ISOLATION OF BACTERIAL STRAINS CAPABLE OF EFFICIENT CONVERSION OF N-ALKANES INTO VALUE ADDED PRODUCTS.

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ABSTRACT

Bacteria that are capable of degrading alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, were isolated from oil-contaminated soil collected from the petrochemical company, CALTEX Refineries, South Africa. A total of twenty-three environmental strains were isolated. A preliminary procedure, Nile Blue A straining suggested that twelve of the twenty-three environmental strains might accumulate polyhydroxyalkanoates (PHAs) in their cytoplasm, which is a good candidate for biodegradable plastics. The gene that catalyzes PHA polymerization, *phaC*, was detected using PCR in some of the environmental strains. The strains of interest were identified and characterized biochemically using various techniques and later sequenced by 16S rDNA PCR. The environmental isolate 2 showed a 99 % identity to *Pseudomonas aeruginosa* BHP7-6 and was for that reason given a name, *Pseudomonas aeruginosa* MB2SA. *P. aeruginosa* MB2SA was shown to possess a 0.5 kb internal fragment corresponding to the *phaC* gene and capable of degrading the alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, effectively. *P. aeruginosa* MB2SA was shown to grow optimally in the long alkane fraction, C_{14}-C_{17}, and was further grown in pure alkanes, n-dodecane, n-tetradecane, and hexadecane for comparison. In addition, the strain, *P. aeruginosa* MB2SA, was grown in an appropriate medium for PHA synthesis and high yields of PHA were observed when both the long alkane fraction, C_{14}-C_{17}, and pure alkane, hexadecane, were employed as sole carbon sources respectively.

The 0.5 kb internal fragment of the *phaC* gene in *P. aeruginosa* MB2SA was cloned in pGEM-T Easy vector and transformed in *E. coli* DH5-alpha cells. Southern hybridization revealed that the cloned *phaC* gene was isolated from *P. aeruginosa* MB2SA. The

deduced PhaC amino acid sequence in *P. aeruginosa* MB2SA was found to have a 99 % identity to the PhaC amino acid sequences in both *P. aeruginosa* UBPP-PA14 and *P. aeruginosa* PAO1. The PhaC in *P. aeruginosa* MB2SA was compared with both the PhaC1 and PhaC2 in *P. aeruginosa* PAO1 showing 60.3 % and 98.7 % identity at the amino acid level respectively. It was therefore concluded that *P. aeruginosa* MB2SA has a PhaC2 synthase which is a type II PHA synthase, and could be novel as a result of the difference in some amino acid residues compared to the published PhaC2 sequences.

CHAPTER 1

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1 GENERAL INTRODUCTION

Petrochemical companies are faced with the challenge of dealing with hydrocarbon chemical wastes. SASOL is one of the biggest petrochemical companies producing hydrocarbon wastes in large quantities contributing to a huge environmental pollution problem faced worldwide. These wastes should be disposed of properly or else they might frustrate the lives of animals, plants, birds, fish, as well as humans. One way of getting rid of these wastes is to convert them to non-hazardous products. Bioconversion of these wastes to value added products might minimize their quantity.

One of the known by-products of these petrochemical hydrocarbons are straight chain alkanes. Alkanes might range from C_4 to C_{18}, and most microorganisms have been shown to be capable of utilizing these alkane fractions as carbon sources in order to survive

(Dinamarca *et al.*, 2003). In addition, these carbon sources could be converted into useful products such as polyhydroxyalkanoates (PHAs).

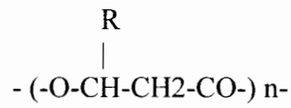
The objective of this study is to isolate microorganisms that are capable of degrading the alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, and that would at the same time produce a product of potential commercial value. The discovery of such organisms would be important because a viable solution to the waste problem faced by petrochemical companies would be established and wastes converted into useful product. Possible alkane by-products that can be commercialised are polyhydroxyalkanoates (PHAs), which are used to manufacture biodegradable plastics. It is a well-known fact that South Africa is currently selling retail plastic bags in an attempt to minimise plastic wastes and encourage recycling. This strategy seems to be working temporarily in reducing plastic waste but has not eliminated the waste problem completely. This study is focussed on the isolation of novel compounds derived from petrochemicals to develop biodegradable plastics, which will eliminate the waste crisis.

Biodegradable plastics can be produced from polyesters of hydroxyalkanoic acids, which are synthesized by many types of bacterial strains that build up these water insoluble polyhydroxyalkanoic acids (PHA) in their cytoplasm as inclusions, as energy and carbon reserve materials. These inclusions are referred to as PHA granules (Anderson and Dawes, 1990; Steinbüchel and Valentin, 1995; Steinbüchel and Füchtenbusch, 1999). PHA granules accumulate when nutrients such as nitrogen or phosphorus are limiting (Anderson and Dawes, 1990). The thermoplastic or elastic properties of PHA polymers are quite diverse depending on their chemical composition (Byrom, 1987; Lee, 1996).

The attention of research has been drawn towards their potential use as a biodegradable alternative to petroleum-based synthesic plastics such as polypropylene (PP) and polyethylene (PE) (Holmes, 1985; Witholt and Kessler, 1999). The most important barrier to the industrial utilization of PHAs is their high price, which currently is more than 10 times the price of their synthetic counterparts (Poirier *et al.*, 1995). Microbial fermentation of pure glucose or organic acids results in a very high PHA content (70-75 % w/w) in dry cell mass. The high production cost of PHAs is mainly due to the carbonaceous raw materials and polymer recovery (Choi and Lee, 1997). A decrease in cellular PHA biomass would drastically decrease the cost of polymer recovery (Du and Yu, 2002).

In order for cells to accumulate PHAs as part of their biomass, an appropriate carbon substrate is required. Polyhydroxyalkanoates (PHAs) are synthesized by diverting central intermediates of the carbon substrate metabolism or derivatives from precursor substrates. These intermediates are provided as carbon source for the growth of the bacterium and subsequently converted to hydroxyacyl-CoA thioesters. Thioesters are then polymerised by PHA synthases that are present on the surface of PHA granules together with other proteins (Steinbüchel and Hein, 2001).

The class of polyesters below represents the general structure of PHAs:



where, R=side chain substituent: its chemical structure depends on the stereospecificity of biosynthetic enzymes, the chiral centres have the (R) stereochemical configuration, so that they are optically active (Akita *et al.*, 1976; Alper *et al.*, 1963).

There are approximately 130 diverse hydroxyalkanoic acids known to be constituents of the bacterial storage polyesters (Steinbüchel and Valentin, 1995). Examples of polyesters existing in living organisms include polymalic acid, which is a water soluble polyester occurring only in lower eukaryotic organisms, suderin and cutin which are water insoluble polyesters in plants (Liu and Steinbüchel, 1996).

PHAs occur in the form of homo- or heteropolyesters. Fermentation of PHAs has been shown to be made possible by various types of bacteria as observed in the following studies, *Pseudomonas* (Doudoroff and Stanier, 1959; Delafield *et al.*, 1965), *Hydrogenomonas* (Schlegel *et al.*, 1961), C*hromatium* (Schlegel, 1962), Cyanobacterium (Jensen and Sicko, 1971), *Azotobacter beijerinckii* (Senior and Dawes, 1973), *Ralstonia eutropha* (Haywood *et al.*, 1988a), *Zoogloea ramigera* (Nishimura *et al.*, 1978), *Rhodococcus ruber* (Pieper-Fürst *et al.*, 1994) and *Methylobacterium rhodesianum* (Mothes *et al.*, 1997). Fermentation processes known to produce large volumes of PHAs

from reusable substrates have been developed. The chemical properties of PHAs are the same as those of other polyolefins and they are stored as granules by many bacterial species. The anaerobic degradation of an unknown substance resulted in the production of 3-hydroxybutyric acids in a soil bacillus similar to *B. megaterium* (Macrae and Wilkinson, 1958). *Azotobacter chroococcum* and *B. megaterium* KM cells were proven to accumulate poly-β-hydroxybutyric acid (PHB), which is a form of PHA when grown under favourable conditions (Griebel and Merrick, 1971; Senior *et al.*, 1972). PHA was noticed to be produced from alkanes by organisms such as *Candida* sp, a yeast, though their metabolic pathways that are fairly different compared to those occurring in bacteria (Fukui and Tanaka, 1981).

2 Enzymology of PHA synthase

PHA synthases are the key enzymes of PHA biosynthesis. These enzymes catalyse the covalent linkage between the hydroxyl group of one and the carboxyl group of another hydroxyalkanoic acid. The substrates of PHA synthases are coenzyme A thioesters of hydroxyalkanoic acids; there is no evidence that PHA synthases can utilize either free hydroxyalkanoic acids or other derivatives of hydroxyalkanoic acids. The three different enzymes for PHA synthesis have been observed, namely PHA synthase I, II, and III (Anderson *et al.*, 1992).

Research concerning poly (β-hydroxybutyric acid) PHB synthesis in *Alcaligenes eutrophus* (*Ralstonia eutropha*) revealed that PHB is synthesized from acetyl-CoA (Gottschalk, 1964). Three enzymes were identified that catalysed the conversion of

acetyl-CoA to PHB in *A. eutrophus* (Fig. 1). The enzyme, β-ketothiolase catalyses the formation of carbon-carbon bonds through the condensation of two acetyl-CoA moieties.

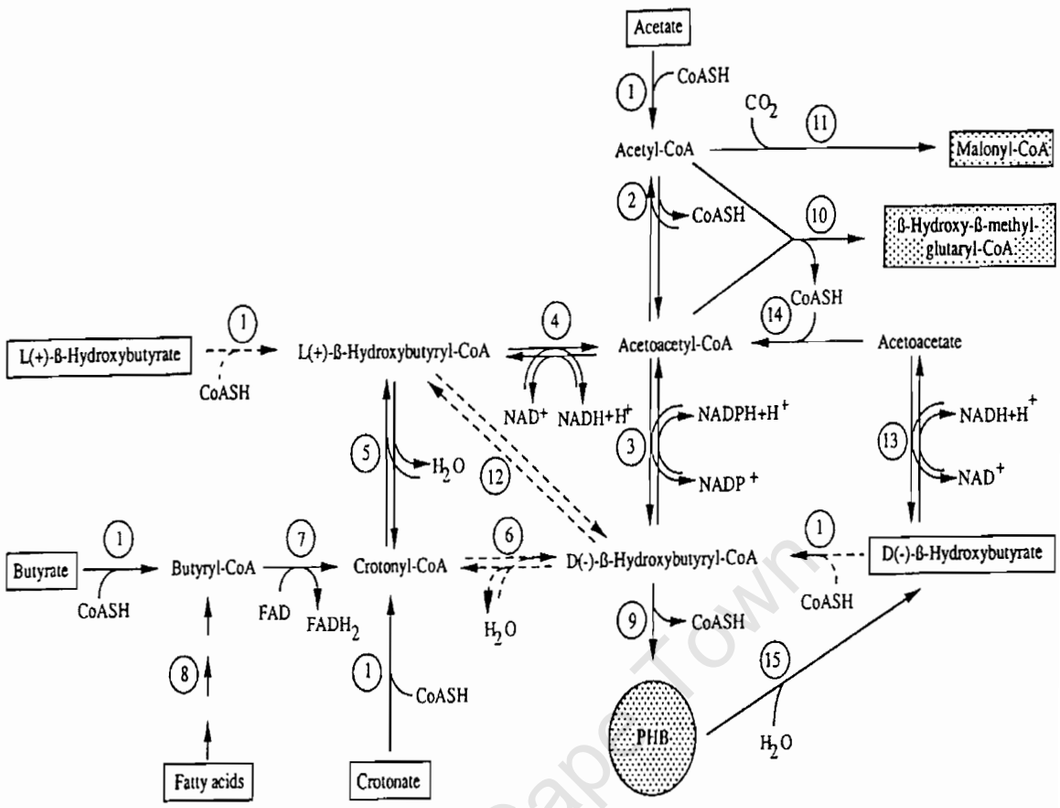


Fig. 1: Pathway of PHB synthesis and related reaction steps in *A. eutrophus*. 1, Acyl-CoA synthetases (it is uncertain whether specific synthetases exist for all the substrates); 2, β-ketothiolase; 3, acetoacetyl-CoA reductase (NADPH-dependent); 4, acetoacetyl-CoA reductase (NADH-dependent); 5, enoyl-CoA hydratase (forming L-(+)-β-hydroxybutyryl-CoA); 6, enoyl-CoA hydratase (forming D-(-)-β-hydroxybutyryl-CoA); 7, butyryl-CoA dehydrogenase; 8, enzymes involved in the β-oxidation pathway; 9, PHB synthase; 10, hydroxymethylglutaryl-CoA synthase; 11, acetyl-CoA carboxylase; 12, β-hydroxybutyryl-CoA epimerase; 13, D-(-)-hydroxybutyrate dehydrogenase (NAD-dependent); 14, CoA transferase; 15, PHB depolymerase. Broken lines indicate reactions, which are uncertain to occur in *A. eutrophus*. [The figure versed by Schubert *et al.* (1988)].

NADPH-dependent acetoacetyl-CoA reductase catalyses the reduction of acetoacetyl-CoA formed in the first reaction to D-(-)-β-hydroxybutyryl-CoA. The PHB synthase links the D-(-)-β-hydroxybutyryl moiety with an existing polyester molecule by an ester bond (Steinbüchel and Schlegel, 1991). The biosynthetic NADPH-dependent acetoacetyl-CoA reductase and β-ketothiolase are soluble proteins. These proteins are soluble in the absence of PHB accumulation. The particulate PHB synthase consists of the soluble enzyme combined with PHB (Fukui *et al.*, 1976; Haywood *et al.*, 1989a). The biosynthetic thiolase, the NADPH-dependent reductase and the soluble PHB synthase prefer C_4-substrates, which lead to the formation of polyesters of β-hydroxybutyrate. With C_5-substrates, all three enzymes exhibit only minor activities, the reductase showing evidence of even lower activity with C_6-substrates (Haywood *et al.*, 1988a, b; 1989a).

In addition, a second β-ketothiolase and an NADPH-dependent acetoacetyl-CoA reductase were also detected in *A. eutrophus* (Haywood *et al.*, 1988a, b). These two enzymes might play a role in the β-oxidation of fatty acids and participate in the conversion of butyrate, crotonate and β-hydroxybutyrate to acetyl-CoA (Fig. 1). It is not known which β-ketothiolase contributes to the synthesis of β-hydroxy-β-methylglutaryl-CoA. Both the degradative β-ketothiolase and the degradative reductase are not restricted to short-chain CoA thioesters (Steinbüchel and Hein, 2001).

2.1 Sizes and structure of PHA synthases

Type I- and type II-PHA synthases are distinguished from type III-PHA synthases because of their different sizes and structure. Type I- and type II-PHA synthases consist of only one type of subunit and type III-PHA synthases have two different types of subunits. Types I and II were shown to be present in *Ralstonia eutropha* and *Pseudomonas oleovorans* respectively, and consist of a PhaC subunit, which is encoded by $phaC_{Re}$ and $phaC_{PO}$ genes respectively (Prieto *et al.*, 1999). The type I synthases polymerises monomers with 3-5 carbons preferably (Haywood *et al.*, 1989a) and type II prefers monomers with more that 5 carbons (Brandl *et al.*, 1988; de Smet *et al.*, 1983).

Type III-PHA synthase was shown to be presente in *Chromatium vinosum* and encoded by $phaEC_{Cv}$. It consists of the two different subunits, $PhaC_{Cv}$ and $PhaE_{Cv}$ (Liebergesell *et al.*, 1992; Liebergesell *et al.*, 1994; Liebergesell *et al.*, 2000). Type III has been shown to polymerise the same chain length carbon monomer as type I synthase (3-5 carbons) (Liebergesell *et al*, 1994).

2.2 Structural relationships of PHA synthases

A comparison of the PHA synthases of microorganisms capable of producing PHAs revealed some important similarities. Feng and Doolittle (1987) aligned the amino acid sequences of 36 PHA synthases (*phaC* gene products), and showed that highly conserved regions were present in all PHA synthases (Fig. 2). These conserved regions have been successfully used to identify *phaC* genes from diverse bacteria such as *A. eutrophus*,

Alcaligenes hydrogenophilus ATCC 33178, *A. latus* ATCC 29714, *Comamonas (Delftia) acidovorans* ATCC 15668, *Hydrogenophaga palleronii* ATCC 17724, *H. pseudoflava* ATCC 33668, *Pseudomonas alcaligenes* ATCC 14909, *P. aureofaciens* ATCC 13986, *P. citronellolis* ATCC 13674, *P. fluorescens* ATCC 12983, *P. (Burkholderia) glathei* ATCC 29195, *P. mendocina* ATCC 25411, *P. oleovorans* ATCC 8062, *P. pseudoalcaligenes* ATCC 17440, *P. putida* ATCC 12633, *Sphaerotilus natans* ATCC 15291, *Azotobacter chroococcum* ATCC 9043, *A. vinelandii* ATCC 478 and *B. megaterium* CCRC 11595, using degenerate primers capable of amplifying these regions (Sheu *et al*., 2000).

Figure 2 shows that most of the Pseudomonas PHA synthases, form one cluster, and this is in accordance with the substrate specificities of these enzymes. There is an obvious phylogenetic difference between the PhaC1 and PhaC2 proteins produced by these Pseudomonas species. The top part of Fig. 2 reveals a close relationship between the PHA synthases of *Nocardia corallina* and *Rhodococcus ruber*, which are Gram-positive bacteria, to the PHA synthases from the pseudomonads. A second cluster, at the bottom section of Fig. 2, represents the PHA synthases from *Synechocystis* sp. PCC6803 and phototrophic sulfur bacteria. A close relationship was also noticed between PhaC1 from *Rickettsia prowazekii* and the PHA synthases from *Bacillus megaterium* (Steinbüchel and Hein, 2001).

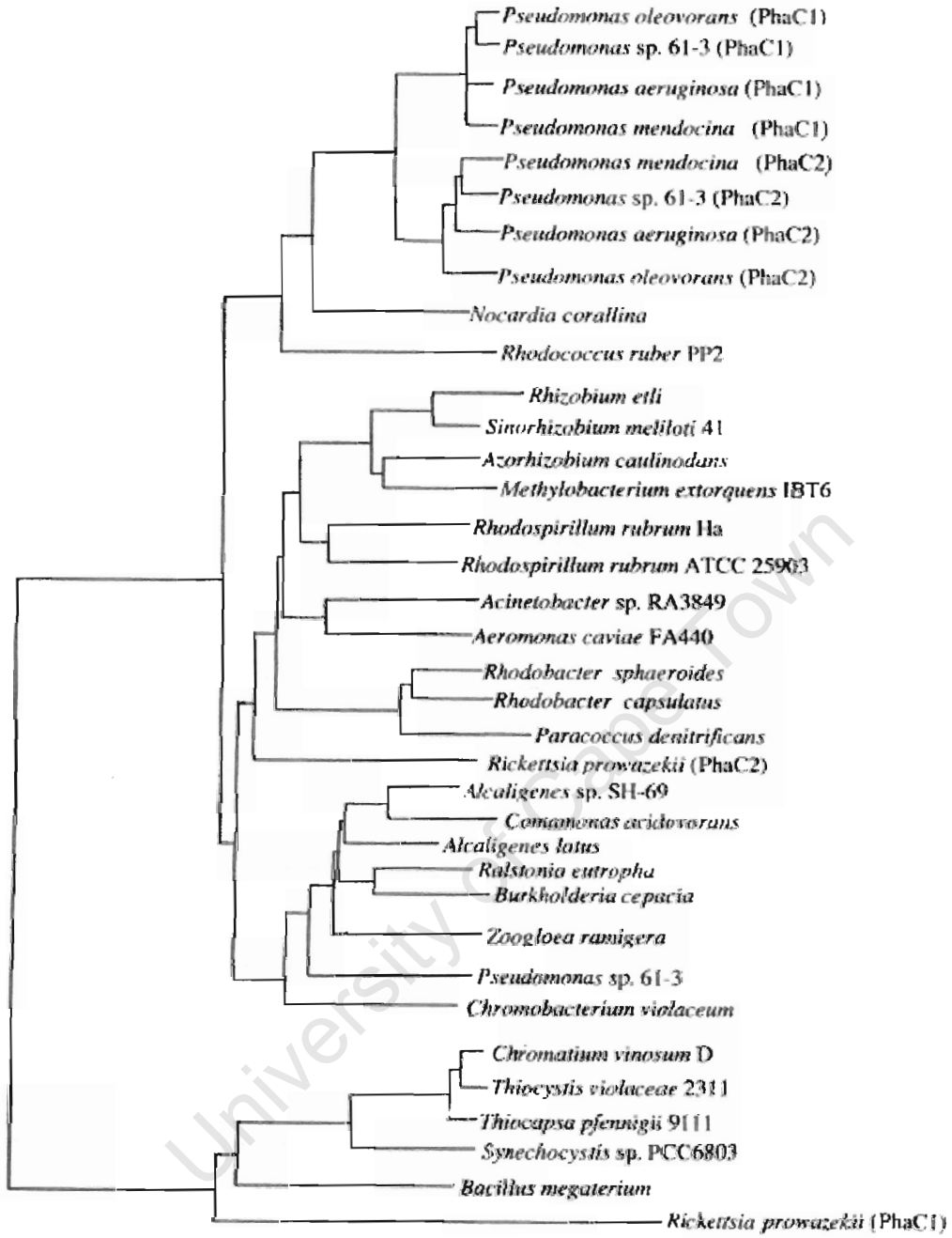


Fig. 2: Phylogenetic tree of 36 PHA synthases. The branching order and the distance score were calculated by the program TREE as previously described (Feng and Doolittle, 1987).

3 Catalysis by PHA synthases

A contemporary hypothesis for the reaction mechanism of PHA synthases was proposed based on a model discovered by Griebel *et al.*, (1968a, b) with two thiolates taking part in the covalent catalysis of polyester synthesis (Anderson *et al.*, 1992). The PHA synthesis take place at the thiolate groups (S_1H and S_2H) of the PHA synthases [E], where one thiolate was participating as a loading site and the second thiolate acted as the elongation site. This mechanism suggested that one thiol group [S_1H] received a hydroxyalkanoic acid from coenzyme A thioester which covalently bounded to the thiol group [E-S_1-CO-Alkyl-OH] and the coenzyme A being released, however, the growing polyester chain remain bound to the second thiol group to form [E-S_2-poly $(PH)_n$-OH]. This complex, [E-S_2-poly $(PH)_n$-OH], is then transferred to the free hydroxyl group upon a nucleophilic attack of the hydroxyl oxygen atom on the carbonyl carbon atom giving rise to [E-S_1-poly $(HA)_{n+1}$-OH]. A subsequent transesterification of the elongated polyester chain from S_1 to S_2 results in [E-S_2-poly $(HA)_{n+1}$-OH] + [E-S_1H], and the latter can now accept the next hydroxyalkanoic acid from a coenzyme A thioester. This catalytic process should be repeated several thousand time, otherwise the very high molecular weights of the polyesters being formed could not be explained. The catalytic cycle may be destroyed if the elongating polyester chain is released from [E-S_2-poly (HA)-OH] by the nucleophilic attack of a hydroxyl group of a molecule not attached to [E], for example, water. It has been proven that exogenous hydroxyl-compounds, such as glycerol, polyethylene glycol or 1,3-propanediol caused such chain terminating hydroxyl group which lead to a PHA molecule that has the respective hydroxyl-compound covalently connected to the PHA molecules (Madden *et al.*, 1999; Sheu *et al.*, 2000).

One highly conserved cysteine residue with regard to this catalytic cycle occurs in all PHA synthases. This cysteine is present as Cys-319 in the PHA synthase of *R. eutropha* (Gerngross *et al.*, 1994) and as Cys-149 in the PHA synthase of *C. vinosum* (Müh *et al.*, 1999). The activity of the PHA synthase enzymes is lost when this cysteine residue is replaced by another amino acid. It is possible that this cysteine residue provides one of the thiolate groups for covalent catalysis. It also appears that this cysteine residue is the only one conserved in all PHA synthases (Gerngross *et al.*, 1994).

Inhibitors, or activators, of pathways are often metabolic intermediates. However, this is not the case with PHA synthases. There was only one reported exception where the PHA synthases of *R. eutropha*, *C. vinosum* and *P. aeruginosa* were inhibited by coenzyme A at very low concentrations (Jossek and Steinbüchel, 1998; Qi *et al.*, 2000). The physiological relevance of this kind of inhibition is not yet understood. *In vitro* PHA biosynthesis processes must consider this inhibition by coenzyme A, and it was suggested that the coenzyme should be recycled to minimize costs and also improve PHA production. In addition, many different non-physiological chemicals were observed to inhibit PHA synthases, including phenazine methosulfonate, a known inhibitor of PHA synthases. These chemicals do not inhibit PHA synthases specifically (Steinbüchel and Hein, 2001).

4 Cellular localization of PHA synthase

PHA synthases were studied in detail in microorganisms such as *R. europha*. PHA synthases from *Zoogloea ramigera* and *R. eutropha* occur in soluble form in the cytoplasm in the absence of PHA accumulation in the cell, however, at the beginning of PHA synthesis these enzymes are bound to the granules. Immunological methods using gold-labelled antibodies indicated that the PHA synthases present in *R. eutropha* (Gerngross *et al*., 1993), *C. vinosum* (Liebergesell *et al*., 1994) and *P. oleovorans* were attached to the PHA granules and are located at the surface of the granules (Stuart *et al*., 1996).

5 Gene arrangement

Previous research has revealed at least 59 PHA synthases from 45 different bacterial strains and their nucleoticle sequences have been obtained (Rehm, 2003). The number of PHA synthase genes per genome varies, but they mainly occur in two or more copies in different bacterial strains. The PHA synthase genes form a cluster with the structural genes for β-ketothiolase and for acetoacetyl-CoA reductase in certain bacteria possessing type I PHA synthases. However, there are reports of the *phaC* gene occurring in isolation from other genes related to PHA biosynthesis in some bacteria possessing this type of PHA synthase (Steinbüchel and Hein, 2001).

Certain PHA accumulating bacteria possess a type I-PHA synthase cluster of genes, composed of the structural genes for PHA synthase (*phaC*), β-ketothiolase (*phaA*), and acetoacetyl-CoA reductase (*phaB*) which occur in varying arrangements, as observed in

R. eutropha, A. latus, B. cepacia, Acinetobacter sp. RA3849, and *Pseudomonas* sp. 61-3 (Matsusaki *et al.*, 1998; Valentin and Steinbüchel, 1993; Choi *et al.*, 1998; Rodrigues *et al.*, 2000). In certain bacterial strains these three genes constitute an operon, and in other bacterial strains additional genes relevant to PHA metabolism are inserted in these clusters (Matsusaki *et al.*, 1998; Rehm and Steinbüchel, 1999; Satoh *et al.*, 2002). However, in the majority of PHA accumulating bacteria having type I-PHA synthase, the PHA synthase genes were not positioned close to each other (Qi and Rehm, 2001; Maehara *et al.*, 1999; Valentin and Steinbüchel, 1993).

In a number of Pseudomonas species two different PHA synthase genes are clustered in the genome and are in the same orientation though separated by a gene that encodes an intracellular PHA depolymerase (*phaZ*) as revealed in *P. aeruginosa*, *P. oleovorans*, *P. mendocina* and *P. putida* strain U (Garcia *et al.*, 1999), (Fig. 3).

A similar set of genes is present in *Pseudomonas* sp. 61-3 (Fig. 3), and in addition, this bacterial strain has a third PHA synthase gene clustered together with the structural genes for a β-ketothiolase and an acetoacetyl-CoA reductase elsewhere on the chromosome. *Pseudomonas* sp. strain GP4BH1 possesses three different PHA synthases (Timm *et al.*, 1990; Preusting *et al.*, 1992; Steinbüchel and Wiese, 1992), and *Rickettsia prowazekii* has two PHA synthase structural genes (Feng and Doolittle, 1987); however their organization is different to those originating from Pseudomonads (Steinbüchel and Hein, 2001).

Most of the studied structural genes are located on the chromosome. Southern hybridization techniques, employing the cloned PHA synthase structural gene of *Acinetobacter* sp. led to the discovery of two copies of *phaC*, with one encoded by a plasmid. This is the first known gene concerned with PHA biosynthesis that is encoded by a plasmid (Schembri *et al.*, 1994).

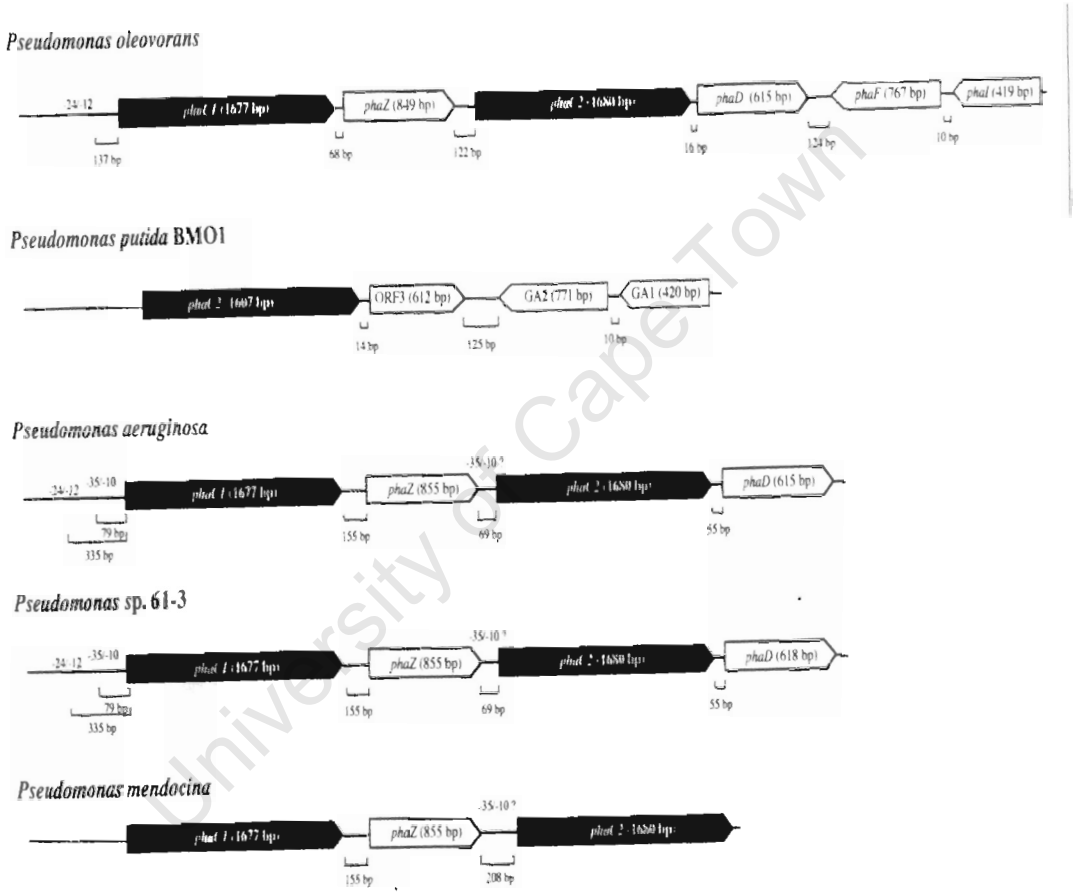


Fig. 3: Molecular organisation of genes encoding PHA synthases of type II, which are co-localized with PHA depolymerase genes in *Pseudomonas* species. *PhaC1/C2*, genes encoding PHA synthase; *phaZ*, gene encoding PHA depolymerase; *phaD*, an open reading frame with unknown function and ORF, another open reading frames with unknown function. PHA structural genes (*phaC1/C2*), and genes for phasins (*phaF*), that are active granule-bound proteins (Steinbüchel and Hein, 2001).

6 Gene regulation during PHA synthesis

Although many *phaC* genes have been cloned and sequenced, very little is known of the regulation of PHA biosynthesis. Recently, Maehara *et al*., (2002) suggested a model that involved PhaR in PHA biosynthesis (Fig. 4) in *Paracoccus denitrificans*. Four genes were discovered on the *PhaC* locus of *P. denitrificans.* These genes were, *phaZ, phaC, phaP, phaR*, encoding an intracellular PHA depolymerase, a PHA synthase, a phasin, and a PHA-responsive repressor PhaR, respectively. Phasins are proteins that are found accosiated with the PHA granule, and have recently been shown to have regulatory role in PHA synthesis (York *et al*., 2001). It was shown that *P. denitrificans* cells were unable to produce PHA when PhaC was produced in excess, presumably because the substrates for PHA biosynthesis had become deficient (Maehara *et al*., 2002).

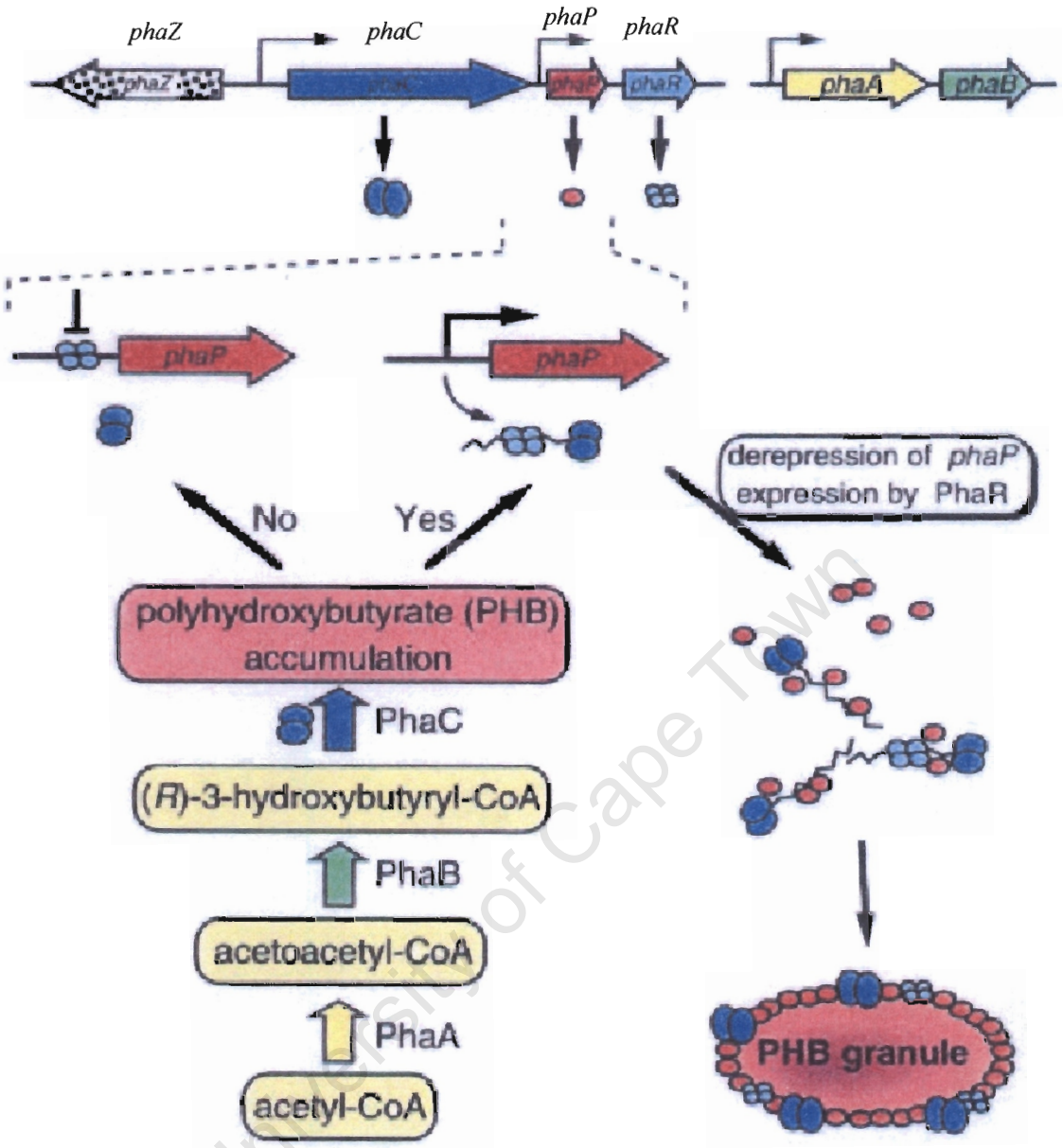


Fig. 4: Putative schematic mechanism of PhaR-mediated *phaP* expression system (Maehara *et al.*, 2001).

The PHA granule-associated protein, PhaP, is not necessary to be synthesized when cells are not accumulating PHAs. In this state, the repressor, PhaR, prevents the production of PhaP, by directly binding on the upstream region of p*haP* PhaR controls its own production when PhaR is produced in excess, by means of PhaR autoregulation. When the substrate (actyl-CoA) for PHA production is sufficient, the cell starts accumulating PHAs, PhaR recognises and binds to the PHA polymer chains. The PhaR-PHA polymer complex induces the expression of *phaP* by the dissociation of PhaR from the upstream region of the *phaP*. The PHA granules increase in size when the PHA polymer chains become longer and the PhaP expressed binds with the PHA granules covering the surface of the PHA granules before the binding of other proteins including PhaR. Excessive PhaR molecules associate with the upstream region of the *phaP* thus preventing the expression of both *phaP* and *phaR* when PHA synthesis discontinues. Prieto *et al.*, (1999) also demonstrated that the PhaF, a PHA-granule-associated protein in *Pseudomonas oleovorans* GPo1 regulates the expression of *pha* genes. PhaF binds to both the PHA granules and to the DNA, turning off the expression of PhaC1, which was shown to be a PHA polymerase.

7 Gene organization in hydrocarbon degradation

Very little is known about the gene regulation of hydrocarbon degradation. The genes for hydrocarbon degradation have only been studied in a minority of microorganisms. The *OCT* plasmid that was discovered in *Pseudomonas putida* PpG6 (ATCC 17633), the most well studied system in bacterial organisms. This plasmid encodes proteins that are active

in the growth of bacteria on C_6 to C_{10} n-alkanes (Chakrabarty *et al.*, 1973; Nieder and Shapiro, 1975; Grund *et al.*, 1975; Benson *et al.*, 1977).

The n-alkanes undergo a mono-oxygenation reaction catalysed by an alkane hydroxylase to produce the corresponding 1-ol, which is oxidised to the corresponding aldehyde by an alcohol dehydrogenase (Fig. 5). Thereafter the aldehyde is converted to the respective fatty acid by means of an aldehyde dehydrogenase, and the fatty acid then enters the β-oxidation pathway where it is further metabolised. A number of loci were discovered on the plasmid and on the chromosome of *Pseudomonas aeruginosa*, which are involved in the β-oxidation pathway (Fennewald and Shapiro, 1977, 1979; Fennewald *et al.*, 1979). The loci were designated as follows: *alk* for alkane hydroxylase; *ald* for aldehyde dehydrogenase; *alc* for alcohol dehydrogenase; *oic* for locus involved in the β-oxidation pathway. Chromosomal genes were; *alc A* for soluble alcohol dehydrogenase which prefer substrate range of C_7 and greater; *alc B* for soluble alcohol dehydrogenase with a preferred substrate range of C_3 to C_6; *ald A* and *ald B* encoding the corresponding aldehyde dehydrogenase.

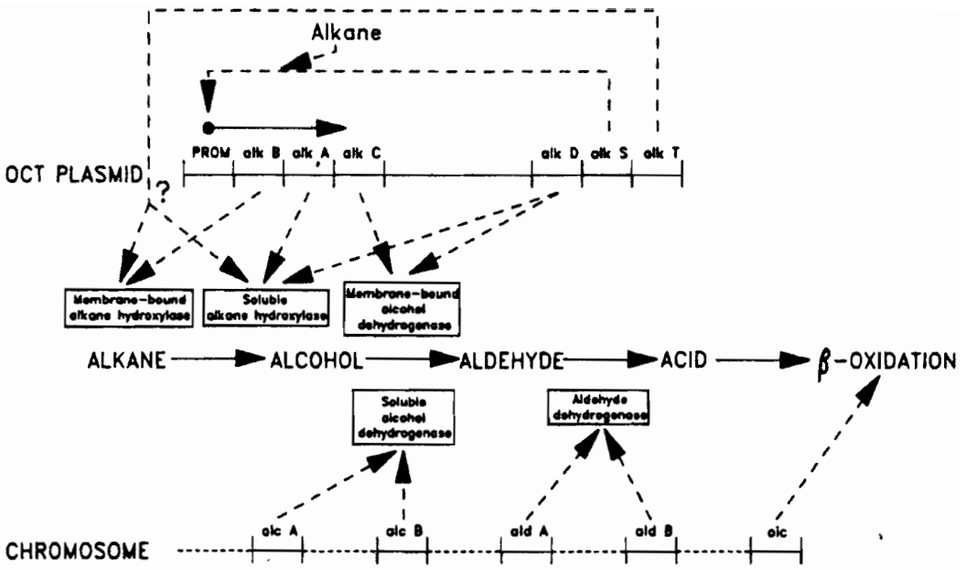


Fig. 5: Structure and function of the *OCT* plasmid and associated chromosomal genes in *Pseudomonas putida* PpG6. The *alk S* and *alk R* loci correspond to the area identified as the *alk R* regulatory locus. The *alk S* gene product is a regulatory protein that controls transcription of the *alk BAC* operon that is induced by n-alkanes. The role of the *alk T* gene product is unclear but it appears to be part of the alkane hydroxylase enzyme complexes. Chromosomal genes code for the soluble alcohol dehydrogenase, the aldehyde dehydrogenase and β-oxidation enzymes [Singer and Finnerty (1984); Eggink *et al.*, (1987, 1988)].

The detailed operation of the OCT system was constructed after intensive research (Fig. 5). The OCT DNA region containing the promoter, *alk BAC* operon and the regulatory locus (then known as *alk R*), was cloned in *E. coli* and a *P. putida* strain which does not degrade alkanes (Eggink *et al.*, 1987a). The genes expressed had all the required information for alkane hydroxylase and alcohol dehydrogenase activity. Six proteins were detected after the translation of the genes of the *alk BAC* operon in *E. coli*, four of which had the following functions: membrane-bound alkane dehydrogenase (41 kDa),

subunits of soluble alkane hydroxylase (15 kDa and 49 kDa), and the other membrane-bound, alcohol dehydrogenase (59 kDa; 20 kDa). The 41 kDa protein was shown to be associated with the *alk B* locus, the soluble alkane hydroxylase subunits with the *alk A* locus and the *alk C* locus appeared to code for by the 58 kDa polypeptide (Kok *et al.*, 1989). Two cistrons designated *alk S* and *alk T*, were discovered by Eggink *et al.*, (1988). The *alk S* encoded a 99-kDa polypeptide, which directly promoted transcription of the *alk BAC* operon. The *alk T* encoding a 48-kDa protein of unknown function though required for alkane hydroxylase activity but did not act as a regulator of gene expression. This protein was assumed to be associated to the alkane hydroxylase complex (Eggink *et al.*, 1988).

8 Applications of Polyhydroxyalkanoates

PHAs have various applications other than serving as a source of biodegradable plastic (Anderson and Dawes, 1990; Hankermeyer and Tjeerdema 1999; Madison and Huisman, 1999). For example, poly (3-hydroxybutyrate) (PHB) serves as a suitable substrate in solid-phase denitrification application for waste purification (Hiraishi and Khan, 2003).

8.1 Plastics

There is a need for biodegradable plastic to replace oil-based polymers because of the adverse effects of oil-based polymers in society. An interesting advantage of biodegradable plastics is that they are fully and rapidly biodegradable under appropriate conditions as opposed to the petroleum-based polymers (Brandl *et al.*, 1990).

Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is available commercially having the trade name, BIOPOL®. BIOPOL® is presently been produced in smaller amounts annually than synthetic polymers, therefore biodegradable plastic has not yet replaced conventional plastics in a significant way. A possible reason for this is that biodegradable plastic production was found to be costly, amounting to about seventeen times the price of the synthetic plastic (Luzier, 1992; Snell and Peoples, 2002). Biodegradable plastics have a number of applications in many industries such as packaging industry, medicine, pharmacy, agriculture, food industry and can also be used as raw materials for paint manufacturing (Anderson and Dawes, 1990).

Researchers are currently attempting to produce PHAs from plant crops. Genes for PHA synthesis are being expressed in plants adding to their agricultural and economical value (Snell and Peoples, 2002). PHA production in plants appears to be another low-cost alternative, although intensive research is needed in this area (Snell and Peoples, 2002). In addition, plants need to be manipulated to acquire high PHA yields, but a challenge has always been trying to express various genes in one plant at the same time. Snell and Peoples (2002) reported that multiple PHA gene expression in plants appears to be possible but more investigations needs to be done to successfully clone these genes.

8.2 Conversion of waste substrates to value-added products

New technologies are being used to improve the production of PHAs from organic matter in wastewater (Chua and Yu, 1999), industrial wastes (Rusendi and Sheppard, 1995; Wong and Lee, 1998), and municipal wastes (Lee and Yu, 1997; Chua *et al.*, 2003).

There are many other benefits to the environment, in addition to sustainable development in producing biodegradable thermoplastics from organic wastes. Organic wastes exist mainly in complex forms, which are not easily utilised by PHA-producing species such as *Ralstonia eutropha*, a bacterium shown to be capable of synthesising large quantities of PHAs (Anderson and Dawes, 1990). Hydrolysis and acidogenesis are the first steps in converting the wastes to short-chain volatile fatty acids such as acetic, propionic, and butyric acids that can be further utilised by PHA-producing bacteria such as *R. eutropha* (Lee and Yu, 1997; Du, 2001). Industrial applications have therefore been developed which couple the anaerobic waste acidogenesis with the aerobic PHA synthesis. A direct method was employed by Chua and Yu (1999) and Yu *et al.* (1999), where both acid-producing and PHA-producing organisms were cultivated in a mixed culture. The second organism directly uses the acids secreted by the initial organism. However, due to the presence of nonbiodegradable contents in the waste and the biomass of those cells not accumulating PHAs, the harvested PHA biomass was not high enough (10-30 wt %) (Chua and Yu, 1999; Yu *et al.*, 1999), as compared to the very high PHA content (70-75 wt %) in the cell mass from pure glucose fermentation (Choi and Lee, 1997; Ryu *et al.*, 1997). High acid concentrations produced by the acidogenesis microbial organisms hamper the activity of the microbial organisms producing PHAs (Yu and Wang, 2001). Indirect coupling of the two types of microbial organisms may be more effective. A more suitable system would be the use of two distinct bioreactors to favour the different physiologies and metabolic activities of two types of microbial population, one for anaerobic acidogenesis of organic wastes and another for enriched culture of aerobic PHA–producing species, such as *R. eutropha*. The fermentative acids produced from the

anaerobic digester should then be transferred to the aerobic reactor, and a biomass of very high polymer content would be produced (Yu and Si, 2001).

Production processes were developed where the recovery of the fermentative acids was the first part of the process, followed by concentration of the diluted acids by evaporation and ion exchange and then the introduction of *R. eutropha* for polymer production (Hassan *et al.*, 1997; Jin *et al.*, 1999). The PHA content (50-60 wt %) of the biomass from acids is similar to that obtained from pure acid fermentation (Wang and Yu, 2001). The high cost of recovery of acids could not be avoided due to the dilute acid concentrations (<50 g/L) in a complex aqueous medium (Du and Yu, 2002).

The use of microfiltration coupling the two reactors was investigated. The fermentative acids were brought by convective hydraulic flow across the filter membrane into the polymer-synthesizing reactor. Yu and Si (2001) revealed that the cells that were washed out carried quite low PHA content possibly due to their very slow growth rate. Another study adopted a novel approach based on acid molecular diffusion coupling the two bioreactors. The first anaerobic digester facilitated the digestion of food scraps to produce the fermentative acids, which were then transferred to the second PHA-producing reactor by means of molecular diffusion. A very high PHA content of cell mass, comparable to the PHA content from pure glucose fermentation, was accumulated in the second reactor with the enrichment culture of *R. eutropha* (Du and Yu, 2002).

8.3 Polyhydroxyalkanoates in water and wastewater treatment

Polyhydroxyalkanoates (PHAs) and other biodegradable polymers have several applications in many areas of biotechnology. Hiraishi and Khan (2003) investigated the use of PHAs as a candidate of solid-phase denitrification in water and wastewater treatment.

Nitrate is reduced to dinitrogen gas through nitrite, nitric oxide, and nitrous oxide by a series of distinct bioenergetic reactions called denitrification (Knowles, 1982; Zumft, 1997). A large range of microorganisms take part in these biochemical processes, which are key steps in the nitrogen cycle (Steinbüchel and Hein, 2001). Biological denitrification plays a major role in the removal of nutrients in water and wastewater treatment processes. Wastewater treatment technology has already developed a nitrogen removal system. However, this system is challenged with the problem that the amount of organic matter as electron donor for denitrification is apt to become much lower than required. To deal with this challenge, acetate and methanol that are some of the simple organic compounds (Hallin *et al.*, 1996) or sludge hydrolysate (Aravinthan *et al.*, 2001) are added to the systems. The risk carried by this application is overdosing, which would encourage deterioration of effluent quality, but need a very complex and expensive control system, (Hiraishi and Khan, 2003). Lately, a solid substrate was suggested as a form of denitrification system in water and wastewater treatment. This type of denitrification was named, "solid-phase denitrification". This technology tested a number of various solid substrates, such as straw, bark, wood, hydrolysed birch wood and some other biopolymers. PHAs formed part of the most favourable solid substrate, since PHAs

form part of the storage materials in microorganisms and therefore regarded to be metabolised without difficulty by a larger range of microorganisms under denitrification, and aerobic environments (Müller *et al.*, 1981).

PHAs are readily biodegradable though they are insoluble in water; and serve as constant sources of reducing power of denitrification. Extracellular PHA degradation in denitrification reactors is relatively similar to nitrate reductions under carbon-limited conditions (Müller and Seebach, 1993). PHAs also serve as solid matrices suitable for the development of microbial films as compared to traditional systems, where some inert material is employed as the surface for microbial growth (Boley *et al.*, 2000). High rates of nitrogen removal have been reported using PHAs in the denitrification processes (Khan *et al.*, 2002; Mergaert *et al.*, 2001). PHB-utilising denitrification systems cost an estimated amount of 21-37 Euro $kg^{-1}NO_3$-N, which is about 10 times higher than the cost for a traditional system that employ methanol (Boley *et al.*, 2000). Aliphatic polyester, for instance poly (E-caprolactone) (PCL), is economically more desirable. PCL production cost is almost half that of PHB, yet the denitrification rate obtained with the former polymer was lower (Boley *et al.*, 2000). This data may be incorrect since the cost estimated is based only on the quantity of the substrate used and other requirements of a conventional system are not included, such as process control cost. The comparison of the cost effectiveness between PHB-utilising denitrification processes and other systems is required (Hiraishi and Khan, 2003), although the environment benefits of the PHB system are evident.

9 Research aims of this Thesis

The aim of this project is to isolate microorganisms that are capable of converting hydrocarbons from petrochemical wastes (i.e. alkane fractions) into a high value product. The discovery of microorganisms that could synthesise PHAs from these wastes would be identified and characterized with respect to their growth and production of PHAs, with a view to developing environmental or commercial applications.

To isolate *phaC* genes by PCR from relevant bacteria in order to compare to published genes, to establish whether the PHAs would be novel.

CHAPTER 2

Isolation of alkane-utilizing bacteria that produce polyhydroxyalkanoates (PHAs)

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2 SUMMARY

Novel South African bacteria capable of efficient degradation of the long chain alkane waste fractions and accumulating PHAs were isolated from crude oil contaminated soil. Of the twenty-three (23) environmental isolates, twelve (12) were capable of degrading alkanes and showed positive results on Nile Blue A stain, which is a preliminary technique to screen for PHA accumulating bacteria. To verify the Nile Blue A staining results, the *phaC* gene responsible for PHA polymerization was screened in all the 23 environmental strains employing the appropriate degenerate primers. A range of biochemical tests were carried out on the environmental isolates that seemed to be different in terms of their phenotype. It was observed that 83 % of the isolated environmental strains were falling under the *Pseudomonas* species. The 16S rDNA sequencing was carried out on five of the nineteen environmental strains that showed interesting biochemical characteristics to determine their genus names. Good yield 16S rDNA amplification was obtained from the chromosomal DNA of the environmental isolate 2. Environmental isolate 2 had 99 % identity to *Pseudomonas aeruginosa* BHP7-6, and therefore given a name *Pseudomonas aeruginosa* MB2SA. *P. aeruginosa* MB2SA has been shown to possess the 0.5 kb PCR fragment corresponding to *phaC* gene responsible for PHA polymerization.

2.1 INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polymeric esters that function as an energy and carbon reserve in prokaryotic cells. They are a chemically diverse group of compounds of which poly-β-hydroxybutyrate (PHB) was one of the first to be reported and is a form of a biodegradable thermoplastic which has been shown to accumulate in many bacteria. PHB is accumulated into discrete inclusions or granules in the cell (Dunlop and Robards, 1973; Ellar *et al.*, 1968; Merrick and Doudoroff, 1964). These PHA granules accumulate when nutrients such as nitrogen or phosphorus are limiting and excess carbon source is available (Anderson and Dawes, 1990). The PHB granules have been recognized by their affinity for the dye Sudan Black B (Burdon, 1946), which is a presumptive test for the presence of PHB (Schlegel *et al.*, 1970).

The presence of PHB has been used as a taxonomic criterion for the classification of bacteria for some time, since this characteristic appears to be stable. The amount of PHB produced is highly variable and dependent upon culture conditions (Stockdale *et al.*, 1968).

Recently, many phenotypic detection methods have been developed to screen bacteria for intracellular PHA granules, including Sudan Black B staining (Schlegel *et al.*, 1970) and Nile Blue A staining (Ostle and Holt, 1982). These stains indicate the presence of PHA by dark blue and fluorescent granules respectively. Nile Blue A has been used as an alternative stain for PHB granules in bacteria and is in fact superior to Sudan Black B for this purpose (Ostle and Holt, 1982). Nile Blue A appears to stain many more PHB granules than Sudan Black B does and is not as easily washed from the cell by

decolorisation procedures. Chemical analysis should still be performed to confirm the presence of PHB (Ostle and Holt, 1982), because Nile Blue A staining alone is not sufficient since it was shown to stains any lipid compounds which may be present in the cell (Sheu *et al.*, 2000).

Although, methods such as Nile Blue A staining are quite sensitive, it is rather time-consuming and labor-intensive to screen a large number of environmental isolates. Alternative methods have recently been developed for directly staining colonies (Kranz *et al.*, 1997) or growing bacteria on plates containing Nile Blue A or Nile Red (Spiekermann *et al.*, 1999), resulting in fluorescent colonies that can be visualized by UV illumination. These colony-staining methods are more suitable for screening a large number of strains. However, appropriate carbon sources should be used and a long culture time (3 days) is required for PHA granule accumulation (Sheu *et al.*, 2000). In addition, these methods cannot distinguish between bacteria that accumulate PHA granules and those that accumulate lipids compounds and therefore more molecular investigations were applied in this study to confirm the presence of the *pha C* gene, the PHA synthase encoding gene responsible for PHA polymerization.

The aim of this work was to isolate novel South African strains capable of efficient degradation of the long chain alkane waste fractions, C_{12}-C_{13} and C_{14}-C_{17}, while accumulating significant quantities of PHAs.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of the bacterial strains

The bacterial strains used in these investigations were isolated from crude oil contaminated soil collected from Milnerton Caltex Refineries, Cape Town. Crude oil had been leaking from its vessels for sometime, making it likely that the soil would be inhabited predominantly by oil-degrading organisms or by organisms that prefer the by-products of crude oil as their source of carbon.

An approximately 5-10 cm opening was dug in the ground to avoid carrying most of the bacterial strains normally predominantly found on the soil surface rather than the bacteria growing on crude oil or by products of crude oil such as alkane fractions. The environmental samples were collected aseptically using sterile stainless steel spoons and 500ml screwed-top bottles. The collected soil samples were stored at room temperature for approximately 1-2 hours before analysis.

Enrichment cultures were set up as described below using a minimal salt medium (MSM, A.2.1.1), with alkane fractions, 1 % v/v C_{12}-C_{13} and 1 % v/v C_{14}-C_{17}, as the sole carbon sources.

Four (4) 500ml Erlenmeyer flasks, each containing 100ml MSM [1 g $(NH_4)_2SO_4$/L] (A.2.1.6), were autoclaved and cooled to room temperature. The 10N NaOH was used to adjust the pH to 7.0 just before the MSM was autoclaved. The carbon sources, C_{12}-C_{13} and C_{14}-C_{17} were not included in the MSM during autoclaving, but filter-sterilized C_{12}-

C_{13} and C_{14}-C_{17} alkane fractions were aseptically injected into the culture with syringe. A 5g-soil sample was inoculated into the MSM and 0.01g/L of amphotericin was included to prevent the growth of fungi. Duplicate cultures were prepared for each of the two-alkane fractions. The bacterial mixed cultures were incubated at 30 ^{0}C with shaking at 250 rpm. After four (4) days incubation, 10 % volume was inoculated into fresh medium and incubated at 30 ^{0}C. This step gets rid of the soil particles.

The bacterial populations were quantitatively determined by standard plate counts. The bacterial strains were initially characterized based on their morphology and motility after having been grown on nutrient agar (NA) plates (A.2.1.3), overnight at 30 ^{o}C.

2.2.2 Other bacterial strains used in this study

Two strains, namely, *Pseudomonas oleovorans* (ATCC 29347) and *Bacillus megaterium* (laboratory strain, UCT), were used as positive controls since they are known to accumulate PHAs, and have been shown to have the *phaC* gene (Sheu *et al.*, 2000). *Escherichia coli* JM109 strain served as a negative control in this study because it does not accumulate PHAs in the cytoplasm. *P. oleovorans* was grown on modified medium E (A.2.1.1), and *B. megaterium* on nutrient agar (NA) (A.2.1.3). An *E. coli* JM109 culture was maintained on 2xYT agar (A.2.1.4) or NA (A.2.1.3) with the appropriate antibiotic selection where applicable. For growth in liquid medium, 2xYT broth (A.2.1.5) or Luria Broth (LB) (A.2.1.2) with appropriate additives were used.

2.2.3 Growth of environmental strains on alkane fractions

Environmental isolates were routinely grown in MSM broth with two different alkane fractions, 1 % v/v C_{12}-C_{13} and 1 % v/v C_{14}-C_{17}, which were obtained from SASOL. Each bacterial strain was aseptically sub-inoculated into fresh MSM broth at 10 % volume after every four days of growth in order to maintain these strains during the course of this study. Stock cultures were maintained on MSM agar containing alkanes at 4 ^{0}C.

2.2.4 Biochemical identification of isolates

The cells were grown in Nutrient Broth overnight at 30 ^{0}C with agitation. A range of biochemical tests were applied on the selected environmental isolates that grew well on alkane fractions. The preliminary biochemical tests included Gram-staining, catalase, oxidase, and Oxidation-Fermentation (O-F) tests (Cowan and Steel, 1965). Morphology and motility were also determined. These tests were only applied to the environmental strains that were able to degrade the alkane fractions in a minimal salt medium (MSM).

These preliminary tests allowed the identification of the bacterial isolates to genus level and were followed by 16S rDNA sequencing of the strains of interest to determine the species.

2.2.5 Culture medium for PHA production on alkane fractions

PHA accumulation by the bacteria was enhanced through growth in a limiting MSM (A.2.1.6) with alkane fractions serving as the carbon source. In this case, nitrogen was limiting [0.4g $(NH_4)_2SO_4$/L], which has been shown previously to favor PHA accumulation (Anderson and Dawes, 1990). The 10 % volume of each culture from the MSM was harvested and centrifuged at 8000 rpm (Beckmann) to remove excess MSM from the bacterial cells. Cell pellets were re-suspended in the original volume of limiting MSM and inoculated into 500ml Erlenmeyer flasks containing 100ml of nitrogen limiting MSM with 5 % v/v C_{12}-C_{13} alkane fraction or 5 % v/v C_{14}-C_{17} alkane fraction. Growth and pH were monitored at 12-hour intervals for 96 hours. Similar cultures containing 5 % of pure alkanes, n-dodecane (Sigma), n-tetradecane (Sigma) and hexadecane (Fluka) were inoculated and grown in the same way for comparison with the results from the mixed fractions.

The bacterial cells growing on nitrogen limiting MSM were tested for PHA accumulation in their cytoplasm by staining them with Nile Blue A stain.

2.2.6 Nile Blue A Staining

P. oleovorans, *P. fluorescens*, *B. megaterium*, *E. coli* cells serving as controls, and the environmental isolates were separately grown under the appropriate conditions for PHA production. After three days of bacterial growth, which was an ideal period for sufficient amounts of PHAs to accumulate in the bacterial cytoplasm (Sheu *et al.*, 2000). Two milliliters (2ml) of the cell culture was centrifuged in a 2ml eppendorf tube at 8000 rpm

using a desktop centrifuge at room temperature. The supernatant was discarded to remove the excess alkane fractions. The cells were washed twice with the nitrogen limiting MSM to make sure that most of the alkane fractions are removed. Bacterial cells were resuspended into 1ml of limiting MSM to concentrate the cells. A loop full of cells was smeared onto a glass microscope slide and allowed to dry at room temperature.

Heat-fixed bacterial cells smears were stained with the Nile Blue A stain (A.2.1.7) at 55 ^{0}C for 10 minutes in a staining jar. Then the stained cells were washed with tap water. These steps remove excess stain on the slides and also washed again with 8 % aqueous acetic acid for 1 minute. Air dried stained bacterial cells were remoistened with tap water, covered with no. 1 glass cover slip and viewed under oil on a fluorescent microscope. The oil immersion would extract some of the fluorescent dye (Nile Blue A) and made it difficult to see because of a general yellow fluorescence on the field. The cover slip prevents this extraction from happening. The stained cells were observed with a Nikon Labphot microscope connected to episcopic fluorescence. An excitation wavelength of 460 nm was utilized (Ostle and Holt, 1982).

2.2.7 DNA Extraction for detection of the PHA synthase gene

The chromosomal DNA extraction was the initial step in the process of PHA synthase gene detection by Polymerase Chain Reaction (PCR) technique. Various techniques were employed in an attempt to discover the method suitable for DNA extraction to a range of environmental bacterial strains.

2.2.7.1 Pure colonies as a DNA Template

The colonies that were capable of utilizing alkane fractions as the carbon source were grown on nutrient agar (NA) plates. Pure colonies were attained using the streak method. The bacterial strains were incubated at 30 ^{0}C overnight. Pure colonies of approximately 1 mm in diameter were picked up with a sterile toothpick and directly transferred into the PCR tube, where lysis would occur during the initial 96 ^{0}C cycle, releasing chromosomal DNA templates. The PCR was carried out as described later in this study.

2.2.7.2 Boiling method of DNA extraction

Five milliliters (5ml) of sterile nutrient broth was inoculated with the bacterial strains. Bacterial strains were incubated at 30 ^{0}C, overnight, with shaking at 250 rpm. One milliliter of the bacterial cultures was microfuged in a 2ml eppendorf tube at 8000 rpm using a desktop microfuge. The pellets were washed with 1ml Tris-EDTA (pH 8.0) and re-suspended in 0.5ml TE. The cells were boiled for 10 minutes in a waterbath to release the chromosomal DNA from the cells. The tubes were cooled, microfuged for 10 seconds to remove the cell debris. The supernatant (450µl) containing DNA was collected making sure that the pellet was undisturbed. Two microliters (2µl) was used as DNA template in a 50µl PCR reaction. The rest of the DNA sample was stored at 4 ^{0}C for future use.

2.2.7.3 DNA extraction by phenol/chloroform

The bacterial cells were grown in 10ml of nutrient broth, incubated overnight at 30 ^{0}C in a 250 rpm shaker incubator. The cells were centrifuged, at 5000 rpm in a desktop Eppendorf centrifuge, at 4 ^{0}C for 10 min. The pellet was retained and resuspended in 1ml of TE buffer (pH 8.0). The mixture was then centrifuged again at 5000 rpm, 4 ^{0}C for 10 min. The washed pellet was resuspended into 1ml of the lysis buffer [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 10 % SDS, 20 mg/ml proteinase K]. A smidgen of dry lysozyme was added to every tube. The mixture was incubated in a 37 ^{0}C waterbath for 1 hour. Five hundred microliters (500 µl) of saturated NaCl was added. The mixture was centrifuged at 18 000 rpm at room temperature for 15 min. The supernatant was transferred into a fresh tube. DNA was then purified by phenol/chloroform extraction followed by ethanol precipitation. The air-dried DNA pellet was resuspended in MilliQ water or TE buffer (Molecular biology technique manual, UCT, 2002).

2.2.7.4 High Pure PCR Template Preparation Kit

The High Pure PCR Template Preparation Kit by Roche was used following the manufacture's instruction manual (2002).

2.2.8 PCR of the *phaC* gene from isolated environmental strains

Polymerase chain reaction technique was applied to the 23 isolated bacterial strains to confirm the presence of the *phaC* gene, encoding the PHA synthase enzyme.

2.2.8.1 PCR primers

Two 26-mer degenerate primers were designed which differed slightly from the ones published by Sheu *et al*., (2000); reason being that the machine (Beckman Instruments Inc. Oligo 1000M DNA Synthesizer) used to synthesize our primers was unable to produce exactly the same primers as those synthesized by Sheu *et al*., (2000). Their sequences were as follows,

5'-ATCAACAA(GGG/A)T(TT/A)CTAC(AA/G)TC(CC/T)T(CC/G)GACCT-3' (designated phaCF1, corresponding to nt 741-766 of *Ralstonia eutropha phaC*) and 5'-AGGTAGTTGT(TT/C)GAC(CCC/GG)(AAA/CC)(AAA/CC)(GGG/A)TAG(TTT/G)TC CA-3' (designated phaCR4, corresponding to nt 1237-1212 of *R. eutropha phaC*), (Sheu *et al*., 2000). The modified primers were 5'-ATC AAC AA(GA) T(TA)C TAC (AG)TC (CT)T(CG) GAC CT-3' (designated phaCF1, corresponding to nt 741-766 of *R. eutropha phaC*) and 5'-AGG TAG TTG T(TC)G AC(CG) (AC)(AC)(GA) TAG (TG)TC CA-3' (designated phaCR4, corresponding to nt 1237-1212 of *R. eutropha phaC*), respectively (this study).

2.2.8.2 The optimized PCR

The PCR reaction mixture consisted of 1x PCR buffer, 2.0, 2.5 and 3.0 mM $MgCl_2$, 2.5 mM each deoxynucleotide triphosphate, 5.0 µM each of primer, 0.3 µl of 5 U/µl Super-Therm DNA polymerase in a 50 µl PCR reaction mixture. A final concentration of 3 % dimethyl sulfoxide (DMSO) as PCR additive was also added to the reaction mixture. The final DNA concentrations of 50 ng/µl were prepared. Hundred nanograms (100 ng) of DNA was used as template when the bacterial DNA had been extracted by either the phenol or by the High Pure PCR Template Preparation Kit method. The thermal cycle program was run on a GeneAmp PCR system 9700 (Applied Biosystems) consisted of one cycle of 96 ^{0}C for 10min, 51 ^{0}C for 2min, 72 ^{0}C for 2min, and 35 cycles of 94 ^{0}C for 20 s, 57 ^{0}C for 45s (decreasing by 1s per cycle), and 72 ^{0}C for 1 min, and then incubation at 72 ^{0}C for 5 min, and a final incubation at 4 ^{0}C (Sheu *et al.*, 2000). The PCR product was observed on a 0.8 % agarose gel.

2.2.9 The 16S rDNA Sequencing

The primer sets (Sheu *et al.*, 2000) that were used for the 16S rDNA sequencing amplifies the conserved region of the 16S rRNA gene from a large range of bacterial species. Internal primers were employed to sequence the internal regions of the 16S rDNA region in this study. The sequencing was carried out using the MegaBace DNA sequencer (Department of Molecular and Cellular Biology, UCT). The multiple sequence alignment of these four products was determined using the DNAMAN program (Lynnon Biosoft). The consensus sequence was compared with the published sequences to

determine the genus and species name of the bacterial strains by using the NCBI Database (www.ncbi.nlm.nih.gov/blast).

2.3 RESULTS AND DISCUSSION

2.3.1 Isolated environmental strains

The environmental isolates capable of efficient degradation of hydrocarbons in the form of alkanes were isolated from oil contaminated soil samples collected from the Caltex Refineries. Soil samples were collected from below the surface of the ground as it was presumed that high populations of the organisms of interest would occur in the crude oil that has sunken into the soil over a period of many years.

Twenty-three (23) presumably different bacterial strains based on phenotypic characteristics, utilized the alkane fractions effectively when grown on a minimal salt medium (MSM), (Table 1). All strains were evaluated for alkane utilization by growing them for 72 hours in MSM and alkanes. Twelve (12) of the environmental isolates grew well on the enrichment MSM, and eleven (11) grew less well, suggesting that these strains were unable to degrade alkane fractions (C_{12}-C_{13} and C_{14}-C_{17}) efficiently, as shown in Table 1. These bacteria might be capable of degrading some of the alkane fractions that are present in the alkane mixture, but may not be able to optimally use all of the fractions provided.

Table 1: The environmental isolates grown in MSM broth containing either the C_{12}-C_{13} or C_{14}-C_{17} alkane fractions. Growth was estimated from turbidity after 72-hour incubation at 30 ^{0}C.

Isolate no.	Alkane fractions	
	1%C_{12}-C_{13}	1%C_{14}-C_{17}
1	+++	+++
2	+++++	+++++
3	++++	+++
4	+++	++
5	++++	++
6	++++	++
7	+++	++
8	+	+
9	+++	++
10	++++	+++
11	+	+
12	+	+
13	+	+
14	+++	++
15	+	+
16	++++	+++
17	+	+
18	+	+
19	+++	++
20	+	+
21	+	+
22	+	+
23	+	+

Symbols:

Excellent,	+++++
Very good,	++++
Good,	+++
Poor,	++
Very poor,	+

2.3.2 PHA accumulation by environmental isolates

The twenty-three (23) new environmental isolates were later grown in nitrogen-limiting MSM broth supplemented with 5 % v/v alkane fractions for 72 hours to investigate whether these strains accumulate polyhydroxyalkanoate (PHA) inclusion bodies in their cytoplasm. Nile Blue A staining technique was a preliminary step to differentiate which of the 23 strains are possible PHA accumulators as shown in Table 2.

In this study, *Pseudomonas fluorescens*, *B. megaterium*, and *P. oleovorans* served as positive controls (Sheu *et al.*, 2000), and were confirmed to accumulate PHA inclusion bodies. *E. coli* JM109 served as a negative control, not accumulating inclusion bodies in its cytoplasm, Fig. 1. The photograph of *E. coli* in Fig. 1 was taken using the normal light microscope since the cells do not fluoresce under the fluorescent light microscope.

Table 2: Environmental isolates grown in nitrogen-limiting MSM for 72 hours at 30 ^{0}C in the presence of alkane fractions and stained with Nile Blue A and Gram stains.

Isolate no.	Nile Blue	Gram Stain	Morphology
1	+++++	negative	rods
2	+++++	negative	rods
3	++++	negative	rods
4	+++	negative	rods
5	+++++	negative	rods
6	+++++	negative	rods
7	+	negative	rods
8	negative	negative	rods
9	+	negative	rods
10	+++++	negative	rods
11	negative	negative	rods
12	negative	negative	rods
13	negative	negative	rods
14	+++	positive	cocci
15	negative	negative	rods
16	+++++	negative	rods
17	negative	negative	rods
18	negative	negative	rods
19	negative	negative	rods
20	negative	negative	rods
21	negative	negative	rods
22	negative	negative	rods
23	negative	negative	rods

Twelve (12) of the 23 isolated environmental strains showed a positive reaction with Nile Blue A after growth with alkane fractions on the nitrogen limiting MSM. These strains might be accumulating PHAs in their cytoplasm. Nile Blue A stains any lipid compounds which may include other components of the cell membrane, therefore not all of the twelve (12) Nile Blue A positive strains in Table 2 necessarily mean they have accumulated PHAs.

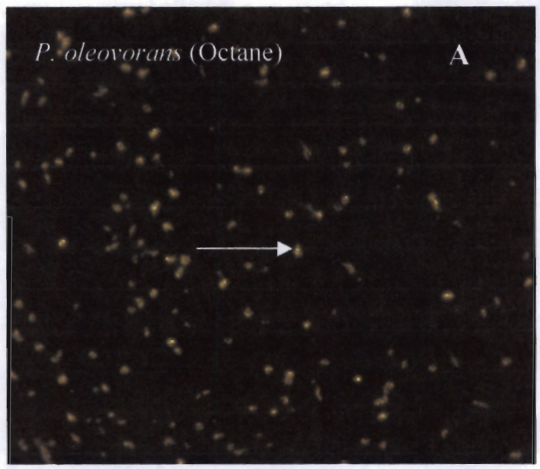
Environmental isolates 2, 5, 6, 10, and 16 were shown to accumulate PHA inclusion bodies after the Nile Blue A staining procedure, although differences in their fluorescence were observed (Fig. 1). Environmental isolate 2 was observed to give consistently good fluorescence when stained with Nile Blue A stain. This bacterial strain appeared to be accumulating large amounts of PHA granules in its cytoplasm compared to the positive controls, however this was hard to quantify since it was not easy to compare different bacterial slides because of a number of factors which influenced the Nile Blue A staining procedure as discussed later.

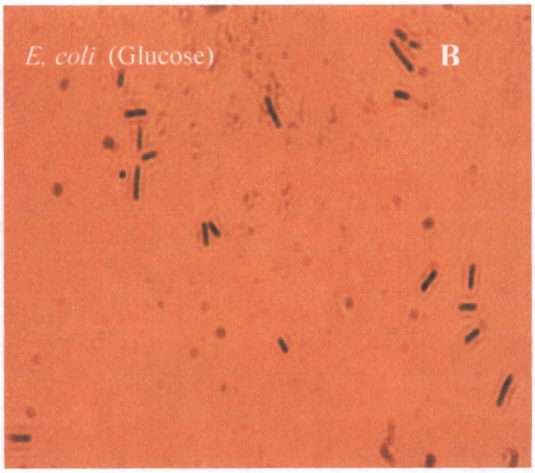
While comparing *P. fluorescens* grown on glucose and alkanes, good fluorescence was observed when glucose was used as carbon source. Presumably, *P. fluorescens* accumulated large amounts of PHA when grown on glucose as a carbon source compared to when alkane fractions were utilized as carbon source. The differences in fluorescence intensities after Nile Blue A staining were observed (Fig. 1C and F).

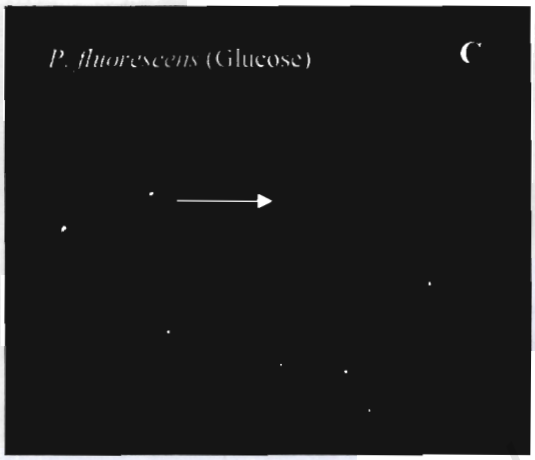
Some interesting observations were made with respect to the staining technique, which gave inconsistent results between experiments. The stain (Nile Blue A) was found to fade when the bacterial cells were exposed for a long time to the fluorescence light. A microscope slide that had more cells clustered on one spot would fluoresce much better than the slide with fewer bacterial cells, so it was necessary to make very even bacterial smears before staining.

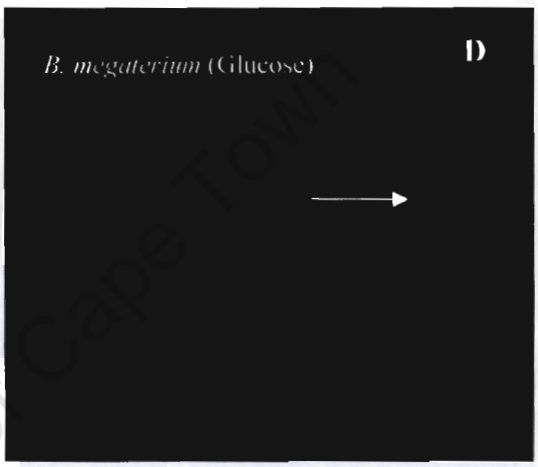
The residual alkane fractions in the medium and the wax-like substance produced by these bacterial strains after three-day incubation were also found to interfere with the staining technique. Bacterial strains did not stick tightly onto the microscope slide, and tended to wash off very easily during the course of the technique. In addition, high background was observed on the slides that had the wax and alkane residues, obscuring the view under the microscope. All these factors make it difficult to compare the amount of PHA produced by different strains, and more quantitative methods would need to be used for this purpose.

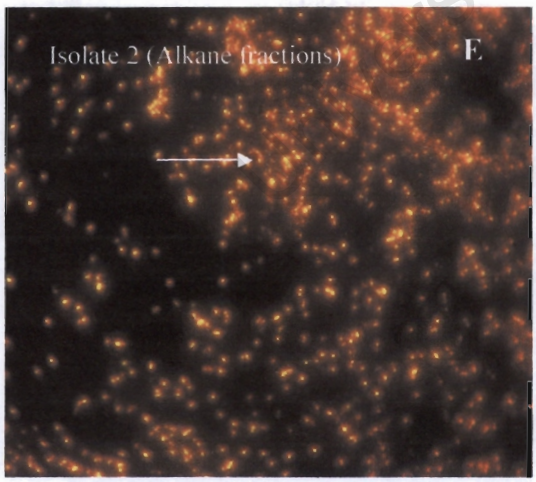
Although Nile Blue A stain is an accepted stain to detect PHA inclusion bodies, it would also detect other lipid compounds, and cell walls and the cell membranes of the bacterial strains are also stained because these organelles contain lipid components.

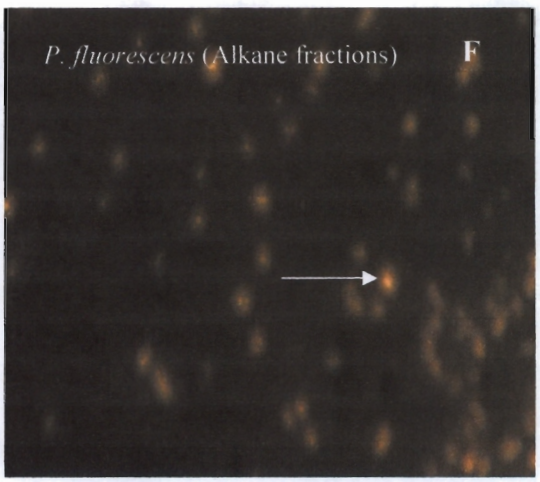












(see more illustrations on the next page)

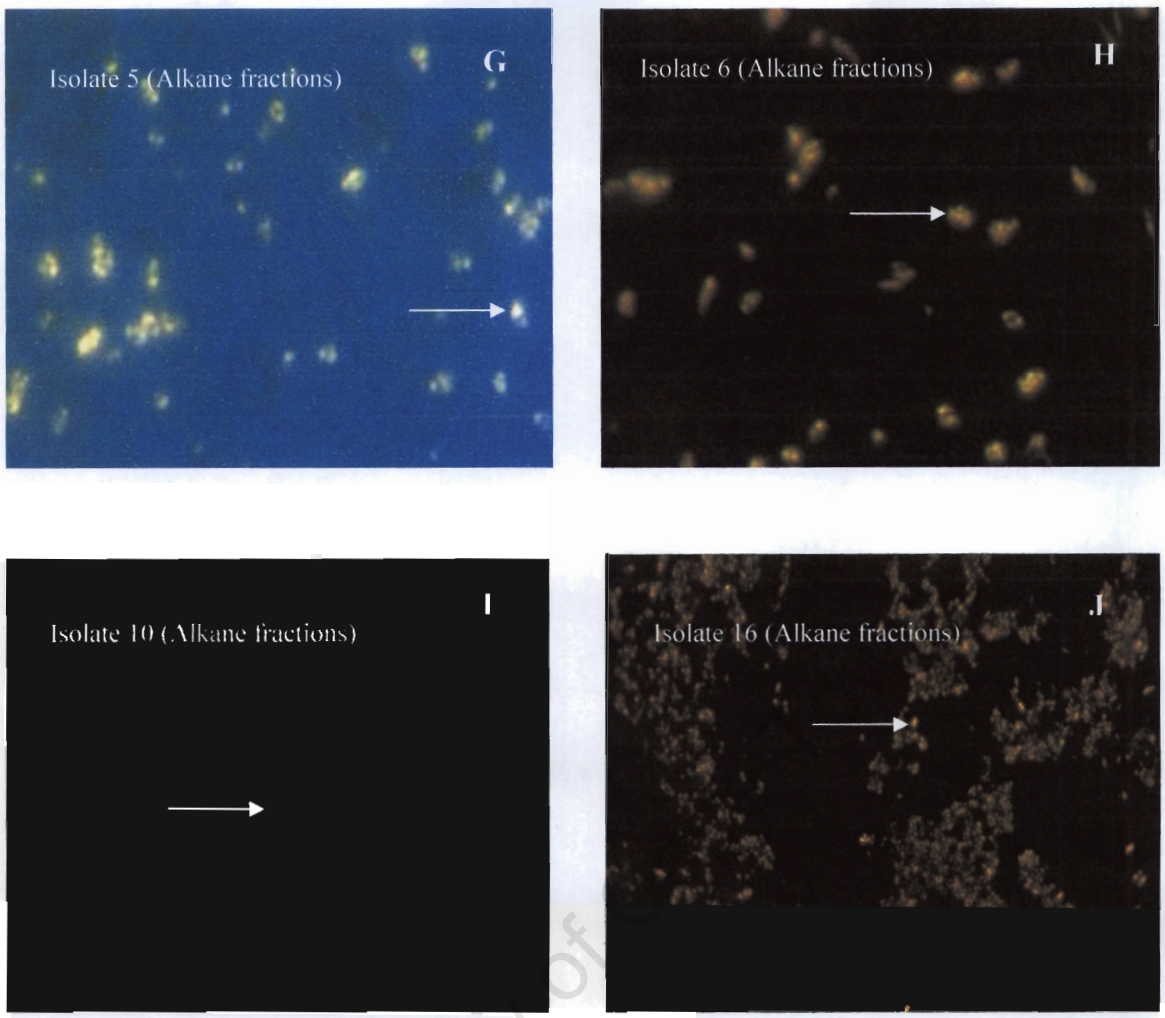


Figure 1: Nile Blue A staining of the different bacterial strains, A: *P. oleovorans*, B: *E. coli*, C: *P. fluorescens*, D: *B. megaterium*, E: Environmental isolate 2, F: *P. fluorescens*, G: Environmental isolate 5, H: Environmental isolate 6, I: Environmental isolate 10, and J: Environmental isolate 16. Strains were all grown in the presence of alkanes, except for *E. coli*, *P. fluorescens* and *B. megaterium*, which were grown on glucose, and *P. oleovorans* which was grown on octane. *P. fluorescens* was grown also on alkanes for comparison. White arrows show the inclusion bodies fluorescing in the cytoplasm except for *E. coli*, which represents the bacterial cells without any fluorescence.

It could not be confirmed conclusively that Nile Blue A positive bacteria accumulate PHAs but this method is a useful preliminary step to identify those bacteria that may be PHA accumulating. A molecular investigation is required to make this kind of a conclusion, such as, the genetic or molecular detection of the gene responsible for PHA polymerization, which was *phaC* gene (Anderson and Dawes, 1990; Poirier *et al.*, 1995).

Similarly, those bacteria which are negative after Nile Blue A staining may still possess the genetic ability to produce PHAs, which are not expressed under these conditions. The other possibility was that, because the cultures were grown for 72 hours before staining, the bacteria could have accumulated PHAs earlier and have subsequently utilized them as energy sources.

2.3.3 Identification of bacterial isolates using biochemical tests

Preliminary biochemical tests were carried out on only twelve (12) of the 23 strains (Table 3) which grew well on alkane fractions and fluoresced on Nile Blue A stain, so as to reduce the number of these environmental isolates. These preliminary biochemical tests gave the probable genus to which the alkane degraders belong and allowed insight into the bacterial types isolated. This means that the investigator would be careful when handling and storing isolates such as *Pseudomonas* species, which is a well known nosocomial pathogen (Quinn, 1998) and resistant to multiple drugs (Alonso *et al.*, 1999; Kohler *et al.*, 1997; Jalal *et al.*, 2000; Ziha-Zarifi *et al.*, 1999).

Table 3: The preliminary biochemical tests, morphology and motility determined on the bacterial strains that grew best on alkane fractions

Isolate no.	Gram-strain	Catalase	Oxidase	O-F	Morphology	Motility	Suggested Genus
1	Negative	+	+	O	Rods	+	*Pseudomonas*
2	Negative	+	+	O	Rods	+	*Pseudomonas*
3	Negative	+	+	O	Rods	+	*Pseudomonas*
4	Negative	+	+	O	Rods	+	*Pseudomonas*
5	Negative	+	+	O	Rods	+	*Pseudomonas*
6	Negative	+	+	O	Rods	+	*Pseudomonas*
7	Negative	+	+	O	Rods	+	*Pseudomonas*
9	Negative	+	+	O	Rods	+	*Pseudomonas*
10	Negative	+	+	O	Rods	+	*Pseudomonas*
14	Positive	+	-	F	Cocci	+	*Klebsiella*
16	Negative	+	+	O	Rods	+	*Pseudomonas*
19	Variable	-	-	O	Rods	+	Unknown

Eighty-three percent (83 %) of the environmental species were found to be pseudomonads, suggesting that they predominate in the alkane degrading bacteria found in oil-polluted South African sites. All these strains are Gram-negative except for Isolate 19 which is Gram-variable. Isolate 19 was the only strain negative on the catalase test which means it does not possess the enzyme, catalase, which destroys the toxic H_2O_2 products formed during metabolism. Anaerobes lack the two enzymes, superoxide dismutase and catalase hence they cannot tolerate O_2. Isolate 19 could therefore be a facultative or aerotolerant anaerobe; it showed very poor growth on alkane fractions and stopped growing aerobically later in this study.

On the Oxidase test, Isolate 14 and 19 were both negative. These environmental strains lack the enzyme, oxidase, which is essential in the electron transport system throughout aerobic respiration. Isolate number fourteen (14) utilized glucose through fermentation rather than by oxidation when the O-F test was done on these environmental strains. This strain also grew poorly on alkanes later in the study.

More information about these bacterial strains is required before a species can be assigned. Further biochemical tests could be carried out or other methods such as 16S rDNA PCR could be employed to obtain a definite identification of the strains.

2.3.4 16S rDNA sequencing of the selected environmental strains

Five environmental strains were selected based on their ability to degrade alkanes, accumulate PHA inclusion bodies as shown by the Nile Blue A staining technique and had a 0.5 kb PCR product corresponding to the *phaC* gene fragment. These strains had shown some interesting differences on the biochemical tests. Isolate 2, 9, and 16 were all Gram-negative rods and Isolate 14, was a Gram-negative coccus. Isolate 19 showed a variable Gram reaction and was rod-shaped. Isolate 14 was negative on oxidase test and Isolate 19 was negative on both the oxidase and the catalase tests, and was probably an aerotolerant anaerobe.

The 16S rDNA sequences of these strains were investigated to obtain a definitive identification. The sequence alignments of these strains were compared to the published sequences using the NCBI Database (www.ncbi.nlm.nih.gov/blast).

Table 4: The 16S sequences found to be most similar to the environmental strains

Isolate no.	Organism	Identity (bp)	Identity (%)	Score E
2	*Pseudomonas aeruginosa* BHP7-6	1302/1310	99	2533 0.0
9	*Pseudomonas* sp. LAB-27	198/200	99	373 e-101
14	*Klebsiella planticola* ATCC	202/203	99	387 e-105
16	*Pseudomonas* sp.	30/30	100	60 7e-08
19	*Pseudomonas mediterranea*	137/139	98	252 1e-64

The DNA sequence obtained from the PCR products was not very good in most cases, possibly due to the poor amplification of 16S rDNA region of these strains by the universal primers (Table 4, see the identity values). However, the percentage identities of the regions obtained gave a good indication of the genus of the environmental strains investigated. Four of the sequenced environmental strains showed 98-100 % identity to *Pseudomonas* spp, which agrees with the identifications assigned as a result of the biochemical tests. Isolate 14 was identified as *Klebsiella planticola*, and showed 99 % identity over a region of 202bp. Larger regions of 16S rDNA should be obtained for this strain in order to get a better identification. Isolate 19 showed 98 % identity to *Pseudomonas mediterranea*, but this was probably a contaminant of the original strain, therefore classified as unknown. The original strain was catalase negative, so could not be *Pseudomonas*, but grew poorly in air and was probably overgrown in this experiment.

Isolate 2 was subjected to more rigorous DNA sequencing, because it consistently gave good yields of PHA after Nile Blue A staining. This environmental strain had a 99 % identity to *Pseudomonas* spp. over a region of 1302bp in eight (8) out of ten (10) strains detected by a BLAST search as shown on Table 5. This strain was therefore given the name *Pseudomonas aeruginosa* MB2SA since most of these 10 top strains obtained after BLAST search fall under the *Pseudomonas aeruginosa*. It might be slightly different from any of these ten published strains when more molecular investigations are done.

Table 5: The ten top strains from NCBI BLAST similar to environmental Isolate 2.

Sequences producing significant alignments:		**Score**	**E (Bits) value**
gi[27372785]gb[AY162139.1]	*Pseudomonas aeruginosa* strain BHP7-6	2533	0.0
gi[27372784]gb[AY162138.1]	*Pseudomonas aeruginosa* strain MO2	2533	0.0
gi[9950500]gb[AE004844.1]	*Pseudomonas aeruginosa* strain PAO1	2533	0.0
gi[9946537]gb[AE004501.1]	*Pseudomonas aeruginosa* strain PAO1	2533	0.0
gi[22297309]gb[AF531099.1]	*Pseudomonas aeruginosa*	2533	0.0
gi[22218209]gb[AF529330.1]	Uncultured gamma proteobacterium	2533	0.0
gi[12641786]emb[AJ309500.1]PAE309500	*Pseudomonas aeruginosa*	2533	0.0
gi[6723936]emb[AJ387904.1]PSP387904	*Pseudomonas* sp.	2533	0.0
gi[5912532]emb[AJ249451.1]PAE249451	*Pseudomonas aeruginosa*	2533	0.0
gi[7110444]gb[AF227866.1]AF227866	*Bacterium* str. 61716	2533	0.0

2.3.5 The detection of the *phaC* gene by PCR

The use of PCR to amplify the *phaC* gene was suggested by Sheu *et al.*, (2000), who proposed that this technique was effective as confirmation of PHA production by unknown bacterial species. This method was shown by Sheu *et al.*, (2000) to produce a desired PCR product of about 0.5 kb when a degenerate set of primers was employed on a range of different bacterial strains. A slight modification was made to the set of primers based on those published by Sheu *et al.*, (2000), for use in this study. The denaturation temperature was also raised from 94 ^{0}C (Sheu *et al.*, 2000) to 96 ^{0}C (this study) accommodating the range of strains isolated in this study, since the bacterial strains might have a high G+C content that might hinder PCR amplification (Sheu *et al.*, 2000) as well as tougher cell membranes due to the environmental habitats of the bacterial strains. The 3 % DMSO was used as an additive to overcome the problem of high G+C content (Sheu *et al.*, 2000).

2.3.6 DNA extraction

Various DNA extraction methods were investigated to create a simple and efficient method for DNA extraction for PCR from a range of different bacterial strains. The method should be effective for both the large and small number of bacterial strains. Four methods of DNA extraction were compared with respect to the quality of the PCR amplification. The methods were: (1) Pure colonies serving as DNA template, (2) The boiling method, (3) Phenol extraction of bacterial DNA, and (4) DNA extraction by the High Pure PCR Template Preparation Kit.

2.3.6.1 Pure colonies as a DNA Template

In the pure colony method, colonies of a specific diameter are picked directly from solid medium and are used as DNA templates in the PCR tubes, where lysis of the bacteria is achieved by the first heating step at 96 ^{0}C. The procedure minimizes DNA loss because the bacterial cells are directly inoculated into the tube and the DNA itself is not manipulated in any way. The cells are directly lysed and the cell membranes are ruptured in a single incubation step. The bacterial cells are not required to be alive. The only disadvantage with this method is that it is more successful with a large number of bacterial cells. When DNA was extracted from a small number of bacterial cells, the DNA yield was less because it was found that the cells did not burst and release DNA consistently (Zhao *et al.*, 2001), hence affecting the quality of the PCR product.

The *phaC* gene was detected from nine (9) environmental strains by this technique (Table 6). The primers used in this investigation were namely, phaCF1 and phaCR4, forward and reverse respectively, which should detect an internal fragment of the *phaC* gene on a 0.5 kb PCR fragment, from a range of bacteria, both Gram-positive and Gram-negative (Sheu *et al.*, 2000). *P. oleovorans* and *B. megaterium* were used as positive controls which should contain the *phaC* gene in their chromosomal DNA, and *E. coli* served as a negative control. The expected 0.5 kb band could be detected in the PCR of *P. oleovorans* (Fig. 2A). *B. megaterium* seemed to be negative when using this primer pair. *B. megaterium* was proven to contain the *phaC* gene by Sheu *et al.*, (2000) when another set of primers were used in a semi-nested PCR following colony PCR. The other reason *B. megaterium* does not show a PCR product is possibly because the cell envelope of this

organism seems to be tough to break, thus making it difficult to release the DNA to be amplified, since there was no evidence of chromosomal DNA in lane 4, 5 and 6. PCR using *E. coli* DNA did not yield a 0.5 kb fragment, corresponding to the *phaC* gene. This confirms the results from the Nile Blue A stain where *E. coli* did not fluoresce, while *P. oleovorans* and *B. megaterium* were both positive.

Further inspection of the gel shown in Fig. 2A, indicates that there were a lot of primer-dimers suggesting that the primers did not anneal to the DNA. There is also a large amount of DNA trapped in the wells of the agarose gel. We can conclude that the primers did not anneal because sufficient DNA denaturation did not occur, possibly due to the presence of cell debris. In addition, this method was not suitable for a number of the environmental strains because of the DNA degradation observed in lanes such as 3, 4, 7, 8, 9, 13, 14, and 16 in Figure 2B, and lane 3, 4, 7, 8, 13, 16 and 19 in Figure 2C, however, good bands can be observed confirming the presence of the *phaC* gene in lanes such as 1, 2, 3 (Fig. 2A), lane 1, 2, 5, 16 (Fig. 2B), and lane 3, 8, 10, 13, 14, and 15 (Fig. 2C, Table 6 and Table 7).

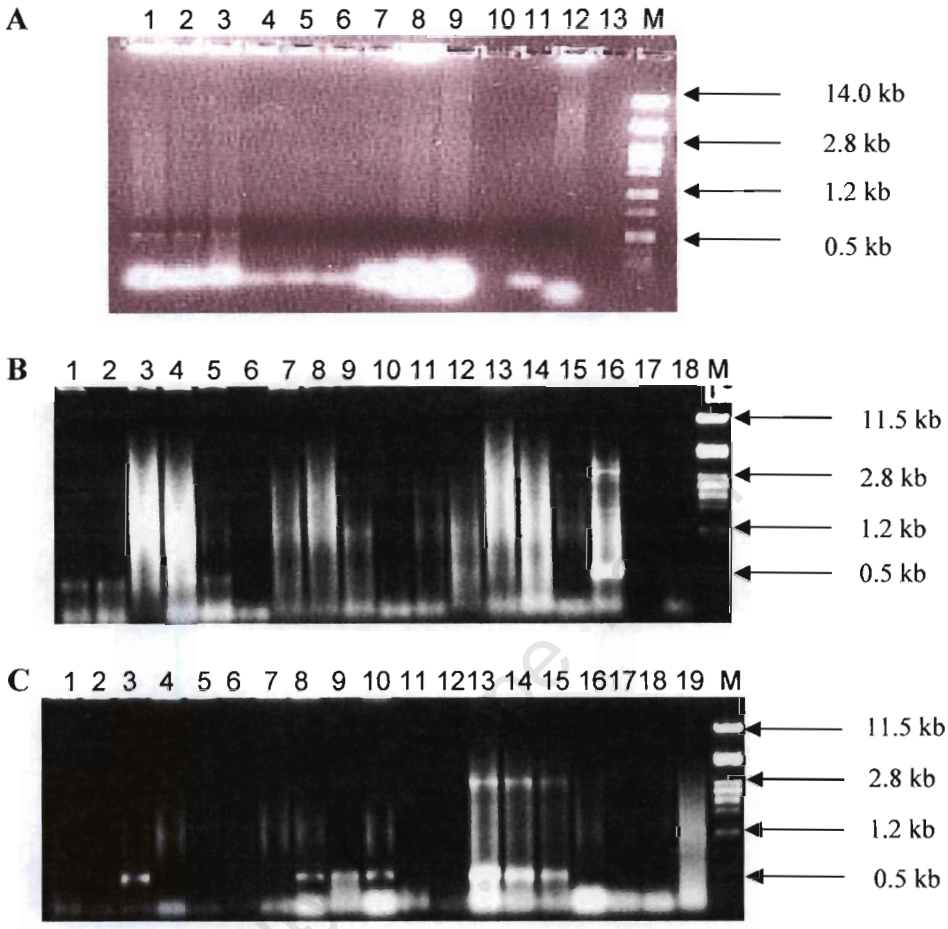


Figure 2: The colony PCR of the PHA-positive strains and the isolated environmental strains amplified with the primer pair phaCF1-phaCR4. The PCR products viewed on a 0.8 % agarose gel. A: Lane 1: *P. oleovorans* 2.0 mM $MgCl_2$, 2: *P. oleovorans* 2.5 mM $MgCl_2$, 3: *P. oleovorans* 3.0 mM $MgCl_2$, 4: *B. megaterium* 2.0 mM $MgCl_2$, 5: *B. megaterium* 2.5 mM $MgCl_2$, 6: *B. megaterium* 3.0 mM $MgCl_2$, 7: *E. coli* 2.0 mM $MgCl_2$, 8: *E. coli* 2.5 mM $MgCl_2$, 9: *E. coli* 3.0 mM $MgCl_2$, 10 Empty, 11: No DNA, 12: Negative control, 13: Empty, M: Molecular size marker (λ*Pst*I).
B: Lane 1: Isolate 1, 2: Isolate 2, 3: Isolate 3, 4: Isolate 4, 5: Isolate 5, 6: Isolate 6, 7: Isolate 7, 8: Isolate 8, 9: Isolate 9, 10: Isolate 10, 11: Isolate 11, 12: Isolate 12, 13: Isolate 13, 14: Isolate 14, 15: Isolate 15, 16: Isolate 16, 17: Empty, 18: No DNA, M: Molecular size marker (λ*Pst*I).
C: Lane 1: Isolate 17, 2: Isolate 18, 3: Isolate 19, 4: Isolate 20, 5: Isolate 21, 6: Isolate22, 7: Isolate 23, 8: *P. oleovorans* 2.5 mM $MgCl_2$, 9: *P. fluorescence* 2.5 mM $MgCl_2$, 10: *P. oleovorans* 2.5 mM $MgCl_2$, 11: *E. coli* 2.5 mM $MgCl_2$, 12: No DNA, 13: *P. oleovorans* 2.0 mM $MgCl_2$, 14: *P. oleovorans* 2.5 mM $MgCl_2$, 15: *P. oleovorans* 3.0 mM $MgCl_2$, 16: *E. coli* 2.0 mM $MgCl_2$, 17: *E. coli* 2.5 mM $MgCl_2$, 18: *E. coli* 3.0 mM $MgCl_2$, 19 *E. coli* 3.5 mM $MgCl_2$, M: Molecular size marker (λ*Pst*I).

The utilization of the whole colony or many entire bacterial cells into the PCR tube results in differing amounts of DNA in the tubes, some of which is quite degraded, as indicated by the smear of DNA down the gel. DNA concentration is very important in PCR; it requires small amounts of DNA, 50-100 nanograms, which has been shown to be sufficient for the double strand amplification. The whole colony method therefore, may have introduced more that the required amounts of DNA in the PCR tubes, and may not be ideal for the isolation of undegraded DNA.

To confirm whether high temperature (96 ^{0}C) and high DNA concentrations were the causes of poor PCR results, a different method of DNA preparation, the "boiling method" was exploited.

2.3.6.2 DNA template preparation by the "boiling method"

The boiling method comprises breaking of the bacterial cells to release DNA from the cytoplasm at a high temperature. DNA was released by boiling the bacterial cells at approximately 100 ^{0}C. The boiling method does not have a DNA purification step; therefore DNA material would not be lost. The characteristics of the isolated environmental bacterial strains were not known, hence the disadvantage of this method might be that the bacterial cells that could withstand temperatures higher than 100 ^{0}C would not break open resulting in very low yields of DNA released from the bacterial cells. The cells are required to be in early stationary stage and alive. Bacterial samples that have been stored for longer periods which might lead to the death of the bacterial cells or to hardened cell walls, cannot be used in this method, because the cells will not break open easily.

The boiling method was employed for the extraction of DNA for PCR purposes. The advantages to this procedure were that the method was quick and less expensive since, the technique involves boiling of the bacterial cells to release DNA from the cytoplasm and centrifugation to pellet the cell debris. The DNA extraction by this method was simple and fast, but it was not effective on a range of different bacterial strains. The DNA of the controls in Figure 3 was degraded when visualized under the UV light following the PCR procedure, as observed in lane 2 and 3 (Fig. 3), and no 0.5 kb PCR product was obtained. These outcomes substantiate the fact that high temperature and high DNA concentration in the presence of proteins and cell debris might have been the cause of DNA degradation, as it was suggested earlier in this study.

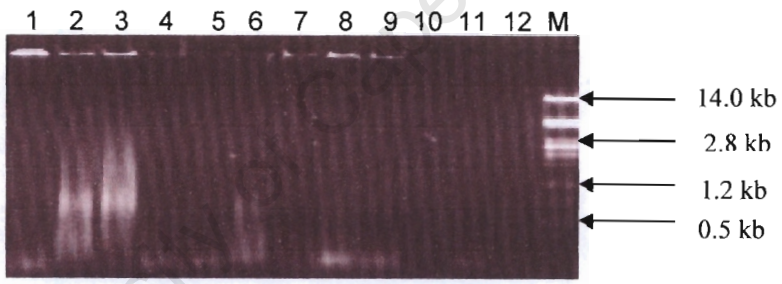


Figure 3: PCR amplification of the *phaC* gene using the primer pair phaCF1-phaCR4. The DNA template was prepared using the boiling method. The PCR products viewed on a 0.8 % agarose gel. Lane 1: *P. oleovorans* 2.0 mM $MgCl_2$, 2: *P. oleovorans* 2.5 mM $MgCl_2$, 3: *P. oleovorans* 3.0 mM $MgCl_2$, 4: *B. Megaterium* 2.0 mM $MgCl_2$, 5: *B. megaterium* 2.5 mM $MgCl_2$, 6: *B. megaterium* 3.0 mM $MgCl_2$, 7: Empty, 8: *E.coli* 2.0 mM $MgCl_2$, 9: *E.coli* 2.5 mM $MgCl_2$, 10: Empty, 11: No DNA, 12: Empty, M: Molecular size marker (λ*PstI*).

2.3.6.3 The phenol-chloroform DNA extraction method

The third method, bacterial DNA purification using phenol-chloroform extraction, has some important advantages. The optimum conditions have been predetermined for DNA extraction from a range of bacteria, although this does not mean that the method would be suitable for the environmental bacterial strains isolated in this study. Extraction with phenol-chloroform would remove the cell debris and many of the cellular proteins from the DNA preparation, resulting in good quality DNA with fewer interfering substances for use in other molecular analyses such as PCR.

This method was found to be effective and reliable as opposed to the colony PCR and the boiling method, although there was some degradation of the DNA from four of the environmental strains (lane 2, 6, and 11 in Figure 4A and lane 6 of Figure 4B). Positive PCR reactions were obtained in nine (9) out of twenty-three (23) environmental strains tried with this method (Table 6). In all cases, the DNA band observed was 0.5 kb. However, no PCR product was obtained in fourteen (14) environmental strains, therefore a further method utilizing a combination of the High Pure PCR Template Preparation Kit (Roche) and glass beads was attempted.

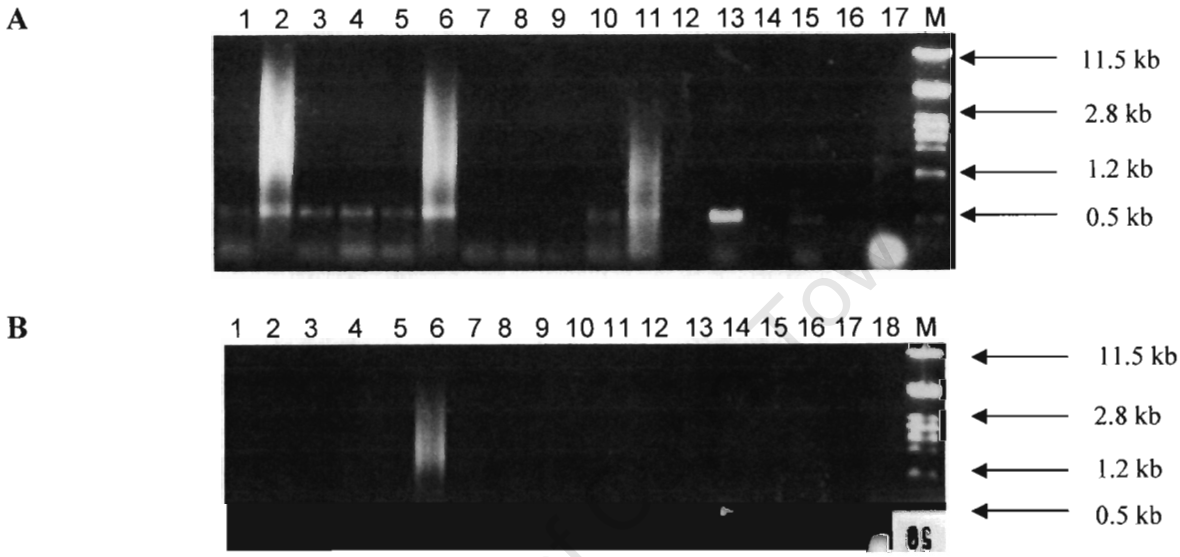


Figure 4: PCR amplification of the *phaC* gene from the PHA-positive and the PHA-negative strains with the primer pair phaCF1-phaCR4. The DNA template was prepared using the bacterial DNA extraction and purification by phenol: chloroform method. The PCR products viewed on a 0.8 % agarose gel. A: Lane 1: Isolate 1, 2: Isolate 2, 3: Isolate 3, 4: Isolate 4, 5: Isolate 5, 6: Isolate 6, 7: Isolate 7, 8: Isolate 8, 9: Isolate 9, 10: Isolate 10, 11: Isolate 11, 12: Isolate 12, 13: Isolate 13, 14: Isolate 14, 15: Isolate 15, 16: Isolate 16, 17: No DNA, M: Molecular size marker (λ*Pst*I).
B: Lane 1: Isolate 17, 2: Isolate 18, 3: Isolate 19, 4: Isolate 20, 5: Isolate 21, 6: Isolate 22, 7: Isolate 23, 8: *P. oleovorans* 2.5 mM $MgCl_2$, 9: *P. fluorescence* 2.5 mM $MgCl_2$, 10: *P. oleovorans* 2.5 mM $MgCl_2$, 11: *E. coli* 2.5 mM $MgCl_2$, 12: No DNA, 13: *P. oleovorans* 2.0 mM $MgCl_2$, 14: *P. oleovorans* 2.5 mM $MgCl_2$, 15: *P. oleovorans* 3.0 mM $MgCl_2$, 16: *E. coli* 2.0 mM $MgCl_2$, 17: Empty, 18: No DNA, M: Molecular size marker (λ*Pst*I).

2.3.6.4 High Pure PCR Template Preparation Kit

The last method involved rupturing the bacterial cell walls by means of glass beads followed by DNA purification using the High Pure PCR Template Preparation Kit (Roche). The DNA extraction by glass beads is one of the most commonly employed methods for DNA extraction from environmental bacterial strains, which may be harder to rupture. The only disadvantage of this method is that the bacteria with weaker cell walls would release their DNA quickly, exposing it to mechanical pressure exerted by the glass beads that could cause shearing of the DNA.

The technique was effective and reliable when dealing with different bacterial species that require specialized DNA extraction procedures, and yielded DNA of good quality (results not shown). It was evident that this method resulted in insignificant DNA degradation although glass beads were employed, (Figure 5A and 5B). DNA extraction by this method can be carried out on more than one bacterial strain simultaneously, and uses minimal volumes of its reagents, making it efficient for numerous DNA extractions.

In Fig 5A, lanes 1, 2, and 3 show the absence of a PCR product to the *phaC* gene in *B. megaterium* when primer pair phaCF1- phaCR4 was used. This confirms the negative results obtained for this strain in the colony PCR method. A PCR product of 0.5 kb was observed in *P. oleovorans*, (Figure 5A in lane 7, 8 and 9). A non-specific band (white arrow, Figure 5A, in lane 7) was observed when the $MgCl_2$ concentration of 2.0 mM was used although the largest amount of PCR product was obtained under these conditions (lane 7, Figure 5A). This DNA fragment, presumably arising from the primers annealing to a different region of the chromosome, was not evident when either 2.5 mM or 3.0 mM

$MgCl_2$ was used. The PCR product was optimized at 2.0 mM $MgCl_2$ rather than at 2.5 and 3.0 mM $MgCl_2$, therefore this concentration of 2.0 mM was applied for PCR of the *phaC* gene from all of the environmental isolates, shown in Figure 5B. The different intensities of the PCR products suggest that the different bacterial strains may require slightly different $MgCl_2$ concentrations or require slight alterations in the PCR protocol in order to optimize the reaction in each case. A good yield of PCR product was seen in lane 8 for the environmental isolate 7 in Figure 5B, however, this strain was not a good alkane degrader and grew slowly in MSM and alkanes as shown earlier. The PCR product for the environmental isolate 2 was faint (lane 3, Figure 5B) but this strain has shown to be a good alkane degrader for both C_{12}-C_{13} and C_{14}-C_{17} alkane fractions and consistently showed a strong positive reaction to Nile Blue A staining, suggesting that the DNA extraction conditions are not optimal for PCR from this strain.

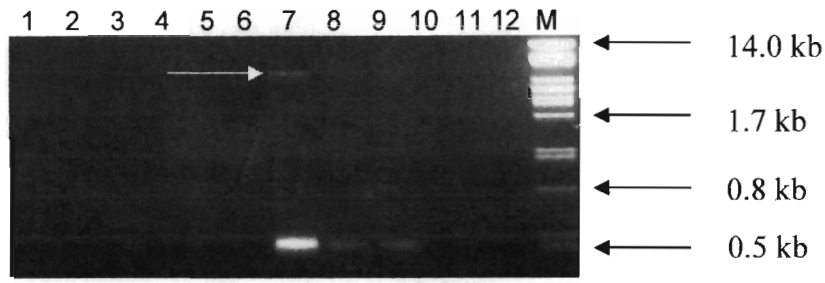
In general, a high yield of good DNA was obtained using this method. It presumably has less protein contamination and the yield is sufficient to be used for other molecular techniques such as cloning and southern blotting. Table 6 shows some observations made when different DNA extraction techniques were used. The PCR product of 0.5 kb was consistently observed in the environmental isolate 1, 2, 3, 4, 5, 6, 14, 16, and 19 throughout the different extraction methods except when the boiling method was used (Table 6). Only the positive controls, *P. oleovorans*, *B. megaterium*, and *E. coli* were tested by this method because of the disadvantages discussed earlier. Isolate 7 gave positive PCR results only when its DNA was extracted using High Pure PCR Template Preparation Kit (Roche) (Table 6).

Table 6: The presence of the *phaC* gene product when different DNA extraction methods were tested. The primer set phaCF1-phaCR4 was used.

	DNA Extraction Method			
Strain	Colony	Boiling	Phenol	Kit
P. oleovorans	+	+	+	+
B. megaterium	-	-	-	-
E. coli	-	-	-	-
1	+	ND	+	+
2	+	ND	+	+
3	+	ND	+	+
4	+	ND	+	+
5	+	ND	+	+
6	+	ND	+	+
7	-	ND	-	+
8	-	ND	-	-
9	-	ND	-	-
10	-	ND	-	-
11	-	ND	-	-
12	-	ND	-	-
13	-	ND	+	-
14	+	ND	+	+
15	-	ND	-	-
16	+	ND	+	+
17	-	ND	-	-
18	-	ND	-	-
19	+	ND	+	+
20	-	ND	-	-
21	-	ND	-	-
22	-	ND	-	-
23	-	ND	-	-

Symbols: + *phaC* gene present
- *phaC* gene absent
ND not done

A



B

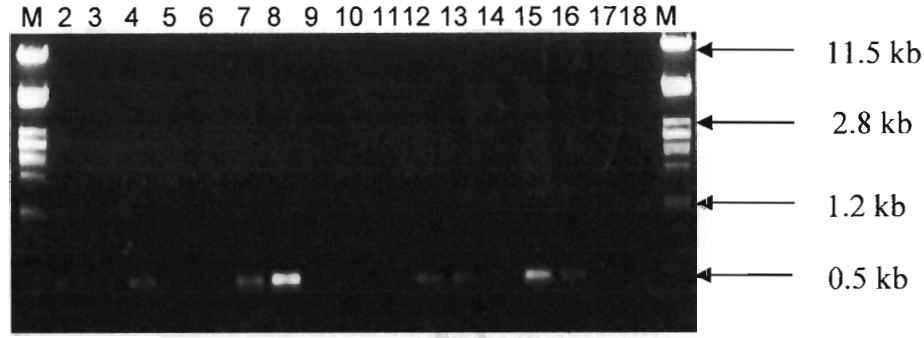


Figure 5: PCR amplification of the *phaC* gene from the PHA-positive, PHA-negative and the environmental strains with the primer pair phaCF1-phaCR4. The DNA template prepared using the High Pure PCR Template Preparation Kit. The PCR products viewed on a 0.8 % agarose gel. A: Lane 1: *B. megaterium* 2.0 mM $MgCl_2$, 2: *B. megaterium* 2.5 mM $MgCl_2$, 3: *B. megaterium* 3.0 mM $MgCl_2$, 4: *E. coli* 2.0 mM $MgCl_2$, 5: *E. coli* 2.5 mM $MgCl_2$, 6: *E. coli* 3.0 mM $MgCl_2$, 7: *P. oleovorans* 2.0 mM $MgCl_2$, 8: *P. oleovorans* 2.5 mM $MgCl_2$, 9: *P. oleovorans* 3.0 mM $MgCl_2$, 10: Empty, 11: No DNA, 12: Empty, M: Molecular size marker (λ*Pst*I).
B: Lane M: Molecular size marker (λ*Pst*I), 2: Isolate1, 3: Isolate 2, 4: Isolate 3, 5: Isolate 4, 6: Isolate 5, 7: Isolate 6, 8: Isolate 7, 9: Isolate 9, 10: Isolate 10, 11: Isolate 14, 12: Isolate 16, 13: Isolate 19, 14: Empty, 15: *P. oleovorans* 2.0 mM $MgCl_2$, 16: *P. oleovorans* 2.0 mM $MgCl_2$, 17: Empty, 18: *E. coli* 2.0 mM $MgCl_2$, M: Molecular size marker (λ*Pst*I).

Table 7: Summary of the relevant characteristics of bacterial strains isolated in this study capable of utilizing alkane fractions as carbon source.

Isolate no.	Nile Blue	PCR	Gram Stain	Morphology
1	+++++	+	negative	rods
2	+++++	+	negative	rods
3	++++	+	negative	rods
4	+++	+	negative	rods
5	+++++	+	negative	rods
6	+++++	+	negative	rods
7	+	+	negative	rods
8	negative	neg	negative	rods
9	+	neg	negative	rods
10	+++++	neg	negative	rods
11	negative	neg	negative	rods
12	negative	neg	negative	rods
13	negative	+	negative	rods
14	+++	+	positive	cocci
15	negative	neg	negative	rods
16	+++++	+	negative	rods
17	negative	neg	negative	rods
18	negative	neg	negative	rods
19	negative	+	variable	rods
20	negative	neg	negative	rods
21	negative	neg	negative	rods
22	negative	neg	negative	rods
23	negative	neg	negative	rods

2.4 Final remarks

In summary (Table 7), a total number of twenty-three (23) environmental bacterial strains were isolated, which were capable of degrading alkane fractions. Nine (9) of these bacterial strains were positive on Nile Blue A after (+++ to +++++) 3 days of growth in the presence of alkanes and showed the presence of the *phaC* gene as detected by PCR.

Ten (10) bacterial strains were negative on Nile Blue A after growth on alkanes and did not possess the *phaC* gene. Two of the bacterial strains were positive after Nile Blue A staining, but did not show the PCR fragment corresponding to the *phaC* gene (isolate 9 and 10), whereas two of the environmental isolates showed the presence of the *phaC* gene by PCR but did not show fluorescence after Nile Blue A staining (isolate 13 and 19). This could be a result of non-optimal growth conditions for PHA accumulation by these strains, or may be due to sampling technique at the wrong time in the growth cycle. The bacteria may have degraded PHAs after 72-hour growth. It should also be noted that *B. megaterium* did not give a PCR product using these primers, despite the fact that it does accumulate PHA inclusion bodies, as reported by Sheu *et al.* (2000). It is possible that the two strains which did not give a PCR product although they were positive for the Nile Blue A staining, have different genes involved in PHA biosynthesis which would not be detected by these particular PCR primers.

CHAPTER 3

The Physiology of *Pseudomonas aeruginosa* MB2SA

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3 SUMMARY

The environmental bacterial strain, *P. aeruginosa* MB2SA, was grown on the two-alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}. The bacterial growths were compared in these two conditions to determine the one favorable for optimum growth. Different methods to determine the bacterial cell numbers were carried out and a suitable method in terms of estimating the cell numbers was employed throughout this study to plot accurate growth curves and pH. Growth curves and pH were monitored every six hours to make sure that anything different between the two conditions might be noticed. *P. aeruginosa* MB2SA grew optimally when the longer alkane fraction, C_{14}-C_{17}, was utilized as a sole carbon source. This environmental bacterium, *P. aeruginosa* MB2SA, grew faster in the longer

alkane fraction than in the shorter alkane fraction. *P. aeruginosa* MB2SA was further grown on three pure alkanes, namely, n-dodecane (C_{12}), n-tetradecane (C_{14}) and hexadecane (C_{16}), and the growths were compared to when the alkane fraction were utilized as sole carbon sources.

Later, *P. aeruginosa* MB2SA was grown in an appropriate medium for PHA production. The PHA yields were compared between a range of carbon sources, two alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, three pure alkanes, n-dodecane (C_{12}), n-tetradecane (C_{14}) and hexadecane (C_{16}) respectively. High yield of PHA was observed when a longer alkane fraction, C_{14}-C_{17}, was utilized as sole carbon source, which was comparable to when the bigger pure alkane, hexadecane (C_{16}), was employed. *P. aeruginosa* MB2SA was proven to have the ability to degrade the alkane fraction wastes efficiently and converting them to value added products such as PHAs.

3.1 INTRODUCTION

Our country, South Africa and the world at large are faced with the current problem of the accumulation of low-value by-products from the petrochemical industries. This important concern has encouraged interesting research in the development and production of biodegradable polymers from these waste products (Ojumu and Solomon, 2003).

Long-chain alkanes, C_{13} to C_{44}, which form part of the petrochemical wastes have been shown to be a potential carbon substrates for polyhydroxyalkanoate (PHA) production (Geißdörfer *et al.*, 1999). These long-chain alkanes could be converted to PHAs by many different bacteria including *Pseudomonas aeruginosa* (Smits *et al.*, 2002). PHA

production from the low-cost alkane wastes would be advantageous as compared to other expensive counterparts which amount to about 70 percent of the PHA production operation costs (Choi and Lee, 1997). These industrial wastes have been shown to give a good PHA yield when used in bacterial growth media and could therefore be economically important (Lee and Gilmore, 2004). Lemoigne (1926) showed that PHAs could be accumulated as energy storage compounds, when one of the non-carboneus nutrients such as nitrogen is a limiting factor.

Apart from nitrogen, there are some factors such carbon source, temperature and pH that affect PHA production by bacteria (Marangoni *et al.*, 2001). The effect of culture conditions namely, nitrogen, phosphorus, oxygen and temperature were investigated in favor of polymer accumulation relative to the carbon source provided (Marangoni *et al.*, 2001).

Wang and Lee (1997) investigated the effect of nitrogen limiting, phosphorus, magnesium, or sulfur in flask cultures and it was discovered that nitrogen limitation was the best condition that enhanced PHB (an example of PHA) production; therefore nitrogen-limiting conditions were employed in this study. It was observed that the metabolic activity of the cells was decreased because of high PHB accumulation (Wang and Lee, 1997; Kim *et al.*, 1994).

High PHA production has been reported when the temperature and pH were controlled at 30 ^{0}C and 6.8 respectively (Wang and Lee, 1997). Temperatures, 30 ^{0}C and 34 ^{0}C were investigated by Marangoni and co-workers (2001), and there was no significant difference in the bacterial growth when the two temperatures were compared. Contrary

to this fact, increased temperatures were shown to decrease PHA production rate because of increased anabolic rates at higher temperatures (Krishna and Van Loosdercht, 1999) hence, 30 ^{0}C was employed in this study as opposed to 34 ^{0}C or 37 ^{0}C.

The carbon source (sucrose) uptake by the most studied PHA producing bacterium, *Alcaligenes eutrophus*, increased in nitrogen limiting conditions as compared to nitrogen sufficient conditions implying that the metabolic rate of the cells increases in nitrogen limiting conditions, hence PHA synthesis was enhanced (Wang and Lee, 1997).

Physiological studies were carried out on the selected strain, *Pseudomonas aeruginosa* MB2SA, since it is capable of degrading alkane fractions effectively and accumulate substantial amounts of PHA-inclusion bodies in its cytoplasm. It therefore is a good candidate for this study, where the objective is to utilize petrochemical waste products such as alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, as a low-cost carbon source to produce a value-added product.

3.2 MATERIALS AND METHODS

3.2.1 Growth studies

One environmental bacterial strain, *Pseudomonas aeruginosa* MB2SA, was selected based on its ability to degrade both C_{12}-C_{13} and C_{14}-C_{17} alkane fractions effectively. This strain also showed to be accumulating PHAs when stained with Nile Blue A stain. *P. aeruginosa* MB2SA was grown on the minimal salt medium (MSM) with the appropriate alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, as carbon source for the duration of this study. The bacterial strain was maintained on the MSM agar plates with the appropriate carbon substrate.

P. aeruginosa MB2SA was routinely grown in MSM broth with two different alkane fractions, 5 % C_{12}-C_{13} and 5 % C_{14}-C_{17}, which were obtained from SASOL. A single pure colony of *P. aeruginosa* MB2SA was aseptically picked using a sterile inoculating loop and inoculated into 100ml MSM (A.2.1.6) broth, having the alkane fractions 5 % C_{12}-C_{13} and 5 % C_{14}-C_{17} as the sole carbon sources. The MSM contained per liter: 1g $(NH_4)_2SO_4$, 1g K_2HPO_4, 0.5g KH_2PO_4 and 10ml salt solution. The salt solution has, per liter: 25g $MgSO_4.7H_2O$, 28g $FeSO_4.7H_2O$, 1.7g $MnSO_4$, 0.6g NaCl, 0.1g $Na_2MoO_4.2H_2O$, 0.1g $ZnSO_4.7H_2O$ and 8.54ml of 32 % HCl solution. The 10N NaOH was used to adjust the pH to 7.0. The carbon sources, C_{12}-C_{13} and C_{14}-C_{17} were not included in the MSM during autoclaving, but filter-sterilized C_{12}-C_{13} and C_{14}-C_{17} alkane fractions were aseptically injected directly into the culture with the syringe. The flasks were incubated at 30 ^{0}C with shaking at 250 rpm to make sure that the strain is viable. This test was done in duplicates. After four (4) days incubation, 10 % volume was

inoculated into two fresh 100ml MSM, containing appropriate carbon sources as describe above and incubated at 30 ^{0}C. Triplicates cultures were prepared for the two-alkane fractions for the duration of this study. Growth and pH were monitored at 6-hour intervals for 90-96 hours. Similar cultures containing 5 % of pure alkanes, n-dodecane (Sigma), n-tetradecane (Sigma) and hexadecane (Fluka) were inoculated and grown in the same way for comparison with the results from the mixed fractions.

The bacterial populations were quantitatively determined applying four different methods. These methods were compared in order to deduce the method effective for this purpose. Cell counts were investigated spectrophotometerically, microscopically, dry weight measurements and using the standard plate counts.

3.2.1.1 Determination of bacterial cell numbers

3.2.1.1.1 Absorbance readings by spectrophotometer

Two milliliters of the *P. aeruginosa* MB2SA was harvested aseptically at 6-hour intervals to monitor both the growth of the organism and the pH of the medium during growth. The sampling was done in triplicate and the average was used to plot the growth curve. Before taking the absorbance reading, the bacterial cells were left at room temperature for two minutes to let the remaining carbon source in the medium migrate to the top of the medium. One-milliliter sample was collected from below of the alkane layer into a one-milliliter plastic cuvette. The absorbance was read at 660nm, on a Beckman DU 530 spectrophotometer.

3.2.1.1.2 Microscopic cell counts

A known amount of the bacterial cell sample was harvested aseptically every six hours from the growth culture. The bacterial cells were counted using a Thoma counting chamber under the normal light microscope, using a 100x oil objective. The numbers were expressed as number of cells per volume.

3.2.1.1.3 Dry Weight Measurement

A known volume of the bacterial culture was harvested aseptically every 6 hours. Washed cell suspension was dried in an oven at 180 ^{0}C using pre-weighed glass dishes. The weight of the cells was obtained in triplicate for each time interval.

3.2.1.1.4 Standard Plate Counts

The bacterial cell growth was determined using the standard plate counts. The bacterial strain was grown on the nutrient agar (NA) plates (A.2.1.3).

3.2.2 Polyhydroxyalkanoate (PHA) Production

The *P. aeruginosa* MB2SA was grown on nitrogen limiting [0.4g $(NH_4)_2SO_4$/L] minimal salt medium (MSM) with 5 % alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, as carbon substrate. A nitrogen limiting MSM has been shown to enhance the production of PHAs (Sheu *et al.*, 2000). Pure alkanes, n-dodecane (Sigma), n-tetradecane (Sigma) and hexadecane (Fluka) were also employed as carbon substrates, each at 5 % v/v, to make a constructive comparison with the results from the alkanes.

The 10 % volume of the bacterial sample from full-strength MSM was harvested and centrifuged at 8000 rpm (Beckmann) to remove excess MSM. Cell pellets were re-suspended into an equal volume of nitrogen limiting MSM [0.4g/L of $(NH_4)_2SO_4$] and the whole cell suspension was inoculated into 5L Erlenmeyer flasks containing 1L of nitrogen limiting MSM with 5 % v/v C_{12}-C_{13} alkane fraction or 5 % v/v C_{14}-C_{17} alkane fraction. This test was carried out in duplicate. The bacterial strain was incubated at 30 ^{0}C with shaking at 250 rpm, for 3 days to allow the accumulation of PHAs in the cytoplasm of the bacterial cells (Sheu *et al.*, 2000). After 3 days the bacterial cells were stained with Nile Blue A stain to check for the presence of PHAs in their cytoplasm before determining the cell dry weight and PHA extraction.

3.2.2.1 Cell Dry Weights

After 72-hour (3 days) incubation, the pH was determined and 500 ml of the bacterial cells was harvested. The cells were centrifuged at 8000 rpm (Beckmann) for 20 minutes, at 4 ^{0}C. The cell pellets were then washed twice with the nitrogen limiting MSM to remove excess alkane fractions. The cell pellets were lyophilized for 2 days before the dry cell weights were determined prior to PHA extraction.

3.2.2.2 Polyhydroxyalkanoate (PHA) Extraction

Five hundred (500) milliliters of hot chloroform was used to extract PHAs from the lyophilized cells using the Soxhlet apparatus. The extraction was done continuously for 6 hours (Ballistreri *et al.*, 2001; Brandl *et al.*, 1988). The extracted polymer was allowed to cool at room temperature. The polymer solution was filtered and then concentrated using

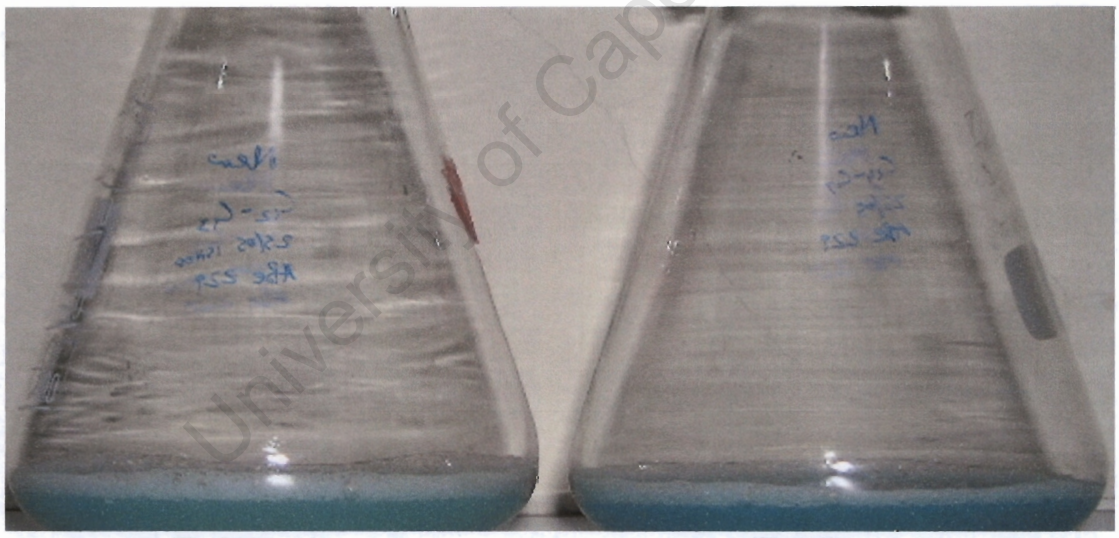
the rotary evaporator. The concentrated solution was gradually decanted into ten volume of cold methanol, which was stirred vigorously. The precipitated polymer was separated by centrifugation (Beckman J2-21; JA-20 rotor, 20 ^{0}C; 9000 x g). The polymer was washed twice with 96 % ethanol, and dried under vacuum (1 mmHg) at room temperature. To improve the quality of the product, the extracted crude PHA was purified by repeated precipitation, dropping it into cold methanol, which was stirred vigorously. The precipitation was repeated at least three times. The weight of the polymer was determined.

3.3 RESULTS AND DISCUSSION

3.3.1 Growth on alkane fractions

Cell growth by Spectrophotometer

The presence of hydrophobic substances in the culture medium can cause problems with some of the methods of measuring growth. The bacterial cell numbers were initially determined using a spectrophotometer, which measures the turbidity of the medium as a result of the cell concentration when cells multiply. The bacterial cell growth in this method should be directly proportional to the turbidity of the medium, indicated by absorbance readings at 660nm on the spectrophotometer.



Flask A **Flask B**

Figure 3.1: *P. aeruginosa* MB2SA grown for 72 hours in a minimal salt medium (MSM) with 5 % v/v alkane fractions as a carbon source in 30 ^{0}C shaker incubator. Flask A: C_{12}-C_{13} alkane fraction and, Flask B: C_{14}-C_{17} alkane fraction. The medium changed color to green in both flasks A and B.

P. aeruginosa has been shown to be capable of producing a variety of water-soluble pigments. One of these pigments was shown to be pyoverdin, which is a yellow-green or yellow-brown fluorescent pigment. A bright green color has been observed when both pyoverdin and the water-soluble blue pigment, pyocyanin, were produced by *P. aeruginosa* (Rahme *et al.*, 1997). Sanchez *et al*, (2002) showed that pyoverdin and pyocyanin pigments could also be evaluated by measuring their absorbances at 400 and 690 nm respectively. A similar kind of color change was also observed in this study (Fig. 3.1), when *P. aeruginosa* MB2SA was grown on alkane fractions for 72 hours. This color change interfered with the absorbance readings at 660nm on the spectrophotometer, and there was an immediate increase in the absorbance reading after the color change was noted in the medium (Fig. 3.2). The color change occurred between 42 and 66 hours of incubation when C_{12}-C_{13} and C_{14}-C_{17} served as carbon substrates respectively (Fig. 3.2). A drop in pH accompanied the green-colored product produced. The pH dropped from 7.0 to 5.0 in cultures of the bacterium growing on the long alkane fraction, C_{14}-C_{17}, between 35 and 42 hours of growth and later, at about 66 hours of growth, in cultures of the bacterium utilizing a shorter alkane fraction, C_{12}-C_{13} (Fig. 3.2), suggesting that the longer alkane fraction is a preferred carbon source.

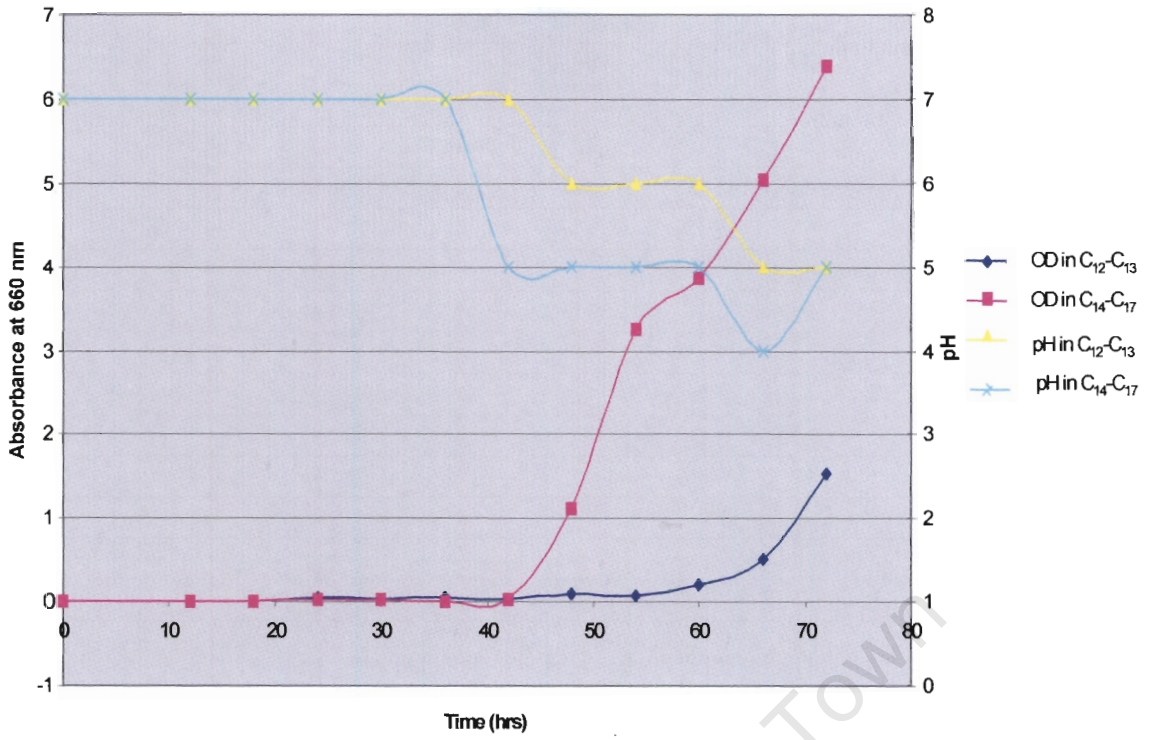


Figure 3.2: The absorbance readings of cultures of *Pseudomonas aeruginosa* MB2SA when growing on the minimal salt medium (MSM) with 5 % alkane fractions serving as carbon substrate.

Attempts were made to wash the cells by centrifuging and resuspending in the fresh medium before the absorbance was measured. However, difficulties were encountered with this method because the cells tended to bind to the alkane droplets making it difficult to pellet these cells even if the centrifugation period was increased. This cell-to-alkane binding suggests that the outer membrane of the cells should be hydrophobic which makes it easier for the cells to bind to the alkanes, which are also hydrophobic. Norman and co-workers (2002) found that utilization of n-alkanes by *P. aeruginosa* depends on direct cell-substrate contact, and the low aqueous solubility of n-alkanes (brownish layer on top of the medium, Fig. 3.1) could affect the ability of *P. aeruginosa* to oxidize these fractions of crude oil (Norman *et al.*, 2002). The ability of the bacteria to

utilize these hydrophobic compounds appears to require cell surface adaptation to allow the interaction between the cell and their hydrophobic substrates. The bacterial outer membrane is the first part of the cell to make contact with the substrate, and growth conditions, for instance temperature, pH, and nutrient availability, could affect the composition of the outer membrane and therefore the ability to bind hydrophobic compounds (Costerton, 1974).

It was concluded that this method could not be employed to determine the number of cells, since the bacterial cells migrated to the surface of the medium together with the alkane droplets (brownish layer) immediately after centrifugation. The other reason the cells were difficult to centrifuge was because the cells were very small, and therefore resuspended immediately and the cell pellet after centrifugation would not give a true reflection of the cell number due to these factors.

Cell growth by Microscopic cell counts

A different method, microscopic cell counts, was carried out in an attempt to determine cell numbers more accurately. However, there were some disadvantages to this method. The method was found to be unreliable because the cells were too small to count even at 100x magnification, and it was almost impossible to distinguish between two or more cells that were close to one another. Here also cells tend to stick to the alkane droplets because of the hydrophobicity of the cell surface. The more the alkane droplets on the microscope field the more cells were present, therefore the cell distribution on the microscopic slide was uneven. The cells could not be centrifuged since some are lost due to the centrifugation and washing process. The other challenge encountered was that, an

investigator might count one cell more than once, due to the movement of the cells from one position to another on the glass slide. Cooling the cells on ice prior counting prevented the cell motility, but because the cells were minute it became more difficult to count. This method was therefore abandoned in favor of more accurate and reliable methods.

Dry Weights

In this method, the cells were directly collected from the Erlenmeyer flasks in which they were grown and then dried at a high temperature. The problem with this method was that *P. aeruginosa* MB2SA produced a substance that caused the medium (MSM) to change color to brownish-yellow and a whitish substance was also observed after drying these cells, which probably contributed to the weight of the sample. The other observation made was that, the residual alkane fractions in the medium could not be completely dried by this process, which clearly introduced an error in the measurement of total biomass of the cells. The graph using these measurements was similar to the one discussed in Fig. 3.2 earlier (not shown). For the above reasons, this method was not used to determine the cell numbers.

3.3.2 Growth studies of *P. aeruginosa* MB2SA on alkane fractions

The use of either spectrophotometer readings, direct microscopic cell counts or cell dry weights had various disadvantages that made these methods not suitable in this study. Standard plate counts on nutrient agar (NA) plates were employed to determine the number of viable cells in the culture flasks. This method was found to be more suitable

for estimating growth and was more reliable than OD readings, microscopic cell counts and cell dry weights. Viable counts also only represent the living cells (Fig. 3.3), whereas the other measurements detect total cell numbers. The accurate growth curves were therefore plotted using the viable cell counts expressed as colony forming units (cfu). This method was convenient and resulted in repeatable measurements, but was very labor intensive.

There may still be an underestimation of the true number of viable cells because of clumping in oil media as previously mentioned; however, samples were well shaken before plating to minimize this. The isolated strain appears to be growing well in the two-alkane fractions as seen on Figure 3.3, A and B. The growth conditions of the two experiments were identical, and the medium was prepared in one container and then divided into two, before adding alkane substrate to each. The Erlenmeyer flasks representing the two different growth conditions in duplicate were incubated at the same time at specified temperature (30 ^{0}C). The pH and growth measurements were also checked at the same time. The Gram-stain was done frequently to check for any contamination of the bacterial strain.

There were some significant differences noted during the growth studies. The growth rate and pH of this bacterial strain was monitored every six (6) hours to ensure that everything happening in the two growth environments was observed. *P. aeruginosa* MB2SA appeared to prefer the longer chain alkanes (C_{14}-C_{17}) for its optimum growth (Fig. 3.3 B). The growth of this bacterium on C_{14}-C_{17} increased exponentially during the first 6-18

hours of incubation, reaching 1.0 X 10^8 cfu/ml at 24 hour. During this time the pH dropped from 7.0 to 5.5. The number of viable cells increased slightly over the next time period reaching its maximum value of 5 X 10^8 cfu/ml after 30-48 hours of growth. The pH dropped further to 5.3 during this stationary phase.

In nitrogen limiting MSM with a shorter alkane fraction, C_{12}-C_{13}, the growth of *P. aeruginosa* MB2SA seemed to be biphasic, where an exponential phase was observed between the sixth and the eighteenth hour and a second exponential phase was evident after 30 hours (Fig. 3.3 A). At the same time that the cell number suddenly increased after 30 hours, the pH dropped to about 5.0. This biphasic growth might have been due to the utilization of another substrate by the organism (Marangoni *et al.*, 2001). Findings from this study suggest that in the early hours of growth, the bacterium utilized part of the carbon substrate, since the fraction had two constituents. After 18 hours, the preferred constituent became deficient forcing the bacterium to switch to another carbon source. This can be observed on Figure 3.3 A. Growth rate decreased slightly just before the second phase, suggesting that the organism was forced to switch to another substrate. It is also possible that toxic substances might have accumulated in the medium, which would cause a decrease in growth. In this case, the organism managed to recover by switching on genes that might have assisted in the degradation of these toxic substances due to the presence of membrane transporters called drug resistance efflux pumps in *P. aeruginosa* (Lomovskaya., 2001).

The biphasic growth pattern is not observed in the presence of the C_{14}-C_{17} alkane fraction. Here the viable cell numbers increased exponentially until 24 hours, where it reached a maximum of 5 X 10^8 cfu/ml after 48 hours. A decrease in pH accompanied the cell growth, and over this time period the pH dropped from 7.0 reaching 5.2 by 24 hours. These results suggest that *P. aeruginosa* MB2SA prefers the longer alkane fraction, C_{14}-C_{17}, because the bacterium's growth rate was faster on this substrate compared to when the shorter alkane fraction, C_{12}-C_{13}, was employed as sole carbon source. In addition, the pH of the medium dropped consistently during exponential growth reaching between 5.2 and 5.3 in both alkane fractions, and remaining constant until the end of a 90-hour incubation, implying that the similar acidic products were produced in both instances (Fig. 3.3 A and B).

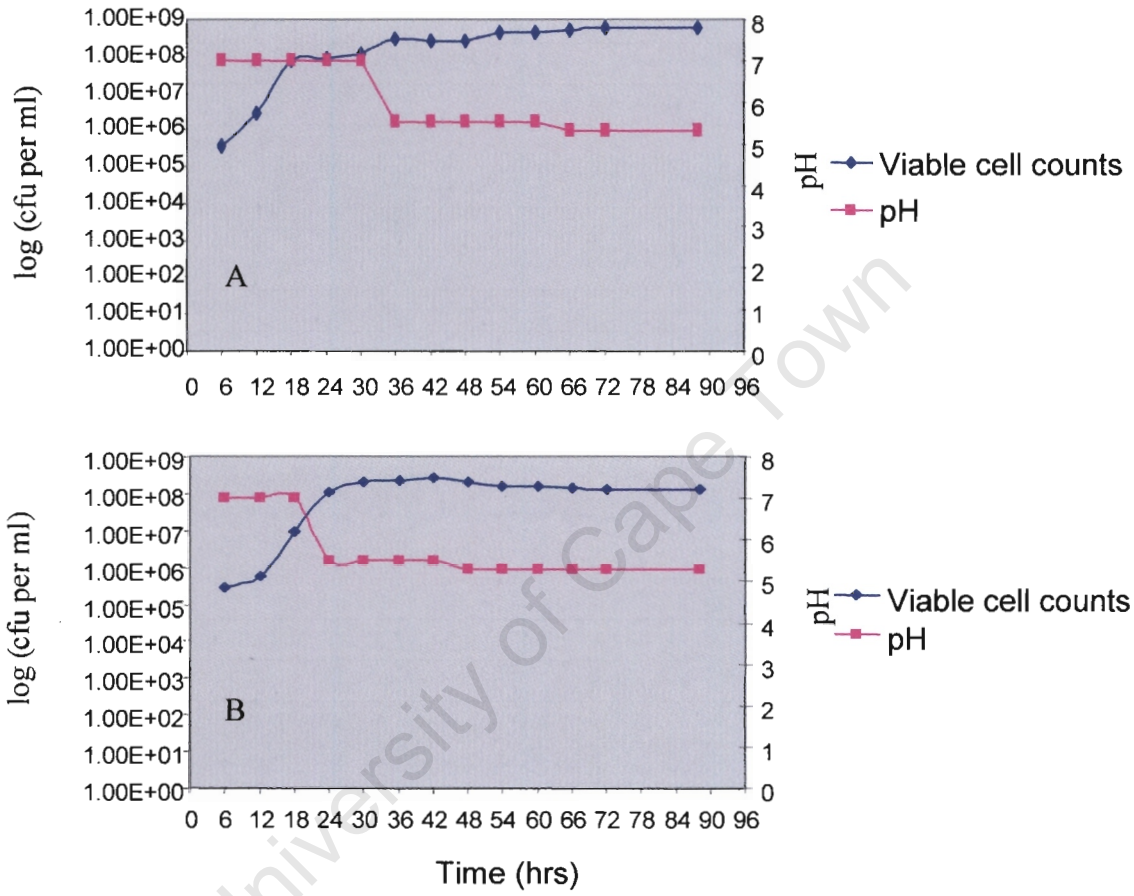


Figure 3.3: Growth curve of *P. aeruginosa* MB2SA grown in a nitrogen limiting MSM with 5 % alkane fractions (C_{12}-C_{13} and C_{14}-C_{17}) as carbon substrate, showing changes in viable cell number at different times. A: C_{12}-C_{13} as a carbon substrate, and B: C_{14}-C_{17} as carbon substrates.

3.3.3 Growth on n-dodecane, n-tetradecane and hexadecane as substrates

The ability of *P. aeruginosa* MB2SA to utilize the pure alkane substrate, n-dodecane (C_{12}), n-tetradecane (C_{14}) and hexadecane (C_{16}) was determined by growth studies in nitrogen limiting MSM with 5 % pure alkanes as sole carbon source (Fig. 3.4 A, B and C). The growth and pH were monitored at 12-hour intervals, for 96 hours; however, it was decreased to 6-hour intervals towards the end of 96 hours to monitor any sudden changes.

P. aeruginosa MB2SA grew well in the medium containing 5 % n-dodecane. The bacterium grew exponentially reaching 1.0 $X10^8$ cfu/ml after 36 hours of incubation at 30 ^{0}C with shaking at 250 rpm. During this time, the pH remained at 7.0. Growth increased slightly to 5 X 10^8 cfu/ml when pH suddenly dropped from 7.0 to 6.0. Stationary phase was reached after 48 hours, and the pH dropped further to 5.0 after 60 hours of incubation with a slight increase in growth to 8 X 10^8 cfu/ml and remained stationary until 96 hours of incubation.

The cell dry weight increased slowly to 0.7 g/L between the 12^{th} and 60^{th} hour of growth with the pH decreasing from 7.0 to 6.0. A maximum dry weight of 2.8 g/L was observed after 72 hours when the pH drastically decreased from 6.0 to 5.0. The dry weight remained stationary at 2.8 g/L for the next 18 hours, and then decreased rapidly from 2.8 to 0.8 g/L after 90 hours (Fig. 3.4 A). It is evident that after 60 hours of growth, the biomass and viable cell number of *P. aeruginosa* MB2SA had increased, while the

production of acidic substances accumulated in the medium caused a pH drop from 6.0 to 5.0 (Fig. 3.4 A). The sudden increase in biomass after 60 hours, which is not accompanied by a similar large increase in viable cells, suggests that the strain, *P. aeruginosa* MB2SA started accumulating PHAs in the cytoplasm of these cells (Fig. 3.4 A). These PHAs would subsequently be used as an alternative energy source (Sheu *et al.*, 2000), indicated by decreased biomass accumulation after 72 hours of growth while the viable cell counts increase (Fig. 3.4 A). A subsequent decrease in biomass after 90 hours implies that the strain might have exhausted most of its carbon substrate, n-dodecane, and started using PHAs which were present within the cells.

Exponential growth of *P. aeruginosa* MB2SA was observed in the first 36 hours when n-tetradecane was used as a carbon substrate, and the pH remained at 7.0 during this time. The pH value suddenly decreased from 7.0 to 5.0 after 48 hours of growth with a slight increase in viable cell counts from 4 X 10^8 to 7 X 10^8 cfu/ml. After 48 hours growth remained stationary at 7 X 10^8 cfu/ml and the pH remained unchanged at 5.0 until the end of this experiment (Fig. 3.4 B).

Dry weight increased slowly from 0.1 to 0.6 g/L between 12 and 36 hours of growth, and then increased significantly to about 2.0 g/L after 48 hours (Fig. 3.4 B). A decrease from 2.0 to 1.1 g/L was observed after 60 hours of growth, which was accompanied by a similar decrease in the viable cell counts suggesting that the strain started using the stored PHAs as energy source (Fig. 3.4 B). This could imply that the strain, *P. aeruginosa* MB2SA, had utilized almost the entire alkane carbon source by 48 hours, causing a

decrease in the viable cell numbers. A switch to utilizing the stored PHAs as an energy source would result in a decrease in biomass (Fig. 3.4 B).

A similar pattern of growth and pH decrease was obtained when *P. aeruginosa* MB2SA was grown on the longer alkane fraction, hexadecane, reaching a maximum value of 8 X 10^9 cfu/ml in 48 hours (Fig 3.4 C). The pH had dropped to 6.0 after 36 hours and further dropped to 5.0 after 48 hours with the viable cell counts increasing from 8 X 10^9 to 9 X 10^9 cfu/ml. The pH remained stationary at the value of 5.0 with the viable cell counts decreasing slightly to 6 X 10^9 cfu/ml for the duration of the experiment.

The cell dry weight reached a maximum value of 1.2 g/L after 36 hours of growth at the pH between 7.0 and 6.0, decreasing gradually when the pH dropped to 5.0 (Fig. 3.4 C). This suggests that the PHAs might have been accumulating when the pH of the medium was at the value of 5.0 and the strain, *P. aeruginosa* MB2SA, immediately started using the stored PHAs as an alternative energy source.

Similar growth patterns were observed when comparing the results obtained when *P. aeruginosa* MB2SA was grown on n-dodecane and the alkane fraction, C_{12}-C_{13} respectively (Figs. 3.3 A and 3.4 A). On both of these carbon sources, a maximum value of 1.0 X 10^8 cfu/ml at the end of exponential growth was reached when the pH was still 7.0.

P. aeruginosa MB2SA reached 1.0 X 10^8 cfu/ml after 18 hours of growth in the alkane fraction, C_{12}-C_{13}, however, the same number of viable cells was only reached after 36 hours when n-dodecane was used as a carbon source. This suggests that the strain grows

faster when the alkane fraction, C_{12}-C_{13}, was utilized as compared to n-dodecane, possibly because of the presence of another longer chain constituent such as C_{13}, in the alkane fraction.

If we consider the longer chain substrates, viable cell counts increased exponentially in 36-40 hours when *P. aeruginosa* MB2SA was utilizing n-tetradecane (Fig. 3.4 B) or hexadecane (Fig. 3.4 C) and in 24 hours with the longer alkane fraction, C_{14}-C_{17} (Fig. 3.3 B). During this time the pH is maintained at 7.0, however, it starts dropping after 18 hours of growth in the longer alkane fraction, C_{14}-C_{17}, and dropped almost to the same value after 36 hours in the pure alkane, n-tetradecane. This suggests that exponential growth of this bacterium is associated with a drop in pH of the medium and that the strain grew much faster in the longer alkane fraction as compared to n-tetradecane or hexadecane. This fast growth might have been enhanced by the presence of other constituents in the longer alkane fraction, C_{14}-C_{17}, such as C_{15}, C_{16} and C_{17}.

The link between exponential growth and the lowering of the pH of the medium is interesting. The pH of the medium dropped from a neutral pH of 7.0 to a slightly acidic pH of 5.5 (Fig. 3.3 A and B). This suggests that one of the products might be carrying an acidic side chain. The change in the acidity of the medium was also noticed when the same organism was grown on the pure alkanes, with definite correlation between rapid growth and drop in pH.

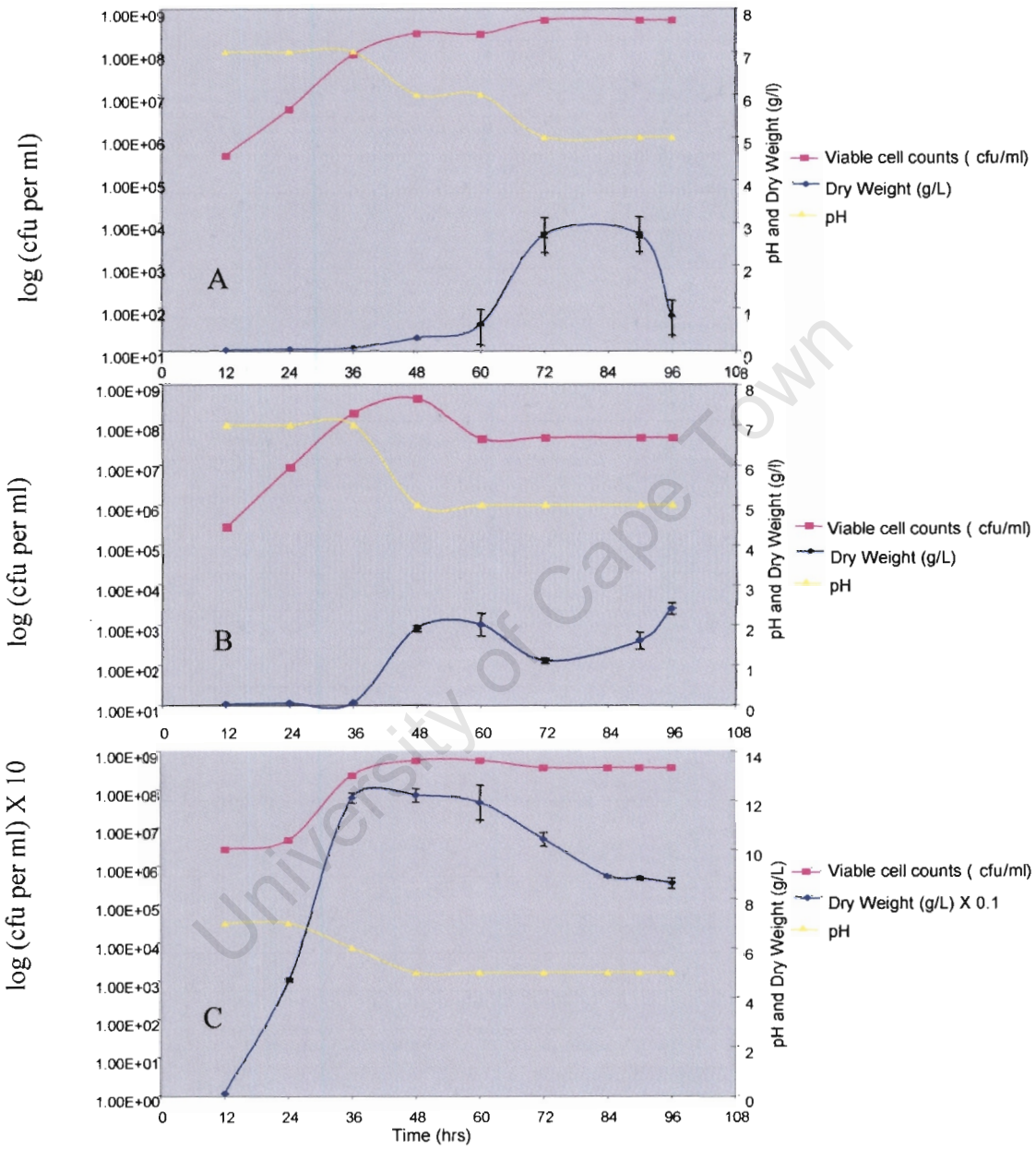


Figure 3.4: Viable cell counts when *P. aeruginosa* MB2SA was grown in a nitrogen limiting minimal salt medium with pure alkanes, n-dodecane, n-tetradecane and hexadecane as carbon substrate. A: 5 % n-dodecane, B: 5 % n-tetradecane and C: 5 % hexadecane as sole carbon source.

Generally, the biomass decreased in the later stages while the viable cell number remained constant, suggesting that the cells were decreasing in weight, rather than dying and lysing. The observation that cells decreased in weight suggested that they have started to use the energy that was stored in the form of PHAs because they had run out of other nutrients in the medium such as the nitrogen since Wang and Lee, (1997) reported that the metabolic rate of the bacteria increased under nitrogen limiting conditions. It would be interesting to quantitate the amount of PHA obtained at different time intervals to confirm whether there is a correlation between decrease in biomass and increase in PHA content.

The bacterial strain produces a wax ester, which floats on the surface of the medium (Fig. 3.1). The wax might hinder a proper mixing of the medium, primarily decreasing the oxygen transfer rate to the cells causing the slower growth rate after many hours of growth. In addition, alkanes are fairly hydrophobic substances and would interfere with oxygen transfer to the cells in the growth medium. *P. aeruginosa* MB2SA is highly aerobic, and the strain may start dying because of oxygen stress, a possible reason for the decreased viable cell counts after 48 hours (Fig. 3.4 B).

3.3.4 PHA production after growth on different substrates

It has been reported that PHA granules accumulate when nutrients such as nitrogen or phosphorus are limiting and excess carbon source (Anderson and Dawes, 1990). In this case nitrogen was limiting at 0.4g $(NH_4)_2SO_4$/L and carbon source was supplied at 5 % v/v.

The bacterial strain, *P. aeruginosa* MB2SA, was allowed to grow for 72 hours in order to accumulate maximum amounts of PHAs (Stockdale *et al.*, 1968). Some environmental isolates namely, CS5, GS28, GG45 and GG47, have been shown to have accumulated PHA after about 72 hours (Sheu *et al.*, 2000). Majority of the new isolates described in this work were Nile Blue A positive. After 72 hours, the cells were harvested and PHAs extracted using the chloroform method (Ballistreri *et al.*, 2001; Brandl *et al.*, 1988).

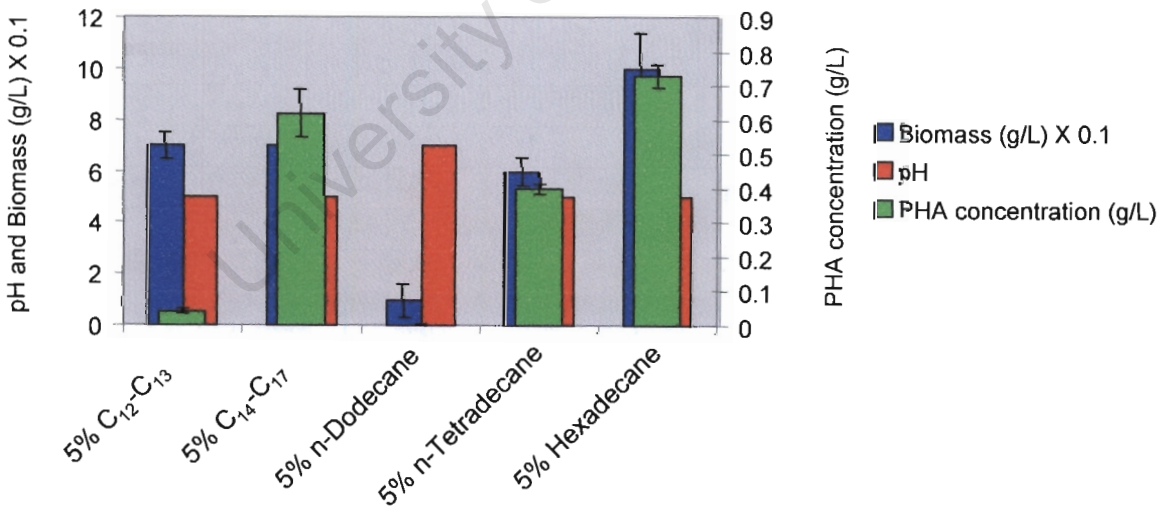


Figure 3.5: Production of biomass and PHA by the environmental isolate, *P. aeruginosa* MB2SA, grown in nitrogen limiting minimal salt medium (MSM) containing various carbon substrates.

P. aeruginosa MB2SA was grown on nitrogen limiting MSM containing different pure alkanes, such as n-dodecane, n-tetradecane and hexadecane, or the alkane fractions, C_{12}-C_{13} or C_{14}-C_{17}. Cultures were incubated at 30 ^{0}C with shaking at 250 rpm for 72 hours, when cells were harvested.

In this instance, poor growth was observed when n-dodecane was utilized as a carbon substrate, resulting in reduced biomass and only one percent (1 %) PHA content, (Table 3.3.1). This result may have been due to the increased culture volume (1 liter as opposed to 1000ml previously). Oxygen transfer to the cells might have been less efficient in the higher volumes (Fig. 3.5). Decreased biomass can also be observed when other alkanes were used in large culture volumes (Fig. 3.5 compared to Fig. 3.4).

In all cases the pH dropped from 7.0 to 5.0 and polyhydroxyalkanoate (PHA) production was high in all the carbon substrates except in the alkane fraction, C_{12}-C_{13}, and n-dodecane, which were 4 and 1 percent respectively (Table 3.3.1). It was at this pH that the production of yellow-green or yellow-brown pigment was produced as previously explained. Production of this pigment could also be used to indicate the period at which PHA is produced. Maximum biomass of 1.0 g/L was produced in a longer alkane, hexadecane, followed by C_{12}-C_{13} and C_{14}-C_{17} both about 0.7 g/L. The least biomass was observed in n-tetradecane and n-dodecane, 0.6 and 0.1 g/L respectively (Fig. 3.5).

The PHA accumulation was comparable in n-tetradecane, hexadecane and C_{14}-C_{17}, and was 63, 68 and 74 percent respectively. The percentage PHA was found to be slightly higher in the longer alkane fraction, C_{14}-C_{17}, than in n-tetradecane and hexadecane, 74 % in C_{14}-C_{17}, 63 % in n-tetradecane and 68 % in hexadecane. However, this experiment

could only be carried out once due to time constrains, and the differences may be due to errors in the biomass measurements or loss of PHA during extraction. *P. aeruginosa* MB2SA clearly grows better with a longer alkane fraction, C_{14}-C_{17}, as a carbon substrate, because the presence of even longer constituent (C_{17}) in the longer alkane fraction, C_{14}-C_{17}, might explain these slight differences (Fig. 3.5). Table 3.3.1 summarizes the PHA production by *P. aeruginosa* MB2SA.

Table 3.3.1: Summary showing biomass, pH and polyhydroxyalkanoate (PHA) produced when the environmental strain, *P. aeruginosa* MB2SA, was grown in different carbon substrates in nitrogen limiting minimal salt medium (MSM), for 72 hours.

5 % Carbon substrate	Biomass (g/L)	pH	PHA concentration (g/L)	PHA content (%)
C_{12}-C_{13}	0.7	5	0.030	4
C_{14}-C_{17}	0.7	5	0.520	74
n-dodecane	0.1	7	0.001	1
n-tetradecane	0.6	5	0.380	63
hexadecane	1.0	5	0.680	68

PHA content was calculated as follows:

PHA content (%) = PHA concentration (g/L) / Biomass (g/L) X 100

3.4 FINAL REMARKS

It is important that the specific growth rate in the first growth phase be high, especially when a toxic carbons source such as alkanes are used. An optimal amount of nutrients and specific carbon substrate concentrations are required to achieve optimal PHA productivity. Zinn (2003) discovered that limiting both the carbon and nitrogen during bacterial growth is a suitable way of producing PHA from a toxic carbon source since the toxicity of the substrate depends on its concentration. The growth rate would not be affected if the substrate is completely metabolized, keeping the toxicity levels of the substrate below threshold (Jung *et al.*, 2001).

The change of pH in the medium during growth could directly affect the growth, which in turn would affect the product production. Specific conditions are required by various bacterial strains for optimum growth (pH 7.0, in this study). PHA production has been shown to change the pH of the medium from 7.0 to about 5.2 (Mahishi *et al.*, 2003), which was also observed in this study. Other strains prefer acidic or basic conditions for their optimum growth and increased product. Environmental isolates used in this study seemed to grow optimally at pH 7.0 and the formation of the product was assumed to be around pH 5.0-5.2, also observed by Mahishi and co-workers (2003), since the cell biomass increased at this point in most cases in this study suggesting that PHA accumulated in the cytoplasm of the *P. aeruginosa* MB2SA between 48 and 72 hours. The organism does not increase in number at below pH 5.0, implying that low pH values affect the optimum growth rate of *P. aeruginosa* MB2SA. The pH might also affect the stability of the product formed (PHAs), and this needs to be investigated.

Temperature also plays a vital role in the survival of the bacterial strains. The environmental strain isolated in this study grew well at 30 ^{0}C. Marangoni *et al.* (2001), showed that the bacterial growth rate of *Ralstonia eutropha* did not change at temperatures, 30 ^{0}C and 34 ^{0}C, however increased temperatures have been shown to hinder PHA production rate in sequencing batch reactor cultures because of increased anabolic rate at high temperatures (Krishna and Van Loosdercht, 1999). In addition, it was shown that the metabolic activity of the cells decreases when high PHA was accumulated (Wang and Lee, 1997; Kim *et al.*, 1994).

This study showed that polyhydroxyalkanoates (PHAs) could be produced from either pure alkanes or petrochemical wastes, such as alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}. These wastes were effectively degraded by an environmental strain, *P. aeruginosa* MB2SA, as low-cost carbon sources, in a nitrogen-limiting medium. Marangoni *et al.* (2001) reported that a nitrogen source in the form of corn-steep liquor gave similar results with regard to specific growth rates when compared to the commercial counter part, ammonium sulphate. This is interesting because the use of a low-cost nitrogen source such as corn-steep liquor together with the petrochemical waste alkane fractions might result in a similar or even better PHA yield, thus producing PHAs at a very low-cost.

The next section of this study concentrates on the *phaC* gene, which encodes the PHA synthase responsible for PHA polymerization. The sequence of the *phaC* gene from *P. aeruginosa* MB2SA will be compared to published *phaC* genes, to determine the closest related genes and to try and predict the nature of the PHA products.

CHAPTER 4

Molecular analysis of the PCR product corresponding to the *phaC* gene encoding PHA synthase

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4 SUMMARY

In this section, the 0.5 kb PCR fragment corresponding to an internal region of the *phaC* gene detected in *P. aeruginosa* MB2SA (Chapter 2), was cloned in pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5-alpha cells. This 0.5 kb internal fragment of *phaC* gene was sequenced using appropriate primers. The deduced amino acid sequence of this gene was compared to the published PHA synthase (PhaC) sequences. The amino acid sequence in *P. aeruginosa* MB2SA had a 98 % identity to both the PhaC in *P. aeruginosa* UCBPP-PA14 and *P. aeruginosa* PAO1. The PHA synthase in *P. aeruginosa* MB2SA had 60.3 % and 98.7 % identity to the amino acid sequences of the PHA synthase 1 and PHA synthase 2 in *P. aeruginosa* PAO1 respectively after carrying out a two-sequence alignment. Therefore, it was concluded that *P. aeruginosa* MB2SA possesses a *phaC* gene fragment encoding the PHA synthase 2 (PhaC2). Restriction enzyme analysis and Southern hybridization of the *phaC* gene region in *P. aeruginosa*

MB2SA revealed that the *phaC* gene lies on chromosomal fragments of a different size to those which occur in *P. aeruginosa* PAO1, suggesting that *P. aeruginosa* MB2SA is distinctly different to other alkane-degrading *Pseudomonas* strains.

4.1 INTRODUCTION

There have been various attempts to increase the amount of PHA biosynthesis by the use of recombinant DNA techniques. The overexpression of the PHA synthase gene (*phaC*) should result in increased yields of PHA, and recombinant *E. coli* strains have been reported to produce high yields of PHA from low-cost carbon substrates, such as sugars (Lee, 1996a, b; Sudesh *et al.*, 2000), decanoate and dodecanoate (Kichise *et al.*, 2002; Langenbach *et al.*, 1997; Park *et al.*, 2002). However, literature has shown that there were some challenges regarding PHA production from other carbon substrates in recombinant *E. coli* strains (Klinke *et al.*, 1999; Rehm *et al.*, 2001; Rehm and Steinbuchel, 2001).

There are advantages of expressing the *phaC* gene in non-virulent, biotechnologically engineered strains, such as *E. coli*.

Although *P. aeruginosa* MB2SA (this study) has some important genetic capabilities enabling this strain to produce PHAs, such as the *phaC* gene, *P. aeruginosa* is recognized as an opportunistic pathogen (Stover *et al.*, 2000). Instead of employing a possible pathogenic strain for PHA production, engineered recombinant strains of bacteria such as *E. coli* could be used to express the *phaC* gene from *P. aeruginosa* MB2SA and accumulate PHAs.

Previous studies have shown that when the *tes*A gene which codes for a modified *E. coli* thioesterase, together with PHA synthase gene (*phaC1*) from *Pseudomonas oleovorans*, were co-expressed in *E. coli* (Nomura *et al.*, 2004), large amounts of PHA accumulated (Klinke *et al.*, 1999). Co-expression of the thioesterase, *tes*A, and the *phaC1* genes from *Umbellularia californica* and *P. oleovorans* respectively, in *E. coli* showed different PHAs being synthesized (Klinke *et al.*, 1999). Another investigation was carried out where the *phaG* gene coding for a putative transacylase from *Pseudomonas putida*, and the *phaC1* gene from *P. aeruginosa* were expressed together in *E. coli* in the presence of the enoyl-ACP reductase inhibitor, triclosan. This gave yields of 2 to 3 % (cellular dry weight) poly-3-hydroxydecanoate homopolymer (Rehm *et al.*, 2001).

The aim of this experiment was to clone the *phaC* PCR fragment from the environmental strain, *P. aeruginosa* MB2SA, sequence and compare it to already published *phaC* gene sequences to deduce whether the *phaC* gene fragment detected in this study is novel, and whether it would be useful as an alternative *phaC* gene that could be expressed in other harmless bacterial strains such as recombinant *E. coli* (Nomura *et al.*, 2004).

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strain, plasmid, and culture conditions

The environmental strain, *Pseudomonas aeruginosa* MB2SA (this study), was isolated from crude oil-contaminated soil, Milnerton Caltex Refineries, Cape Town, South Africa. This strain was maintained in 20 % glycerol with appropriate alkane fractions at -70 ^{0}C. The bacterial strain was activated by thawing the glycerol stock culture and was grown on minimal salt medium agar with appropriate alkane fractions or nutrient agar at 30 ^{0}C, overnight, and used as inoculum. *P. aeruginosa* MB2SA was cultured in 100 ml nutrient broth at 30 ^{0}C in a shaker incubator at 250 rpm, overnight.

PCR of the internal fragment of the *phaC* gene using the primers phaCF1 and phaCR4 (Sheu *et al*., 2000) was carried out as in Chapter 2. The pGEM-T Easy vector system was employed in this study as a cloning host, following the instructions in the manufacture's technical manual (Promega). The pGEM-T Easy vector containing the 0.5 kb PCR product was transformed into an *E.coli* DH5-alpha strain. The transformants were routinely grown at 37 ^{0}C on X-gal (LB/ampicillin/IPTG/X-gal) plates containing ampicillin (100 µg/ml) for plasmid selection.

4.2.2 DNA extractions and general DNA manipulations

4.2.2.1 Chromosomal and larger-scale plasmid DNA extractions

P. aeruginosa MB2SA was grown in 100 ml nutrient broth at 30 ^{0}C in a shaker incubator, for 16-18 hours. The cells were centrifuged at 5000 rpm at 4 ^{0}C for 10 minutes. The supernatant was discarded. Cells were resuspended in 10 ml TE, (10 mM Tris, pH 8; 1 mM EDTA, pH 8). The cells were collected by centrifugation, 5000 rpm, at 4 ^{0}C for 10 minutes. The pellet was resuspended in 10 ml lysis solution, which contained, 9.5 ml TE (pH 8), 0.5 ml of 10 % SDS, 50 µl of 20 mg/ml proteinase K and a pinch of dry lysozyme. The cells were mixed gently. The mixture was incubated for an hour in a waterbath at 37 ^{0}C. After incubation, 5 ml of saturated NaCl was added to the mixture, which was mixed gently by inverting, and then centrifuged at 18000 rpm, at room temperature for 15 minutes. The supernatant was transferred into a fresh tube. Proteins and RNA were removed by treatment with phenol:chloroform, and with ribonuclease A (Sigma) respectively. Protein-free DNA was precipitated by adding 2.5 volume of 100 % cold ethanol into the supernatant, mixed gently and incubated at –20 ^{0}C for an hour. DNA was collected by centrifugation at 5000 rpm, at 4 ^{0}C for 30 seconds and dried carefully. The chromosomal DNA was resuspended in sterile distilled water and stored at 4 ^{0}C. All the large-scale (midi-prep) plasmid DNA extractions were carried out using the Plasmid MAXI Prep Kit (Quagen) following the manufacturer's instructions.

4.2.2.2 Small-scale (mini-prep) plasmid DNA extractions

The recombinant *E. coli* DH5-alpha cells were grown in 5 ml LB with appropriate antibiotic selection at 37 ^{0}C with subsequent shaking at 250 rpm for 16-18 hours. Two milliliters (2 ml) culture was centrifuged at 8000 rpm for 30 seconds. The supernatant was discarded. The cell pellet was resuspended in 300µl TENS [1M Tris-Cl (pH 8), 0.5M EDTA (pH 8), 10N NaOH and 25 % SDS] and 150µl of 3M sodium acetate at pH 5.4 was added, mixed well briefly and microfuged for 5 minutes. Proteins in the DNA solution were removed by treatment with ribonuclease A (Sigma) or by phenol:chloroform treatment. The supernatant was transferred into a new microfuge tube where 0.9 ml of 100 % ethanol was added and incubated at –20 ^{0}C for 10 minutes. The mixture was microfuged for 30 minutes at room temperature and the pellet was washed with 0.3 ml of 70 % ethanol. The DNA was dried carefully, resuspended in 30µl sterile distilled water and stored at 4 ^{0}C. For sequencing of the *phaC* gene, plasmid DNA extraction was carried out using the Perfectprep Mini Plasmid Isolation Kit (Eppendorf) as described by the manufacturer.

Restriction endonuclease digestions and cloning procedures were performed according to the manufacturer's recommendations (Promega). The DNA fragment of interest to be sequenced was gel purified using High Pure PCR Product Purification Kit (Roche) following the manufacturer's instruction manual. All DNA gel fractionations were done in 0.8 % w/v agarose gels using Tris-Acetate EDTA buffer.

4.2.3 Competent cells preparation and transformation

Competent *E. coli* DH5-alpha cells were prepared by $CaCl_2$ shock treatment. The strain was grown overnight (16-24 hours) in 200 ml 2XYT broth in a standard container at 37 ^{0}C with subsequent shaking at 150 rpm. Overnight culture was diluted to 25, 50 or 100 ml in 2xYT broth in flasks at 10x culture volume (i.e. 25 ml in 250 ml flask etc.) and grown to early log phase (OD_{600} = 0.3-0.6). Cells were collected by centrifugation at 5000xg for 5 minutes at 4 ^{0}C. The cells were kept ice cold in all steps. Cells were resuspended in ice cold 0.1M $MgCl_2$ (1 culture volume) and left on ice for about 1 minutes.

Cells were collected by centrifugation as before and resuspended in ½ culture volume of 0.1M $CaCl_2$. The culture was incubated on ice for a minimum of 30 minutes, preferably 1-2 hours. Cells were collected by centrifugation at 5000xg for 5 minutes at 4 ^{0}C and gently resuspended in 1/10 culture volume 0.1M $CaCl_2$. To transform, 0.1 ml aliquots were mixed with DNA (1-10ng) and left on ice for 10 minutes and heat shocked at 42 ^{0}C for 2 minutes or 37 ^{0}C for 5 minutes. For expression, 0.9 ml 2xYT broth was added to the cells and incubated at 37 ^{0}C for 30-60 minutes before plating. The competent DH5-alpha cells were stored by adding 1 volume ice-cold sterile glycerol, mixed and frozen at -70 ^{0}C.

For transformation of plasmid ligations, approximately 50µl frozen *E. coli* DH5-alpha cells was defrosted and kept on ice throughout. The entire ligation (i.e. pGEM-T Easy vector containing the 0.5 kb PCR fragment) mix was transferred into the DH5-alpha cells at the ratio of 1:10. The mixture was left on ice for 25 minutes. The cells were heat shocked at 37 ^{0}C for 4 minutes and cooled on ice for 1 minute. LB (Luria Broth) was added to 1 ml total volume and incubated at 37 ^{0}C for ½-1hour. Hundred microliters (100 µl) was plated onto duplicated LB/ampicillin/IPTG/X-Gal plates. The rest of the cells were centrifuged at 8000 rpm for 1minute, resuspended in 200µl LB and 100µl was plated on each of the two plates for high number of colonies. Plates were incubated at 37 ^{0}C, for 16-24 hours.

4.2.4 Southern hybridization

Southern hybridization was carried out using the non-radioactive DIG DNA-labeling and detection kit (Boehringer Mannheim), following the manufacturer's instructions. Plasmid and chromosomal DNA were digested to completion with the appropriate restriction endonucleases, and fractioned by gel electrophoresis. The DNA was transferred by capillary action onto a Hybond-N+ nylon hybridization transfer membrane (Amersham Pharmacia Biotech) and fixed accordingly. DNA fragments selected as probes were gel purified, and labeled with Digoxigenin-11-dUTP by random primed labeling as described in the instruction manual (Boehringer Mannheim). Hybridization was performed using denatured DIG-labeled probes at 68 ^{0}C overnight. Membranes were washed under stringent conditions, followed by chemiluminescent detection with disodium 3-(4-

methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4- yl) phenyl phosphate (CSDP) (Roche).

4.2.5 Sequencing and analysis of a 0.5 kb PCR fragment

The PCR fragment was initially cloned into pGEM-T Easy vector (Promega) and then sequenced using T7 promoter primer or the pUC/M13 forward primer (Promega). The generated DNA fragment was analyzed on NCBI Database by comparing it with the published sequences, using the BLAST programme. DNA analysis was performed using DNAMAN (Lynnon Biosoft).

4.3 RESULTS AND DISCUSSION

4.3.1 Cloning of the PCR fragment corresponding to the *phaC* gene

The PCR primers designed by Sheu *et al.* (2000), were used to amplify a 0.5 kb internal fragment of the *phaC* gene of *P. aeruginosa* MB2SA (see Chapter 2). The 0.5 kb PCR fragment corresponding to the *phaC* gene was cloned into pGEM-T Easy vector (Fig. 4.4.1), following the manufacturer's technical manual (Promega) and transformed into *E.coli* DH5-alpha. The recombinants were screened by growing them on X-gal (blue/white colony selection) plates for the presence of the PCR fragment.

After the small-scale (mini-prep) plasmid DNA extraction from two blue-colored and six white-colored colonies, the plasmid DNAs were digested with *Eco*RI to release the 0.5 kb (PCR fragment) insert from the pGEM-T Easy vector (Fig. 4.4.2).

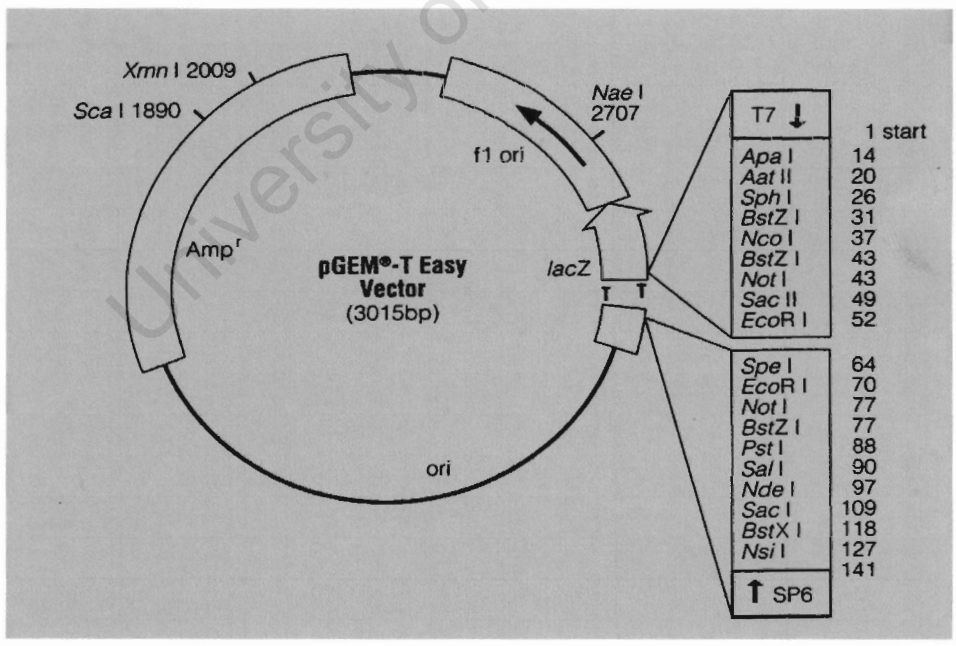


Figure 4.4.1: pGEM-T Easy vector used for cloning *phaC* PCR fragment detected from *P. aeruginosa* MB2SA.

The correct PCR fragment was observed in lane 8, 9, 12 and 13 (Fig. 4.4.2). The sizes of these four DNA fragments were confirmed to be of the same size as the 0.5 kb PCR fragment corresponding to the internal fragment of the *phaC* gene in lane 6, shown black arrow (Fig. 4.4.2). An interesting observation was that the cloned PCR fragment corresponding to the *phaC* gene was present not only in white colonies (lane 9, 12 and 13, Fig. 4.4.2.) but also in a blue colony (lane 8, Fig. 4.4.2), suggesting that in this case, the inserted DNA was in frame to the *lacZ* gene, resulting in the correct translation of the *lacZ* gene. Two bands were observed in lane 10 (Fig. 4.4.2), suggesting that an arbitrary fragment containing an *Eco*RI site had been cloned in this recombinant, giving two bands.

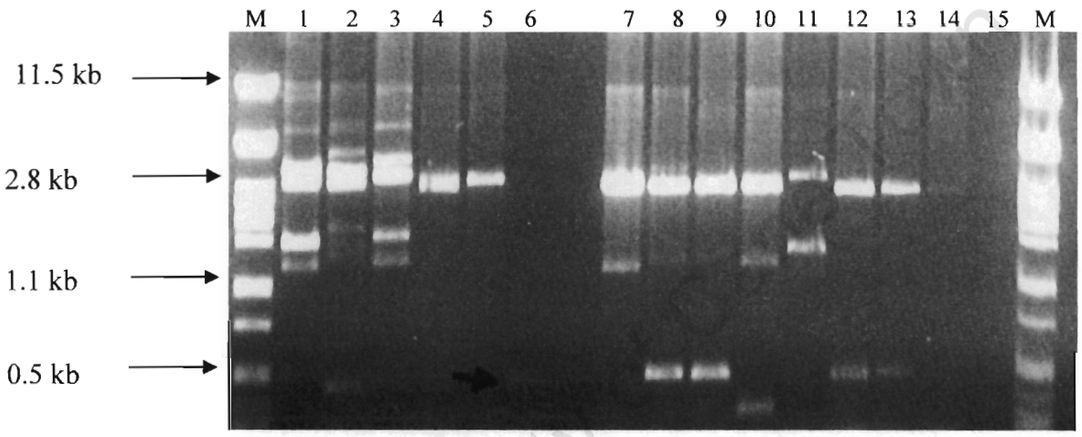


Figure 4.4.2: Plasmid mini-preps of *E.coli* DH5-alpha containing the 0.5 kb PCR fragment corresponding to the *phaC* gene cloned into pGEM-T Easy vector. Lane M: Molecular marker, λ *Pst*I, 1-2: Recombinant plasmid DNA from blue colonies not digested with *Eco*RI, 3-5: Recombinant plasmid DNA from white colonies not digested with *Eco*RI, 6: PCR fragment corresponding to the *phaC* gene, 7-8: Recombinant plasmid DNA from blue colonies digested with *Eco*RI, 9-14: Recombinant plasmid DNA from white colonies digested with *Eco*RI, 15: PCR fragment corresponding to the *phaC* gene digested with *Pst*I.

4.3.2 Analysis of the cloned PCR fragment

An *E. coli* recombinant colony containing the 0.5 kb fragment was grown in 5 ml of LB with appropriate antibiotic selection at 37 ^{0}C overnight. Plasmid DNA was extracted employing the Perfectprep Mini Plasmid Isolation Kit (Eppendorf). An appropriate plasmid DNA concentration was digested with *Eco*RI to show the presence of the 0.5 kb insert (PCR fragment) before sequencing. The gel-purified plasmid DNA was sequenced in both directions using the T7 promoter primer or the M13 forward primer (Promega), using the MegaBace DNA sequencer (Department of Molecular and Cellular Biology, UCT).

The deduced amino acid sequence of the 0.5 kb fragment was analyzed by comparing it with the published DNA sequences on NCBI Database. The top four (4) amino acid sequences, which show similarity to the cloned 0.5 kb PCR fragment, are shown in Table 1.

The deduced amino acid sequence from the cloned 0.5 kb PCR fragment was observed to be very similar to the PHA synthetase in *P. aeruginosa* UCBPP-PA14 and PHA synthase 2 in *P. aeruginosa* PAO1, both at 98 % identity. In addition, the 0.5 kb PCR fragment showed significant identity to the PHA synthase 2 in *P. resinovorans* and *Pseudomonas* sp. 61-3 at 77 % and 76 % respectively (Table 1). There are two PHA synthase proteins present in *P. aeruginosa* PAO1 reported by Timm and Steinbuchel (1990), namely, PhaC1 and PhaC2. To verify the type of PHA synthase encoded in the fragment isolated from *P. aeruginosa* MB2SA, its protein sequence (*P. aeruginosa* MB2SA) was compared to both the PhaC1 (Fig. 4.4.3 A) and PhaC2 (Fig. 4.4.3 B) protein sequences of *P.*

aeruginosa PAO1 using two-sequence alignments on DNAMAN program (Lynnon Biosoft). The protein sequence from *P. aeruginosa* MB2SA was confirmed to be more similar to PhaC2 (Fig. 4.4.3 B) from *P. aeruginosa* PAO1 (98.67 %), and only showed 60.26 % identity to the deduced PhaC1 protein (Fig. 4.4.3A). Therefore it was concluded that the *phaC* gene fragment cloned from *P. aeruginosa* MB2SA (Chapter 2) encodes the PhaC2 protein responsible for the PHA polymerization. In addition, these results confirm that the environmental strain isolated in this study is indeed *P. aeruginosa* as discussed in Chapter 2 and also that the deduced protein sequence from *P. aeruginosa* MB2SA was proven to be closely related to the protein sequences of the two *P. aeruginosa* strains (Table 1). It is possible that a second *phaC* gene, encoding the PhaC1 synthase, is also present in the genome of *P. aeruginosa* MB2SA, but has not been detected due to only one recombinant clone being sequenced. More constructs could be sequenced to determine whether this is the case.

Table 1: Four sequences, producing significant alignments with the deduced amino acid sequence of the 0.5 kb PCR fragment amplified from *P. aeruginosa* MB2SA.

Sequences producing significant alignments:	**Identity(%)**
gi[32044433]ref[ZP_00141534.1] *Pseudomonas aeruginosa* UCBPP-PA14, PHA synthetase	98
gi[15600251]ref[NP_253745.1] *Pseudomonas aeruginosa* PAO1, PHA synthase 2	98
gi[10835920]gb[AAD26367.2] *Pseudomonas resinovorans*, PHA synthase 2	77
gi[4062970]dbj[BAA36202.1] *Pseudomonas* sp. 61-3, PHA synthase 2	76

The PhaC2 protein from *P. aeruginosa* MB2SA (this study) was compared to other published PHA synthase proteins to determine whether it is novel or not by doing a multiple sequence alignment (Fig. 4.4.4). Most of the amino acid residues are conserved

in all the PHA synthases from different bacteria (Steinbüchel and Hein, 2001), including those of the *Pseudomonas* species and *Ralstonia metallidurans*, which is a different species used for comparison in this study (Fig. 4.4.4 shown by shaded areas). The amino acid residues shown in navy-blue areas are highly conserved amino acid regions present in all the bacterial strains used in this study (represented by the consensus sequence at the bottom), the ones shown in purple areas are less conserved followed by the ones shown in sky-blue areas which are least conserved (Fig. 4.4.4). Steinbüchel and Hein, (2001) observed a similar type of a pattern obtained in Figure 4.4.4. When taking a closer look at the PHA synthase 2 identified in *P. aeruginosa* MB2SA (this study) and the PHA synthase 2 in *P. aeruginosa* PAO1, it was observed that there are differences that may suggest that the PHA synthase 2 in *P. aeruginosa* MB2SA (this study) might be novel. When comparing these two *P. aeruginosa* strains, *P. aeruginosa* PAO1 and *P. aeruginosa* MB2SA, and the amino acid residue I (Ile) in *P. aeruginosa* PAO1 has been replaced by V (Val) in *P. aeruginosa* MB2SA at position 2. In addition, R (Arg) replaced by S (Ser), W (Trp) replaced by G (Gly) and N (Asn) replaced by D (Asp) at positions 123, 152 and 154 respectively (Fig. 4.4.4).

```
Fast alignment of protein sequences Mb2sa and phaC1

Ktuple=2  Gap_penalty=4

Upper line: Mb2sa, from 1 to 152
Lower line: phaC1, from 229 to 379

Mb2sa:phaC1 identity= 60.26%(91/151) gap=0.66%(1/152)
1     YVFDLSPEKSFVQYALKNNLQVFVISWRNPDAQHREWGLSTYVEALDQAIEVSREITGSR
      ||||||| ||     | |  | |  |||||    ||||| || |||  ||||   ||||
229   YVFDLSPDKSLARFCLRNGVQTFIVSWRNPTKSQREWGLTTYIEALKEAIEVVLSITGSK

61    SVNLAGACAGGLTVAALLGHLQVRRQLRKVSSVTYLVSLLDSQMESPAMLFADEQTLESS
        || ||| || | | | ||         ||    | ||| ||         ||||| |||
289   DLNLLGACSGGITTATLVGHYVASGEK.KVNAFTQLVSVLDFELNTQVALFADEKTLEAA

121   KRSSYQHGVLDGRDMAKVFAWMRPNDLIWNYG
      || ||| ||| | ||||||||||||||||
348   KRRSYQSGVLEGKDMAKVFAWMRPNDLIWNYW
```

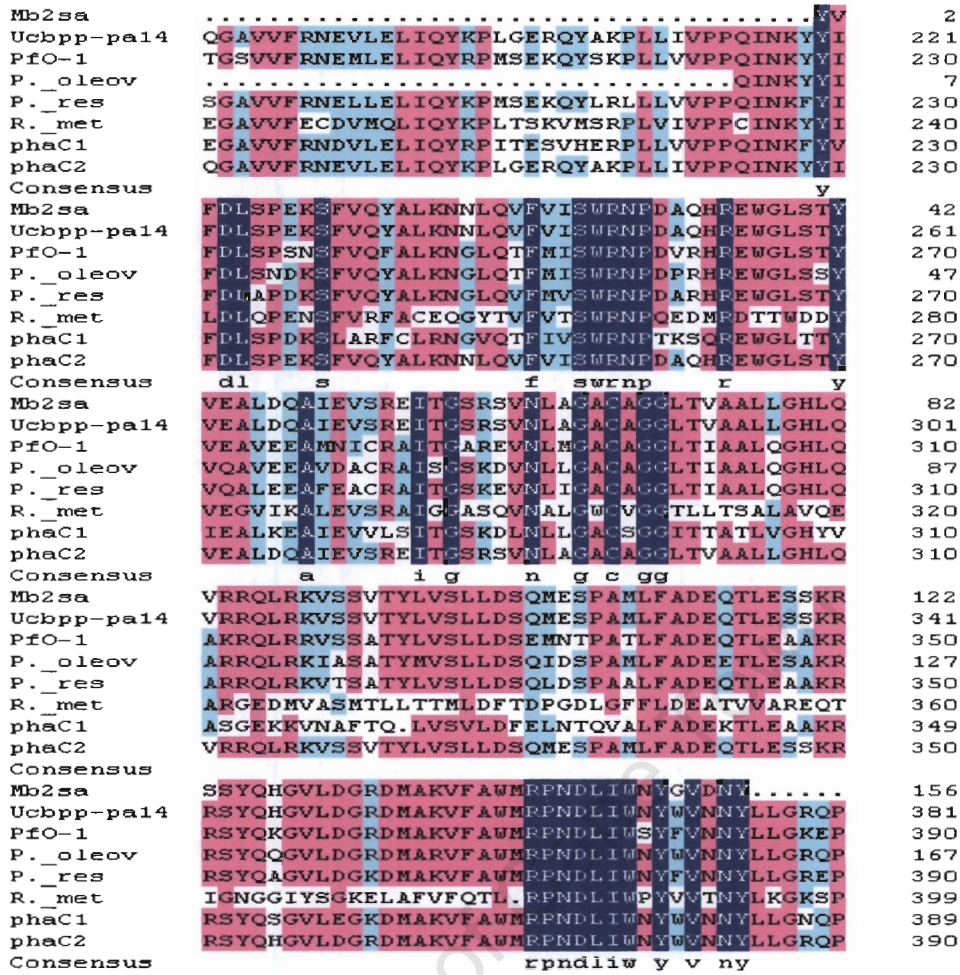
A

```
Fast alignment of protein sequences Mb2sa and phaC2

Ktuple=2  Gap_penalty=4

Upper line: Mb2sa, from 3 to 152
Lower line: phaC2, from 231 to 380

Mb2sa:phaC2 identity= 98.67%(148/150) gap=0.00%(0/150)
3     FDLSPEKSFVQYALKNNLQVFVISWRNPDAQHREWGLSTYVEALDQAIEVSREITGSRSV
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
231   FDLSPEKSFVQYALKNNLQVFVISWRNPDAQHREWGLSTYVEALDQAIEVSREITGSRSV

63    NLAGACAGGLTVAALLGHLQVRRQLRKVSSVTYLVSLLDSQMESPAMLFADEQTLESSKR
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
291   NLAGACAGGLTVAALLGHLQVRRQLRKVSSVTYLVSLLDSQMESPAMLFADEQTLESSKR

123   SSYQHGVLDGRDMAKVFAWMRPNDLIWNYG
       ||||||||||||||||||||||||||||
351   RSYQHGVLDGRDMAKVFAWMRPNDLIWNYW
```

B

Keys: Mbsa, *P. aeruginosa* MB2SA internal fragment of the *phaC* gene (this study); phaC1, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthase 1 (NP_253743); and phaC2, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthetase 2 (NP_253745).

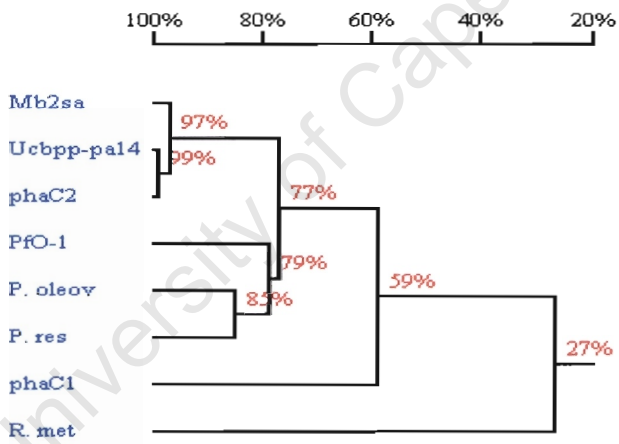
Figure 4.4.3: Two-sequence alignments comparing the amino acid sequence in *P. aeruginosa* MB2SA to the two PHA synthases identified in *P. aeruginosa* PAO1. A, PHA synthase 1 (PhaC1) and B, PHA synthase 2 (PhaC2).



Keys: Mbsa, *P. aeruginosa* MB2SA internal fragment of the *phaC* gene (this study); Ucbpp-pa14, *P. aeruginosa* UCBPP-PA14 poly (3-hydroxyalkanoate) synthetase (ZP_00141534); PfO-1, *P. fluorescens* PfO-1 poly (3-hydroxyalkanoate) synthetase (ZP_00084251); P. oleov, *P. oleovorans* PHA synthase 2 (AAK06657); P. res, *P. resinovorans* PHA synthase 2 (AAD26367); R. met, *R. metallidurans* poly (3-hydroxyalkanoate) synthetase (ZP_00022376); phaC1, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthase 1 (NP_253743); and phaC2, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthetase 2 (NP_253745).

Figure 4.4.4: Multiple sequence alignment comparing the PhaC amino acid sequences from different bacterial strains that are related to *P. aeruginosa* MB2SA. The *R. metallidurans* was included in this comparison so as to identify the most conserved amino acid regions in all the bacteria possessing the PhaC proteins.

The PHA synthase 2 in *P. aeruginosa* MB2SA is closely related to the one present in *P. aeruginosa* UCBPP-PA14 and *P. aeruginosa* PAO1, at 97.4 % and 97.4 % respectively, as shown by the homology tree (Fig. 4.4.5) and homology and distance matrix (Fig. 4.4.6), although slightly different from other *Pseudomonas* species namely, *P. oleovorans*, *P. resinovorans* and *P. fluorescens* PfO-1, at 75.6 %, 79.5 % and 76.3 % respectively (Figs. 4.4.5 and 4.4.6). The PhaC1 in *P. aeruginosa* PAO1 was observed to be less related to the PhaC2 in *P. aeruginosa* MB2SA at 58.7 % (Figs. 4.4.5 and 4.4.6). *R. metallidurans* PHA synthase showed a least relatedness to PhaC2 in *P. aeruginosa* MB2SA, as shown at the bottom of the homology tree (Fig. 4.4.5) and at 31.6 % (Fig. 4.4.6).



Keys: Mbsa, *P. aeruginosa* MB2SA internal fragment of the *phaC* gene (this study); Ucbpp-pa14, *P. aeruginosa* UCBPP-PA14 poly (3-hydroxyalkanoate) synthetase (ZP_00141534); PfO-1, *P. fluorescens* PfO-1 poly (3-hydroxyalkanoate) synthetase (ZP_00084251); P. oleov, *P. oleovorans* PHA synthase 2 (AAK06657); P. res, *P. resinovorans* PHA synthase 2 (AAD26367); R. met, *R. metallidurans* poly (3-hydroxyalkanoate) synthetase (ZP_00022376); phaC1, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthase 1 (NP_253743); and phaC2, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthetase 2 (NP_253745).

Figure 4.4.5: The homology tree comparing the PHA synthases from different *Pseudomonas* strains and *R. metallidurans*, which is of different species was included to compare with the other bacterial strains carrying the PHA synthases.

```
Homology matrix of 8 sequences
Mb2sa                100%
Ucbpp-pa14           97.4% 100%
PfO-1                76.3% 73.0% 100%
P._oleov             75.6% 80.0% 80.6% 100%
P._res               79.5% 76.8% 77.1% 85.3% 100%
R._met               31.6% 26.9% 26.1% 32.0% 25.3% 100%
phaC1                58.7% 58.7% 55.5% 62.1% 59.6% 23.9% 100%
phaC2                97.4% 99.5% 72.5% 80.0% 76.4% 26.6% 58.0% 100%

Distance matrix of the 8 sequences
Mb2sa                0
Ucbpp-pa14           0.026 0
PfO-1                0.237 0.270 0
P._oleov             0.244 0.200 0.194 0
P._res               0.205 0.232 0.229 0.147 0
R._met               0.684 0.731 0.739 0.680 0.747 0
phaC1                0.413 0.413 0.445 0.379 0.404 0.761 0
phaC2                0.026 0.005 0.275 0.200 0.236 0.734 0.420 0
```

Keys: Mbsa, *P. aeruginosa* MB2SA internal fragment of the *phaC* gene (this study); Ucbpp-pa14, *P. aeruginosa* UCBPP-PA14 poly (3-hydroxyalkanoate) synthetase (ZP_00141534); PfO-1, *P. fluorescens* PfO-1 poly (3-hydroxyalkanoate) synthetase (ZP_00084251); P. oleov, *P. oleovorans* PHA synthase 2 (AAK06657); P. res, *P. resinovorans* PHA synthase 2 (AAD26367); R. met, *R. metallidurans* poly (3-hydroxyalkanoate) synthetase (ZP_00022376); phaC1, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthase 1 (NP_253743); and phaC2, *P. aeruginosa* PAO1 poly (3-hydroxyalkanoate) synthetase 2 (NP_253745).

Figure 4.4.6: Homology and distance matrixes comparing different bacterial strains possessing the PHA synthase.

4.3.3 Restriction site analysis on the PCR fragment

Restriction enzyme analysis was performed on the 0.5 kb DNA sequence using DNAMAN (Lunnon Biosoft). This gene fragment contains different restriction enzyme sites, which might be useful for cloning and further analysis of the *phaC* gene. In addition, two (2) *Pst*I restriction enzyme sites were observed within the *phaC* gene (underlined in black, Fig. 4.4.7). To confirm the presence of these sites, the PCR fragment corresponding to the *phaC* gene was digested with *Pst*I (lane 15, Fig. 4.4.2) and compared to lane 6, containing the 0.5 kb undigested PCR fragment (Fig. 4.4.2). The absence of the 0.5 kb fragment in lane 15 (Fig. 4.4.2) implies that the *Pst*I restriction

enzyme digested the PCR fragment into smaller fragments that could not be visualized on a 0.8 % agarose gel (Fig. 4.4.2, lane 15).

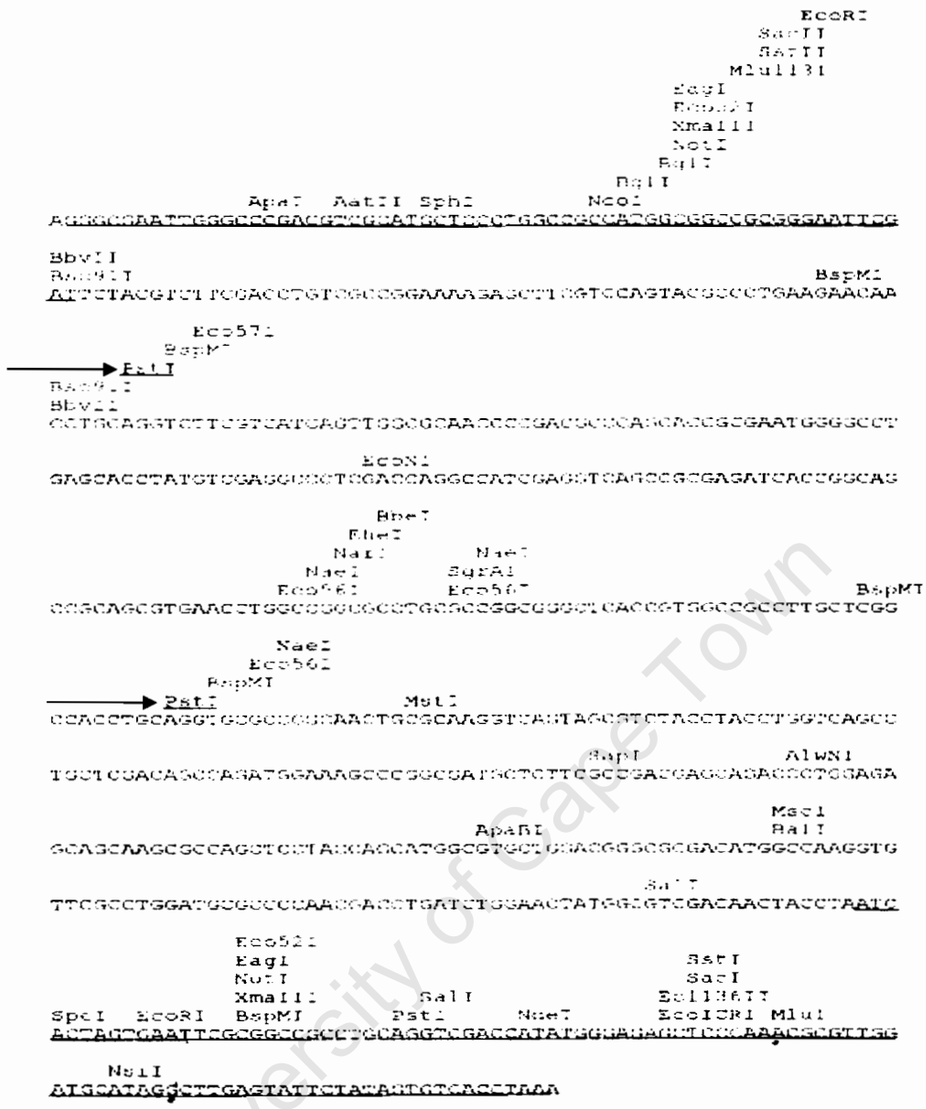


Figure 4.4.7: Restriction site analysis of the 0.5 kb cloned PCR fragment. Two *Pst*I restriction enzyme site located within the *phaC* gene fragment are shown with black arrows. The underlined sequence represents part of the pGEM-T Easy vector where the *phaC* gene fragment was cloned from *P. aeruginosa* MB2SA.

4.3.4 Southern Hybridization

To confirm that the 0.5 kb DNA fragment successfully cloned in section 4.3.1, did in fact originate from *P. aeruginosa* MB2SA, Southern hybridization of the chromosomal DNA isolated from *P. aeruginosa* MB2SA was performed using the 0.5 kb insert as a probe. *P. aeruginosa* MB2SA chromosomal DNA was extracted using the chromosomal DNA extraction method and digested with eight commercial restriction enzymes, *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pvu*II, *Xba*I, *Sma*I and *Xho*I, which cut outside the 0.5 kb PCR fragment corresponding to *phaC* gene. Of the eight, *Pvu*II, *Sma*I and *Xho*I digested the chromosomal DNA sufficiently for hybridization analysis. The chromosomal DNA digested with *Pvu*II, *Sma*I and *Xho*I was gel fractioned on a 0.8 % agarose (Fig. 4.4.8). The recombinant plasmid, pGEM-T Easy containing the 0.5 kb PCR fragment was isolated from the relevant host using the Plasmid MAXI Prep. Kit (Quagen) and digested with *Eco*RI to release the 0.5 kb insert (Fig. 4.4.8), containing the internal fragment of the *phaC* gene to use as a probe.

After PCR, the 0.5 kb fragment was purified by gel electrophoresis and was randomly labeled using non-radioactive DIG DNA-labeling (Roche). This probe was used in Southern hybridization to detect the complete *phaC* gene located on the chromosomal DNA of our environmental strain, *P. aeruginosa* MB2SA, using the DIG detection system (Roche).

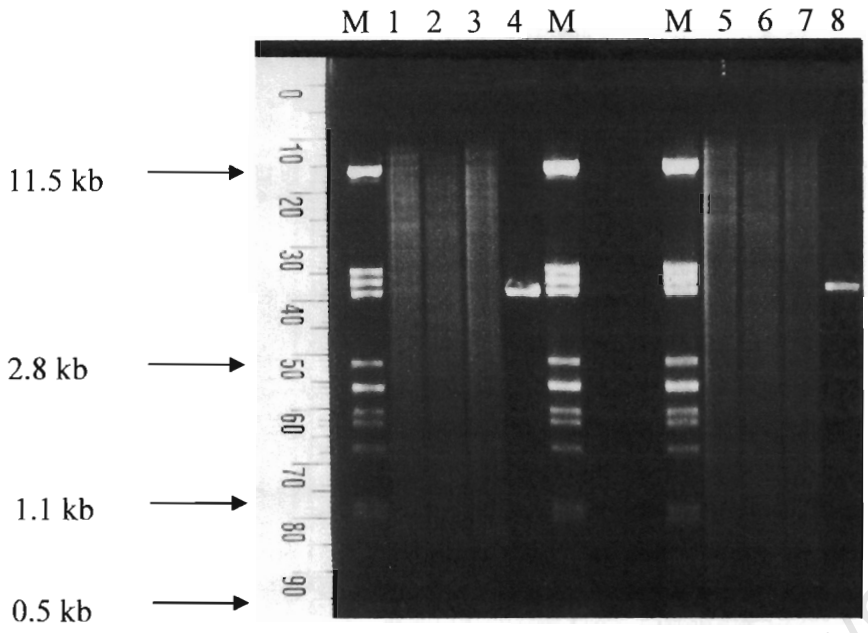


Figure 4.4.8: *P. aeruginosa* MB2SA chromosomal DNA digested with *Pvu*II, *Sma*I, and *Xho*I, for Southern hybridization. Lane 1 and 5: Chromosomal DNA digested with *Pvu*II; 2 and 6: Chromosomal DNA digested with *Sma*I; 3 and 7: Chromosomal DNA digested with X*ho*I; 4 and 8: pGEM-T Easy vector containing a 0.5 kb insert digested with *Eco*RI, M: Molecular marker, λ *Pst*I.

Different sized signals were detected in the chromosomal DNA digested with all three different restriction enzymes (Fig. 4.4.9), confirming that the *phaC* gene encoding PHA synthase responsible for PHA synthesis is present in *P. aeruginosa* MB2SA. The DIG-labeled probe identified a band at 8.0 kb in the chromosomal DNA digested with *Pvu*II, a *Sma*I fragment at 1.4 kb and a *Xho*I fragment at 7.7 kb (Fig. 4.4.9). All these signals represent the *phaC* gene located within the chromosomal DNA of the environmental strain, *P. aeruginosa* MB2SA. Different restriction enzymes digested the chromosomal DNA at different sites hence different DNA signal sizes observed after Southern hybridization (Fig. 4.4.9), however these sizes cannot be confirmed until further DNA sequence of these regions is obtained. Analysis of the DNA sequence of *P. aeruginosa* PAO1 indicates that the *phaC2* gene would lay on DNA fragments of 4.2 kb and 7.0 kb

for *Pvu*II and *Xho*I respectively. This is further proof that *P. aeruginosa* MB2SA is not identical to *P. aeruginosa* PAO1, although it is very closely related.

These results confirm that *phaC* gene encoding the PHA synthase responsible for PHA polymerization is present in the chromosomal DNA of *P. aeruginosa* MB2SA.

Figure 4.4.9: Southern hybridization of chromosomal DNA extracted from *P. aeruginosa* MB2SA digested with three different restriction enzymes. Lane 1: *Pvu*II fragment, 2: *Sma*I fragment, 3: *Xho*I fragment, and 4: pGEM-T Easy vector containing the cloned 0.5 kb fragment digested with *Eco*RI to release the 0.5 kb insert. The band at 3.0 kb suggests that the insert was incompletely excised from the vector.

CHAPTER 5

5 General Conclusions

5.1 Isolated Environmental Strains

Twelve environmental strains capable of degrading alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, were isolated in this study (Chapter 2). Most of these bacterial strains were found to be pseudomonads, which are medically important. The strain that was further studied in this work was identified to be *P. aeruginosa* and given a name *P. aeruginosa* MB2SA after performing 16S rDNA sequencing. *P. aeruginosa* has been reported to be a nosocomial pathogen (Quinn, 1998) and it was shown to be resistant to most drugs (Alonso *et al.*, 1999; Kohler *et al.*, 1997; Jalal *et al.*, 2000; Ziha-Zarifi *et al.*, 1999).

5.2 Growth studies, preferred substrate and PHA production by *P. aeruginosa* MB2SA

High yield of PHAs were obtained after growth of *P. aeruginosa* MB2SA when a long alkane fraction, C_{14}-C_{17}, was employed as carbon source (Chapter 3). This study showed that *P. aeruginosa* MB2SA might have incorporated the medium-carbon-chain length hydroxyalkanoic acids (HA_{MCL}) carrying 5 or even up to 16 carbon atoms into the PHAs produced (Steinbüchel and Hein, 2001) since this strain grew optimally when the long alkane fraction, C_{14}-C_{17}, was provided (Chapter 3). PHA synthases either incorporate short-carbon chain alkanes (Type I and Type III) or medium to long-carbon chain alkanes (Type II) (Steinbüchel and Hein, 2001). The PHA synthase produced by *P. aeruginosa* MB2SA would therefore appear to be a

Type II-synthase, which is consistent with reports of other *Pseudomonas* PHAs (Steinbüchel and Hein, 2001). Investigation needs to be done to characterize types of PHA the *P. aeruginosa* MB2SA accumulate in its cytoplasm.

In addition, *P. aeruginosa* MB2SA was observed to be secreting a substance that caused the medium to change colour at difference stages of growth (Chapter 3). This has also been reported by Rahme *et al.*, (1997). It would be interesting to investigate the growth (in the C_{14}-C_{17} alkane fractions) of other *P. aeruginosa* strains to determine whether they have similar characteristics to *P. aeruginosa* MB2SA. This colour change might be used in future to indicate a period of PHA production during large scale growth studies.

5.3 The *phaC* gene detected in *P. aeruginosa* MB2SA

P. aeruginosa MB2SA was shown to have the gene encoding the PhaC2 protein which was found to be closely related to the PhaC2 protein of *P. aeruginosa* PAO1, although slight differences in their amino acid sequences suggest that the PhaC2 in *P. aeruginosa* MB2SA might be novel (Chapter 4). These interesting observations encourage further investigation on this protein, PhaC2, to confirm these differences. Additionally, sequencing the bands obtained when performing the Southern hybridization would provide more insight to this matter (Chapter 4).

Steinbüchel and Hein (2001) reported that all pseudomonads carry two different PHA synthase genes and are closely related in their genomes. These structural genes were showed to be associated with the structural genes of β-ketothiolase and acetoacetyl-CoA reductase. The same gene cluster with the two PHA synthase genes occurring in the same orientation, separated by a gene coding for an intracellular PHA_{MCL}

depolymerase, has been observed in *P. aeruginosa*, *P. oleovorans*, and *P. mendocina*. A similar order of genes was observed in *P. putida* strain U (Garcia *et al*., 1999) and the Southern hybridization done by Schembri *et al*. (1994) revealed that two *phaC* genes in *Acinetobacter* sp. may occur in both the plasmid and the chromosomal DNA. Therefore, the DNA flanking the *phaC* gene present in *P. aeruginosa* MB2SA (this study) needs to be sequenced further both upstream and downstream to determine whether the other genes and the order observed by Steinbüchel and Hein (2001), and Garcia *et al*. (1999) is conserved in strain *P. aeruginosa* MB2SA. In addition, the location of this gene, *phaC*, could be investigated to determined whether it occurs in both the plasmid and the chromosomal DNA (Schembri *et al*., 1994) since it was shown to be present in the chromosomal DNA of *P. aeruginosa* MB2SA (this study). This would provide more information about the PHA structural gene organization and location in *P. aeruginosa* MB2SA.

P. aeruginosa MB2SA was shown to possess the *phaC* gene (Chapter 2) and this strain has the ability to degrade the alkane fraction wastes produced by SASOL petrochemical company. In addition, *P. aeruginosa* MB2SA was found to be capable of converting these alkane wastes to value added product such as PHAs, which are candidates for the manufacture of biodegradable plastics. These plastics would solve the challenge of dealing with the plastic waste which is accumulating in vast amounts. The current plastic does not decompose naturally hence survives in the environment for longer periods. This non-decomposing plastic has caused accidental deaths of millions of mammals, birds, reptiles and fish every year after being entangled or ingested. Research carried out on 50 South African beaches indicated that there are over 3500 plastic particles per square kilometre in the Eastern Cape and Cape Town coast. By 1989 plastic pollution in the Eastern Cape and Cape Town has increased to

about 190 % and 90 % respectively, of which was contributed by plastic identified at these beaches. Plastic is now all over the South African beaches including the most remote areas (www.botany.uwc.ac.za).

Biodegradable plastic is in great demand nationally as well as world wide, however, its production is costly. Some of the factors contributing to this cost include the carbon and the nitrogen sources. This study has shown that a cheaper carbon source in the form of waste alkane fractions, C_{12}-C_{13} and C_{14}-C_{17}, could be used to produce PHAs using the bacterium, *P. aeruginosa* MB2SA. By supplementing with corn steep liquor, which was suggested to be a cheaper nitrogen source more suited to industrial production processes (Marangoni *et al.*, 2001), the production cost of biodegradable plastic could be sufficiently reduced to make it economically viable.

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APPENDIX

MEDIA

A.2 MEDIA

A.2.1 Growth media

A.2.1.1 Modified medium E

Modified medium E contained, per liter, 3.5 g of $NaNH_4PO_4.4H_2O$, 7.5 g of K_2HPO_4, 3.7 g of KH_2PO_4, and 2.9 g of Na_3 citrate.$2H_2O$. The pH was adjusted to 7.1 with 10mM NaOH. This mineral medium was autoclaved and then supplemented with 1 ml of filter-sterilized $MgSO_4$ (1 M) and 1 ml of MT trace element stock solution, which contained, per liter, 2.78 g of $FeSO_4.7H_2O$, 1.47 g of $CaCl_2.2H_2O$, 1.98 g of $MnCl_2.4H_2O$, 2.81 g of $CoSO_4.7H_2O$, 0.17 g of $CuCl_2.2H_2O$, and 0.29 g of $ZnSO_4.7H_2O$ in 1M HCl (modified from Brandl *et al.*, 1988).

A.2.1.2 Luria-Bertani (LB) broth

Ingredients	**% Final concentration**	Gram (g) per liter
Tryptone	1.0	10
Yeast Extract	0.5	5
NaCl	0.5	5

Make up with distilled water and autoclave.

A.2.1.3 Nutrient Agar (NA)

Ingredients	**% Final concentration**	Gram (g) per liter
Meat Extract	1.0	10
Peptone	0.5	5
Yeast Extract	0.2	2
NaCl	0.8	8
Agar	1.5	15

Make up with distilled water and autoclave.

A.2.1.4 2X Yeast-tryptone Agar (2xYT Agar)

Ingredients	**% Final concentration**	Gram (g) per liter
Tryptone	1.6	16
Yeast Extract	1.0	10
NaCl	0.5	5
Agar	1.3	13

Make up in distilled water and autoclave. Cool to 50 ^{0}C prior to antibiotic addition (if appropriate)

A.2.1.5 2X Yeast-tryptone broth (2xYT broth)

Ingredients	**% Final concentration**	Gram (g) per liter
Tryptone	1.6	16
Yeast Extract	1.0	10
NaCl	0.5	5

A.2.1.6 Nitrogen limiting Minimal Salt Medium (MSM)

The nitrogen limiting minimal salt medium contained, per liter: 0.4 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 0.5 g KH_2PO_4 and 10 ml salt. The salt solution has, per liter: 25 g $MgSO_4.7H_2O$, 28 g $FeSO_4.7H_2O$, 1.7 $MnSO_4$, 0.6 g NaCl, 0.1 g $Na_2MoO_4.2H_2O$, 0.1 g $ZnSO_4.7H_2O$ and 8.54 ml of 32 % HCl solution. The pH was adjusted to 7.0 with 10N NaOH. After autoclaving, the medium was cooled to room temperature and an appropriate carbon source was added (modified from Brandl *et al.*, 1988).

A.2.1.7 Nile Blue A stain

The Nile Blue A (make) was prepared by dissolving 1 % w/v of Nile Blue A stain in a distilled water and filtered prior to use. To dissolve the stain completely, a slight heating may be required (Ostle and Holt, 1982).