL sample of culture fluid was centrifuged at 5000g for 30 minutes.
- The resulting supernatant was diluted to 20mL with Milli-Q water in a 25mL volumetric flask.
- 2mL of a 0.25M ferric ammonium sulfate solution in 9M nitric acid was added to the flask, followed by 2mL of a saturated solution of mercuric isothiocyanate in ethanol.
- The solution was mixed thoroughly and the colour allowed to develop for 10 minutes.
- The absorbance was then measured against water at 450nm in a Shimadzu UV-250 spectrophotometer.
- The chloride concentration was determined from a calibration curve.

The blank consisted of the growth medium plus substrate, and was treated in the same manner as the sample. The method was an adaptation of that used by Bergmann and Sanik (1957).

3.8.3.2 Chloride electrode method

This method was used for dehalogenation assays where many samples were analysed at short time intervals.

- 500 μ L of sample was diluted to 5mL with Milli-Q water in a 10mL beaker.
- 50 μ L of ionic strength adjusting (ISA) solution was added to the above. The ISA solution was 1M NaNO₃.

- The chloride concentration of the solution was measured using a pHoenix Chloride Ion Combination Glass Electrode (pHoenix Electrode Company, USA) fitted to an Orion SA 520 pH/mV Meter (Orion Research Incorporated, USA).
- Chloride concentration was read from a calibration curve prepared anew for each experiment.

3.9 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was used in the partial characterisation of enzymes during this study.

3.9.1 SDS-PAGE

All SDS-PAGE gels were run using a Mini-PROTEAN II Dual Slab Cell (Bio-Rad Laboratories, Richmond, CA, USA).

<u>Separating Gel Reagents</u>		<u>Stacking Gel Reagents</u>	
4.85mL	Distilled water	6.1mL	Distilled water
2.5mL	1.5M Tris-HCl, pH 8.8	2.5mL	0.5M Tris-HCl, pH 6.8
100µL	10% (w/v) SDS	100µL	10% (w/v) SDS
2.5mL	30% Acrylamide/Bis	1.3mL	30% Acrylamide/Bis
50µL	10% Ammonium persulfate	50µL	10% Ammonium persulfate
5µL	TEMED	10µL	TEMED

To prepare the monomer solution for the separating gel, all the reagents were combined except the ammonium persulfate and the TEMED. The solution was then degassed under vacuum for 15 minutes. The ammonium persulfate and TEMED were then added and the gel immediately poured and overlain with water.

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The separating gel was then left to polymerise overnight. The water was then removed before the stacking gel was poured on top. The stacking gel was prepared as for the separating gel, using the reagents listed above and allowed to polymerise for 45 minutes.

The gel system was set up as per manufacturers instructions and the 5 μ L samples and standards (both containing 10% glycerol and a drop of bromophenol blue marker dye) were loaded into the wells with a Hamilton syringe.

The gel was then run at a constant 200 volts until the marker dye was 5mm from the end of the plate.

The gel was then removed and stained for 30 minutes with 0.1% Coomassie blue R-250 in 40% methanol and 10% acetic acid. The gel was then destained for 2 hours with 40% methanol/10% acetic acid.

CHAPTER 4

ISOLATION AND IDENTIFICATION OF PURPLE NONSULFUR BACTERIA CAPABLE OF GROWTH ON PHENOLIC COMPOUNDS

4.1 Introduction

Members of the purple nonsulfur bacteria have been isolated from a large variety of environments, but are rarely found in high concentrations due to being in constant competition with chemoorganotrophic organisms. Their requirement for organic compounds as photoassimilable carbon sources means the purple nonsulfur bacteria are predominantly found in environments with high populations of heterotrophic organisms that provide the necessary carbon sources. The purple nonsulfur bacteria are unable to utilise polymers such as starch and cellulose

The purple nonsulfur bacteria are found in waters that are very rich in organic matter, such as wastewater stabilisation lagoons (Siefert *et al.* 1979). Other environments commonly containing purple nonsulfur bacteria include the sediments of ponds, drainage ditches, rice fields, and a number of estuarine and marine environments. Work performed in this laboratory has also shown the prevalence of thermotolerant strains of these organisms in thermal sulfide springs (Charlton, unpublished data).

Part A of this chapter describes the isolation and identification of a number of purple nonsulfur bacteria strains from a variety of environments. Emphasis was placed on the enrichment of organisms capable of utilising aromatic compounds as carbon sources for growth. Part B describes growth studies of type-strains and isolated strains of purple nonsulfur bacteria on phenolic compounds.

Part A: Isolation of Purple Nonsulfur Bacteria

4.2 Sampling sites

Selection Criteria

(a) Presence of anaerobic sediment.

The sediment at each site was required to be of a consistency that would ensure anaerobicity within 1 cm from the sediment/water interface. Mud or very fine silty material has proven to be an ideal source for the enrichment and isolation of purple nonsulfur bacteria (McGrath, unpublished data). Sites with sandy or gravelly sediments were not considered.

(b) Site and sediment accessibility

Sampling sites had to be easily accessible and the sediment itself easily sampled. The maximum depth of water mass overlaying the sediment and still allowing for precise sampling was found to be 1 m.

(c) Availability of light

Each sampling site was required to be fully exposed to sunlight. Shaded sites were not considered. The depth and clarity of the overlaying water mass was also a consideration. A visual assessment of the maximum depth for water of a certain clarity still allowing ample light to reach the sediment was made at each sampling site.

(d) Availability of nutrients

Selected sites were those known to be high in available nutrients, either from influent feed or from macrophytes and macrofauna.

(e) Influent characteristics

The characteristics of the influent fed to sampling sites were an important criterion for selection. Selected sites were those receiving influent likely to contain a variety of nutrient and recalcitrant compounds.

Particular emphasis was placed on selecting sites with a known or suspected aromatic compound content in the influent feed.

Site Information

Dairy Shed Effluent Pond

An oxidation pond on a Te Kawa farm receiving dairy shed effluent. The sources of recalcitrant compounds are mainly from detergent products used for milk-line cleaning. Samples of sediment were taken in 0.7 m deep water of reasonable clarity. The sediment consisted primarily of faecal material with some mud content.

Farm Pond

Typical farm 'duck pond' situated in Te Kawa and receiving water via a farm drain system and from direct pasture runoff from an adjacent hillside. The sources of recalcitrant compounds are mainly from herbicides applied periodically to pastures. The pond perimeter is sprayed regularly with herbicide to control grass and blackberry growth. Samples of sediment were taken in 0.5 m deep water of high clarity. The sediment consisted predominantly of mud with a small amount of faecal matter, primarily from a large duck population.

Te Koutu Lake

A sizeable lake situated in a steep-sided gully in Te Koutu Park, Cambridge. The influent feed is primarily stormwater and general drainage. Some of the drainage is from old railway sidings, with areas around the inflow often having visible oil films. Samples were taken in 0.5m of high clarity water. The sediment was predominantly mud with duck faecal matter.

Lake Rotoroa Subsidiary Pond

A small pond situated on the northern side of Lake Rotoroa in central Hamilton City. Essentially stagnant, the pond receives a small influent from road side drainage. Frequent spraying of herbicides around the pond perimeter would contribute to a recalcitrant compound loading. A number of macrophytes grow around and in the pond itself and there is evidence of duck residence. Sampling was performed in 0.2 m of water of reasonable clarity. The sediment was primarily mud overlain with a green algal film.

4.3 Materials and methods

Figure 4.1 outlines the steps employed in the enrichment and isolation of purple nonsulfur bacteria from environmental samples. Each of the individual steps are detailed in the relevant section of Chapter 3.

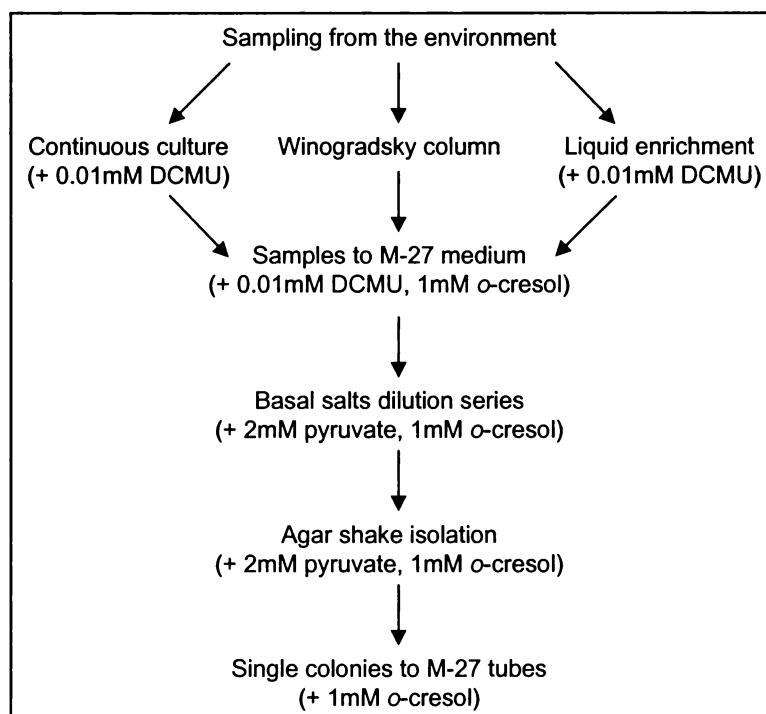


Figure 4.1 Outline of the enrichment and isolation protocol.

4.4 Results

The following results outline the attempts to identify the strains of purple nonsulfur bacteria isolated in this study.

4.4.1 Isolated strains

Three separate methods were used for the enrichment of purple nonsulfur bacteria from sediment samples. Table 4.1 shows the source of the isolated purple nonsulfur bacteria strains and the enrichment method used.

STRAIN	SOURCE	ENRICHMENT
TK1	Te Koutu Lake	Batch (Winogradsky column)
TK2	Te Koutu Lake	Batch (Winogradsky column)
FEP1	Dairy shed effluent pond	Batch (Winogradsky column)
FEP2	Dairy shed effluent pond	Batch (Winogradsky column)
FEP3	Dairy shed effluent pond	Batch (liquid)
Fpond1	Farm pond	Batch (liquid)
Fpond2	Farm pond	Batch (liquid)
CCLRSP1	Lake Rotoroa subsidiary pond	Continuous culture
CCLRSP2	Lake Rotoroa subsidiary pond	Continuous culture
CCLRSP3	Lake Rotoroa subsidiary pond	Continuous culture

Table 4.1 Sources and enrichment methods for the isolated strains.

Winogradsky columns (Section 3.6.2.2) were initially set up for all batch enrichments. However, the time required for colony formation and the difficulties in retrieving the colonies meant that liquid enrichment (Section 3.6.2.1) became the batch method of choice. Due to the set up and running time, continuous culture enrichments (Section 3.6.2.3) were limited to a single sediment sample from the Lake Rotoroa subsidiary pond site. The strain numbers represent bacteria isolated from different sites within the same source via batch enrichment, or in the case of

continuous culture enrichment, bacteria isolated from samples taken at different stages of reactor bloom development.

4.4.2 Morphology

Four morphological features were examined to provide information toward a tentative identification of the strains isolated. Shape, size and motility were examined by light microscopy as described in Section 3.6.4.3. The colour of a photoheterotrophically grown cell suspension of each strain was also noted (Section 3.6.4.4). The morphologies of the purple nonsulfur bacteria strains isolated are shown in Table 4.2.

STRAIN	SHAPE	SIZE* (μm)	MOTILITY	COLOUR
TK1	rod	0.6 x 1.5	+	dark red/brown
TK2	rod	0.6 x 1.5	+	dark red/brown
FEP1	rod/ovoid	0.8 x 1.7	-	light brown/yellow
FEP2	branched rod	1.2 x 2.5	+	pale brown
FEP3	rod	0.7 x 1.2	+	dark red/brown
Fpond1	rod	0.5 x 1.0	+	red/brown
Fpond2	rod	0.5 x 1.3	+	red/brown
CCLRSP1	rod	0.6 x 1.8	+	dark red/brown
CCLRSP2	rod	0.6 x 1.5	+	dark red/brown
CCLRSP3	rod	0.7 x 1.6	+	red

* Average size of cells in single field viewed at x1000 magnification.

Table 4.2 Morphologies of isolated strains.

The overall morphological diversity was small; the majority of the isolates were red to brown, small motile-rods. Cells of strains with this morphology tended to clump together in microscopic rosette-shaped cell aggregates, a characteristic consistent with but not exclusive to the commonly isolated species *Rhodopseudomonas palustris*. The cell suspension colour of strain FEP1 was light brown to yellow and

appeared microscopically as non-motile ovoid to rod-shaped cells. Three purple nonsulfur bacteria, *Rhodobacter blasticus*, *Rhodobacter veldkampii*, and *Rhodovulum adriaticum* share a similar morphology. The latter two require added sulfide and NaCl respectively for growth, whereas strain FEP1 required neither. Strain FEP2 was a pale brown colour and exhibited branching motile-rod morphology. The apparent branching was due to stalked-budding type morphology, a characteristic unique to the genus *Rhodomicrobium*.

4.4.3 Carbon source utilisation

Carbon source utilisation tests were performed in Hungate tubes with basal salts medium (Section 3.3.1) and a substrate concentration of 2mM, except in the case of phenol which was supplied at a concentration of 1mM due to its relative toxicity. Table 4.3 shows the results of these tests, scored as growth (+) or no growth (-). The substrates used were those able to best provide further diagnostic information for tentative classification of the isolates. Benzoate is commonly utilised by the species *Rps. palustris* and *Rmi. vanniellii* (Shoreit and Shabeb 1994), but not by the species *Rba. blasticus*. Strain FEP1 and three of the red-brown coloured isolates did not utilise benzoate at the concentration provided. Two of the more widely used substrates, ethanol and glucose, provided some of the best diagnostic information. *Rba. blasticus* is one of the few purple nonsulfur bacteria unable to use ethanol as a carbon source for growth, the only strain isolated here which was unable to use it was FEP1. Each of the red isolates was able to use ethanol, a trait common with *Rps. palustris* strains but hitherto not seen in species of the morphologically similar *Rhodoplanes* genus. Strain FEP2 was the only isolate unable to utilise glucose as a carbon source. This result was consistent with the morphology of FEP2 resembling that of *Rmi. vanniellii*. The only isolate able to utilise phenol at the concentration provided was CCLRSP3, tentatively making it

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the first known strain of this morphology to utilise the compound as a sole carbon source for growth.

Strain	Benzoate	Citrate	Ethanol	Glucose	Fructose	Lactate	Phenol
TK1	+	-	+	+	-	+	-
TK2	+	-	+	+	-	+	-
FEP1	-	-	-	+	+	+	-
FEP2	+	-	+	-	-	+	-
FEP3	+	-	+	+	-	+	-
Fpond1	-	-	+	+	-	+	-
Fpond2	+	+	+	+	+	+	-
CCLRSP1	+	-	+	+	-	+	-
CCLRSP2	-	-	+	+	-	+	-
CCLRSP3	+	-	+	+	-	+	+

Table 4.3 Carbon source utilisation by the isolated strains.

Based on the morphological and carbon source data obtained tentative species classifications were made for each of the isolates obtained. These classifications are shown in Table 4.4.

TK1	<i>Rps. palustris</i>	Fpond1	<i>Rps. palustris</i>
TK2	<i>Rps. palustris</i>	Fpond2	<i>Rps. palustris</i>
FEP1	<i>Rba. blasticus</i>	CCLRSP1	<i>Rps. palustris</i>
FEP2	<i>Rmi. vanniellii</i>	CCLRSP2	<i>Rps. palustris</i>
FEP3	<i>Rps. palustris</i>	CCLRSP3	<i>Rps. palustris</i>

Table 4.4 Tentative classification of the isolates obtained.

4.4.4 Further classification of strain CCLRSP 3

CCLRSP3 was initially tentatively characterised as a strain of *Rps. palustris*. Hitherto, no strain of *Rps. palustris* has shown the ability to grow on phenol as a carbon source for photoheterotrophic growth; hence it was decided that this strain should be more formally classified. Two further tests were performed on the strain to provide information toward a more conclusive characterisation.

4.4.4.1 Electron microscopy studies

Transmission electron micrographs of strain CCLRSP3 grown photoheterotrophically on phenol were taken in an attempt to identify the structure of the internal photosynthetic membranes. Micrographs of several cells from different fields of view were taken. Figure 4.2 shows a representative cell of strain CCLRSP3 magnified x250000. The internal photosynthetic membrane can be clearly seen as being of a lamellar stack morphology (refer to Section 2.3 and Table 2.4), consistent with that found in *Rps. palustris*.

4.4.4.2 16S rDNA sequence analysis

Further information for the classification of strain CCLRSP3 was obtained by sequencing the 16S rDNA molecule of the organism. The method used is described in Section 3.6.5. Figure 4.3 shows the phylogenetic relationship of CCLRSP3 to other strains of purple nonsulfur bacteria. Of particular note is how it closely clusters with various strains of *Rps. palustris*.

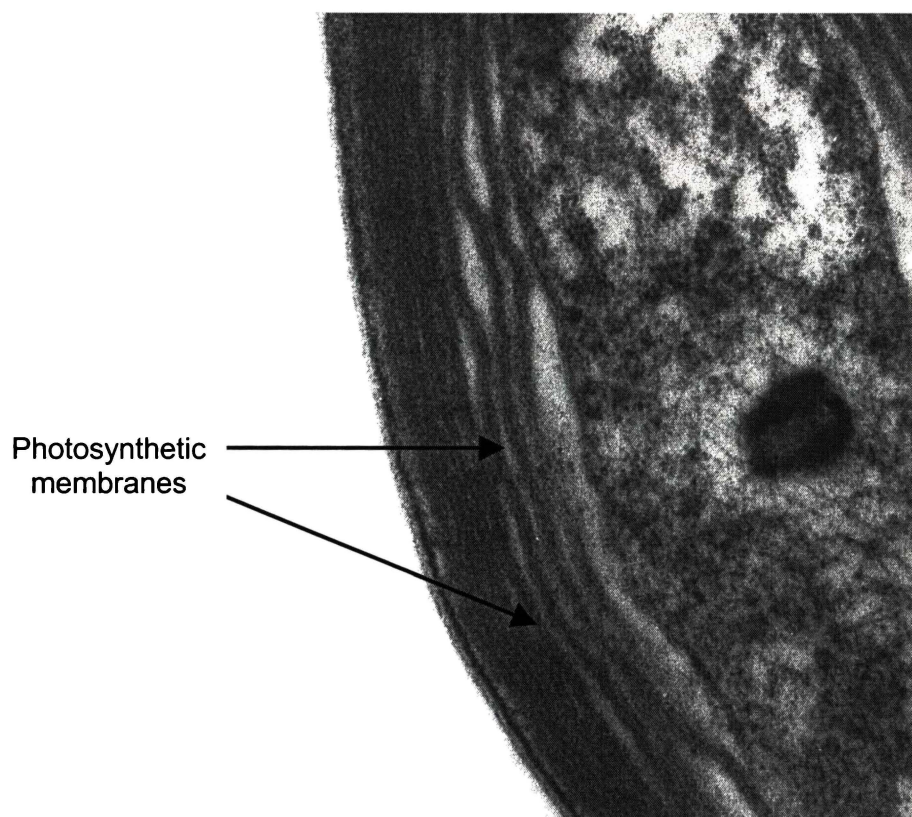


Figure 4.2 Transmission electron micrograph (x250000) of a representative cell of purple nonsulfur bacteria strain CCLRSP 3 showing photosynthetic membranes of the lamellar-stack type.

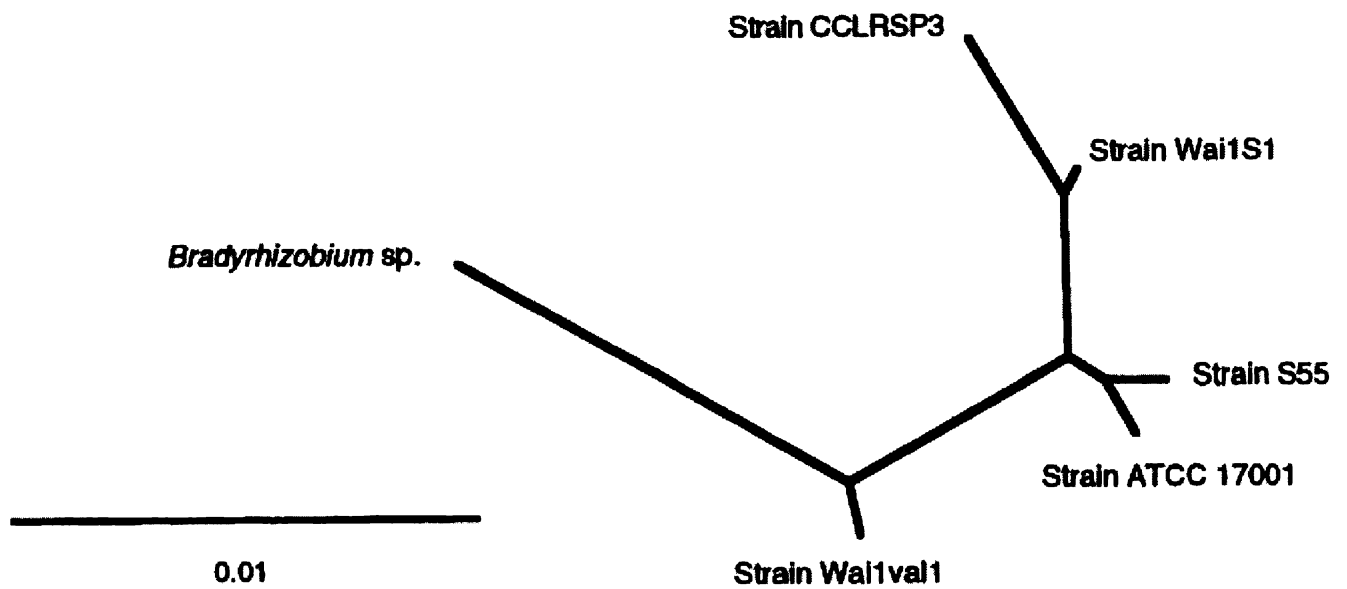


Figure 4.3 Phylogenetic tree representing the relationship of strain CCLRSP3 to strains of *Rps. palustris* and a *Bradyrhizobium* species.

4.5 Discussion

Attempts to isolate strains of purple nonsulfur bacteria capable of growing on phenol as sole carbon source yielded only one strain with this ability, *Rps. palustris* CCLRSP3.

Rps. palustris CCLRSP3 was isolated from the sediment of a subsidiary pond of Lake Rotoroa, Hamilton (Section 4.2) via continuous culture enrichment using *o*-cresol as sole added carbon source. A number of other purple nonsulfur bacteria isolates were obtained from a variety of sources, each representing commonly isolated species and having a generally broad substrate utilisation range, but with no ability to utilise phenol at the concentration tested. 1mM was chosen as a concentration that both allowed for measurable growth and provided the substrate at a level that would be non-toxic to organisms with the capability to metabolise it.

Due to the relative ease of commissioning, far more batch enrichments were performed than continuous culture enrichments. The batch enrichments however, failed to yield a single strain that was capable of growth on phenol, this result could be attributed to a number of reasons.

The main problem with using batch enrichment for microorganisms able to grow on toxic substrates is the supplying of the substrate at a concentration that is both non-toxic and able to support growth over and above that which is endogenous. If the optimal concentration is found, then the selective pressure will be such that the predominant growth in an enrichment will be that of organisms able to metabolise the added substrate. The use of Winogradsky columns proved to be the least effective in all aspects of enrichment. By nature, the Winogradsky column has an inherently high initial concentration of readily metabolisable substrates that can support the growth of a wide variety of microorganisms. In this study, the proliferation of photosynthetic organisms growing only on endogenous substrates

and unable to metabolise phenol lasted for up to six weeks, after which, algae would overrun the enrichment.

Modifications were made to the columns in an effort to improve the probability of enriching for phenolic compound metabolisers. One such modification involved moistening small wads of cottonwool with the neat liquid or a twofold dilution (with water) of various aromatic compounds. Single wads containing single compounds were placed in new enrichments approximately halfway up the sediment column. It was hoped that radiant diffusion from the cottonwool through the sediment mass would set up a concentration gradient and provide microenvironments of suitable substrate concentration that would allow only for growth of organisms capable of growth on, or tolerance toward, the compound. As expected, zones of apparent growth inhibition were observed around the cottonwool wads, with no pigmented growth observed within a 2 cm diameter. Samples were taken from areas of pigmented growth nearest the cottonwool and subsequently handled as described in Figure 4.1. The modification proved unsuccessful with no growth observed on phenol as sole carbon source. In enrichments using readily metabolisable substrates such as malate and ethanol, growth occurred. These organisms could not use phenol.

This result suggests that the diffusion of the substrate through the sediment may have been poor and hence a very steep concentration gradient would have formed with little chance of formation of a microenvironment of ideal concentration. The failure of the Winogradsky column as an enrichment technique in this study may have also been due to the imprecise nature of colony collection from the column. Commonly, a Pasteur pipette is used to extract samples of pigmented growth at the sediment-glass interface. This technique is however imprecise, and collection of other types of bacteria is inevitable. If the organism of interest is present in only small numbers, the chances of growth on a highly selective medium are small due

to toxicity, and the chances of proliferation of unwanted contaminants are high in a non-selective medium containing a more readily metabolisable substrate.

Utilisation of a Winogradsky plate (Charlton, McGrath and Harfoot. 1997), a technique recently developed in this laboratory for phototrophic batch enrichment, would allow for far more precise sampling and hence improved chances of isolating strains of interest.

Liquid batch enrichments also proved to be unsuccessful in obtaining purple nonsulfur bacteria capable of phenol degradation. The most probable reason for the failure was that the initial concentration of purple nonsulfur bacteria in the sediment samples that were capable of phenol degradation was very small; this compounded with the toxicity and unwanted proliferation problems referred to above made the likelihood of obtaining phenol-degrading organisms small.

Continuous culture enrichments gave the only successful isolation of a purple nonsulfur bacteria strain capable of utilising phenol as a sole carbon source. The continuous culture system provides the desired substrate at a very small concentration, and in doing so avoids the toxicity associated with batch enrichments. The problem of endogenous growth is avoided by initially running the system in batch mode to remove readily degradable compounds. When the system was switched to continuous mode the only available substrate was *o*-cresol. The effect of the continuous system was to slowly wash out those organisms unable to grow on the sole carbon source and to allow time for the establishment of those organisms that could. Purple nonsulfur bacteria enriched within the first two months of the continuous culture mode did not show the ability to utilise phenol. The density of growth in the reactor vessel after initial washout of that due to endogenous growth appeared low, suggesting that the strains isolated during this period (CCLRSP 1 and CCLRSP 2) may have been growing on the small amounts of yeast extract (supplied as a vitamin source) in the feed medium.

The gradual increase in growth density corresponding with the isolation of the phenol utilising strain CCLRSP 3 suggests that an adaptation to utilisation of *o*-cresol may have occurred during the period of the enrichment.

The mechanisms of adaptation of bacterial communities to grow on a particular substrate can be classified into three general categories: (i) growth of a small population that is capable of utilising the compound, (ii) delayed induction of enzymes involved in the catabolic pathway, and (iii) genetic change that enables the microorganisms to grow at the expense of the substrate. A short lag period between the exposure of a sample to the substrate and the growth of a population (or degradation of the substrate) suggests that induction of a degradative pathway has occurred. Lag periods of intermediate length that show a gradual rise in growth or degradation suggest that during the lag period growth of a specific population of bacteria capable of utilising the substrate has occurred. Extended lag periods followed by an abrupt increase in growth or degradation rate suggest a genetic change has occurred, allowing a population to utilise the substrate.

The continuous culture system appeared to allow for the gradual establishment of strain CCLRSP 3 as *o*-cresol became the primary substrate, suggesting an adaptation of the type described in (ii) above.

Part B: Growth of Purple Nonsulfur Bacteria on Phenolic Compounds

4.6 Screening for growth on phenols

A number of strains of purple nonsulfur bacteria were screened for their ability to utilise a number of phenolic compounds as sole carbon source for photoheterotrophic growth. A total of 14 strains encompassing a number of genera were each tested for growth on 10 phenols at a concentration of 1 mM; these are listed in Table 4.5.

STRAIN	
<i>Blc. viridis</i>	<i>Rps. acidophila</i>
<i>Phs. molischianum</i>	<i>Rps. palustris</i> ATCC 33872
<i>Rba. blasticus</i>	<i>Rps. palustris</i> CCLRSP 3
<i>Rba. capsulatus</i>	<i>Rps. palustris</i> DSM 123
<i>Rba. sphaeroides</i>	<i>Rsp. photometricum</i>
<i>Rcy. tenuis</i>	<i>Rsp. rubrum</i>
<i>Rmi. vannielii</i>	<i>Rvi. gelatinosus</i>
PHENOLIC COMPOUND	
Phenol	2,4-dichlorophenol (2,4-DCP)
o-cresol	2-nitrophenol (2-NP)
2-chlorophenol (2-CP)	3-nitrophenol (3-NP)
3-chlorophenol (3-CP)	4-nitrophenol (4-NP)
4-chlorophenol (4-CP)	2,4-dinitrophenol (2,4-DNP)

Table 4.5 Organisms and substrates used in the phenol utilisation study.

4.6.1 Materials and methods

Each of the strains was tested for its ability to grow photoheterotrophically on each of the substrates as sole carbon source at a concentration of 1 mM.

Experiments were performed in Hungate tubes containing anaerobic basal salts medium (Section 3.3.1). The substrate was added to the Hungate tube from the

stock solution (Section 3.4) via hypodermic needle and syringe to the required final concentration. Upon mixing, a 0.2 mL inoculum of the required strain from a stock culture was added to the tube. For each of the species tested, a negative control comprising an inoculated tube of medium without substrate was employed, this allowed for a comparison of growth on the substrate with background growth due to the yeast extract in the medium. A positive control comprising an inoculated tube of medium containing 1 mM sodium acetate was also employed.

After inoculation, all tubes were incubated in the dark for 24 hours at 25°C. The tubes were then incubated at 25°C under constant illumination at an approximate light intensity of 800 lux provided by 40W tungsten bulbs. The tubes were inverted regularly to ensure the contents remained well mixed throughout incubation.

Growth was scored as; (+) for growth equivalent to that in the negative control, (++) for growth well in excess of background (usually 0.04 absorbance units), and (-) for no growth. Growth assessments were made turbidometrically as absorbance at 660 nm.

4.6.2 Results

The results of screening for growth of strains of purple nonsulfur bacteria on various phenolic compounds are shown in Table 4.6, and show that very few strains were able to utilise them as sole carbon source for growth at 1 mM concentration. The only strains that showed the ability to grow on any of the phenols were; *Rps. palustris* CCLRSP 3, which showed growth on phenol and 2-chlorophenol; *Rsp. photometricum*, which grew on phenol and *o*-cresol; and *Rsp. rubrum* which showed growth on phenol, *o*-cresol, and 2,4-dinitrophenol. *Rps. palustris* CCLRSP 3 and *Rsp. rubrum* showed resistance to the toxicity of a larger number of phenols than the other strains tested. The strains least resistant to the phenols at 1mM concentration were *Rcy. tenuis* and *Phs. molischanum*.

ISOLATION AND IDENTIFICATION

STRAIN	SUBSTRATE									
	Phenol	o-cresol	2-CP	3-CP	4-CP	2,4-DCP	2-NP	3-NP	4-NP	2,4-DNP
<i>Blc. viridis</i>	+	+	-	-	-	-	-	-	-	-
<i>Phs. molischianum</i>	-	-	-	-	-	-	-	-	-	-
<i>Rba. blasticus</i>	+	+	-	-	-	-	-	-	-	-
<i>Rba. capsulatus</i>	+	+	-	-	-	-	+	-	-	+
<i>Rba. sphaeroides</i>	+	+	-	-	-	-	+	-	-	+
<i>Rcy. tenuis</i>	-	-	-	-	-	-	-	-	-	-
<i>Rmi. vannielii</i>	+	+	-	-	-	-	-	-	-	-
<i>Rps. acidophila</i>	+	+	-	-	-	-	-	-	-	-
<i>Rps. palustris</i> ATCC 33872	+	-	-	-	-	-	-	-	-	-
<i>Rps. palustris</i> CCLRSP 3	++	+	++	-	-	-	+	-	-	+
<i>Rps. palustris</i> DSM 123	+	+	-	-	-	-	+	-	-	+
<i>Rsp. photometricum</i>	++	++	-	-	-	-	+	-	-	-
<i>Rsp. rubrum</i>	++	++	+	-	+	-	+	+	-	++
<i>Rvi. gelatinosus</i>	+	+	-	-	-	-	-	-	-	-

Table 4.6 Growth of selected strains of purple nonsulfur bacteria on phenolic compounds.

The compounds that proved to be both the least inhibitory and the most susceptible to degradation, were phenol and *o*-cresol. The chlorinated phenols proved to be the most inhibitory to growth of the strains tested. In all cases except for *Rps. rubrum*, each of the chlorophenols was toxic to those strains that were unable to utilise them for growth. The *meta* and *para* isomers of the monosubstituted chloro- and nitrophenols, in general proved more toxic than the *ortho* isomers. 2,4-dichlorophenol was inhibitory to all the strains at the concentrations tested, but the corresponding dinitrophenol proved considerably less toxic than the *meta* and *para* mononitro-isomers.

4.7 Substrate disappearance during growth on phenolic compounds

Growth of *Rsp. rubrum* on 2,4-dinitrophenol at a concentration of 1.5 mM was observed by measurement of total protein and by following substrate disappearance with capillary gas chromatography. To ensure that substrate disappearance was due only to microbial action, an uninoculated negative control was run concurrently under identical conditions to the experimental vial. A positive control substituting 1.5 mM sodium acetate for 2,4-dinitrophenol was also run concurrently.

4.7.1 Materials and methods

Experiments were performed in 100 mL serum vials containing; 2 mL of inoculum, 1 mL of bicarbonate solution (Section 3.3.4), 1.5 mL of a 100 mM stock solution of substrate, and basal salts medium to a final volume of 100 mL. The headspace was flushed with oxygen-free nitrogen. At two day intervals during a 28 day incubation in the light at 25°C, total cell protein was measured as described in Section 3.7.3, and a 1 mL sample of the culture fluid was taken for substrate

concentration analysis via capillary gas chromatography (Section 3.8.1.2).

4.7.2 Results

Substrate disappearance during growth of *Rsp. rubrum* on 2,4-dinitrophenol is shown in Figure 4.4. An extensive lag phase before any growth occurred was observed during which almost all of the initial substrate had been converted. The lag phase was likely attributable to a multi-step conversion of the substrate before any energy yielding step.

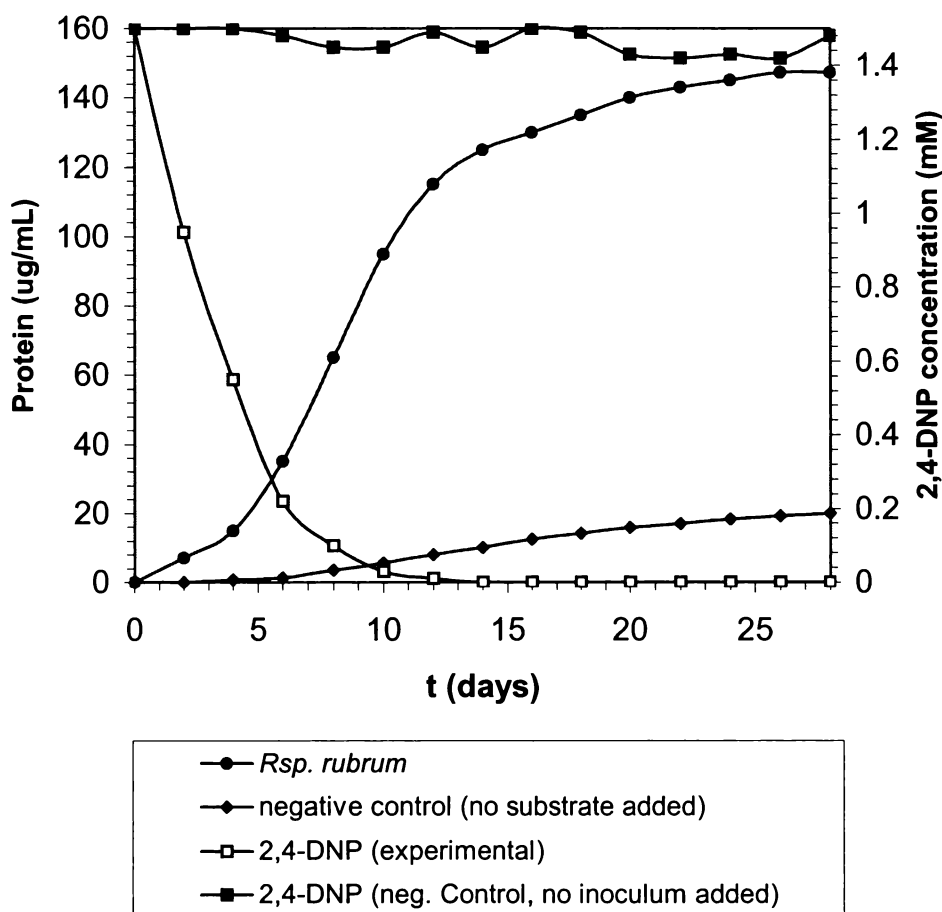


Figure 4.4 Growth of *Rsp. rubrum* on 2,4-dinitrophenol.

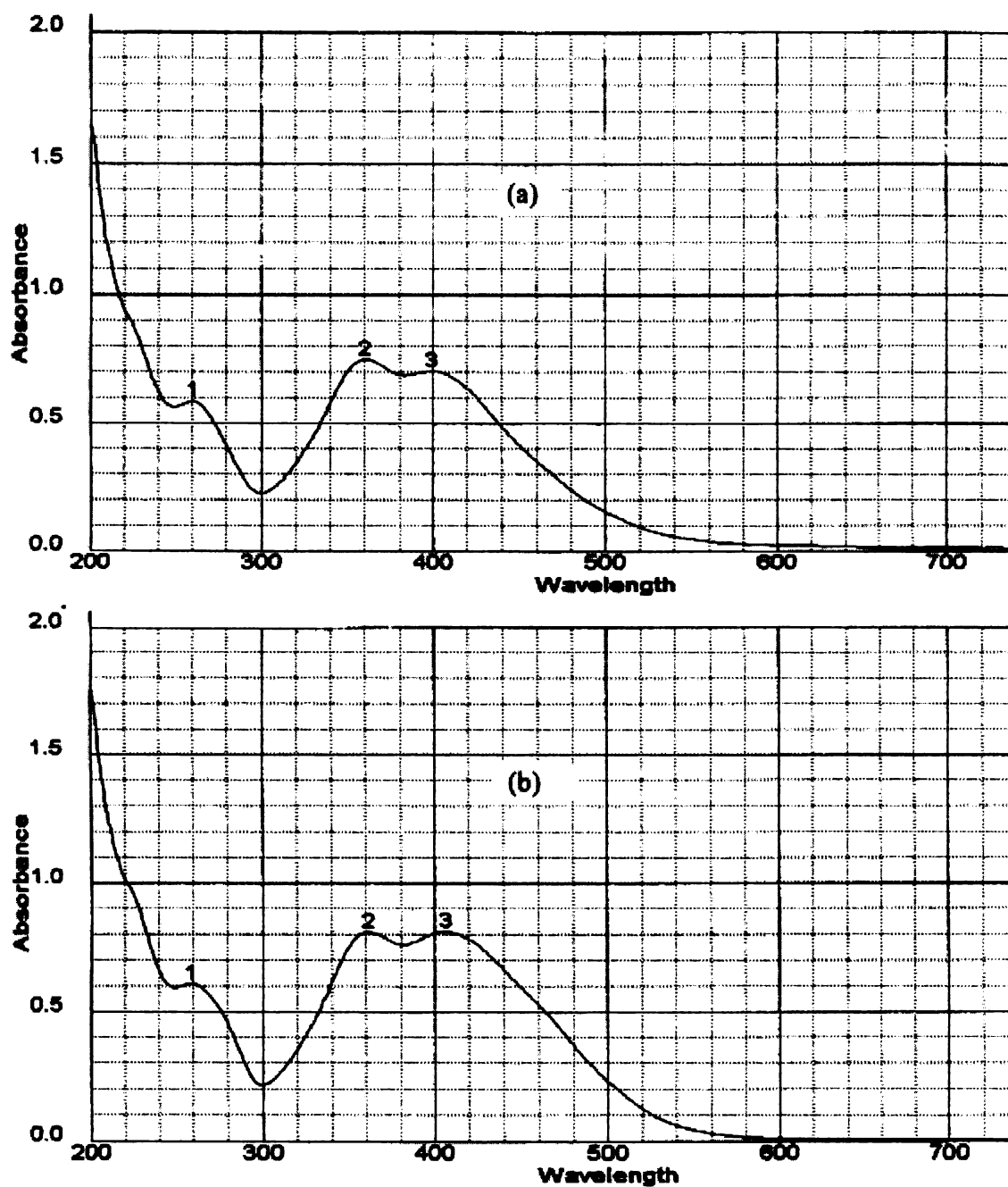


Figure 4.5 UV spectra of (a) growth medium after four days growth of *Rsp. rubrum* on 2,4-dinitrophenol, and (b) a 50:50 mixture of 2,4-dinitrophenol and 2-amino-4-nitrophenol.

Very early in the incubation, the liquid in the experimental serum vial changed from a yellow colour (due to the 2,4-DNP) to a deep orange. This suggested a microbially-mediated transformation of the substrate, as the colour change was not observed in the negative control vial. Gas chromatography analysis showed a rapid loss of the 2,4-DNP in the experimental vial but showed no significant loss of substrate in the negative control vial (Figure 4.4). Blasco and Castillo (1997) showed that a strain of *Rba. capsulatus* degraded 2,4-dinitrophenol to 2-amino-4-nitrophenol phototrophically. The colour change in the experimental vial suggested this as the initial transformation in this case also. In an attempt to prove this, a sample from the experimental vial was taken after four days, when according to gas chromatograph analysis, approximately half of the 2,4-DNP had been transformed. The UV-absorbance spectrum of the sample was compared to that of a 50:50 v/v mixture of 2,4-DNP and 2-amino-4-nitrophenol, using a Pharmacia-Biotech Ultrospec 3000 spectrophotometer, (Pharmacia Co., USA). The two spectra are shown in Figure 4.5.

The similarity in the UV-absorbance spectra would suggest that the assumption that the initial step in the degradation of 2,4-DNP by *Rsp. rubrum* is a reduction of the *ortho*-nitro group to an amino group, is not unreasonable.

4.8 Discussion

The results presented in the above section describe growth of strains of purple nonsulfur bacteria with phenolic compounds present as the only significant carbon source. The negative control results provide good evidence for growth on the phenolic compounds. First, the yeast extract (provided to a final concentration of 0.002%) supported only a very small amount of growth, equivalent to approximately 1/8th that achieved in the experimental growth vessels. Yeast extract at the concentration provided has been shown to be required as a source

for the complex vitamin requirements for the purple nonsulfur bacteria (Lee 1992, McGrath 1995, Charlton 1997). Second, no disappearance of the phenolic compound was observed in the negative control, showing that there is no abiotic mechanism (adsorption, precipitation, or abiotic reduction) taking place that is transforming the substrate. In an attempt to provide further proof and validity to the conclusion that the strains were growing on the phenolic compounds, isotope incorporation experiments involving uptake of ^{13}C -labelled phenol were planned for the strains concerned. However, upon revisiting the cultures for the experimentation, growth results as reported above were unable to be reproduced. Extensive efforts were made to get the cultures to once again grow on phenol as sole carbon source. These involved using inocula from old cultures growing on phenol and from log phase growth of new cultures on acetate. The protocol used for both old and new cultures is described in Figure 4.6.

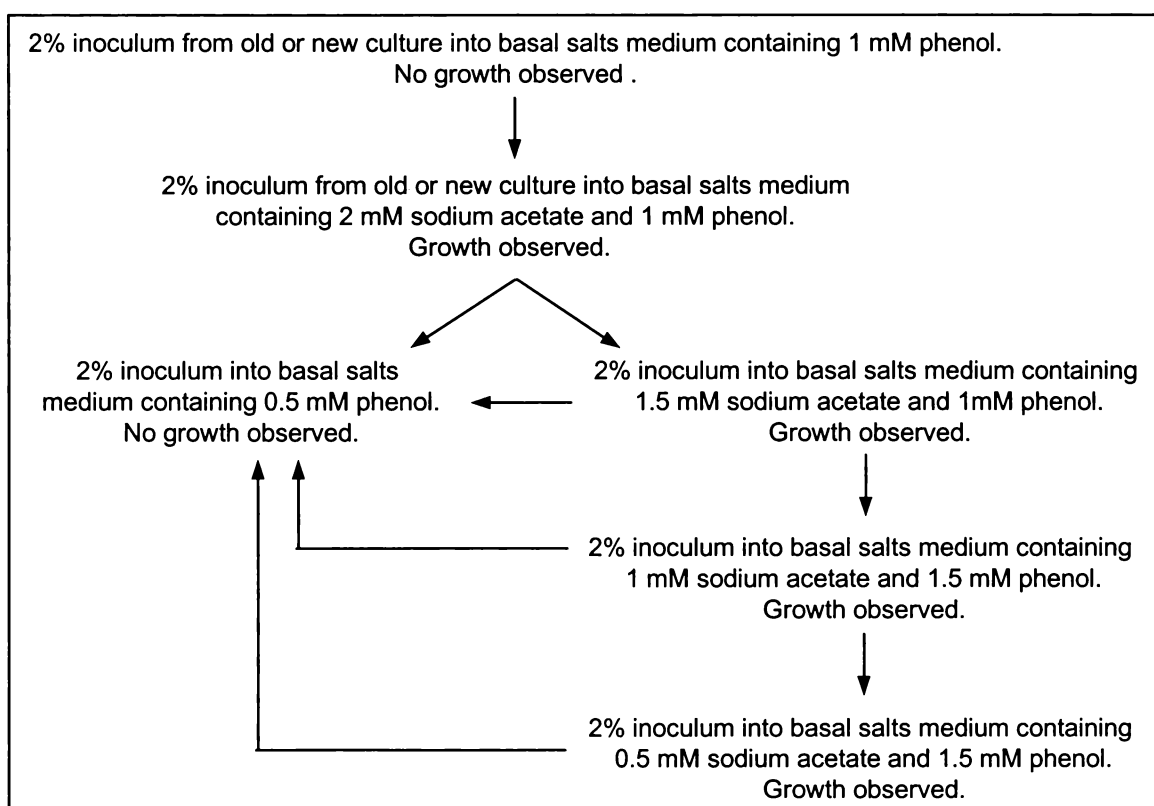


Figure 4.6 Protocol used in attempts to achieve growth on phenol.

The above protocol was repeated using different readily assimilable carbon compounds such as propionate, malate and succinate; each one producing the same results. The protocol was also repeated using *o*-cresol in place of phenol, with the same results. The results showed that neither phenol nor *o*-cresol was toxic to the organisms at the concentrations supplied. Attempts were made to grow the organisms on plates with 100 μ L of either phenol or *o*-cresol at 100 mM placed in a centre well and allowed to radiantly diffuse throughout a basal salts medium containing 5% agar. No growth was observed on these plates, although positive control plates containing the same concentration of acetate in the well showed a halo of growth with about a 1 cm radius from the well. Old cultures of purple nonsulfur bacteria showing positive growth on phenol and *o*-cresol were incubated at low light intensity (approx. 400 lux) with 1 mM phenol or *o*-cresol added. Inocula from these tubes were also tested for growth according to the protocol in Figure 4.6, with the same results.

A reason for the lack of growth upon revisiting the CCLRSP 3 culture is the possibility that it was not actually a completely pure culture, despite the rigorous purification protocol employed throughout. The possibility that a sulfate reducing bacteria contaminant was present throughout, performing at least part of the phenolic degradation and allowing growth of CCLRSP 3 cells, cannot be completely ruled out. Incubation of the revisited CCLRSP 3 culture under anaerobic conditions in the dark with 5 mM sodium acetate and the same basal salts medium, showed no bacterial growth. This indicated that any sulfate reducing bacterial contaminant was no longer viable. Although the CCLRSP 3 culture had lost the ability to grow at the expense of phenolic compounds, possibly as a result of a sulfate reducing bacteria being no longer present or viable, an experiment was performed to determine whether non-catabolic transformation of these compounds was still occurring.

Hungate tubes containing basal salts medium, 0.1 mL of bicarbonate solution and 0.5 mM of phenol or o-cresol were inoculated with 0.5 mL of log-phase CCLRSP 3 culture. An uninoculated control tube was also set-up to determine if any abiotic transformation of the compounds was taking place. 1 mL samples of culture fluid were taken every two days for 14 days. Each sample was centrifuged at 5000x g for 15 minutes and the supernatant analysed by HPLC for phenolic compound concentration. 10 μ L of supernatant was injected onto a C₁₈, 300 x 3.9 mm HPLC column with a 10 μ m particle size. Acetonitrile and 0.05 M phosphate buffer (pH 7.2) was used as the mobile phase at ratio of 65:35. The flow rate was 1.0 mL/minute and detection was by UV at 254 nm. Figure 4.7 shows the retention times of phenol and o-cresol. Concentrations were determined from standard curves for each substrate.

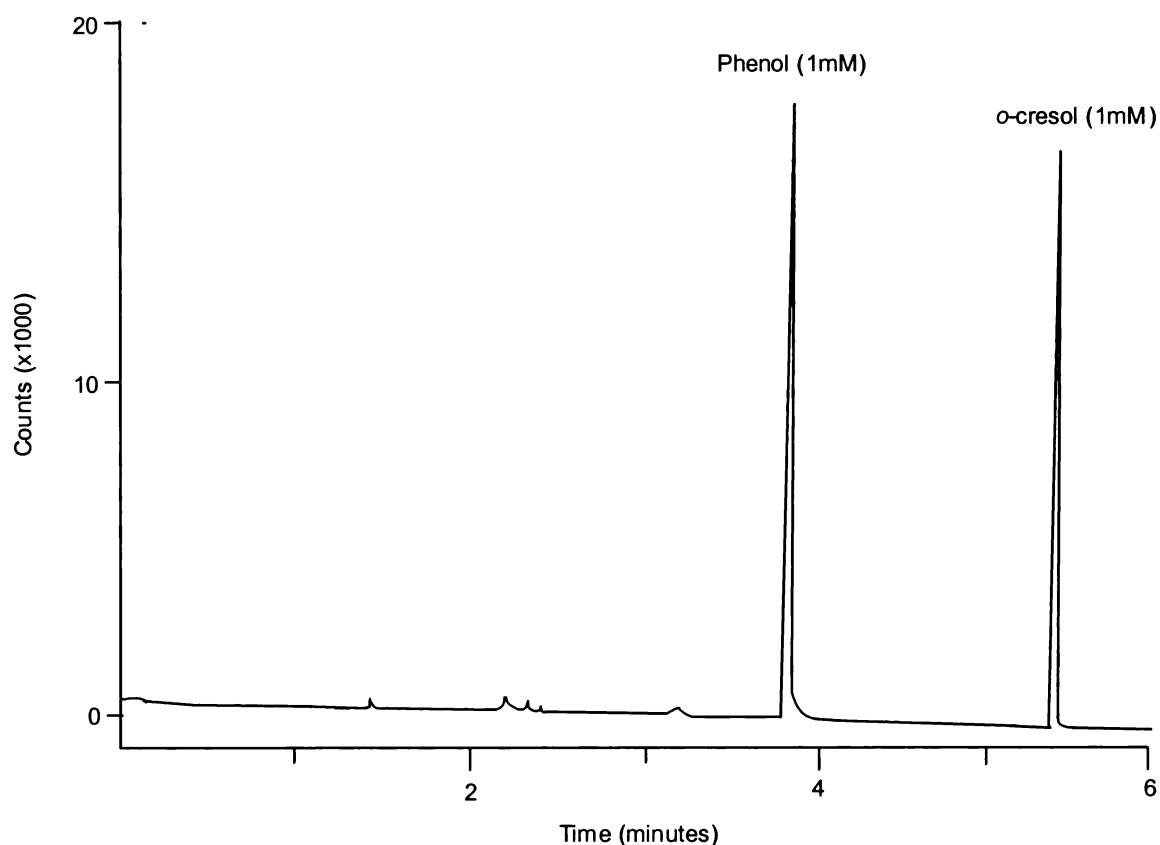


Figure 4.7 HPLC retention times of phenolic substrates

Figure 4.8 shows that there was no appreciable decrease in the concentration of phenol or *o*-cresol during the incubation with CCLRSP 3 cells. This would suggest that there was no transformation of these phenolic compounds. The slight decrease over time is comparable to that in the control tube, and is likely within the detection limits of the analysis.

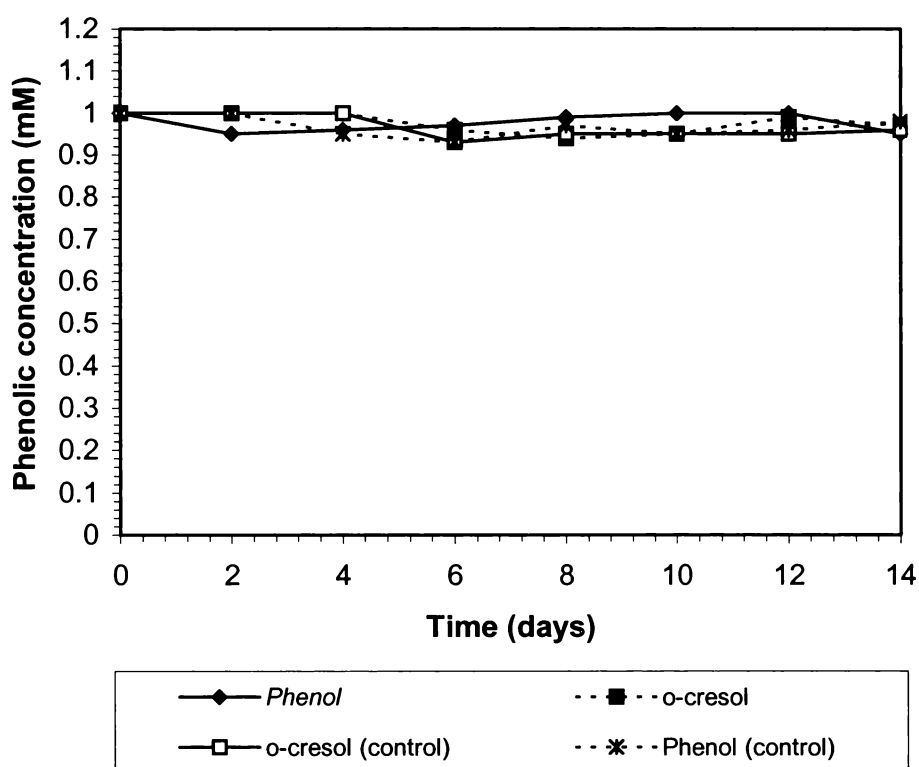


Figure 4.8 Disappearance of phenolic compounds during incubation with CCLRSP 3 cells

Without conclusive proof of substrate uptake by the cells, the results presented here do not allow the conclusion to be drawn that the strains that showed growth far in excess of the negative control were doing so at the expense of the phenolic compound. And in the case of CCLRSP 3, initial positive growth results on phenolic compounds could possibly be due to co-metabolism of the substrates with a sulfate reducing bacterial contaminant.

4.9 Summary

A summary of the findings in this chapter are outlined in Table 4.7

1. A small number of isolated and culture collection strains of purple nonsulfur bacteria were able to grow in the presence of certain phenolic compounds. However, no conclusive proof was obtained that showed they are able to grow on these compounds as sole carbon source for photoheterotrophic growth.
2. *Rhodospirillum rubrum* was shown to initially transform 2,4-dinitrophenol to 2-amino-4-nitrophenol under photoheterotrophic conditions.

Table 4.7 Summary of the findings in Chapter 4.

CHAPTER 5

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

5.1 Introduction

The work described in this chapter reports the metabolism of halogenated carboxylic acids by species of the purple nonsulfur bacteria. The first section describes screening, utilisation, and pathway studies; the second section describes work on optimal and minimum inhibitory concentration identification.

5.2 Growth on halogenated carboxylic acids

A selection of seven strains of purple nonsulfur bacteria were screened for their ability to utilise a number of two- and three-carbon halogenated carboxylic acids (halocarboxylic acids). The species selected encompassed five genera and were chosen on the basis of their known versatility of carbon metabolism. All strains used were type strains and along with the halocarboxylic acids used, they are listed in Table 5.1.

STRAINS	
<i>Rba. capsulatus</i>	<i>Rsp. photometricum</i>
<i>Rcy. purpureus</i>	<i>Rsp. rubrum</i>
<i>Rps. palustris</i> ATCC 33872	<i>Rvi. gelatinosus</i>
<i>Rps. palustris</i> DSM 123	
HALOCARBOXYLIC ACIDS	
Bromoacetic acid	2-Bromopropionic acid
Chloroacetic acid	2-Chloropropionic acid
Fluoroacetic acid	3-Chloropropionic acid
Iodoacetic acid	

Table 5.1 Organisms and substrates used in the halocarboxylic utilisation study.

5.2.1 Materials and methods

Each of the strains were tested for their ability to grow photoheterotrophically on each of the substrates as sole carbon source at concentrations of 1mM and 2mM. Experiments were performed in Hungate tubes containing anaerobic basal salts medium (Section 3.3.1) The substrate was added to the Hungate tube from the stock solution (Section 3.4) via hypodermic needle and syringe to obtained the required final concentration. Upon mixing, a 0.1 mL inoculum of the required strain from a stock culture was added to the tube. For each of the species a negative control comprising an inoculated tube of medium without substrate was employed, this allowed for a comparison of growth on the substrate with background growth due to the yeast extract in the medium. A positive control comprised an inoculated tube of medium containing a substrate known to support good growth of the species. Sodium acetate and sodium propionate were used as the positive control substrates for this experiment.

After inoculation, all tubes were incubated in the dark for 24 hours and 25°C. The tubes were then incubated at 25°C under constant illumination at an approximate light intensity of 800 lux provided by 40W tungsten bulbs. The tubes were inverted

regularly to ensure the contents remained well mixed throughout incubation.

Growth was scored after 20 days of light incubation according to the protocol outlined in Table 5.2.

SCORE	DESCRIPTION OF GROWTH	ABSORBANCE AT 660nm (with respect to negative control)
I	Growth inhibition	< 0.000
-	Equivalent to negative control tube	0.000 - 0.050
+	More growth than negative control	0.050 - 0.100
++	Good growth on substrate	0.100 - 0.300
+++	Excellent growth on substrate	>0.300

Table 5.2 Protocol for the scoring of substrate utilisation.

5.2.2 Results

The results of screening for growth of the selected strains of purple nonsulfur bacteria on two- and three-carbon halocarboxylic acids are shown in Tables 5.3 and 5.4.

With the exception of *Rcy. purpureus* and *Rps. palustris* ATCC 33872, each of the species screened was able to grow at the expense of at least one of the halocarboxylic acids tested. Of the species tested, *Rsp. photometricum* was able to grow on the greatest number of substrates, showing no significant growth on bromoacetic acid and iodoacetic acid only. *Rps. palustris* DSM 123 and *Rsp. rubrum* were the next most versatile species.

Bromoacetic acid and iodoacetic acid supported the growth of the fewest species, the former supporting weak growth of *Rps. palustris* DSM 123 alone at the lower concentration of 1 mM. In the majority of cases, substrates that supported growth

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

at 1 mM supported increased growth at 2 mM, the exceptions being bromoacetic acid and iodoacetic acid which inhibited growth at 2 mM. Bromoacetic acid and iodoacetic acid were the only substrates that proved to be inhibitory at the concentrations tested, with the former proving toxic to most species at 1 mM and to all species at 2 mM.

SPECIES	HALOCARBOXYLIC ACID (1 mM)						
	BAA	CAA	FAA	IAA	2-BPA	CPA	2-CPA
<i>Rcy. purpureus</i>		-	-	-	-	-	-
<i>Rba. capsulatus</i>		-	-	-	+	+	+
<i>Rsp. photometricum</i>		++	+	-	++	++	+
<i>Rsp. rubrum</i>		+	-	+	+	+	+
<i>Rvi. gelatinosus</i>	+	+	+	+	+	+	+
<i>Rps. palustris</i> DSM 123	+	+	+	+	++	++	+
<i>Rps. palustris</i> ATCC 33872	-	-	-	-	-	-	-

Table 5.3 Growth of purple nonsulfur bacteria on 1 mM halocarboxylic acids.

SPECIES	HALOCARBOXYLIC ACID (2 mM)						
	BAA	CAA	FAA	IAA	2-BPA	CPA	2-CPA
<i>Rcy. purpureus</i>		-	-	-	-	-	-
<i>Rba. capsulatus</i>		-	-	-	+	+	+
<i>Rsp. photometricum</i>		++	+		++	+++	+
<i>Rsp. rubrum</i>		+	-		+++	++	+
<i>Rvi. gelatinosus</i>		++	+		++	++	+
<i>Rps. palustris</i> DSM 123		+	+		++	++	++
<i>Rps. palustris</i> ATCC 33872		-	-	-	-	-	-

Table 5.4 Growth of purple nonsulfur bacteria on 2 mM halocarboxylic acids.

5.3 Substrate disappearance and product formation during metabolism of halocarboxylic acids

From the above results, six combinations of species and substrate were selected for further study based primarily on good growth at 2 mM; these are listed in Table 5.5. The disappearance of the substrate and the accumulation of halide were measured concurrently with growth. The accumulation of the intermediary breakdown product of metabolism was measured along with halide production in non-growing degradation tests.

SPECIES	HALOCARBOXYLIC ACID (2 mM)
<i>Rps. palustris</i> DSM 123	2-Bromopropionic acid 2-Chloropropionic acid 3-Chloropropionic acid
<i>Rba. capsulatus</i>	3-Chloropropionic acid
<i>Rsp. photometricum</i>	Chloroacetic acid
<i>Rsp. rubrum</i>	2-Bromopropionic acid

Table 5.5 Species and halocarboxylic acids used for substrate disappearance and product formation studies.

5.3.1 Materials and methods

Growth, substrate disappearance and halide production

Growth experiments where substrate disappearance and halide production were measured concurrently were performed in 100 mL serum vials. A modified basal salts medium with the chloride salts replaced with the corresponding sulfate was used, this decreased the background chloride levels allowing for more accurate halide measurement. The inocula used were from log phase cultures growing photoheterotrophically on a readily metabolisable carbon source.

Each serum vial contained 2 mL of inoculum, 1 mL of bicarbonate solution (Section 3.3.4), 2 mL of a 100 mM stock solution of substrate, and the modified basal salts medium to a final volume of 100 mL. The headspace was flushed with oxygen-free nitrogen. At regular intervals during a 20 day incubation in the light at 25°C, three 1mL samples of the culture fluid were taken for analysis. One sample was measured for total cell protein (Section 3.7.3) and the other two were measured for halide (Section 3.8.3.1) and substrate (Section 3.8.1.1) concentration. Two negative controls per species/substrate concentration were set up and incubated concurrently. Each of the control vials was identical to the experimental vials except that one contained no inoculum and the other contained no substrate; they were included to show there was no abiotic disappearance of substrate and no growth without the added substrate respectively.

Detection of intermediates

The two most common mechanisms of dehalogenation by bacteria growing anaerobically are reductive and hydrolytic dehalogenation (Section 2.9). Of the substrates tested in this study, the intermediary carboxylic acids produced by either reductive or hydrolytic dehalogenation are known to be readily assimilable carbon sources for growth of each of the species. To avoid immediate assimilation of the carboxylic acid and to allow for accumulation to a detectable level, growth of the bacteria was prevented by removal of assimilable-nitrogen sources from the basal salts growth medium. The bacteria used in this experiment were starved of nitrogen by subculturing once into ammonium chloride-free basal salts medium with an argon headspace. Cells were hence forced to use cellular reserves of assimilable nitrogen for growth and hence became nitrogen starved. Log phase cells were then centrifuged at 15000g for 15 minutes and resuspended in the same medium. To test that the cells were not able to grow, an inoculum from the resuspended cells of

each species was placed into ammonium chloride-free basal salts medium along with a common carbon source for growth (sodium propionate). The results of these tests are shown in Figure 5.1 and show a very small amount of growth, probably attributable to utilisation of residual cellular assimilable nitrogen or small amounts of assimilable nitrogen in the yeast extract.

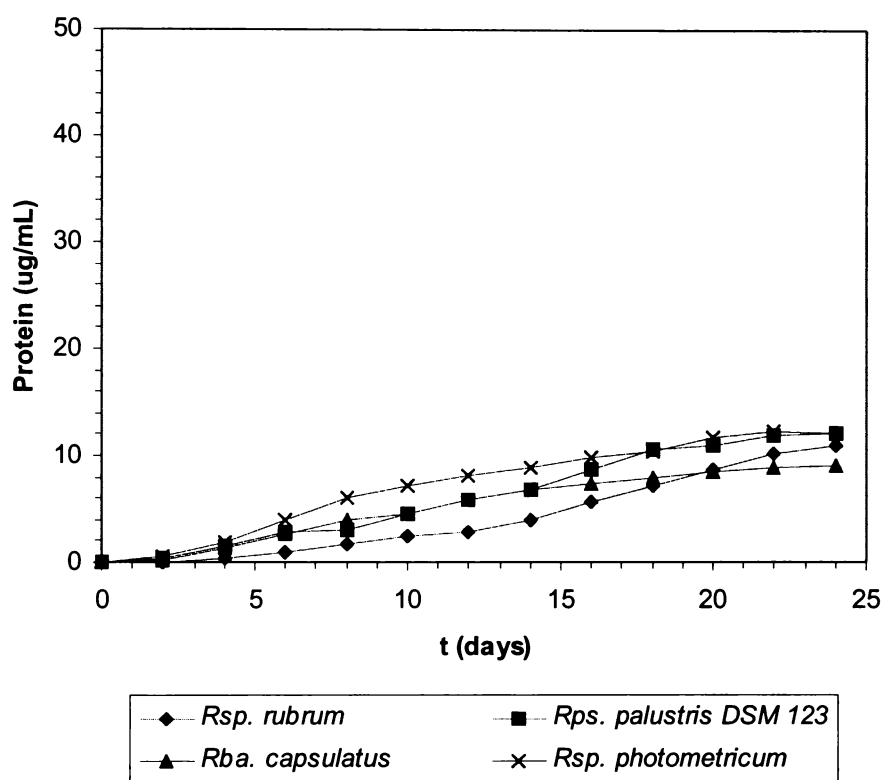


Figure 5.1 Growth of nitrogen-starved purple nonsulfur bacteria in the presence of yeast extract and a readily metabolisable carbon source (sodium propionate).

The appropriate halocarboxylic acid was used as sole substrate in each subculture. Each 100 mL serum vial contained the inoculum (resuspended cells), 1 mL of bicarbonate solution, 2 mL of a 100 mM stock solution of substrate, and the ammonium chloride-free basal salts medium to a final volume of 100 mL.

The headspace was flushed with argon. 1 mL samples were taken regularly throughout a 20 day incubation in the light at 25 °C for each of substrate disappearance, halide production, and intermediate production measurement. Halide concentration was measured as described in Section 3.8.3.1.

Intermediate production was measured by gas chromatography as for substrate disappearance, but subsequent to sonic disruption for 5 x 2 minutes, of the 1 mL sample. A gas chromatogram produced from a mixture of the products and likely intermediates, each at a concentration of 1 mM (in water) is shown in Figure 5.2. Identification of each compound was made by GC analysis of the individual compound at 1 mM in water and matching of retention times. Because each compound was tested alone, internal standards were not used.

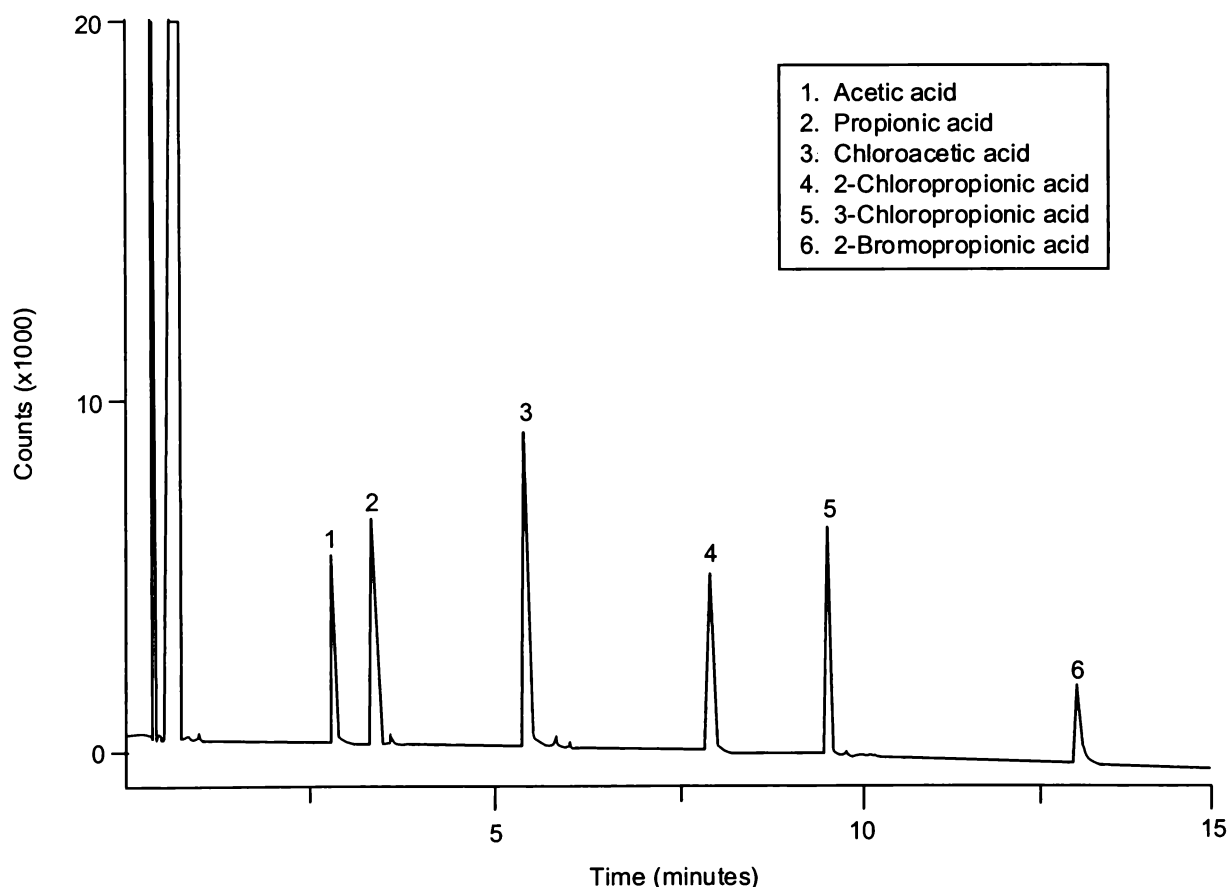


Figure 5.2 Gas chromatogram of mixed halogenated and non-halogenated carboxylic acids.

5.3.2 Results

The results of the experiments described above are shown graphically, with the two graphs for each combination in Table 5.5 representing the results from both experiments respectively.

5.3.2.1 *Rps. palustris* DSM 123 on 2-BPA

The growth of *Rps. palustris* DSM 123 on 2-bromopropionic acid and the associated substrate disappearance and bromide release is shown in Figure 5.3.

After an initial period of growth that can be attributed to metabolism of the substrate in addition to the small amount of yeast extract present in the medium, a lag phase of between two and three days was observed prior to log phase growth. During the lag period the concentration of 2-BPA decreased and the bromide concentration increased, suggesting a small amount of dehalogenation from the beginning of the incubation. The period of highest growth rate occurred immediately subsequent to the highest rates of 2-BPA disappearance and bromide release. The generation time during the log phase of growth was around 72 hours. The stationary phase of growth was reached after 15 days incubation, at which time bromide release and 2-BPA disappearance slowed markedly and halted soon after. The appearance of bromide appeared to be reasonably stoichiometric with the disappearance of 2-BPA with 1.3 mM of bromide released for approximately the same concentration of substrate utilised.

Figure 5.4 shows the disappearance of 2-BPA and the appearance of bromide and a product in a non-growing culture of *Rps. palustris* DSM 123. The product appeared stoichiometrically with the disappearance of 2-BPA and the release of bromide.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

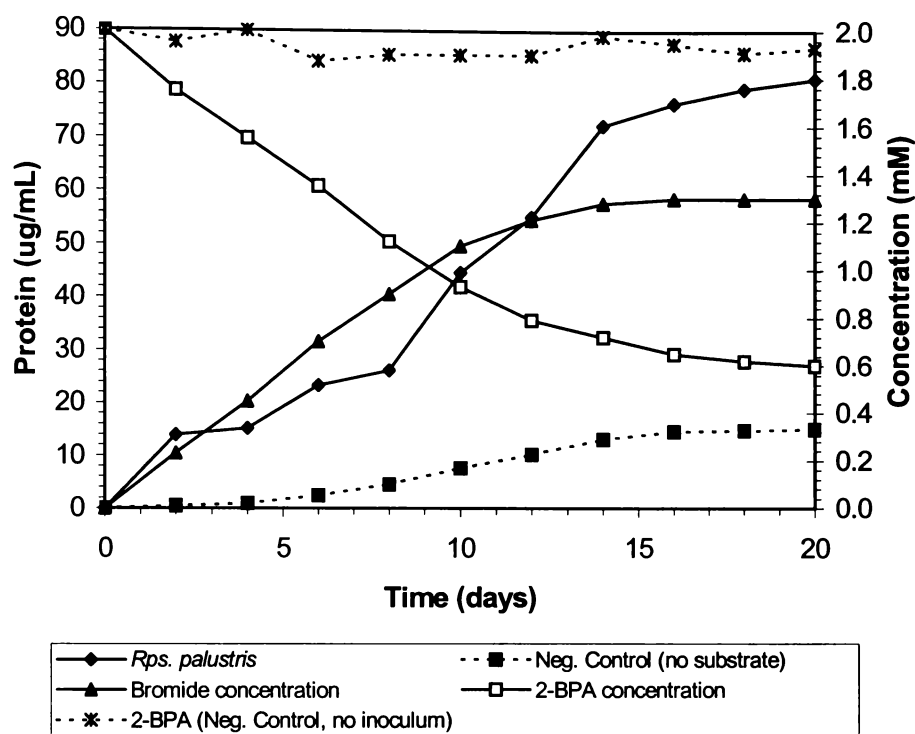


Figure 5.3 Growth of *Rps. palustris* DSM 123 on 2-BPA.

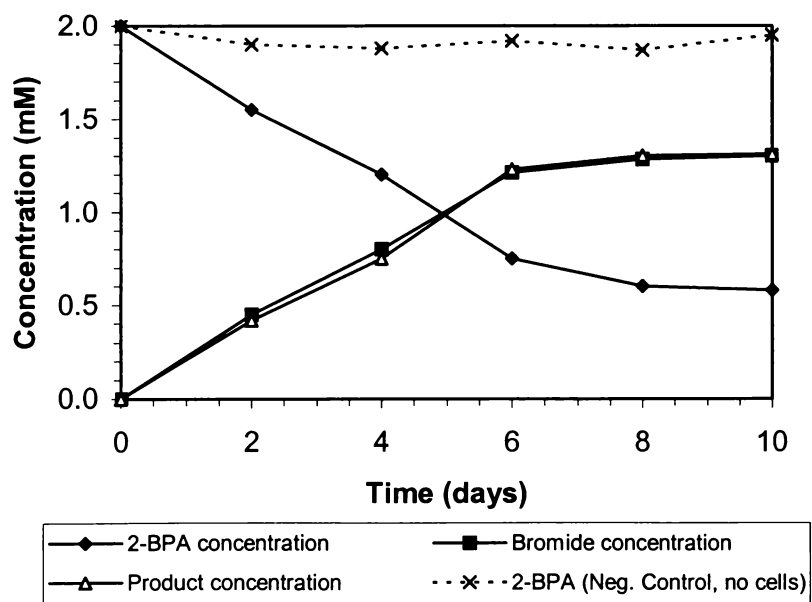


Figure 5.4 Product formation during conversion of 2-BPA by *Rps. palustris* DSM 123.

5.3.2.2 *Rps. palustris* DSM 123 on 2-CPA

Figure 5.5 shows the growth of *Rps. palustris* DSM 123 on 2-chloropropionic acid at a concentration of 2 mM and the associated substrate disappearance and chloride release. Compared to the growth observed by *Rps. palustris* DSM 123 on 2-bromopropionic acid, growth on 2-CPA proceeded with a longer lag period, although during this period the growth rate was appreciably higher than that observed on 2-BPA. Similar to growth on 2-BPA, the log phase was preceded by a sharp increase in halide release and substrate disappearance rate. The generation time during log phase was approximately 72 hours and the final yield was very similar to that observed for growth on 2-BPA. Stationary phase was reached approximately 14 days into the incubation and coincided with a halt in bromide release and substrate disappearance. Bromide appearance and substrate disappearance appeared to be stoichiometric throughout the incubation, with the final concentration of bromide released being 1.6 mM and approximately the same concentration of substrate utilised.

Figure 5.6 shows the disappearance of 2-CPA and the appearance of chloride and a product in a non-growing culture of *Rps. palustris* DSM 123. The substrate disappeared and the products appeared in reasonably stoichiometric amounts throughout the incubation.

5.3.2.3 *Rsp. palustris* DSM 123 on CPA

The growth of *Rps. palustris* DSM 123 on 3-chloropropionic acid at 2 mM is shown in Figure 5.7 along with the substrate disappearance and chloride release. The final growth yield observed was similar to that seen for growth of the same organism on 2-BPA and 3-BPA, although differing in this case, was the complete utilisation of the substrate. The rate of chloride release and substrate disappearance followed very closely that of growth throughout the entire

incubation. Unlike the previous examples, there was no appreciable lag period at the beginning of the incubation and no sharp increase in growth rate signalling the start of the log phase. The generation time during log phase growth averaged approximately 80 hours, which was somewhat longer than that observed for growth on either of the 2-haloacids. The stationary phase was reached after 12 days. Chloride release occurred stoichiometrically with substrate disappearance throughout the incubation, with the final concentration of chloride in the medium being very close to the concentration of substrate utilised.

Figure 5.8 shows the disappearance of CPA and the appearance of bromide and a product in a non-growing culture of *Rps. palustris* DSM 123. The product appeared in reasonable stoichiometry with the disappearance of CPA and the release of chloride.

5.3.2.4 *Rba. capsulatus* on CPA

The growth of *Rba. capsulatus* on 3-chloropropionic acid and the associated substrate disappearance and chloride release is shown in Figure 5.9. An initial lag phase of 2-3 days was observed which coincided with low rates of substrate disappearance and chloride release. The highest growth rate was immediately preceded by the highest rates of both chloride release and substrate disappearance.

The generation time of approximately 48 hours during log phase growth was appreciably shorter than that obtained by *Rps. palustris* DSM 123 growing on the same substrate, although the final yield was significantly lower. Stationary phase was reached after 10-11 days, with only half of the available substrate used. Chloride release and substrate disappearance were reasonably stoichiometric.

Figure 5.10 shows that the disappearance of CPA and the appearance of chloride and product in a non-growing culture of *Rba. capsulatus* is stoichiometric.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

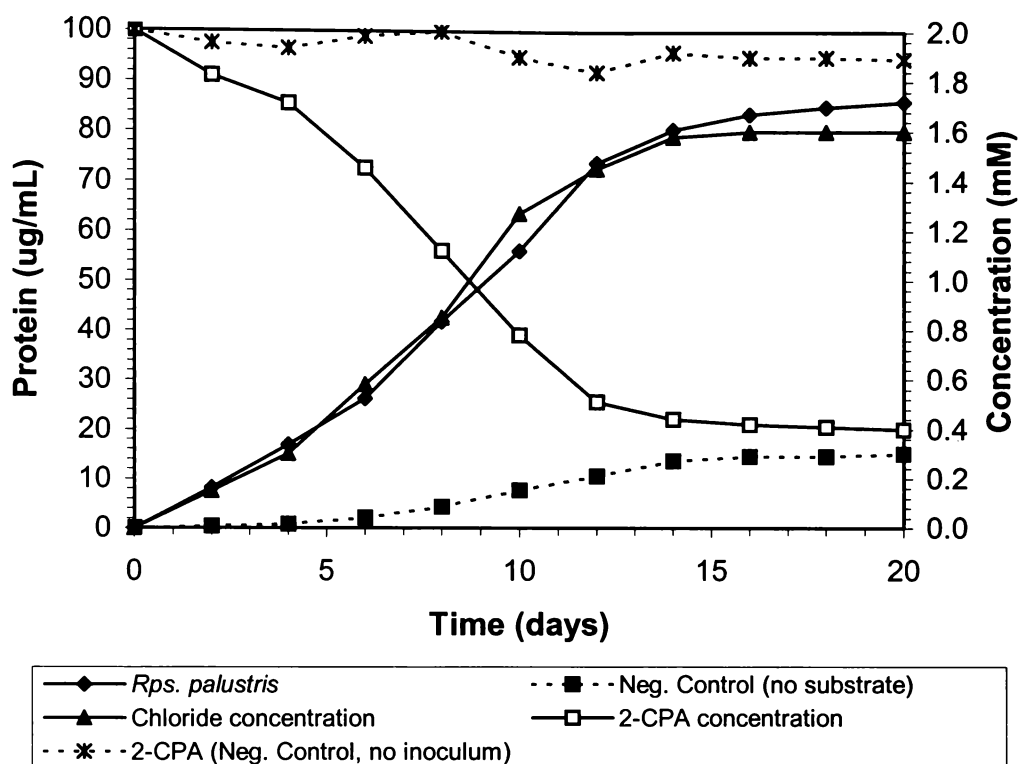


Figure 5.5 Growth of *Rps. palustris* DSM 123 on 2-CPA.

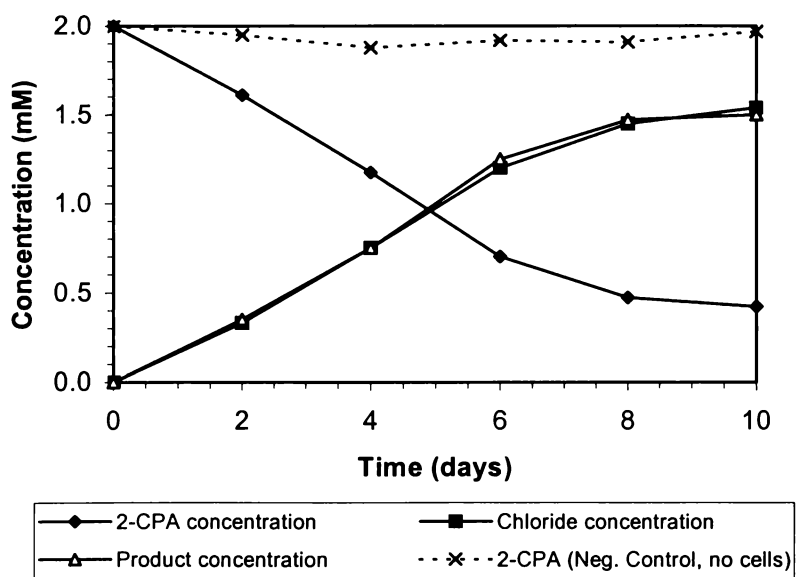


Figure 5.6 Product formation during conversion of 2-CPA by *Rps. palustris* DSM 123.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

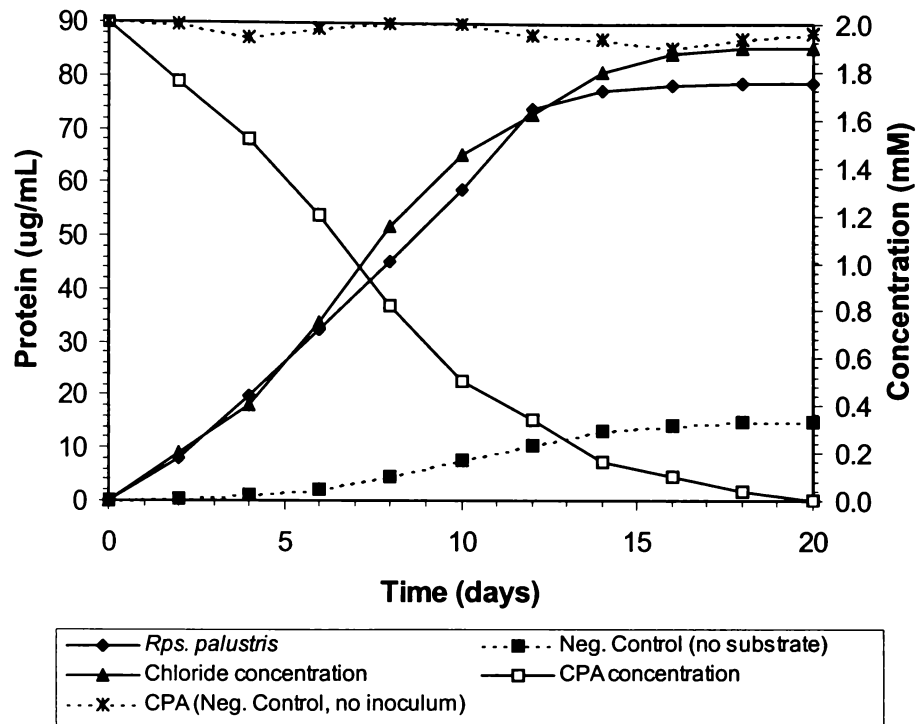


Figure 5.7 Growth of *Rps. palustris* DSM 123 on CPA

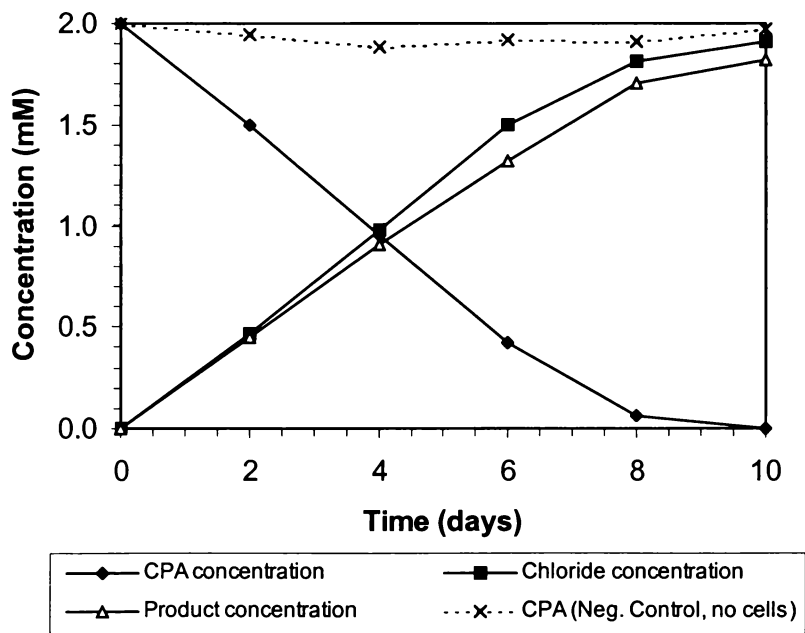


Figure 5.8 Product formation during conversion of CPA by *Rps. palustris* DSM 123.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

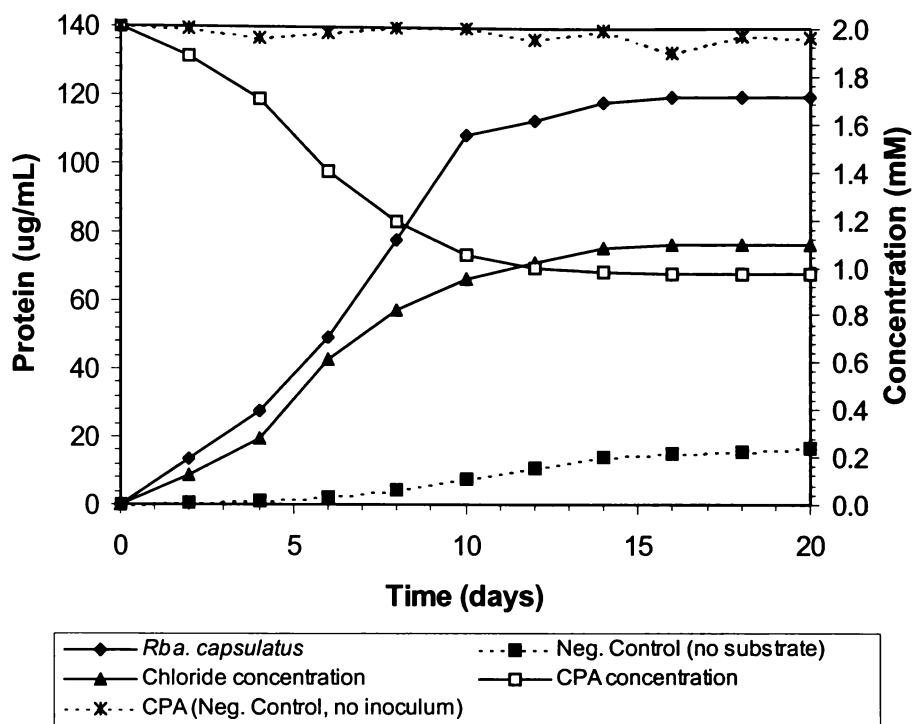


Figure 5.9 Growth of *Rba. capsulatus* on CPA

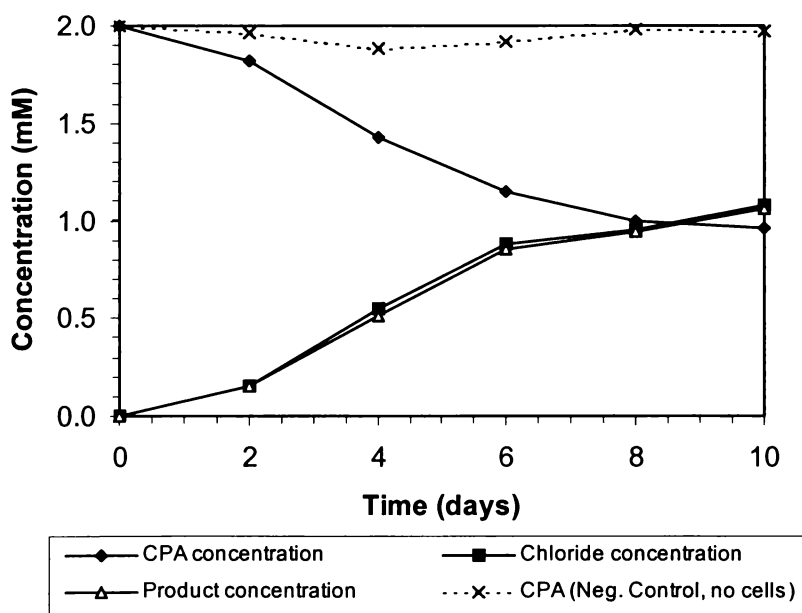


Figure 5.10 Product formation during conversion of CPA by *Rba. capsulatus*.

5.3.2.5 *Rsp. photometricum* on CAA

The growth of *Rsp. photometricum* on chloroacetic acid at 2 mM is shown in Figure 5.11 along with the substrate disappearance and chloride release. Generation times of typically 60 hours were observed during log phase growth and the stationary phase was reached around 15 days from the start of the incubation. The final yield was shown to be similar to those organisms that were grown on three-carbon halocarboxylic acids. Approximately 80% of the available substrate was used and stoichiometric amounts of chloride were released into the medium throughout the incubation. Figure 5.12 shows the disappearance of CAA and the appearance of chloride and a product in a non-growing culture of *Rsp. photometricum*. The substrate disappeared and the products appeared in reasonably stoichiometric amounts throughout the incubation.

5.3.2.6 *Rsp. rubrum* on 2-BPA

The growth of *Rsp. rubrum* on 2-bromopropionic acid and the associated substrate disappearance and bromide release is shown in Figure 5.13. After a lag period of less than 48 hours log phase growth was reached, during which, generation times of around 36 hours were observed. The final yield obtained after 16 days when no further growth occurred was higher than that obtained by other organisms growing on the same substrate. Bromide release into the medium appeared to be stoichiometric with the disappearance of substrate throughout the incubation, with the final bromide concentration of approximately 1.3 mM equalling the concentration of substrate utilised. The slowing in the rate of substrate conversion occurred just prior to the reduction in growth rate of the organism. Figure 5.14 shows the disappearance of 2-BPA and the appearance of bromide and a product in a non-growing culture of *Rsp. rubrum*. The product appeared in reasonable stoichiometry with the disappearance of the substrate and bromide release.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

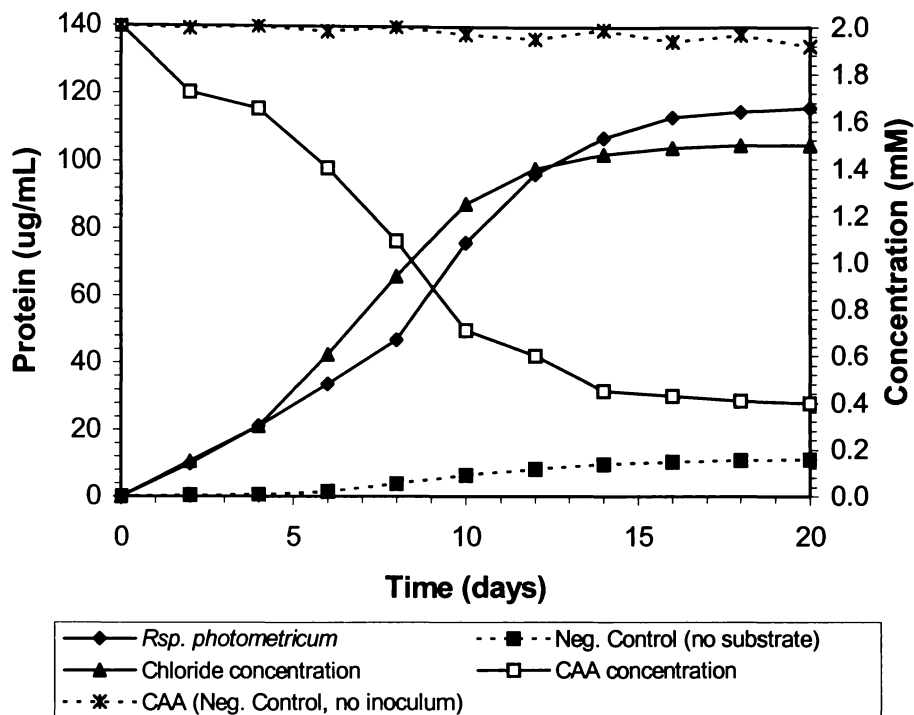


Figure 5.11 Growth of *Rsp. photometricum* on CAA.

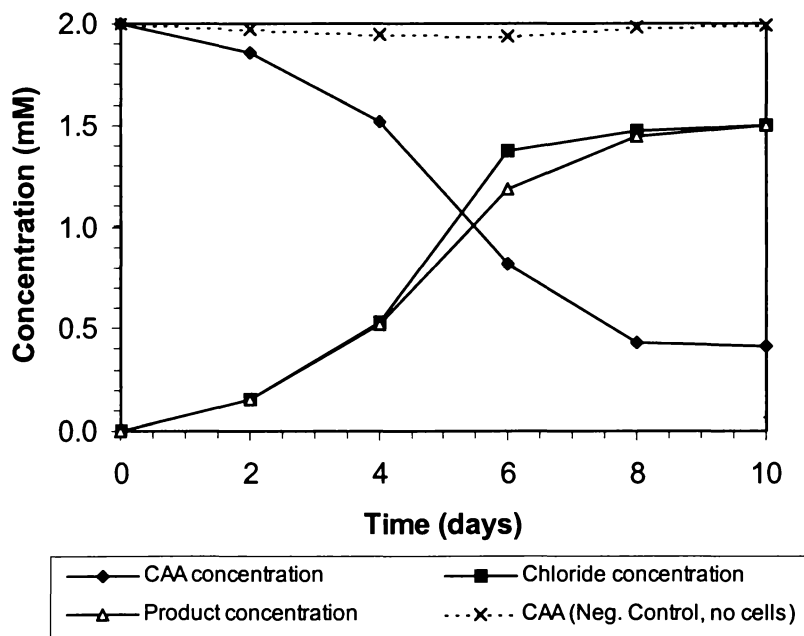


Figure 5.12 Product formation during conversion of CAA by *Rsp. photometricum*.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

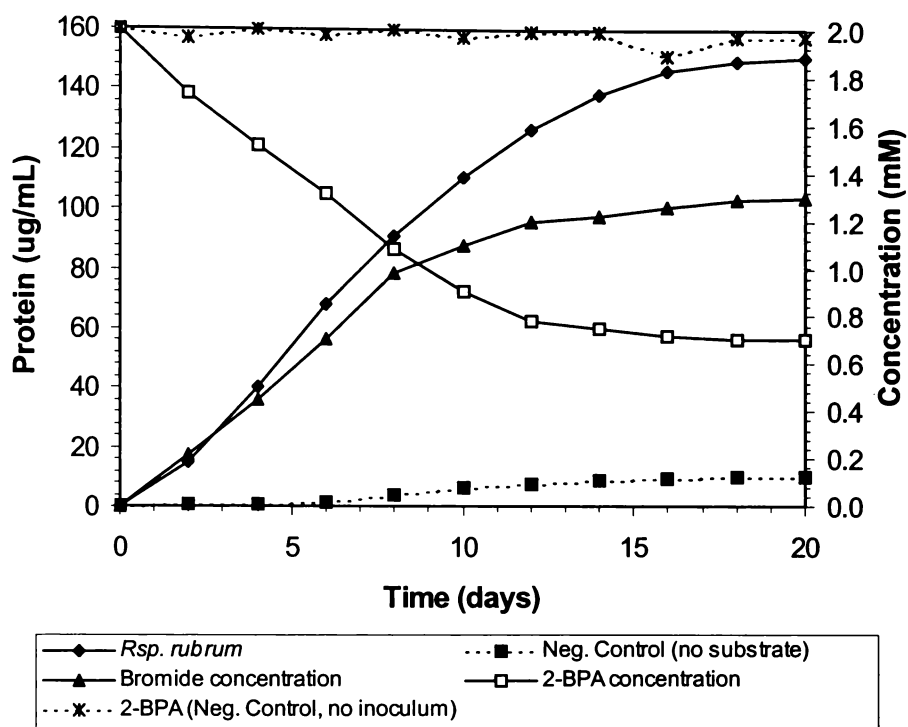


Figure 5.13 Growth of *Rsp. rubrum* on 2-BPA.

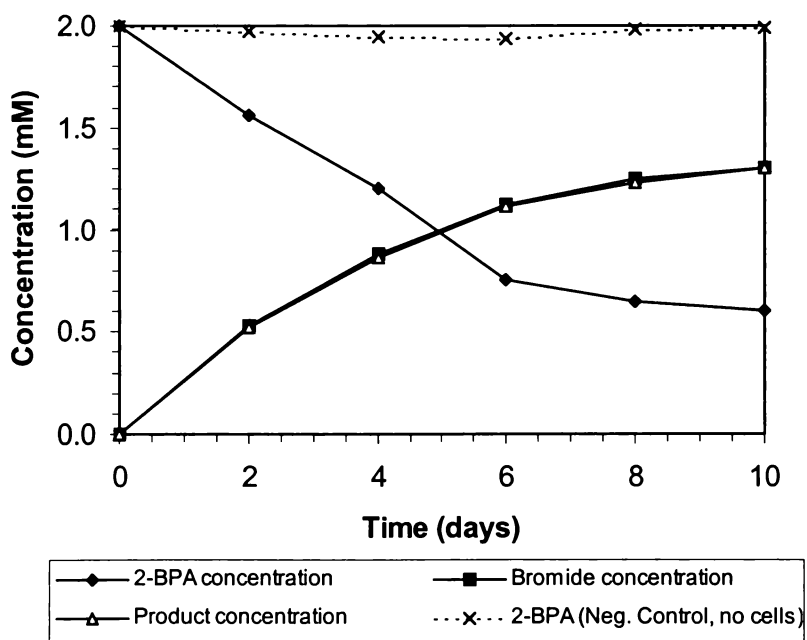


Figure 5.14 Product formation during conversion of 2-BPA by *Rsp. rubrum*.

5.3.2.7 Identification of intermediates

In order to establish the pathway of halocarboxylic acid metabolism by the species of purple nonsulfur bacteria tested, identification of the intermediary compound that accumulated during substrate conversion by non-growing cultures was necessary. Two methods were used for identification; gas chromatography using flame ionisation detection (Section 3.8.1.1) and electrospray mass spectrometry (Section 3.8.2).

Quantitative analysis of intermediate formation also allowed for tentative identification. For the gas chromatographic analysis of the conversion of halogenated acetic and propionic acids, the retention time of the intermediate was compared with that of the non-halogenated analogue of the substrate, i.e. acetic or propionic acid. In each case, the retention time of the detected intermediate was the same as the corresponding non-halogenated acid. Although not quantitative, electrospray mass spectrometry (ESMS) analysis of a culture fluid sample provided confirmation of intermediate identification. The ESMS results showed the formation of a compound with a mass that corresponded to that of the non-halogenated analogue in each of the cases tested. Figure 5.15 shows the ESMS trace of supernatant fluid taken from a negative control vial containing the modified basal salts medium without an added substrate but with non-growing cells as prepared for experimental vials. The control trace shows the background peaks due to the medium and any residual cellular material. All peaks on the trace are represented as a percentage of the most abundant species in the sample. Figures 5.16 and 5.17 are ESMS traces showing the appearance of a compound with a mass equivalent to propionic acid from the conversion of 3-chloropropionic acid by a non-growing culture of *Rps. palustris* DSM 123 and of 2-bromopropionic acid by *Rsp. rubrum*.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

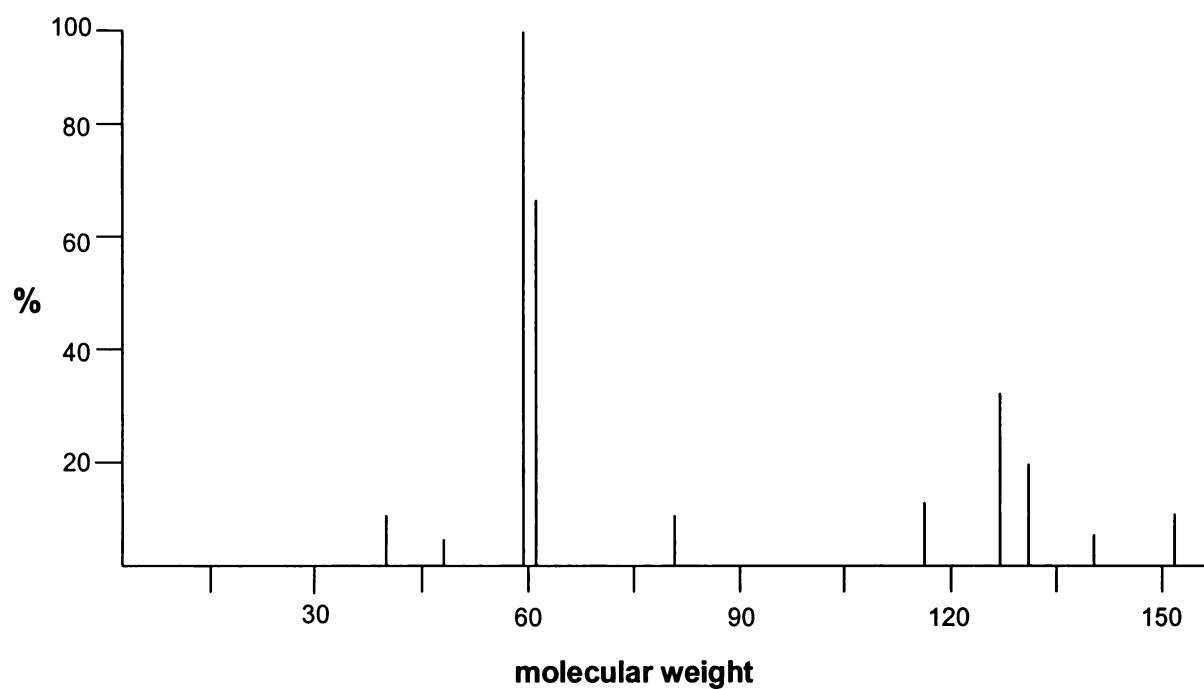


Figure 5.15 ESMS trace showing background peaks from a negative control vial containing non-growing cells in the absence of substrate.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

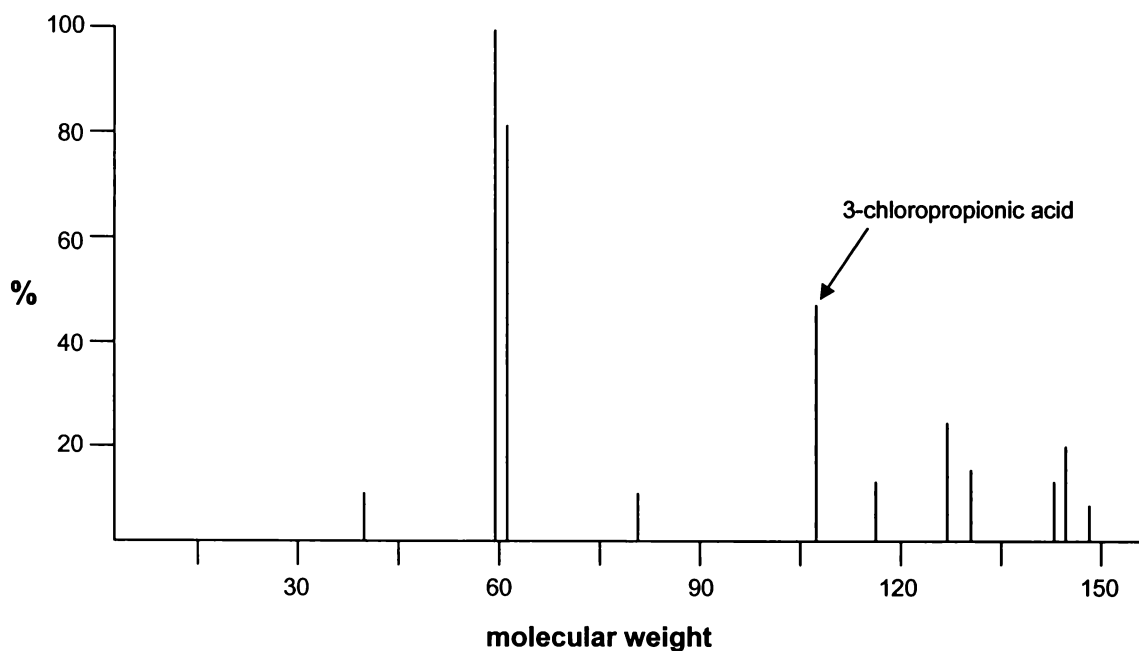


Figure 5.16 ESMS trace of *Rps. palustris* DSM 123 on CPA at time zero.

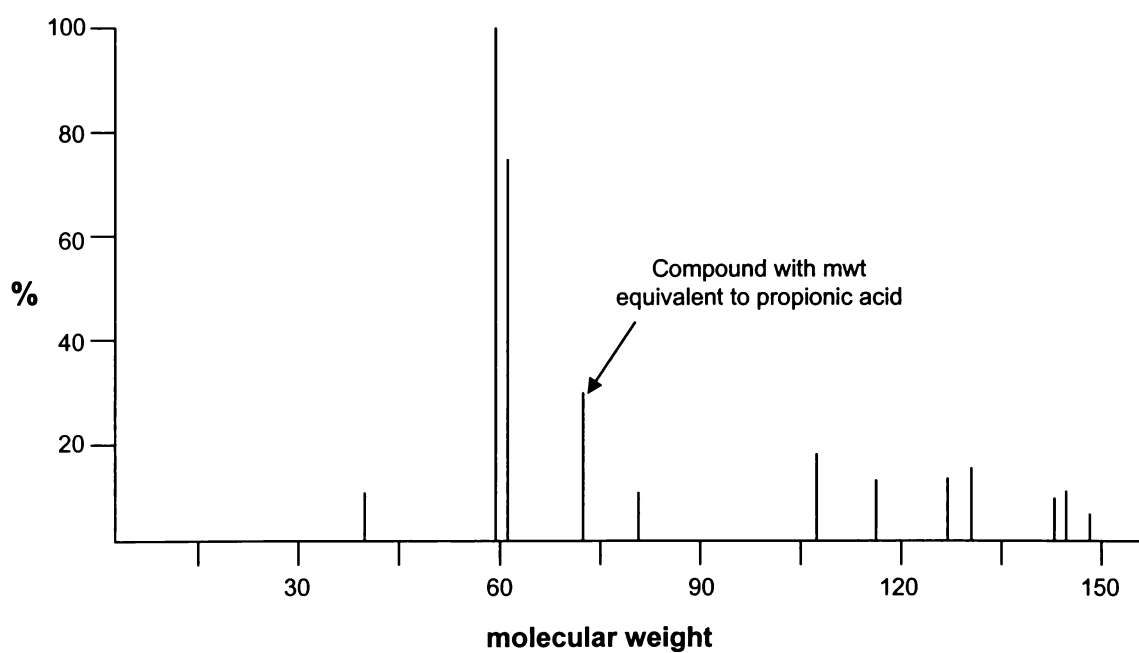


Figure 5.17 ESMS trace of *Rps. palustris* DSM 123 on CPA at 10 days.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

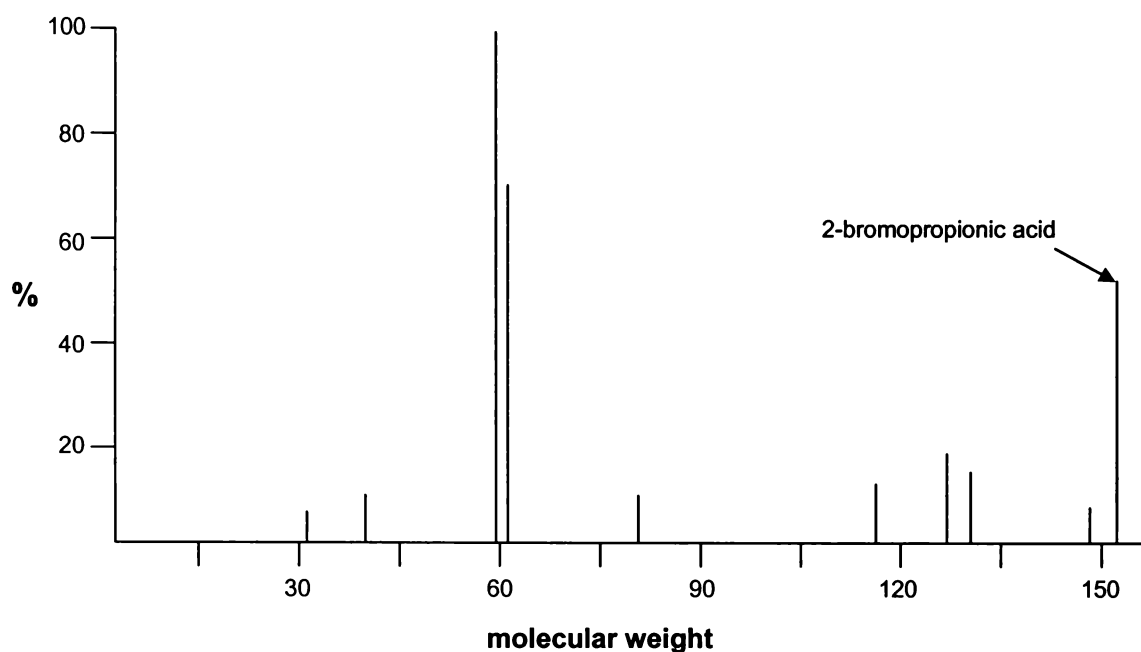


Figure 5.18 ESMS trace of *Rsp. rubrum* on 2-BPA at zero time.

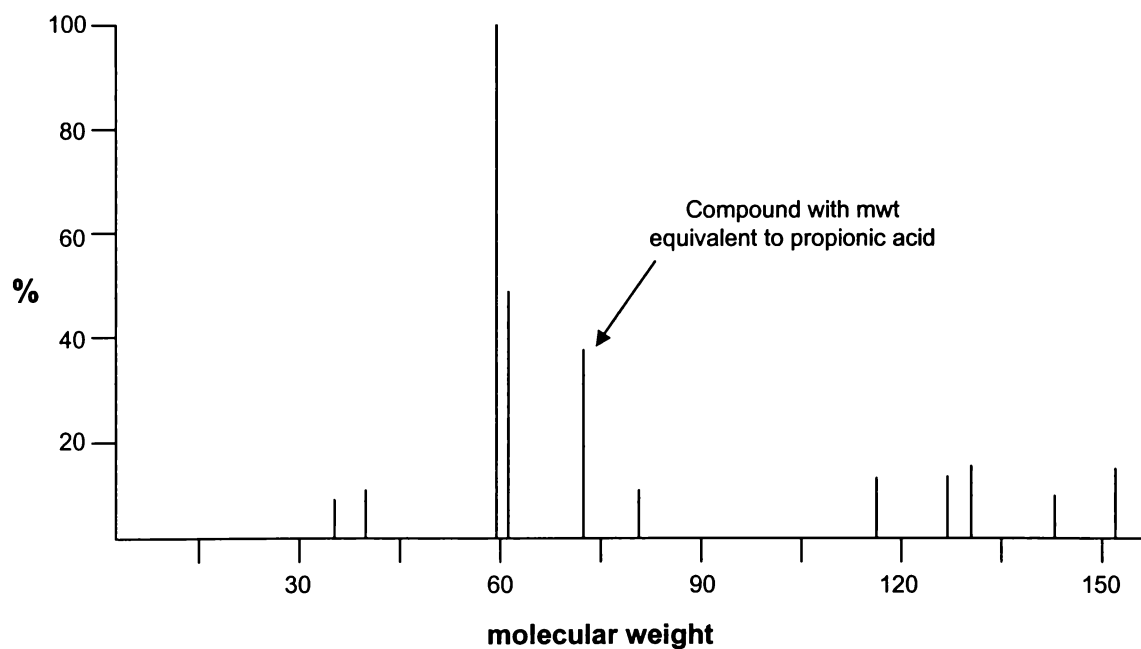


Figure 5.19 ESMS trace of *Rsp. rubrum* on 2-BPA at 10 days.

5.4 Proving halocarboxylic acids are used as sole carbon source

In order to prove that the purple nonsulfur bacteria were growing solely at the expense of the halocarboxylic acid, all carbon sources other than the added acid needed to be excluded from the growth medium. Due to the halocarboxylic acids being less reduced than cell material, there should be no requirement for an electron sink such as bicarbonate. Yeast extract is added to the medium as a source of vitamins; however, it is also an undefined mixture of assimilable carbon. The yeast extract can instead be replaced in the medium with small amounts of the actual vitamins required by the species. Exclusion of both bicarbonate and yeast extract from the growth medium with subsequent growth by the bacteria well in excess of the negative control, would allow one to assume that this growth is solely at expense of the halocarboxylic acid substrate.

5.4.1 Materials and Methods

This experiment was carried out similar to that described in Section 5.3.1 with the following modifications:

- No bicarbonate was added to the serum vials
- The modified basal salts medium contained no yeast extract
- The vitamins were added to the serum vials at the required final concentration; individual species vitamin requirements are shown in Table 5.6 below
- No samples were taken for halide measurement
- Detection of intermediates was not performed

SPECIES	VITAMIN REQUIREMENT
<i>Rps. palustris</i> DSM 123	1 µg/L Biotin, 10 µg/L <i>p</i> -aminobenzoic acid
<i>Rba. capsulatus</i>	50 µg/L Thiamine
<i>Rsp. photometricum</i>	50 µg/L Niacin
<i>Rsp. rubrum</i>	1 µg/L Biotin

Table 5.6 Vitamin requirements for purple nonsulfur bacteria species

5.4.2 Results

Figures 5.20 through 5.25 show that growth well above the negative control was observed in each of the serum vials. The growth rates and final growth yields were shown to be similar to those described in the presence of bicarbonate and yeast extract. The small amount of growth observed in the negative controls is likely attributable to endogenous activity and assimilation of small amounts of residual carbon from the added vitamins.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

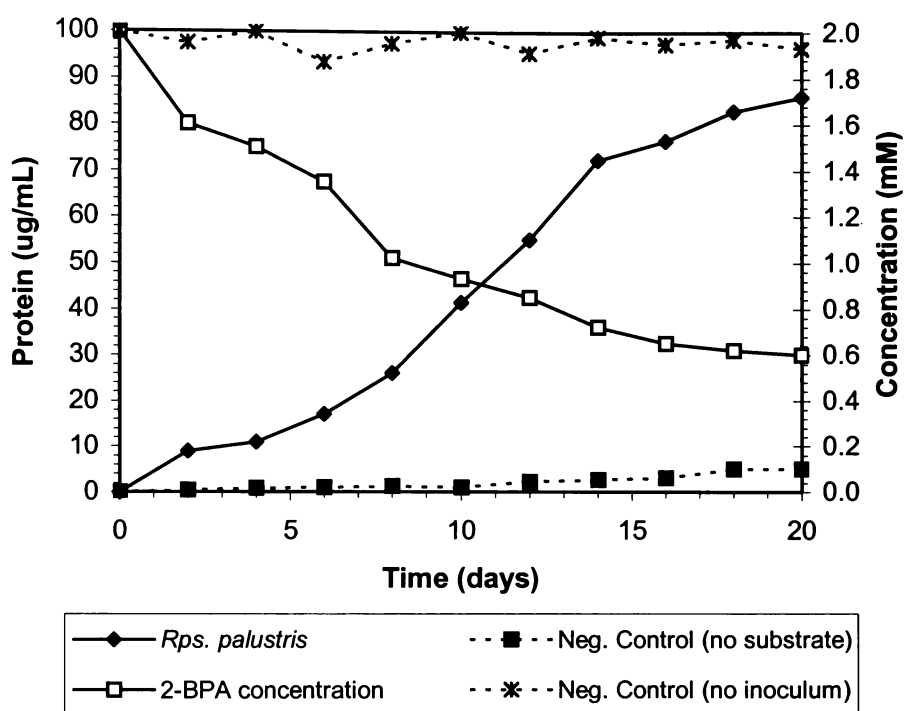


Figure 5.20 Growth of *Rps. palustris* DSM 123 on 2-BPA as sole carbon source.

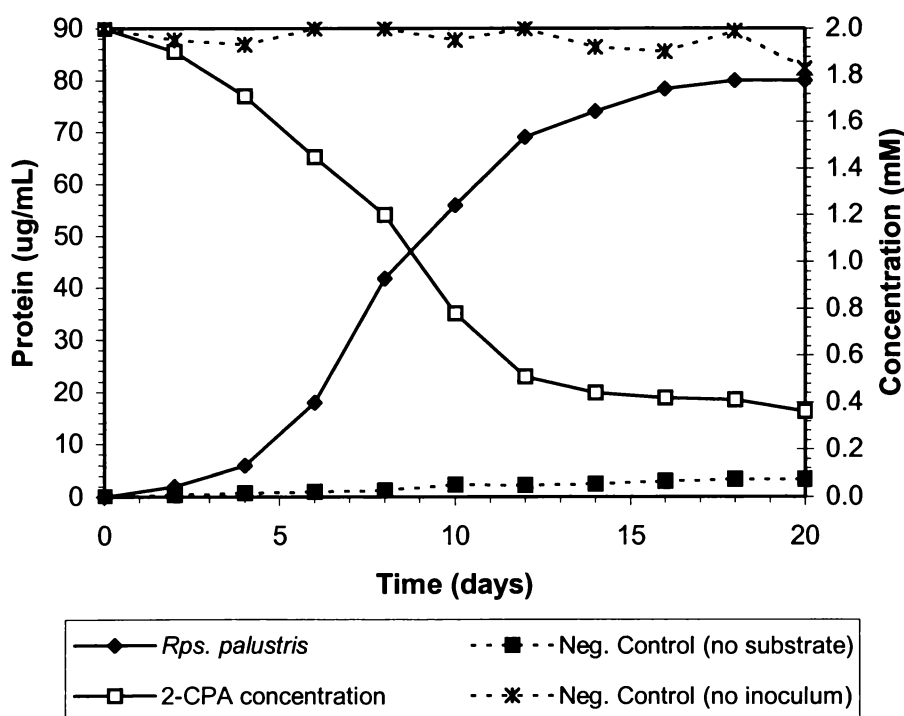


Figure 5.21 Growth of *Rps. palustris* DSM 123 on 2-CPA as sole carbon source.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

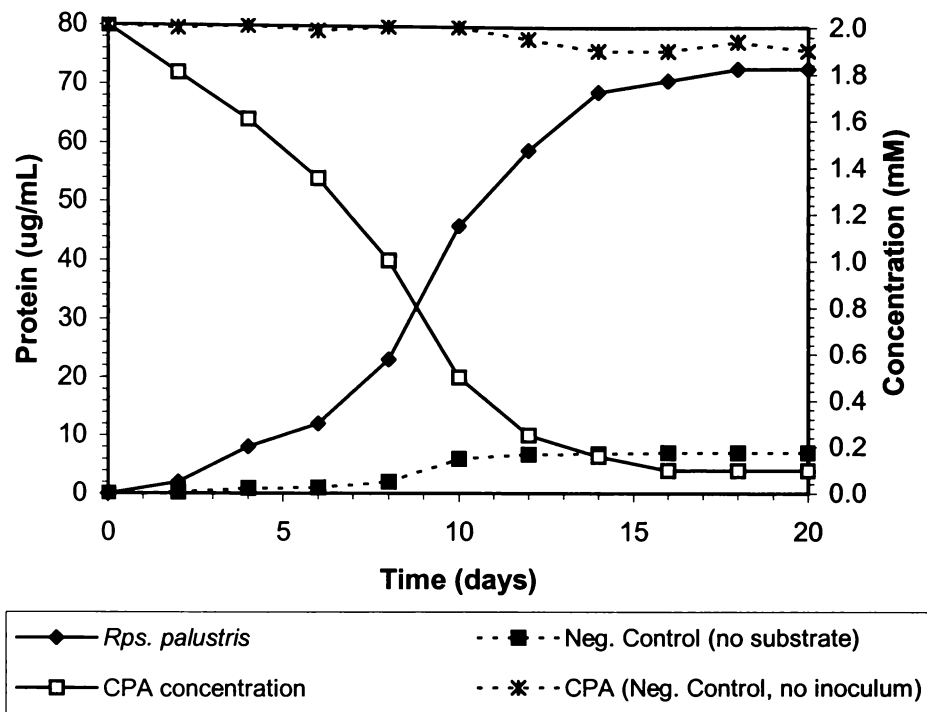


Figure 5.22 Growth of *Rps. palustris* DSM 123 on CPA as sole carbon source.

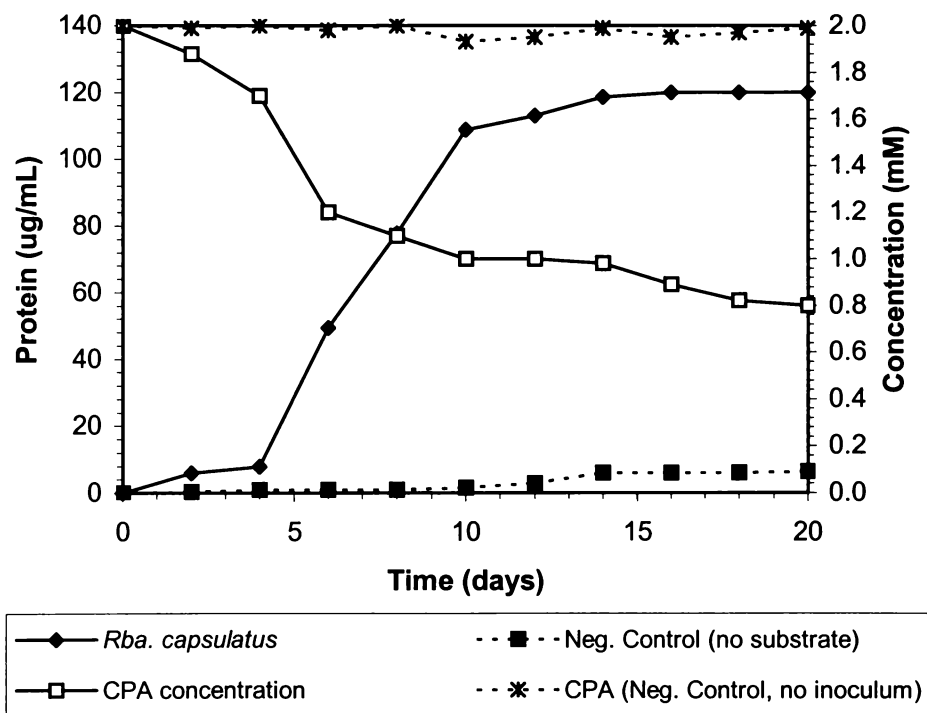


Figure 5.23 Growth of *Rba. capsulatus* on CPA as sole carbon source.

DEGRADATION OF HALOGENATED CARBOXYLIC ACIDS

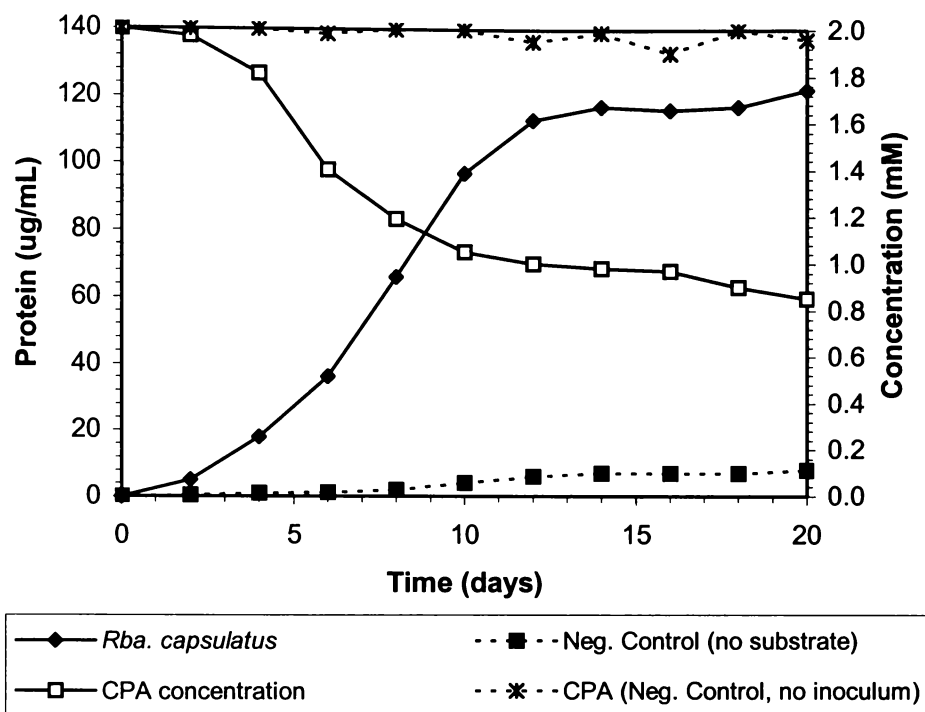


Figure 5.24 Growth of *Rba. capsulatus* on CPA as sole carbon source

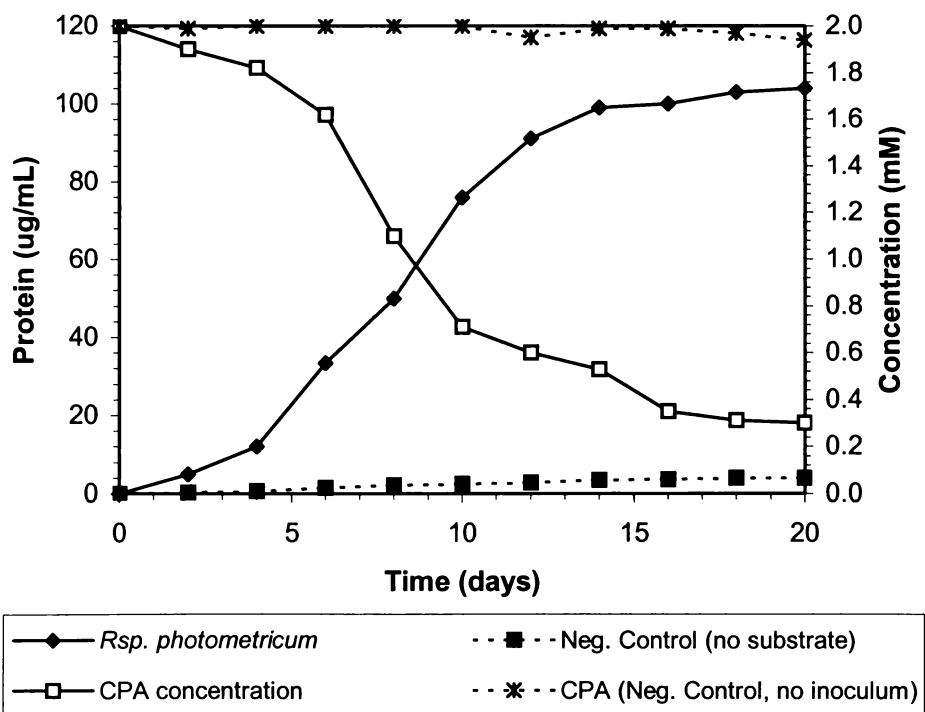


Figure 5.25 Growth of *Rsp. photometricum* on CPA as sole carbon source.

5.5 Optimal and inhibitory concentrations of halocarboxylic acids

Halocarboxylic acids that had proven to be good substrates for growth and some that had supported only poor growth were further studied to establish the optimal growth concentration and minimum inhibitory concentration for selected species of purple nonsulfur bacteria. Table 5.7 shows the combinations of species and substrate used in this section of work.

SPECIES	HALOCARBOXYLIC ACID
<i>Rps. palustris</i> DSM 123	3-Chloropropionic acid 2-Bromopropionic acid
<i>Rsp. photometricum</i>	3-Chloropropionic acid
<i>Rsp. rubrum</i>	2-Bromopropionic acid

Table 5.7 Species and halocarboxylic acids used for optimal and inhibitory concentration studies.

5.5.1 Materials and methods

Growth rates of the bacteria on each of the substrates were determined at eight concentrations. The concentrations (mM) used were: 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, 8.0, and 10.0. The experiments were performed in Hungate tubes which each contained 0.2 mL of inoculum, 0.1 mL of bicarbonate solution, the appropriate volume of 100 mM stock substrate, and basal salts medium to a final volume of 10 mL. Inocula were taken from cultures acclimated to the appropriate substrate by several previous subcultures. For each species tested, a positive control with the corresponding non-halogenated acid at a concentration of 5 mM was employed. Tubes were incubated in the light at 25 °C following a 24 hour dark incubation period. Growth was measured by total protein concentration as described earlier.

5.5.2 Results

The results of the toxicity and optimal concentration studies are represented graphically showing the rate of growth during log phase growth on the halocarboxylic acid at a range of concentrations. Growth rates are represented as generations per day (24 hours). Concentrations at which the substrate is toxic are represented as having a growth rate equal to zero.

5.5.2.1 *Rps. palustris* DSM 123 on CPA

The growth rate of *Rps. palustris* DSM 123 on 3-chloropropionic acid at a range of concentrations is shown in Figure 5.26. Results show that the optimal concentration for growth on the substrate is around 1.5 mM, with a growth rate just above 0.8 generations/day. The growth rate decreases at higher concentrations, with no growth occurring above or at 8 mM. The growth rate obtained at 2 mM with the acclimated inoculum used in this experiment was 0.5 generations/day, considerably higher than the 0.3 generations/day obtained with the non-acclimated inoculum used in the initial growth study (Section 5.3.2.3). Furthermore, the final yield obtained was two-fold higher than that obtained at the same concentration in the initial study.

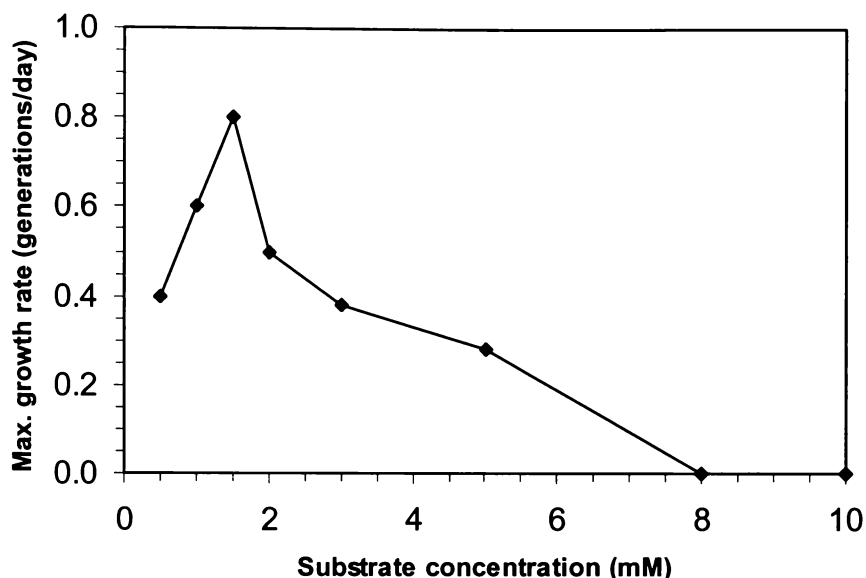


Figure 5.26 Maximum growth rates of *Rps. palustris* DSM 123 at various concentrations of CPA.

5.5.2.2 *Rps. palustris* DSM 123 on 2-BPA

The growth rates of *Rps. palustris* DSM 123 at various concentrations of 2-bromopropionic acid are shown in Figure 5.27. The highest rate of growth, 0.85 generations/day, occurred at a substrate concentration of 1.5 mM, the same result observed for growth of the same organism on 3-chloropropionic acid. Maximum growth rates decreased rapidly as the concentration increased, with 5 mM inhibiting growth of the organism, compared to a considerably higher minimum inhibitory concentration for growth on 3-chloropropionic acid. As above, the growth rate of 0.8 generations/day at 2 mM was far greater than that obtained in the previous growth study using a non-acclimated inoculum, where the maximum was only 0.33. The final growth yield of two-fold that obtained in the previous study mimicked the result for 3-chloropropionic acid.

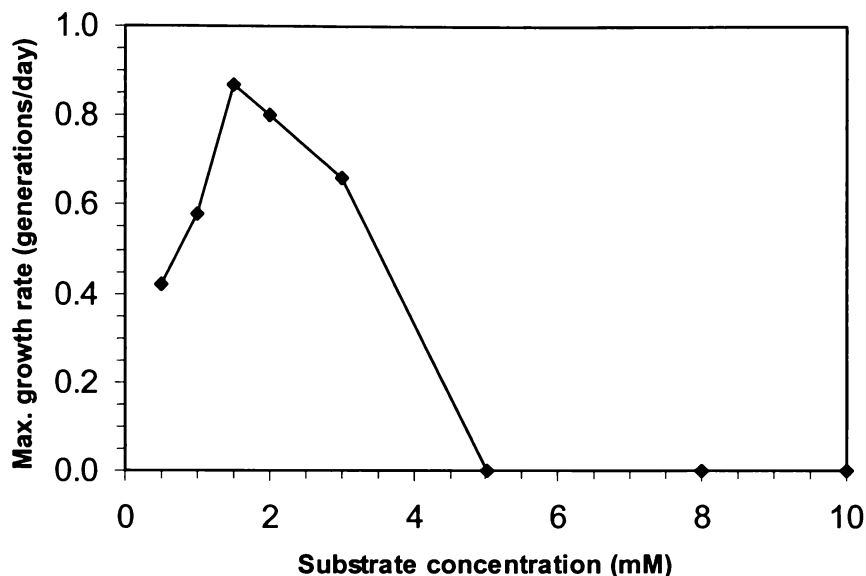


Figure 5.27 Maximum growth rates of *Rps. palustris* DSM 123 at various concentrations of 2-BPA.

5.5.2.3 *Rsp. photometricum* on CPA

Figure 5.28 shows the maximum growth rates obtained by *Rsp. photometricum* at various concentrations of 3-chloropropionic acid. The optimal concentration is shown to be around 3 mM, considerably higher than that of *Rps. palustris* DSM 123 on the same substrate. Growth rates at the optimal concentration are twice that observed for the *Rps. palustris* strain, at over 1.7 generations/day, although the minimum inhibitory concentrations for both species appear to be similar at between 7 and 8 mM. Growth yields obtained in this experiment again followed the pattern of being approximately twice those obtained at a similar concentration in tests performed with non-acclimated cells. In the case of *Rsp. photometricum* growing on CPA, the comparison was made from screening tests described early in this chapter where actual cell density values are not shown.

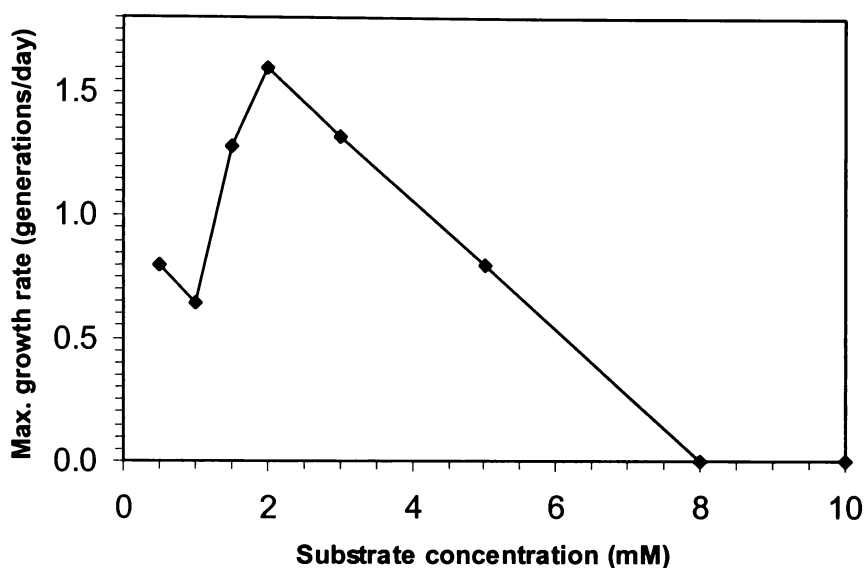


Figure 5.28 Maximum growth rates of *Rsp. photometricum* at various concentrations of CPA.

5.5.2.4 *Rsp. rubrum* on 2-BPA

Growth rates of *Rsp. rubrum* at various concentrations of 2-bromopropionic acid are shown in Figure 5.29. The optimum growth concentration is shown to be 1.5 mM, the same as for *Rps. palustris* DSM 123 on the same substrate, although the growth rate in this case was approximately twice that of the latter at 1.6 generations/day. As with *Rsp. palustris* DSM 123, the optimum is reasonably sharply defined with a rapid drop in rate as the concentration increases, with a minimum inhibitory concentration occurring around 4 mM. The maximum growth rate obtained was considerably higher than the 0.75 generations/day of non-acclimated cells on the same substrate.

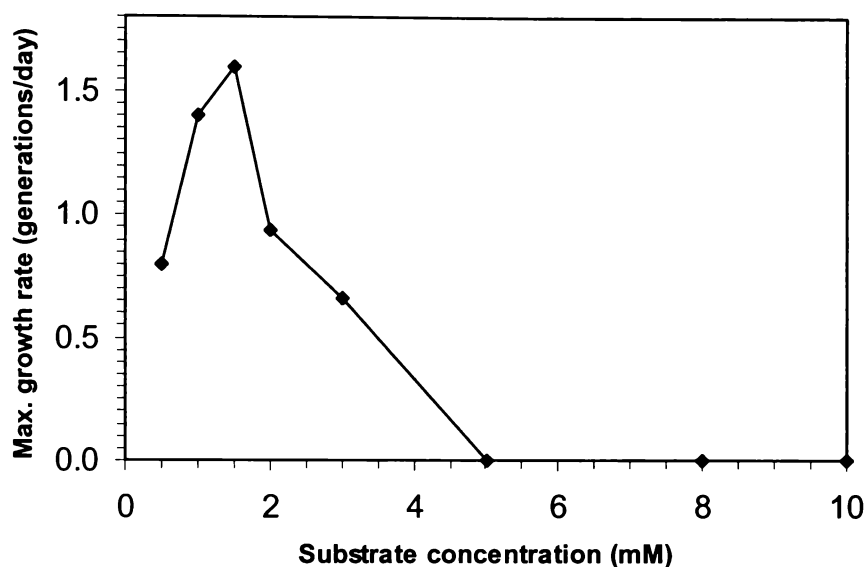


Figure 5.29 Maximum growth rates of *Rsp. rubrum* at various concentrations of 2-BPA.

5.6 Attempts to identify the *Rsp. rubrum* dehalogenase

SDS-PAGE was used in an attempt to identify a dehalogenase from *Rsp. rubrum* by comparing band patterns of total protein from cells grown on 3-chloropropionic acid, 2-bromopropionic acid, and propionic acid.

5.6.1 Materials and methods

Rsp. rubrum was grown photoheterotrophically in separate 100 mL serum vials on 3mM propionic acid, 2-bromopropionic acid, and 3-chloropropionic acid. Log phase cells were harvested by centrifugation and the cell pellet immediately frozen in liquid nitrogen for offsite transport and subsequent breakage by French pressure cell. Each cell pellet was resuspended in 5 mL of anoxic lysis buffer containing 200 mM Tris (pH 8.0), 1mM MgCl₂, and 0.5 mM sodium dithionite. Cells were then

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broken by two passages through an AMINCO French pressure cell (Model J4-3396) mounted in a WABASH hydraulic press (model 12-10S) at a pressure of 60 MPa. The pressure cell was cooled to 4 °C before use. Cell extracts were centrifuged at 9000g for 30 minutes to remove cell debris. The resulting supernatant was loaded onto a polyacrylamide electrophoresis gel of the following composition:

<u>Stock solution</u>	<u>Separating Gel</u>	<u>Stacking Gel</u>
49.5%T(3%C) acrylamide	3.5 mL	365 µL
3M Tris (pH 8.9)	5 m µL	1.14 mL
10%(w/v) SDS	150 µL	45 µL
H2O:glycerol (3.25:2)	6.3 mL	3 mL
10% APS	50 µL	36 µL
TEMED	50 µL	36 µL

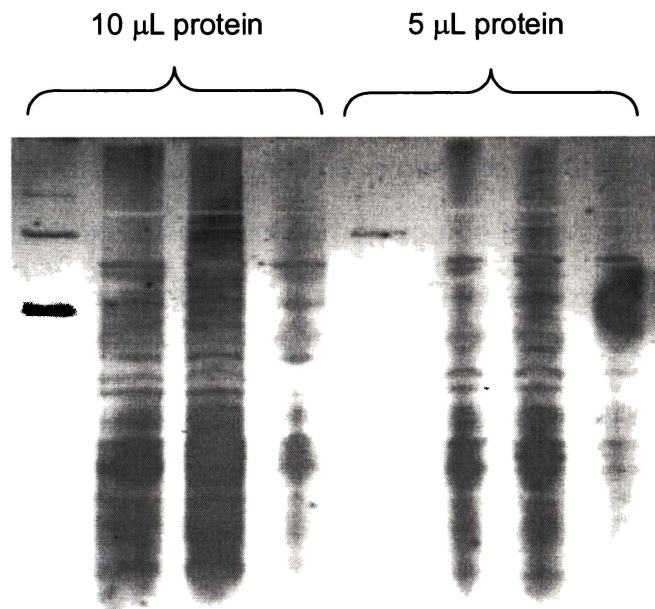
160 mL of 0.1 M Tris, 0.1 M Tricine, and 1.4 mL 10% SDS was added to the upper chamber of the gel apparatus. The lower chamber was filled with 0.2 M Tris (pH 8.9). An equal volume of supernatant was added to a gel-loading buffer (8% SDS, 24% glycerol, 100 mM Tris-Cl (pH 6.8), 4% 2-mercaptoethanol, 0.02% Coomassie Brilliant Blue G-250) and boiled for 10 minutes. 5 µL or 10 µL of this solution was added to the gel lanes. Gels were run at 30 mA and 75-100V for four hours. Gels were then stained by washing in the following silver-staining procedure:

- 30% ethanol, 10% acetic acid, 30 mins
- 30% ethanol, 10-15 mins, three times
- Milli-Q water, 5 mins
- 100 mL 0.1% AgNO₃ plus 150 µL formaldehyde solution, 30 mins

- Milli-Q water rinse, 15 secs
- 100 mL 3% Na₂CO₃ plus 150 μL formaldehyde solution plus 100 μL 1% Na₂S₂O₃·5H₂O until bands develop
- 7% acetic acid, to fix.

5.6.2 Results

Figure 5.30 is a photograph of a gel containing total protein of *Rsp. rubrum* cells grown on propionic acid, 2-bromopropionic acid, and 3-chloropropionic acid. The results show that although good protein separation and reasonable resolution was achieved, there was no distinctly different band in either of the lanes containing protein from cells grown on the halogenated substrate from that which was grown on propionic acid. The result suggests that the proportion of total cell protein that is dehalogenase in actively dehalogenating cells may be low.



Lanes from left to right: Molecular size marker*, 3-chloropropionic acid, propionic acid, 2-bromopropionic acid.

Figure 5.30 SDS-PAGE gel of total protein from *Rsp. rubrum* cells grown on halopropionic acids and propionic acid.

*The protein molecular size marker (Low Molecular Weight Calibration Kit from Amersham-Pharmacia Biotech Ltd) consisted of the following proteins:

Phosphorylase b (MW 94000)	Carbonic anhydrase (MW 30000)
Bovine serum albumin (MW 67000)	Trypsin inhibitor (MW 20100)
Ovalbumin (MW 43000)	α -lactalbumin (MW 14400)

5.7 Discussion

The ability of a number of species of purple nonsulfur bacteria to grow phototrophically at the expense of two- and three-carbon halocarboxylic acids at concentrations of 1 mM and 2 mM was shown. The chlorinated and brominated substrates were more commonly used than the iodo- or fluoro-compounds, with the three-carbon acids expectedly affording the highest growth yields of the substrates tested. Of the seven purple nonsulfur bacteria used in this study, *Rps. palustris* DSM 123, *Rsp. photometricum*, and *Rsp. rubrum* are the three most versatile with respect to their carbon metabolism, and proved to be the most versatile in their ability to use halocarboxylic acids as carbon source for growth.

Three factors that affect whether or not a halogenated compound is metabolisable include the recalcitrance of the compound, the toxicity of the compound, and whether or not the dehalogenated product is a metabolisable carbon source. The higher the electronegativity of the halogen substituent the more recalcitrant is the carbon-halogen bond, which implies that of the halocarboxylic acids used in this study, fluoroacetic acid is likely the most difficult to dehalogenate. This suspected difficulty in the dehalogenation of fluoroacetic acid was confirmed by the few organisms that were able to utilise it for growth, with only the aforementioned three able to do so, and then only at a very slow rate.

Toxicity of halocarboxylic acids is probably due to the intracellular concentration, and hence uptake of the compounds by the bacteria (Pries *et al.* 1994). The toxicity of halocarboxylic acids to the purple nonsulfur bacteria seems to be inversely related to the presence and activity of the appropriate dehalogenase. The compounds that were utilised as carbon sources for growth proved to have minimum inhibitory concentrations far in excess of those that were not metabolised. Fluorine and iodine substituted acids proved to be the most toxic, the former probably because it was not readily dehalogenated, and the latter, although probably dehalogenated, having an appreciable toxicity due to the liberated iodine. In each case where growth by a species of purple nonsulfur bacteria on a halocarboxylic acid was observed, the initial degradation product was the corresponding carboxylic acid, irrespective of the type or position of the halogen substituent (McGrath and Harfoot 1997). The two most common dehalogenation mechanisms employed by anaerobic bacteria are reductive and hydrolytic. Clear evidence of product (acetate or propionate) formation due to the reductive dehalogenation mechanism with no identification of hydrolytically derived products (glycolate or lactate), suggests that the former mechanism is the one employed by the purple nonsulfur bacteria. The pathway for the degradation of halocarboxylic acids as proposed by this study is shown with two examples in Figure 5.31: dehalogenation of 3-chloropropionic acid by *Rps. palustris* DSM 123, and dehalogenation of 2-bromopropionic acid by *Rsp. rubrum*. The dehalogenation product in both cases was propionic acid, although the species and the halogen substituent and position were different.

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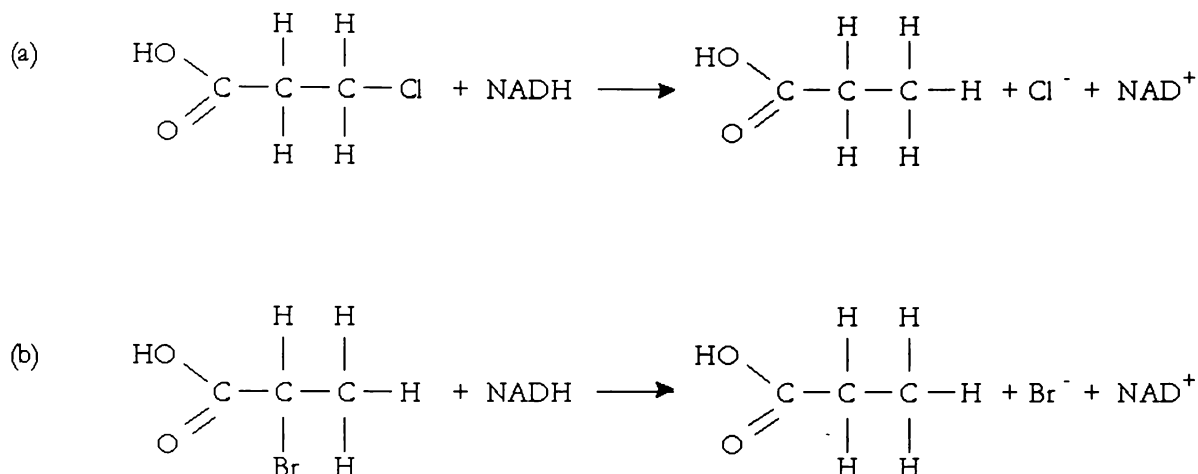


Figure 5.31 Proposed pathway for the dehalogenation of (a) 3-chloropropionic acid by *Rps. palustris* DSM 123; and (b) 2-bromopropionic acid by *Rsp. rubrum*.

Initial growth tests and the initial screening for the ability of purple nonsulfur bacteria to use halocarboxylic acids, were performed with inocula from cultures with no previous exposure to the compounds. The effect of culture acclimation prior to measurement of growth was found to be pronounced when subsequent growth studies were performed using inocula from acclimated cultures. The initial lag phase was markedly reduced when acclimated cells were used, consistent with an increased time require for dehalogenase synthesis. However, the observed lag phases were of no more than 48-72 hours duration in the non-acclimated cultures, suggesting that the genes for dehalogenase synthesis were already present and not acquired through mutation events. The higher growth yields, growth rates, optimum growth concentrations, and minimum inhibitory concentrations obtained with acclimated cultures could be explained by the rate of dehalogenation being

higher than the rate of substrate uptake. Synthesised and active dehalogenases in acclimated cells would rapidly detoxify the intracellular halocarboxylic acids so that they accumulate slowly, hence far higher initial extracellular concentrations can be dealt with. In non-acclimated cells, substrate uptake would cause increasing intracellular concentrations whilst dehalogenase synthesis is taking place, hence initial concentrations in the medium above which that are toxic intracellularly would cause growth inhibition. Lower growth rates during log phase growth of non-acclimated cultures suggests attenuation by the elevated intracellular halocarboxylic acid concentration as compared to that in an acclimated cell. In many cases, it was shown that non-acclimated cultures entered death phase before much of the substrate was utilised, the detection of much of this unused substrate in the culture medium likely due to a combination of permease activity loss and ultimately, cell lysis.

The ability of purple nonsulfur bacteria to utilise halocarboxylic acids as sole carbon sources for growth by way of reductive dehalogenation suggests that these bacteria may have a role to play in the detoxification of such compounds in aquatic habitats such as ponds and estuaries. Identification in this study of dehalogenating ability by the purple nonsulfur bacteria suggests this group of organisms may show dehalogenating activity towards other haloaliphatic compounds. Such compounds as chlorinated alkanes, many of which are used in large volumes as industrial solvents, and other more heavily halogenated carboxylic acids, could be susceptible to degradation by the purple nonsulfur bacteria.

5.8 Summary

A summary of the findings in this chapter are outlined below in Table 5.8

<ol style="list-style-type: none"> 1. Species of purple nonsulfur bacteria were shown to utilise two- and three-carbon monohalocarboxylic acids as sole carbon source for photoheterotrophic growth. 2. <i>Rps. palustris</i> DSM 123 showed the widest overall ability to utilise halocarboxylic acids for growth, including the widely used pesticide monofluoroacetate, suggesting an ability to break the recalcitrant carbon-fluorine bond. 3. Halocarboxylic acids with chlorine or bromine as the halo-substituent were most readily used by the purple nonsulfur bacteria. 4. Optimum growth concentrations ranged from 1.5 to 3.5 depending on strain and substrate; minimum inhibitory concentrations were typically around 4 mM for brominated acids and 7mM for chlorinated acids. 5. Removal of the halogen from the organic moiety by the purple nonsulfur bacteria is postulated to occur via reductive dehalogenation. 6. Acclimation of cells of purple nonsulfur bacteria to halocarboxylic acids results in increased growth rate, growth yield, and resistance to toxicity of the compounds, over non-acclimated cells.

Table 5.8 Summary of the findings in Chapter 5.

CHAPTER 6

GENERAL CONCLUSIONS

6.1 Introduction

This chapter will summarise and draw conclusions from the results obtained in this study. Conclusions will be drawn from each of the chapters and from the study as a whole, addressing such areas as ecological and bioremedial applications, limitations of the results, and suggestions for further work.

6.2 Chapter conclusions

Isolation, characterisation and growth on phenolic compounds

The first aim of the study was to isolate and characterise anaerobic photosynthetic microorganisms capable of degrading phenol and substituted phenolic compounds. A number of isolates of purple nonsulfur bacteria were obtained from sites thought likely to contain aromatic compounds derived from either human activity or naturally from plant material. The majority of the isolates were of the species *Rps. palustris*, which is the most commonly isolated of the purple nonsulfur species. Only one of the isolates was shown to grow with phenol as sole added carbon source. The isolate was characterised and identified as a strain of *Rps. palustris* and was designated the strain name of CCLRSP 3, in reference to the method of enrichment and the site from which it was isolated.

Continuous culture enrichment proved to be the most successful method for isolating organisms of the type desired, although it was extremely time consuming and in practice allowed for enrichment from only one sediment sample at a time.

GENERAL CONCLUSIONS

The work shows that the purple nonsulfur bacteria in the sediment samples probably had to adapt to utilisation of phenolic compounds, hence the difficulty in isolation likely due to substrate concentration and time considerations. The result also suggests that the sampling sites may not have contained concentrations of phenolic compounds that would be significant enough to the resident purple nonsulfur bacteria population to result in *in situ* adaptation.

Once the isolation work was completed, studies into the utilisation of phenolic compounds by the isolated strain of *Rps. palustris* and type strains of a number of species of purple nonsulfur bacteria were undertaken. The isolated strain was deemed to be in pure culture by virtue of it having been isolated repeatedly by single colonies on agar shake tubes or plates and being a non-slime producer, there was no chance for co-culturing with other organisms residing in the slime halo. The 16S rDNA result (represented by an electropherogram in an appendix at the end of this chapter) showed the isolate's DNA to be very pure and free from nuclear material from a co-cultured organism. However, the possibility of a sulfate reducing bacteria contaminant surviving in small numbers throughout the enrichment and isolation procedure could not be completely ruled out. Results showed for the first time a number of strains of purple nonsulfur bacteria capable of growing on phenol and substituted phenolic compounds as sole added carbon sources for photoheterotrophic growth. Very few species were able to utilise the compounds for growth at the concentrations tested; those that could were the type strains of *Rsp. photometricum* and *Rsp. rubrum* and the isolated CCLRSP 3 strain of *Rps. palustris*. The compounds utilised were phenol, 2-chlorophenol, *o*-cresol, and 2,4-dinitrophenol. Growth at the expense of phenolic compounds could not however be proved by isotope incorporation studies, as upon revisiting the cultures it was surprising to find that strains capable of growing on these compounds to high cell density in the past had lost the ability to do so. All

GENERAL CONCLUSIONS

attempts to re-establish this growth failed. Genes for the metabolism of aromatic compounds are commonly carried on self-transmissible plasmids, the role of which in spreading these genes between organisms in nature is undebateable (Shepherd 1997). In anaerobic sediment, it is quite conceivable that resident purple nonsulfur bacteria cells could obtain these genes by horizontal transfer mechanisms. It is also conceivable that over time under laboratory conditions, an isolated strain could lose the ability to degrade certain aromatic compounds if the genes are carried on plasmids. The possibility of a sulfate reducing bacteria being present as a contaminant but actually providing important phenolic degrading steps in co-culture with the *Rps. palustris* strain could not be ruled out. Consequently, the failure to re-establish growth of the culture on phenolic compounds could have been due to the contaminant organism no longer being present. All non-catabolic transformations of the phenolic compounds were also no longer evident.

The degradation of 2,4-dinitrophenol by *Rsp. rubrum* was shown to proceed via an initial step involving the reduction of the *ortho*-nitro group to an amino group.

Growth on, or in the presence of phenolic compounds by the purple nonsulfur bacteria seems to be related to their ability to take up and detoxify the substrate. In the case of nitrophenols, research has shown that a strain of a purple nonsulfur bacterium was able to successfully take up and detoxify 2,4-dinitrophenol but not mononitrophenols at neutral or close to neutral pH (Blasco and Castillo 1992). This seems to be reasonably consistent with the results obtained in this study, as mononitrophenols proved to be more toxic.

Growth on halocarboxylic acids

Growth on mono-halogenated carboxylic acids under photoheterotrophic conditions by a number of species of purple nonsulfur bacteria was reported. The

type strain of *Rps. palustris* was shown to utilise the widest range of halocarboxylic acids including, perhaps most interestingly, monofluoroacetate. Due to the electronegativity of fluorine, cleavage of the fluorine-carbon bond is relatively rare among bacteria when compared to the other halogen-carbon bonds. Monofluoroacetate, or its synonym 1080 poison, is used widely in many countries including New Zealand as a pesticide; hence its degradation in certain anaerobic sediments may involve the commonly occurring *Rps. palustris*. Other purple nonsulfur genera shown to grow at the expense of halocarboxylic acids included *Rhodospirillum*, *Rhodobacter*, and *Rubrivivax*.

Results showed that adaptation to the substrates had a marked effect on growth rate and on the optimum concentration for growth, both of which increased when acclimated inocula was used over non-acclimated. The comparative lag phases suggested that the genes coding for dehalogenases are present in purple nonsulfur bacteria, and that synthesis of the enzymes occurs in the extended lag phase of non-acclimated cultures.

Degradation of halocarboxylic acids by the purple nonsulfur bacteria was shown to likely proceed via an initial reductive dehalogenation to the corresponding free acid, which was subsequently used as a carbon source for growth.

6.3 Limitations of the results

The majority of the conclusions drawn in this study are tentative. No attempt has been made to postulate proposed pathways for the degradation of phenolic compounds by the purple nonsulfur bacteria because the evidence required for such could not be produced. This was due in most part to the cultures losing the ability to degrade the compounds that supported growth earlier in the study. Results for growth on both phenolic compounds and halocarboxylic acids are reported for relatively high concentrations. Hence, results reported as inhibitory or

negative for growth at the concentrations tested may not be accurate at lower concentrations. Hence, the actual range of substrates utilised and the species that use them is probably underestimated by this work.

The enrichment regimes which were dominated by batch techniques in this study, probably did not reflect the true diversity of purple nonsulfur bacteria within the sampling sites that were capable of phenolic degradation. Continuous culture systems would likely better reflect the purple nonsulfur population in these habitats, but the time required to enrich and isolate many strains via this procedure was beyond the bounds of this study.

6.4 Ecological and bioremedial considerations

Substituted phenols and halocarboxylic acids are included in the U.S. Environmental Protection Agency Priority Pollutant List and are known to be contaminants of many habitats. Transformation of substituted phenols in the environment often leads to more toxic and/or recalcitrant products, an example of which is the transformation of 2,4-dinitrophenol to 2-amino-4-nitrophenol. Resident bacteria that can completely mineralise pollutants are of extreme importance in the bioremediation of a contaminated site. This study has shown that members of the purple nonsulfur bacteria, which are common in anaerobic sediments, can degrade certain pollutant compounds. The high concentrations of these compounds that could be tolerated and utilised suggest that in highly contaminated sediments certain species of purple nonsulfur bacteria may proliferate. The treatment of high-strength industrial wastestreams is also a plausible application of the degradative ability of the purple nonsulfur bacteria. Selection of specific strains for treatment of characterised waste under constant illumination using bioreactor technology could be applied to low-volume/high-strength industrial effluents.

6.5 Suggestions for further work

Throughout the study, the results yielded a number of ideas for further work. During the isolation and characterisation section of the work it was shown that continuous culture systems were superior to the other enrichment techniques employed. Because of the pressure on time and equipment resources that continuous culture systems apply, it was only possible to use the technique in limited fashion. Further study using continuous culture as the enrichment procedure of choice, would likely yield a large number and variety of purple nonsulfur bacteria capable of aromatic pollutant degradation. Studies of individual contaminated habitat types such as estuaries or effluent ponds at different sites for their aromatic degrading phototroph population could be performed. Studies such as these, as well as yielding ecological information, could yield organisms of possible value for treatment systems.

Of particular interest in the halocarboxylic acid utilisation studies was growth at the expense of monofluoroacetate by the type strain of *Rps. palustris*. Further characterisation of the metabolism would be useful in determining whether this species is likely to play a major role in degradation of the compound in freshwater sediments. Comparisons of defluorination rates by *Rps. palustris* and other anaerobic bacteria would also yield information as to the importance of the organism in the detoxification of the compound.

Studies into the degradation of phenolic compounds at lower concentrations could not only show a larger number of strains able to transform them, but also a greater variety of compounds susceptible to transformation. The focus of this study was to identify purple nonsulfur bacteria capable of growing at the expense of the compounds tested, whereas a number of strains may show the ability to transform compounds in a non-catabolic manner at much lower concentrations.

GENERAL CONCLUSIONS

Identification of a number of transformation capabilities within the purple nonsulfur bacteria could lead to co-culture studies and possible degradation of more heavily substituted and recalcitrant aromatic pollutants. Another avenue of research could be in the use of solid media to test for degradative capability. This would allow for testing of less soluble compounds such as lindane and 2,4-dichlorophenoxyacetic acid, which were shown to be impractical for liquid medium studies. Further work would need to be undertaken in optimising conditions for growth of purple nonsulfur bacteria on solid medium, as good growth is only observed for a small number of species to date.

The application of a number of specific species inhibitors during enrichment and isolation steps would circumvent possible contamination problems. The use of molybdate in anaerobic sediment enrichments would, for example, rule out the possibility of sulfate reducing bacteria contamination of purple nonsulfur bacteria isolates.

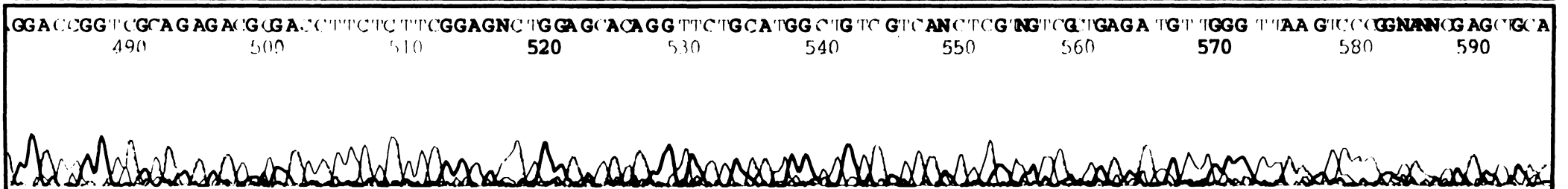
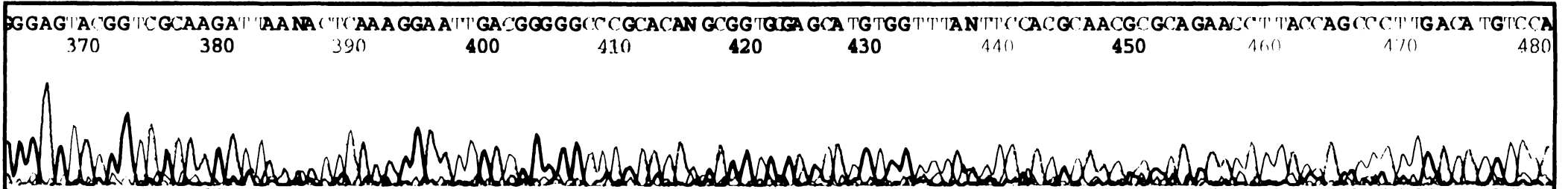
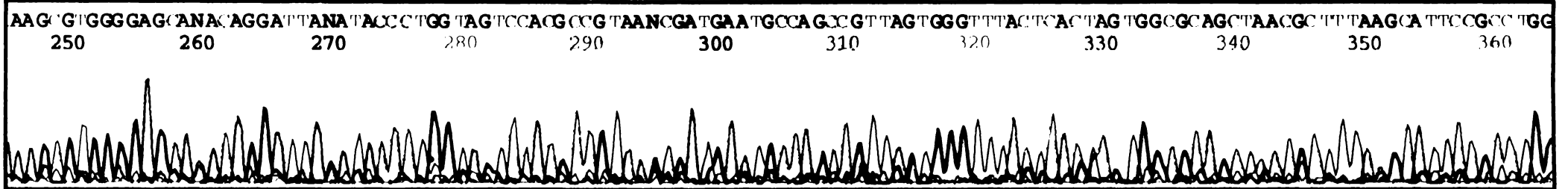
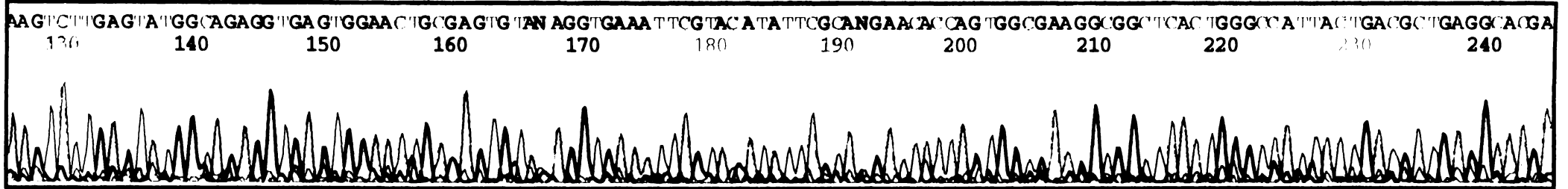
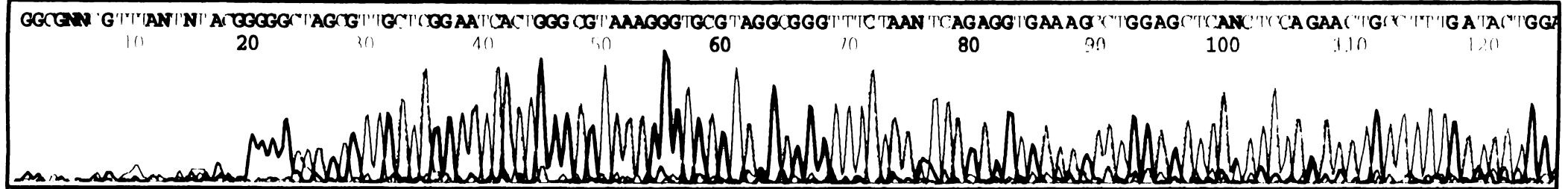


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