AEROBIC UTILIZATION OF SELECTED PHARMACEUTICAL AND PERSONAL CARE PRODUCT BY ESTUARINE HETEROTROPHIC BACTERIA

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DECLARATION

I, Rendani Bridghette Bulannga, declare that

1. The research presented in this thesis, except where otherwise indicated, is my own and has been generated by me as the result of my own original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other person's data, pictures, graphs or other information unless specifically acknowledged.

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ABSTRACT

Pharmaceutical and personal care products (PPCPs) constitute a broad class of organic compounds, some of which belong to the list of the OECD high production volume (HPV) chemicals. These compounds have emerged as environmental contaminants with potentially detrimental effects. They have been detected in various environmental compartments typically in a nano- to microgram range and sewage treatment plants represent the major point source for the aquatic environment. Salicylic acid, a monohydroxybenzoic acid, is widely used in cosmetic and therapeutic products and is listed as HPV chemical. Benzyl salicylate and phenyl salicylate are diaryl ester compounds commonly used in pharmaceutical formulations, fragrances and household detergents. Benzyl salicylate is listed as HPV chemical. The fate of salicylic acid in the environment has been reported previously while those of benzyl salicylate and phenyl salicylate are unknown. Although studies are available on the microbial degradation of aromatic compounds, studies exclusive to the catabolism of PPCPs by marine heterotrophic bacterial isolates are rather limited. Therefore, the aim of this thesis was to characterize PPCPs (salicylic acid, benzyl salicylate and phenyl salicylate) utilizing bacteria from an estuarine environment (Durban Harbour, KwaZulu-Natal, South Africa). Selective enrichments were employed using artificial seawater medium typically supplemented with 2 mM of the target compounds (salicylic, benzyl salicylate or phenyl salicylate). After successive subculturing, bacteria capable of utilizing target compounds as sole carbon and energy source were characterized by morphological and physiological features, 16S rRNA gene sequence and MALDI-TOF MS analysis. Growth kinetics were assessed by monitoring the optical density, cell count and protein formation over time. The utilization of salicylic acid and phenyl salicylate was verified using UV spectroscopy and HPLC and the key reactions involved were verified by determining the specific oxygen uptake rates using resting cells and specific activities of representative enzymes. A Gram-negative coccus shaped bacterium belonging to the genus *Acinetobacte*r degrading salicylic acid and phenyl salicylate, a Gram-negative rod shaped marine bacterium belonging to the genus *Oceanimonas* degrading salicylic acid and phenyl salicylate and a Gram-negative rod shaped bacterium belonging to the genus *Pseudomonas* utilizing benzyl salicylate in the presence and absence of synthetic surfactants (Tween 80) were isolated. The growth of *Acinetobacter* and *Oceanimonas* species was dependent on

salicylic acid and phenyl salicylate as carbon source as growth was only observed when the carbon source was present and the compound was degraded almost to completion. Growth of *Pseudomonas* with benzyl salicylate was enhanced in the presence of surfactant. All three strains did not have an obligate requirement for NaCl. *Acinetobacter* and *Oceanimonas* strains were tolerant to high concentrations of salicylic acid and were inhibited at a concentration above 20 mM while phenyl salicylate did not show toxic effects on the strains; instead growth increased with the increase in concentration. Salicylic acid was utilized via catechol by both strains as they showed high specific oxygen uptake rates and catechol-1, 2-dioxygenase activity for this chemical. Phenyl salicylate was hydrolyzed at the ester bond to phenol and salicylic acid, as these were the metabolites that accumulated during growth with phenyl salicylate. The mono-aromatic metabolites resulting from the hydrolysis of diaryl substrate were further metabolized via catechol. Microbial catabolic activities were solely responsible for the loss of contaminant in the medium as confirmed by abiotic controls. Heterotrophic bacteria can therefore play an important role in the removal of contaminants from marine environments.

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CHAPTER 1

Literature Review

1.1. Introduction

Marine and estuaries are the most productive ecosystem, yet they constitute one of the most threatened environments worldwide (Forbes and Demetriades, 2009; Kennish, 2002). Estuaries provide a wide range of habitat to many species of marine fish and invertebrates; they depend on estuaries as nurseries, feeding and roosting ground. Estuaries are also economically important area as human population relies on the estuaries resources for subsistence and recreational activities. Because of the high population growth and increased urbanization, estuaries are now under pressure due to anthropogenic activities. These pressures contribute to habitat alteration, change in the dynamic of the biotic communities and pollution (Allanson and Baird, 1999; Meire *et al.,* 2005; Zietsman, 2011).

Estuaries are partly enclosed bodies of water along the coastline where freshwater and marine water mix. They are transition zone between the ocean and the land, thus they are the point of accumulation and discharge of a wide range of chemicals (Meire *et al.,* 2005). Land-based activities represent the most important cause of marine and estuarine pollution (Basnyat *et al.,* 1999; Kennish, 2002). It is estimated that 80% of the marine pollution originates from the land and the prime sources being municipal waste, industrial discharge and agricultural practices, they reach the estuaries through direct discharge, river influx and through run-off (Basnyat *et al.,* 1999; Kennish, 2002; Islam and Tanaka, 2004).

Sydney Harbour in Australia is one such estuarine that is heavily urbanized and is reported to receive high pollution loads from storm water runoff and sewer overflow, this consequently had an impact on the microbiological quality and posed a health risk to aquatic organisms (Courtenay *et al.,* 2005). Persistent Organic Compound such as PCB, PAH and DDT were detected from the sediments of Zhujiang estuary in Guangdong Province (China) which is heavily influenced by industrial and commercial activities concentrated around the coast (Yuan *et al.,* 2001). Accumulation of these toxic chemicals in marine organisms was the highest levels of DDT in the liver of the fish *Lateolabrax japanicus* and mussel *Perna viridis* detected in Juilong River estuary

in China (Klumpp *et al.,* 2002). Several estuaries in the United Kingdom suffer from contamination by endocrine disrupting chemicals; these chemicals have potential to bioaccumulate and may cause adverse biological effects on marine organisms. Allen *et al.,* (1999a) reported that the estrogenic materials that were detected in estuarine water caused masculinity in female gastropods and bivalves. Another report by Allen *et al.,* (1999b) stated that the breeding efficiency of flounder fish which spend most of their sensitive life stages in estuaries was negatively affected.

1.2. Durban Harbour

The marine and coastal areas in South Africa are highly valued for human settlement, recreation and resource use. There are 256 functional estuaries in the country which make up to about 1000ha of the most productive habitat (Turpie *et al.,* 2002; Zietsman, 2011). In the past few decades, there have been an increase in resort development, reclamation and increasing human disturbance and exploitation of these environments. Because of these pressures, many estuaries have become functionally degraded (Allanson and Baird, 1999). Recent assessment by the CSIR (2010) revealed that large numbers of South African estuaries are still in good condition. However, those located close to urban area, particularly in KwaZulu-Natal, are mostly in poor condition and their deterioration is mostly due to habitat destruction and pollution.

Durban Harbour (29°52’ S; 31°4’ E) is located south of the eThekwini Metropolitan in KwaZulu-Natal. It is one of the busiest ports in South Africa and Africa. It is an estuary bay that is ranked as the 11th most important estuary out of 256 estuaries in the country. It is regarded as a highly degraded estuary as pollutant accumulation was most significant due to a combination of high pollutant loads and longer retention times of pollutants as the freshwater inflow is reduced (Guastella, 1994; Zietsman, 2011). A large part of the bay has been lost to port infrastructure and industrial developments. The areas around the bay and along the rivers are highly industrialized and urbanized. Therefore, the waste generated by industrial, commercial and residential land use often ends up in the bay. Moreover, port activities have an additional impact of chemical pollution (Brown, 1987; Forbes and Demetriades, 2009).

In South Africa, there is limited information concerning marine pollution. Most of the studies on determining the type of pollutants in marine and estuarine environments focused mainly on trace metals. The organic pollutants that have been reported to be present in South African estuaries are phenol, HCH, PAH, PCB and DDT. DDT was most predominant in KwaZulu-Natal; this might be attributed to the fact that this chemical is widely used as an insecticide in sugarcane industries and malaria control (Brown, 1987; O'Donoghue and Marshall, 2003; Moloney *et al.,* 2013).

1.3. PPCPs as environmental pollutants

Organic pollutants in marine water have not been studied comprehensively because of the limitation in analytical methods available. The available analytical techniques only allowed for determination of limited number of organic pollutants as they are time and cost consuming. It is only over a decade ago that new analytical techniques have been developed that allows detection of new pollutants in aquatic environments even in trace amounts (Daughton and Ternes, 1999; Dachs and Méjanelle, 2010).

One class of toxic chemicals that has received little attention is the group consisting of pharmaceuticals and personal care products (PPCPs). They are classified as emerging contaminants as they are coming to prominence in the last decade for environmental regulators and are considered to be potential threat to the ecosystem and human health (Quintana *et al.,* 2005; Ellis, 2006; Ternes and Joss, 2006). They enter the water system on a continual basis due to their worldwide use and have been detected in the water system typically in range of concentration from nanograms to low micrograms per litre. They are introduced into the environment via individual household use, hospital discharge or disposal of unwanted or out dated drugs and cosmetic products, thus the sewage treatment plant represents the major point of source as commonly used water treatment processes are insufficient or not designed to completely remove PPCP residues. Therefore, the residues that pass a sewage treatment plant ultimately end up in the aquatic environments (Daughton and Ternes, 1999; Ellis, 2006; Ternes and Joss, 2006; Caliman and Gavrilescu, 2009).

Not surprisingly, PPCPs have been detected in marine and estuarine environments in trace amounts. Thomas and Hilton (2004) reported that 14 pharmaceuticals were detected in 5 estuaries in the UK in trace amounts. Hedgespeth *et al.,* (2012) reported that some of the PPCPs that were detected in treated wastewater effluent discharged into Charleston Harbour of South Carolina were also detected in coastal surface water of Charleston Harbour in trace amounts. Benotti and Brownawell (2007) investigated the Jamaica bay in New York City which is heavily impacted by sewage discharge; they detected 12 pharmaceuticals throughout in trace amounts.

1.3.1. Salicylate and its derivatives

- Salicylic acid

Salicylic acid (synonym, 2-hydroxybenzoic acid, Figure 1.1.) is a monohydroxybenzoic acid that is a member of large group of pharmaceuticals listed by the OECD as High Production Volume Chemicals (OECD, 2009). It was first introduced in 1783 for therapeutic purposes, now salicylic and its salicylate derivatives are largely used in the production of cosmetic products and therapeutic drugs. It is used in the treatment of acne, to reduce fever and as an active ingredient in stomach relief agents (Chaniotakis *et al.,* 1989; Castleman, 2009). In addition, its bactericidal properties allow it to be used as a preservative in cosmetic products and in food and also as an antiseptic (Price *et al.,* 2000). It is also naturally produced by plants as a phytohormone playing a key role in the expression of resistance; it can induce resistance after initial attack by plant pathogens. It has been extracted from white willow bark and from the herb meadowsweet (Castleman, 2009; Vicente and Plasencia, 2011). Salicylic acid is also an intermediate produced during microbial degradation of various polycyclic aromatic hydrocarbons (PAH) such as naphthalene (Habe and Omori, 2003).

Salicylic acid is a common environmental pollutant, it has been detect in municipal waste treatment effluent of Kansas City with the average daily output of 8.64 (0.55–28.69) kg over a 10 month period (Hignite and Azarno, 1977) and it was also detected in German river and stream waters up to a concentration of 0.2 µg/L (Ternes, 1998).

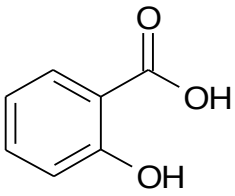

Figure 1.1. Salicylic acid

- Benzyl salicylate

Benzyl salicylate (salicylic acid benzyl ester, Figure 1.2) is a diaryl ester compound consisting of two benzene rings interconnected by an ester bond. It is a clear liquid prepared by an esterification reaction of salicylic acid with benzyl alcohol. Because of its balsamic floral scent it is widely used as a fragrance compound in cosmetic products such as decorative cosmetics, fine fragrances and other toiletries and also in non-cosmetic products such as detergents and as a food preservative. It is listed as HPV chemical with the worldwide use of greater than 1000 metric tonnes per annum. It is also a naturally occurring constituent of essential oils from plants which include *Cananga odorata* (ylang ylang) (Belsito *et al.,* 2007; Lapczynski *et al.,* 2007a; OECD, 2009).

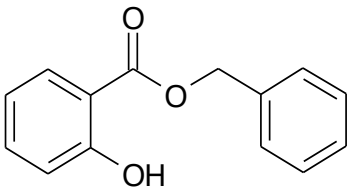

Figure 1.2. Benzyl salicylate

- Phenyl salicylate

Phenyl salicylate (Figure 1.3), also known as Salol, is a salicylic acid derivative that is a diaryl ester with an ester bond connecting the two benzene rings of phenol and salicylic acid. It is synthesized by heating salicylic acid with phenol in the presence of a catalyst (H_2SO_4, HCl, $POCl_3$ or sulfonic acid). Its worldwide use is reported as being in the region of less than 0.1 metric tonnes per annum (Belsito *et al.,* 2007). It is a white powder widely used in manufacturing of plastics, paints and varnishes as it has

ultra violet absorbing properties. Because it has an antibacterial activity, it also finds application in medicine as an antiseptic and as a coating of pills in which the medicine is intended for controlled release. It is also used in detergents, household products and many cosmetic products (Lapczynski *et al.,* 2007b; Dongwei *et al.,* 2009).

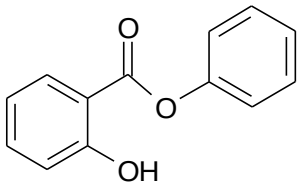

Figure 1.3. Phenyl salicylate

Table 1.1. Molecular properties of salicylic acid, benzyl salicylate and phenyl salicylate

	Salicylic acid	Benzyl salicylate	Phenyl salicylate
Physical form	white crystal	pale yellow liquid	white granular crystals
solubility at 20°C	2 g/L	0.15 mg/L	0.15 g/L
Log K_{OW}	2.26	4.31	3.82
BCF (L/kg wet weight)	3.16	324.8	154
BAC (L/kg wet weight)	12	16.6	26.1
Vapour pressure at 25°C	$8x10^{-5}$ mmHg	$1x10^{-3}$ mmHg	$6x10^{-5}$mmHg
Flash point	157°C	137°C	160°C

The physicochemical characteristics of salicylic acid and salicylate derivatives as calculated by Estimated Program Interface (EPI) suite and from literature (Nordström and Rasmuson, 2006; Lapczynski *et al.,* 2007a; Lapczynski *et al.,* 2007b)

1.3.2. Ecotoxicity of salicylic acid and its derivatives

When chemical pollutants are released into the aquatic environment, the effect they have on aquatic biota varies with concentration and duration of exposure to the toxicant (Láng and Kőhidai, 2012). Aquatic organisms often exhibit a wide range of tolerance to specific toxicants with consequences that a contaminant may have a lethal effect on some species but cause no recognizable effect on other organisms (Pastorok and Bilyard, 1985; Fleeger *et al.,* 2003). Because PPCPs are designed to have a specific biological activity at low concentration causing a preferred physiological response, they undergo stringent regulatory processes before being made accessible to the public (Fent *et al.,* 2006; Christen *et al.,* 2010). The health impact they have on humans depends on their physicochemical properties, concentration and degradation products (Boxall, 2004). At high concentrations benzyl salicylate and phenyl salicylate are skin irritants and are not known to be carcinogenic and mutagenic. These compounds do not accumulate in animal tissue but continual exposure of salicylic acid to human organs may lead to their deterioration and can cause adverse reproductive effect in animals (Belsito *et al.,* 2007).

Salicylic acid and its derivatives pose a toxic effect on non-target organisms even at low concentrations (Daughton and Ternes, 1999; Ginebreda *et al.,* 2010; Láng and Kőhidai, 2012). Salicylic acid derivatives where found to be extremely toxic to aquatic organisms. They induce morphological and physiological change influencing their metabolism, photosynthesis and their breeding efficiency. Ecological toxicity assessment showed that benzyl salicylate has a median lethal concentration (LC_{50}) of 1.03 mg/L (96h) on freshwater fish *Pimephales promelas*, 1.10 mg/L (48h) on *Daphnia magna* and 1.70 mg/L (24h) on algae and phenyl salicylate has an LC_{50} of 0.99 to 1.2 mg/L (96h) on freshwater fish *Pimephales promelas*. Salicylic acid is a *beta*-hydroxy acid which can penetrate through skin and break down fats and lipids causing chemical burn. The LC_{50} on *Daphnia magna* was found to be 112 mg/L (48h) and the half maximum effective concentration (EC_{50}) on algae was found to be >100 mg/L (48h) (Martin and Young, 2001; Camacho-Muńoz *et al.,* 2010). Salicylate compounds have antibacterial properties and thus they may either destroy the microbial community or may induce a number of morphological and physiological alterations such as antibiotic resistance or increase pathogenic virulence. The EC_{50} of salicylic acid is 214 mg/L (5 minutes) on *Phytobacterium* and 43.1 mg/L (15minutes) on *Vibrio*

fischeri. The presence of these compounds in the estuarine marine environment endangers the natural ecosystem due to their toxic effect (Price *et al.,* 2000).

1.4. Abiotic fate of salicylate and its derivatives

When organic compounds enter the environment, they are either subjected to volatilization, photodegradation, hydrolysis, bioaccumulation in sediments or animal tissue or microbial transformation and these determine the fate of pollutants in the environment (Richardson and Bowron, 1985; Calimann and Gavrilescu, 2009).

1.4.1. Volatilization

Volatilization, which is the movement of organic compounds from solid or liquid phase into the atmosphere, can be important in reducing the concentration of the pollutant in an environment. It is related to the aqueous solubility of the substance and is limited to compounds with high vapour pressure (Spencer *et al.,* 2009; Morra *et al.,* 2011). Salicylic acid, which is a carboxylic acid, is characterized by low volatility. It has a solubility of 2 g/L at 20°C in water and a negligible vapour pressure of $8x10^{-5}$ mmHg at 25°C (Table 1.1). Esters, conversely, are typically volatile compounds which have a characteristic aroma. However, since benzyl salicylate has a solubility of ~0.15 mg/L at 20°C and vapour pressure of less than 0.0010 mmHg at 25°C and phenyl salicylate with a solubility of ~0.15 g/L at 20°C and a negligible vapour pressure of 0.0000627 mmHg at 25°C (Table 1.1), volatilization is not expected to be an important environmental fate of these compounds (Sourthworth, 1979; Lapczynski *et al.,* 2007a; Lapczynski *et al.,* 2007b).

1.4.2. Hydrolysis

Hydrolysis is one of the major abiotic processes of contaminant removal in aquatic environments where the organic molecule reacts with water (Miyamoto, 1996). It occurs in esters and amides and the process is catalyzed by hydrogen (H^+) and hydroxyl (OH^-) ions under appropriate pH conditions (Carey, 1994). The pH of

seawater (typically in the range of 7.5 – 8.4) suggests that the hydrolysis may be important in this environment (Neilson and Allard, 2008). Salicylic acid cannot undergo hydrolysis as it has a functional group that is resistant to this process whereas benzyl salicylate and phenyl salicylate are susceptible to hydrolysis of the ester bond. Benzyl salicylate was found to have a hydrolysis half-life of 1.7 years at pH 7 and 63 days at pH 8 and that of phenyl salicylate was 6.6 days at pH 6.3 and below pH 4, phenyl salicylate was stable (Lyman *et al.,* 1990). Although these two compounds may undergo hydrolysis, the contaminant may not be completely transformed as the products that are formed maybe resistant to further hydrolysis. The resultant products of benzyl salicylate and phenyl salicylate hydrolysis are salicylic acid and benzoate and salicylic acid and phenol respectively, these compounds are resistant to hydrolysis.

1.4.3. Photodegradation

The photochemical decomposition of PPCPs in surface water is considered most likely to play a major role in determining their fate (Boreen *et al.,* 2003). The compound is transformed into other substances or even completely degraded by direct or indirect absorption of photons (Mansour, 1985; Hsien *et al.,* 2001; Zhao *et al.,* 2005). The direct photolysis involves the absorption of light by the pollutant typically resulting in oxidation, this process however is limited to compounds that absorb light above 290nm (Zepp and Cline, 1977). Indirect photolysis involves transformation of the pollutants through reaction with transient oxidants such as hydroxyl radicals, singlet oxygen and nitrate ions. Photodegradation of contaminants in aquatic environments is restricted to the surface water layer down to 2m in depth with the sunlight reaching the earth showing a wavelength over 286nm (Wu *et al.,* 2001; Dabrowska *et al.,* 2004). However, photodegradation of vast numbers of PPCPs has not been described (Boreen *et al.,* 2003). Salicylic can undergo photochemical degradation and the photodegradation studies of salicylic acid showed that it has a photolysis half-life of 30 to 47 days and its salicylate ions 40 to 122 days while that of benzyl salicylate was found to be 7.4 hours (Vione *et al.,* 2003). No studies have been done on the photodegradation of phenyl salicylate. On the other hand, when benzyl salicylate and phenyl salicylate are used as UV filters, they are photostable (Kunz *et al.,* 2006).

1.4.4. Bioaccumulation

Depending on their physicochemical properties, contaminants have a potential to accumulate within the cells of an organism, this is known as bioaccumulation. Some PPCPs have been reported to accumulate in aquatic organisms (Farré *et al.,* 2008). This may enhance the persistence of the compound in the environment as it is not exposed to direct physical, chemical or microbial degradation but rather fixed within the cell (Caliman and Gavrilescu, 2001; Hedgespeth *et al.,* 2012). Bioaccumulation is high for compounds that have low water solubility, high lipophilicity and high molecular weight (Streit, 1992; Sharifi and Connell, 2003; Ivanciuc *et al.,* 2006). Contaminants with a bioconcentration factor (BCF) exceeding 1000 and log octanol-water partition coefficient (log K_{OW}) above 5 have potential to accumulate within a tissue. BCF of salicylic acid was found to be 3.16 with log Kow of 2.26, for benzyl salicylate the BCF is 324 and log K_{OW} is 4.31 and the BCF of phenyl salicylate is 154 with log K_{OW} of 3.82 (Table 1.1) (Lapczynski *et al.,* 2007a; Lapczynski *et al.,* 2007b; Mackay and Fraser, 2000). Therefore, bioaccumulation is not considered a significant environmental fate of these compounds.

Though organic pollutants may undergo abiotic degradation, the physicochemical processes involved are generally slow and may result in by-products that are resistant to further degradation or may cause irreversible damage to the ecosystem. Therefore the most successful strategy to remove pollutants from the environment is through biotic mineralization or metabolic processes which contribute to the disappearance of the parent pollutants and also change their physicochemical properties, thus influencing their fate and behaviour in the environment.

1.5. Aerobic biodegradation

It has long been known that microorganisms play a significant role in the elimination of toxic chemicals from the environment (Atlas, 1972; Atlas, 1981; Dìaz, 2004; Dash *et al.,* 2012). The ability of microorganisms to utilize aromatic compounds was first demonstrated by Stömer (1908); he isolated a bacterium that was capable of utilizing toluene and xylol as carbon source. Since then many prokaryotes and eukaryotes capable of mineralizing toxic chemicals have been isolated. The target compound either serve as a source of carbon and energy where it is completely mineralized to

carbon dioxide and water or may be transformed by co-metabolic steps where the target compound is partly mineralized in the presence of a primary substrate (Farré *et al.,* 2008; Hedgespeth *et al.,* 2012).

Bacteria are the most studied microorganisms in biodegradation of organic pollutants. They are classified as chemoorganoheterotrophs as they use the organic pollutant as their carbon and energy source using a variety of aerobic and anaerobic catabolic strategies (Dìaz, 2004; Seo *et al.,* 2009). Because of their high metabolic activity, supreme enzymatic activity and high potential to completely mineralize pollutants, they play a leading role in establishing water quality and determining purification potential of the aquatic ecosystem (Yu *et al.,* 2006; Dìaz, 2008; Cao *et al.,* 2009). Recent review listed 79 bacterial genera that utilize hydrocarbons as their carbon and energy source (Chung and King, 2001; Dìaz, 2004).

Although many bacteria have been isolated from marine and coastal environment, majority of them are related to terrestrial and freshwater microbes. Only few true marine bacteria have been isolated and these mostly belong to the genera of *Vibrio*, *Marinobacter* and *Cycloclasticus* (Geiselbrecht *et al.,* 1996; Hedlund *et al.,* 1999; García *et al.,* 2004; Dash *et al.,* 2012). The species of these genera from marine environments have been reported to mineralize various aromatic hydrocarbons. Moxley and Schmidt (2012 and 2010) isolated a *Vibrio* species and a *Marinobacter* species capable of aerobically utilizing benzoic acid and phenol respectively from estuarine water of Durban Harbour (South Africa). *Cycloclasticus* specie isolated from Eagle Harbour utilized PAHs (naphthalene and phenanthrene), biphenyls and toluene as sole carbon source but showed poor growth in media with no aromatic compounds (Geiselbrecht *et al.,* 1998).

Microbial degradation of PPCPs have been described, this however has mostly focused on the removal of PPCPs during waste water treatment (Quintana *et al.,* 2005; Yu *et al.,* 2006; Gröning *et al.,* 2007; Benotti and Brownawell, 2009). There is limited information on the microbial metabolism of these compounds in freshwater and especially in marine and estuarine environments (Ying and Kookana, 2003). Nonetheless, there is a growing interest on the role that microorganisms play in elimination of contaminants in this particular environment.

Bacterial mineralization of salicylic acid for terrestrial and aquatic environments has been reported and was found to be readily utilized as carbon and energy source by vast number of bacteria (Chakrabarty, 1972; Karegoudar and Kim, 2000). Phenyl salicylate and benzyl salicylate are listed as emerging pollutants (Belsito *et al.,* 2007) and the fate of these two compounds in the environment is not well known. However, bacteria able to utilize these two compounds have been reported. *Micrococcus*, Gram negative cocci bacterium isolated from garden soil utilized several benzyl compounds including benzyl salicylate as sole carbon and energy source (Voet and Schinckel, 1927). Phenanthrene utilizing bacteria, *Bacillus phenanthrenicus bakiensis,* also isolated from soil utilized phenyl salicylate (salol) as sole source of carbon (Tausson, 1928).

1.6. Catabolic sequence of salicylic acid and its derivatives

Bacteria have evolved diverse strategies to degrade organic pollutants as carbon and energy sources both aerobically and anaerobically (Fuchs *et al.,* 2011). The degradation of aromatic compounds is most frequently an aerobic process by use of an oxidative strategy where oxygen serves as an electron acceptor and a reactant and this process is distributed among a large variety of bacterial genera (Gibson and Harwood, 2002). The key step in the degradation of the aromatic compounds is the activation of the inert ring into a limited number of common intermediates which take place through a series of enzymatic reactions followed by ring cleavage pathways to the central metabolism of the cell (Gibson, 1968; Dìaz, 2008).

The observation of organic compound degradation was first made in aerobic organisms (Evans, 1963; Carmona *et al.,* 2009). Oxidoreductase enzymes play a key role in the hydroxylation reaction for the activation of the aromatic ring and the oxygenolytic cleavage of the ring; this includes a monooxygenase enzyme which incorporates one atom of oxygen while the other oxygen atom is reduced to H_2O and a dioxygenases which incorporates two oxygen atoms into the organic substrate (Gibson, 1968; Pérez-Pantoja *et al.,* 2008; Carmona *et al.,* 2009; Dìaz *et al.,* 2013). The aerobic degradation of aromatic compound commonly proceeds via two steps, a peripheral pathway and subsequent pathway linking to central metabolism. The first step involves the destabilization of the ring by an oxygenase enzyme which activates

the stable oxygen molecule to produce a highly electrophilic reagent adding to the stable aromatic ring to form hydroxylated intermediates catechol, protocatechuate, gentisate or 1,2,3-trihydroxybenzene. The second step is the ring cleavage where the hydroxylated intermediates are channelled into the central metabolism through the ortho- or meta-cleavage pathway by oxygenases enzyme forming metabolites that can be incorporated into the tricarboxylic cycle (Harwood and Parales, 1996; Gibson and Harwood, 2002; Jördening and Winter, 2005; Dìaz, 2008; Carmona *et al.,* 2009).

1.6.1. Catabolism of salicylic acid

The aerobic catabolism of salicylic acid and its genetic basis have been described with various bacteria (Chakrabarty, 1972; Karegoudar and Kim, 2000). It has been described to occur via two main routes, the catechol pathway and the gentisate pathway. The first pathway involves decarboxylation of salicylic acid to catechol catalyzed by salicylate-1-hydroxylase. The second pathway involves hydroxylation of salicylic acid at the C5 position yielding gentisic acid catalyzed by salicylate-5-hydroxylase (Filonov *et al.,* 2000; Karegoudar and Kim, 2000; Ishiyama *et al.,* 2004).

Aerobic catechol utilization (Figure 1.4A) – Catechol is cleaved via ortho- (intradiol) cleavage or meta- (extradiol) cleavage. Ortho cleavage is followed by the β-ketoadipate pathway; it is catalyzed by catechol-1,2-dioxygenase which cleaves the ring at the ortho position resulting in *cis,cis*-muconic acid. Muconate is successively transformed by a series of enzymes to β-ketoadipate which is further converted to succinate and acetyl-CoA. These final cleavage end products may then be channelled to Kreb's Cycle to be utilized. The meta cleavage pathway is catalyzed by catechol-2,3-dioxygenase which cleaves catechol between position C2 and C3 to yield 2-hydroxymuconic semialdehyde. Semialdehyde is subsequently cleaved to form 4-hydroxy-2-ketovaleric acid which is further transformed to acetaldehyde and pyruvic acid (Dagley and Gibson, 1965; Gibson, 1968; Harwood and Parales, 1996).

Aerobic gentisate pathway (Figure1.4B) – This pathway occurs when ring cleavage is between a hydroxyl group and carboxyl group catalyzed by gentisate-1, 2-dioxygenase to yield maleyl pyruvic acid. The metabolite can either be directly converted to TCA cycle metabolites, pyruvate and maleate or undergo isomerisation

to fumaryl pyruvate leading to pyruvate and fumurate (Lack, 1959; Harpel and Lipscomb, 1990; Ohmoto *et al.,* 1991; Karegoudar and Kim, 2000).

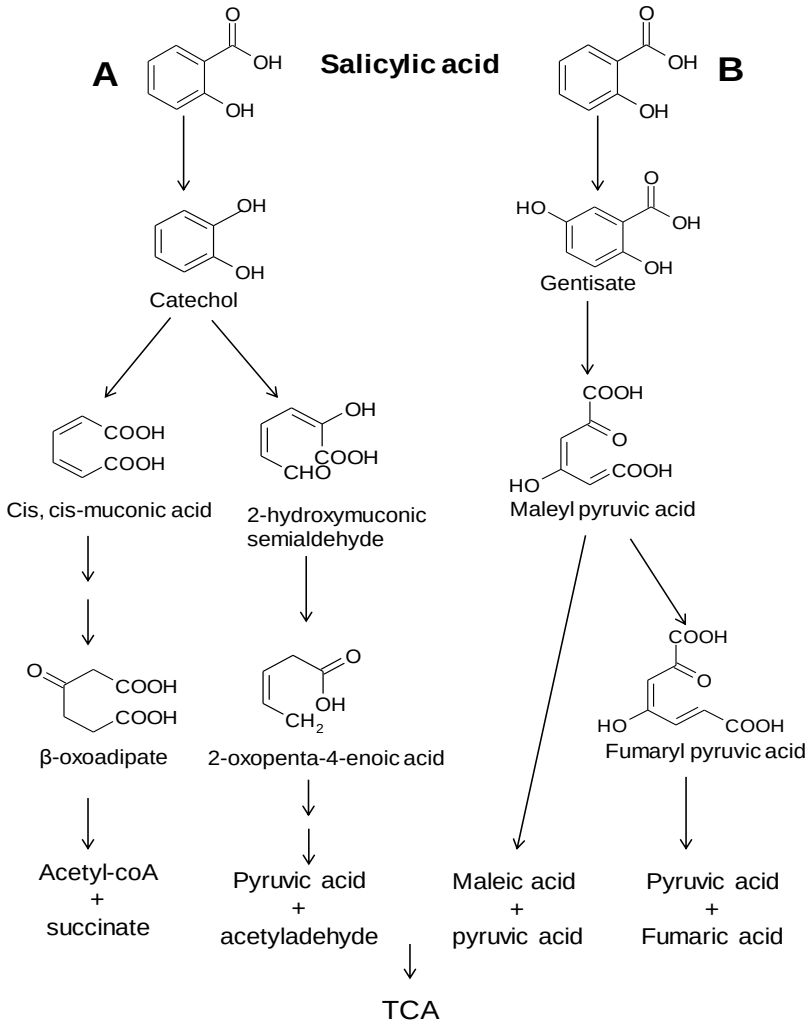

Figure 1.4. Catabolic pathway of salicylic acid. Salicylic acid is either metabolized via the catechol pathway (A) or gentisate pathway (B) to metabolites that can be incorporated into the TCA cycle.

Even a direct ring cleavage of salicylic acid has been reported in *Pseudaminobacter salicylatoxidans*. This unique pathway is catalyzed by salicylate-1,2-dioxygenase which cleave the aromatic ring yielding 2-oxohepta-3,5-dienedioic acid (Hitner *et al.,* 2004). It differs from the catechol or gentisate pathway found in most bacteria as the 1, 2-dioxygenase enzyme does not require the coenzyme ($NADH_2$) as primary electron donor. The enzyme cleave salicylate that carry only a single hydroxyl group and that are not activated by additional electron donating substituent for ring fission reaction (Hitner *et al.,* 2001).

1.6.2. Catabolism of diaryl ester

Diaryl ester compounds are characterized by two aromatic rings interconnected by ester linkage. Bacteria with the ability to utilize these compounds as a sole source of carbon and energy have been isolated. The hydrolysis of these compounds can occur via two pathways; the enzymes either attack the ester bond connecting the two benzene rings resulting in separate aromatic rings or attack one of the rings enabling further enzymatic reaction for catabolism. The attack on the ester linkage, giving rise to the corresponding carboxylic acid and alcohol, is the most common pathway as the ester bond is most susceptible to initial attack by the esterase enzymes. These bacteria typically further metabolize one or both products formed by the hydrolytic cleavage of the ester linkage into intermediates that are transformed by a peripheral pathway to metabolites that are channelled into the tricarboxylic cycle (Bornscheuer, 2002; Schmidt, 2002).

There is limited information available concerning the bacterial degradation of benzyl salicylate and phenyl salicylate. However, studies have been carried out on the degradation of structurally similar compounds such as phenyl benzoate by *Pseudomonas* species and benzyl benzoate by *Acinetobacter* species. These studies showed that the initial attack by esterase enzymes occurs at the bond interconnecting the benzene rings resulting in benzyl alcohol and benzoate for benzyl benzoate and for phenyl benzoate, the intermediate formed were phenol and benzoate (Reich *et al.,* 1999; Göttsching and Schmidt, 2002).

1.7. Factors affecting biodegradation in estuarine environment

The extent to which microorganisms can degrade organic contaminants in the environment is influenced by a number of factors which may have an effect on the activity of the microorganism or affect the rate of biodegradation; these include the molecular properties of the compound and environmental conditions. Therefore, to achieve successful degradation, factors must be adequate to support the activity of microorganisms involved in degradation (Providenti *et al*., 1993; Boopathy, 2000).

1.7.1. Bioavailability

For catabolism to occur, the target contaminant must be accessible to the bacteria. Bioavailability is defined as the amount of the chemical that is available for the cell. Uptake by the cell is the first factor that potentially limits biodegradation. It considers the interaction of the chemical with the bacterial cell and this is influenced by solubility of the substrate in water, volatilization into air, adsorption onto the sediments and bioaccumulation in the tissue (Reid *et al.,* 2000; Semple *et al.,* 2007; Cirja *et al*., 2008) Substrates are only accessible to microbial attack in aqueous solution. It was observed that the growth of *Pseudomonas* strain was related to the amount of dissolved aromatic compounds such as anthracene, naphthalene and phenanthrene (Simoni *et al.,* 2001; Semple *et al.,* 2003). However, many compounds have limited solubility and thus exist in non-aqueous phase in the environment (Boopathy, 2000). Bacterial degradation of these hydrophobic compounds is limited as the mass transfer of the molecule to the bacterial cell is limited (Bosma *et al.,* 1997; Noordam and Janssen, 2002). Bacteria capable of utilizing hydrophobic compounds as their growth substrate have been reported. This was observed during degradation of octadecane by mixed bacterial culture in a liquid medium (Providenti *et al.,* 1993; Barkay *et al.,* 1999; Satpute *et al.,* 2010) and degradation of naphthalene where the cells of *Pseudomonas putida* G7 attached to the surface of the molecule (Law and Aitken, 2003).

The availability or accessibility of the pollutant for bacterial catabolism can be limited by the sorption of the molecule onto hydrophobic matrix such as the sediments (Bosma *et al.,* 1997). Sorption occurs when the pollutants binds to the sediment particles thereby removing the compound from its dissolved state, this is illustrated by

the octanol-water partition coefficient (K_{OW}). Compounds that are characterized by log K_{OW} of lower than 2.5 do not adsorb to the sediment, between log K_{OW} of 2.5 and 4 have moderate sorption and higher than 4 have high sorption potential (Providenti *et al.,* 1993; Cirja *et al*., 2008; Caliman and Gavrilescu, 2009).

When the concentration of the substrate is below their threshold, microorganisms do not grow sufficiently as they do not obtain enough energy to cause substantial growth and degradation. As volatilization of organic compound into the atmosphere reduces the rate of biodegradation, it reduces the concentration of substrate in the solution available for microbial attack (Boethling and Alexander, 1976; Alexander, 1994). Conversely, this can enhance biodegradation as the concentration of the substrate is reduced to below toxic level that the microorganism can tolerate.

Salicylic acid is water soluble (2 g/L at 20°C) with a log K_{OW} value of 2.26 (Table 1.1), thus it is present in aqueous phase for bacterial degradation and it does not tend to adsorb to sediment. Because benzyl salicylate is insoluble (0.15 mg/L at 20°C) in water with a high log K_{OW} of 4.31 and phenyl salicylate is slightly soluble in water (0.15 g/L at 20°C) with log K_{OW} of 3.82 (Table 1.1), these two compounds have limited availability to cell uptake with the potential to adsorb on sediments (Lapczynski *et al.,* 2007a; Lapczynski *et al.,* 2007b).

1.7.2. Nutrients

The nutritional requirements essential for bacteria catabolism include carbon, oxygen, hydrogen, phosphorus, nitrogen, potassium, calcium, magnesium, iron, sulphur and trace elements. Nitrogen, phosphorus and potassium are major elements needed in the bacterial degradation of organic compounds and it was found that the quantity of nutrients available for bacterial uptake was a limiting factor during biodegradation. However, excessive high nutrient availability hinders the growth of microorganisms (Leahy and Colwell, 1990). During the study of aerobic degradation of polycyclic aromatic hydrocarbons (PAH), the optimal microbial growth was found when the ration of C:N:P ranged from 100:15:3 to 120:10:1 (Atlas, 1981; Bamforth and Singleton, 2005). When studying the degradation of phenol in the marine environments, it was observed that at high nitrogen and phosphate concentrations, phenol was degraded via the meta-pathway while the ortho-pathway was used at low concentration of

nitrogen and phosphorus (Le Borgne *et al.,* 2008). Hence the availability of nutrients in the environment plays a major role in determining the rate of biodegradation and the fate of contaminants in the environment. Marine and estuarine environments often lacks supply of nitrogen and phosphorus as some marine organisms such as phytoplankton consume them in competition with the bacteria degrading pollutant (Atlas and Bartha, 1972; Leahy and Colwell, 1990).

1.7.3. Molecular properties of the compound

The physicochemical properties of the target molecule determine its potential for bacterial degradation and have an influence on the degree and the rate of biodegradation. These include the number, type and the position of substituents. As the number of substituents increases the compound becomes more complex, its molecular weight and the recalcitrance increases and therefore ultimately decreases the rate of biodegradation (Leahy and Colwell, 1990).

Hydrocarbons undergo aerobic degradation by wide range of microorganisms. Aliphatic hydrocarbons with chain length of C10 to C20 are easily degraded while those of chain length less than C10 are volatile and toxic to many microorganisms. Those with longer chain length and cycloalkenes are often resistant to biodegradation (Baek *et al.,* 2006). Only a limited number of microorganisms are able to mineralize aromatic compounds and an increase in the number of aromatic rings increases the molecular weight and the octonal-water partition and solubility decreases, thus the bioavailability to bacterial degradation is reduced (Colombo *et al.,* 1996). Halogenated organic compounds are commonly degraded anaerobically by reductive dehalogenation reaction and as the number of halogens increases the rate of biodegradation is reduced. The position of the substituent influences the rate of biodegradation. Substituents located at the para-position are less degraded than the substituent in meta- and ortho-position (Wang *et al.,* 1988).

Toxicity affects the rate of metabolic reaction and it varies with the concentration of the toxicant. The compound may disrupt the structure of the cell or may prevent the enzymes from binding to the substrate; as such it inhibits the functioning of the cell or the process that is vital for the metabolism of the substrate (Price *et al.,* 2000). Salicylic acid and its derivatives have antibacterial properties. Salicylic acid is a

bacteriostatic which prevents DNA replication and therefore cell replication, ultimately preventing bacterial population increase (Block, 2001).

1.7.4. Environmental conditions

The growth and the activity of microorganisms are affected by various environmental factors such as oxygen content, temperature, salinity and pH, thus affecting the degree and rate of biodegradation. The degree of tolerance to these factors differs between strains and outside the tolerated range the microorganisms are unable to degrade the contaminant. Furthermore, these conditions have an effect on the physical properties of the contaminant and its bioavailability (Providenti *et al.,* 1993; Alexander, 1994).

- Oxygen

Hydrocarbon degradation occurs at a faster rate aerobically when compared to degradation under anaerobic condition (Leahy and Colwell, 1990). During the aerobic bacterial degradation of aromatic compounds, oxygen serves as terminal electron acceptor and a reactant during aromatic ring cleavage and hydroxylation catalyzed by oxygenases. In estuarine and marine environments, oxygen is limited by the high salt content. The sediments are anoxic apart from the upper surface of the sediments (Baker and Herson, 1994). Thus the availability of oxygen has an influence on the rate and type of biodegradation and the fate of pollutants.

- Temperature

Temperature has an influence on the degradation of contaminants by affecting the physical state of the contaminant and microbial metabolism of the substrate (Margesin and Schinner, 2001). It was found that the change in temperature at a range between 10°C and 40°C did not have significant effect on the rate of biodegradation, but when the temperature drops to below 10°C the rate of degradation decreases rapidly and at a higher temperature than 40°C biodegradation does not occur for mesophillic bacteria. Only few bacteria are known that can carry out biodegradation above 40°C

(Baker and Henson, 1994; Brakstad and Bonaunet, 2006). The temperature of the ocean water in Durban ranges between 17°C and 28°C which falls within the range of temperature required for degradation by mesophillic bacteria.

Temperature alters the physical state of the molecule by either affecting its solubility, volatilization, viscosity or sorption. Solubility of organic compounds in water was found to increase with increasing temperature. For example, at 20°C the solubility of salicylic acid was 1.8 g/L, at 40°C was 3.7 g/L and at 80°C the solubility was 20.5 g/L (Nordström and Rasmuson, 2006). As the temperature increases, the viscosity of organic compounds decreases which affects the degree of distribution and decreases the diffusion rate. Viscosity of benzyl salicylate is 7.5 Pa.s at 59°C and that of phenyl salicylate is 8.3 Pa.s at 43°C and thus temperature has an influence in the viscosity of organic compounds (Margesin and Schinner, 2001). Therefore, temperature determines the rate and the degree at which the contaminants can be degraded by microorganisms in the environment.

- Salinity

The estuarine region is a transition zone where river-water and seawater mix, thus it is subjected to both seawater influence such as salinity and freshwater. The salinity at coastal and estuarine sites varies as there is a constant mixing of freshwater with seawater ranging from 0 to 3% salinity due to the diluting effect. For the open ocean salinity ranges from 3.2 to 3.8% (Austin, 1988). The bacteria inhabiting these ecosystems are specifically adapted and have developed a strategy to maintain the intracellular environment forming osmotic balance with their external environment (Roberts, 2005). Salt can cause cell lysis when the concentration of solute in the cell is higher than in the surrounding environment as water moves into the cell and causes the cell to expand. To overcome this, the bacteria pump ions (for example H^+) out of the cell thus lowering the concentration of solutes in the cytoplasm to concentrations similar to the external environment (Kapfhammer *et al.,* 2005; Oren, 2008). When the concentration of solutes in the cell is lower than the surrounding environment, the water moves out of the cell causing shrinkage (Leahy and Colwell, 1990; Ventosa *et al.,* 1998). To cope with the increase in salt concentration, the bacteria increase the concentration of compatible solutes in the cytoplasm to concentrations similar to the

surrounding environment by accumulating high concentration of variety of inorganic ions in the cytoplasm (Kapfhammer *et al.,* 2005; Oren, 2008).

- pH

The pH in the estuarine environment is around neutral to slightly alkaline (ranges from 7.5 to 8.4) and this is the pH that most aromatic degrading bacteria favour. The rate of biodegradation was found to increase as the pH of the environment increases up to the pH of 8 (Leahy and Colwell, 1990; Cirja *et al.,* 2008).

1.8. Aim of the thesis

Marine and estuaries are heavily influenced by human activities globally. Chemical pollution is one of the greatest concerns that threatens the health and productivity of these environments. Land base activities are the major contributor of marine pollution as the waste discharge and industrial effluent ends up in estuaries. Durban Harbour is no exception; it is highly degraded and receives high pollution loads from the surrounding industries, wastewater treatments plants and domestic waste. The presence of toxic organic contaminants has been reported in this area. PPCPs are present in the aquatic environments and have the potential to cause adverse effect on aquatic organisms. Removal of these pollutants from the environments is essential as they pose health impact of marine biota. There is limited information on the role that bacteria play in elimination of PPCPs from aquatic environments and especially on marine environments.

The aim of this thesis was to isolate heterotrophic bacteria from estuarine water samples from Durban Harbour (KwaZulu-Natal, South Africa) capable of utilizing selected PPCPs (salicylic acid, benzyl salicylate and phenyl salicylate) as sole carbon and energy source.

The main objectives of this study were:

- To isolate and characterize bacteria from estuarine water samples from Durban Harbour (KZN, South Africa) able to use the target compounds (salicylic acid, benzyl salicylate and phenyl salicylate) as sole carbon and energy source by selective enrichment using artificial seawater based medium.

- To determine growth kinetics of selected strains utilizing target compounds and establishes the degree of substrate utilization.

- To determine the catabolic pathway of utilization and key enzymes involved by HPLC, specific of selected enzymes and the oxygen uptake by resting cells.

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CHAPTER 2

Isolation and characterization of aerobic heterotrophic bacteria utilizing salicylic acid, benzyl salicylate and phenyl salicylate as a sole carbon and energy source from estuarine water

2.1. Introduction

Removal of organic contaminants from the environment by microbial activity is the major biotic mechanism governing their fate. Studies on the biodegradation provide information on the potential fate of the contaminants in the environment (Dìaz, 2004; Gomez *et al.,* 2007). Traditionally, these studies are initiated by isolation of microorganisms from the environment capable of catabolizing the target compound, involving the identification and characterization of the isolated microbe. This provides essential information on the microbial ecology of the contaminated environment and highlights biochemical mechanisms involved in the biotransformation of the chemical (Watanabe, 2001; Kostka *et al.,* 2011).

Since the first reported isolation and characterization of aromatic compounds utilizing bacteria by Stömer (1908); there have been many such studies on a wide range of environmental contaminants (Bushnell and Haas, 1941; Zobell, 1946; Atlas, 1981). Microbial degradation of a wide variety of chemicals is documented; nonetheless, there is still large number of chemicals that have not been studied with an ever increasing production of new chemicals (Gomez *et al.,* 2007). Although most microorganisms have the ability to utilize hydrocarbons as sole carbon and energy source to some degree, bacteria in particular are the most well studied in catabolism of toxic chemicals (Zobell, 1946; Cao *et al.,* 2009). This is mainly due to their high growth efficiency, rapid growth rate and a range of catabolic mechanisms which enables them to utilize a larger range of hydrocarbons (Dìaz, 2004; Kostka *et al.,* 2011).

Microorganisms capable of mineralizing aromatic compounds have been successfully isolated from the environment by selective enrichment in which the hydrocarbon of interest is the only source of carbon and energy present for bacterial growth (Bushnell and Haas, 1941). The principle of the selective enrichment technique is to provide

growth conditions that are favourable to the target organism thus giving advantage to the organism of interest over competing organisms; therefore the microorganism of interest is selected and enriched (Dunbar *et al.,* 1996; Hilyard *et al.,* 2008).

Traditionally, the identification of bacteria was based on phenotypic characteristics. However, this method on its own is not able to distinguish phenotypically very similar isolates. Methods have therefore been developed that are based on chemotaxonomy and even more so on genotypic characteristics, with the small subunit 16S ribosomal RNA being the molecule of choice as it is present in all bacteria and is conserved among species (Janda and Abbott, 2007). Phenotypic and genotypic characteristics are closely related and therefore both should be accounted for when classifying or identifying microorganisms (Clarridge, 2004).

Many bacteria have been isolated and characterized with over 79 genera of bacteria listed as potential degraders. However, the majority of these were isolated from terrestrial and freshwater environments (Dìaz, 2004; Chung and King, 2001). Lesser studies have been carried out in estuarine and marine environments and the bacteria that have been isolated from these environments are mostly related to terrestrial and freshwater microbes. Typical marine bacteria that are well known to utilize aromatic contaminants are *Marinobacter*, *Vibrio* and *Cycloclasticus* (Geiselbrecht *et al.,* 1996; Hedlund *et al.,* 1999; Moxley and Schmidt, 2010; Moxley and Schmidt, 2012).

The aerobic utilization of salicylic acid in terrestrial and aquatic environments has been reported and salicylate was found to be readily utilized as carbon and energy source by many bacteria (Karegoudar and Kim, 2000). Nevertheless, there is limited data for bacteria isolated from estuarine and marine environments (García *et al.,* 2004; Oie *et al.,* 2007). Phenyl salicylate and benzyl salicylate are identified as emerging pollutants (Quintana *et al.,* 2005; Lapczynski *et al.,* 2007a; Lapczynski *et al.,* 2007b), the fate of these compounds in the environment is not well known and there is only limited information available on the aerobic utilization of phenyl salicylate and benzyl salicylate.

Therefore, the aim of this study was to isolate aerobic marine heterotrophic bacteria able to productively utilize salicylic acid, phenyl salicylate and benzyl salicylate from estuarine water samples collected from Durban Harbour in KwaZulu-Natal, South Africa.

2.2. Materials and Methods

2.2.1. Media preparation

2.2.1.1. Artificial Seawater Based Medium (ASW)

ASW was used for initial enrichment and isolation. The medium was prepared by dissolving 9.1 g Tris-HCl, 1.0 g NH_4Cl, 50 mg K_2HPO_4, 10 mL of 0.01 M $FeSO_4$x $7H_2O$, 11.7 g NaCl, 1.2 g $MgSO_4$, 0.75 g KCl and 1.10 g $CaCl_2$ in 1L distilled water. The pH of the medium was adjusted to 7.8 using HCl; it was then autoclaved at 121°C (2 atm) for 15 minutes. For solid media, 20 g/L of bacteriological agar was added.

2.2.1.2. Mineral Salts Medium (MSM)

For analytical purpose, the medium was changed to mineral salts medium. MSM was prepared by dissolving 2.8 g Na_2HPO_4, 1 g K_2HPO_4, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgCl_2$ x $6H_2O$ and 0.05 g $Ca(NO_3)_2$ x $4H_2O$ in 1 L of distilled water. 500 µL of trace element solution per litre of distilled water (containing 5 g EDTA, 3 g $FeSO_4$ x $7H_2O$, 30 mg MnCl x $4H_2O$, 50 mg $Co(II)Cl_2$ x $6H_2O$, 10 mg $CuCl_2$ x $2H_2O$, 20 mg $NiCl_2$ x $6H_2O$, 30 mg Na_2MoO_2, 50 mg $ZnSO_2$ x $7H_2O$ and 20 mg H_3BO_3) was added to 1 L of medium. The pH was adjusted to 7.4 using HCl and the medium was autoclaved at 121°C (2 atm) for 15 minutes. For solid media, 20 g/L of bacteriological agar was added.

2.2.1.3. Addition of carbon source

Salicylic acid, benzyl salicylate and phenyl salicylate were purchased from Sigma-Aldrich (South Africa). In case of salicylic acid, stock solutions of 200mM salicylic acid were prepared by dissolving 6.91 g in 250 mL of 200mM Sodium hydroxide (NaOH). Phenyl salicylate and benzyl salicylate purity (purity >99%) were added directly to the medium to a desired concentration. The carbon sources were added to the cooled sterile medium (≤30°C) to desired concentrations.

2.2.2. Isolation of aerobic heterotrophic bacteria

2.2.2.1. Sample collection

Water samples were collected from Durban Harbour (29°50'51.09"S, 31°1'30.72"E), KwaZulu-Natal using sterile Schott bottles, transported back to the lab on ice and stored in the fridge at 4°C until used.

2.2.2.2. Enrichment and isolation

Aromatic compounds utilizing microorganisms were isolated from the water samples by selective enrichment using 25 mL of ASW in 100 mL Erlenmeyer flasks containing the target compound (2 mM salicylic acid, 2.2 mM benzyl salicylate or 0.5 g/L phenyl salicylate) as sole carbon and energy source. These flasks were inoculated with 1 mL of the water sample and then incubated on a rotary shaker in the dark at 25 $\pm$ 2°C at 200 rpm. After 7 days of incubation, 1 mL of enrichment cultures was transferred into fresh medium; this was repeated 3 times to allow for selection of salicylate, benzyl salicylate and phenyl salicylate utilizing bacteria. After the last transfer, culture samples were streaked onto solid medium containing the target compound as sole carbon and energy source and after successive sub-culturing; pure cultures were established from colonies formed. To ascertain purity, the cultures were routinely streaked on Nutrient agar and analyzed by microscopy.

Pure cultures were then grown in liquid MSM supplemented with 2 mM salicylic acid, 2.2 mM benzyl salicylate or 0.5 g/L phenyl salicylate, 3 strains able to grow on either of the target compounds as a growth substrate were selected for further studies.

To preserve bacterial cultures, the isolated strains were grown in MSM with salicylic acid, benzyl salicylate or phenyl salicylate for 24 hours and then stored in 20% (*v/v*) glycerol (200 µL glycerol and 800 µL liquid culture) at -80°C.

Three strains were selected with the ability to grow on salicylic acid, benzyl salicylate and phenyl salicylate (S19, S21 and B12). These strains were further characterized following microbiological methods of identification.

2.2.3. Microbiological characterization

The characterization of the isolates was carried out by examining colony characteristics, cell morphology and analyzing various biochemical reactions. Additional methods of classification and identification (i.e. analysis of the 16S rRNA gene sequences and MALDI-TOF MS) were carried out to verify the identity of the isolated strains.

2.2.3.1. Morphological characteristics

- Colony characteristics

Isolated strains were streaked on artificial seawater based agar with 2 mM salicylic acid, 2.2 mM benzyl salicylate or 0.5 g/L phenyl salicylate. After 48 hours incubation at ambient temperature, colonies were assessed for color, size, shape, form and appearance.

- Cell characteristics

Light microscopy

The Gram stain was performed according to Corlone *et al.* (1983). The stained bacteria were examined using bright field microscopy (Zeiss Primo Star). To verify the Gram stain, the KOH test was carried out according to the method described by Gregersen (1978). *Bacillus subtillis* ATCC 31293 (Gram positive) and *Escherichia coli* ATCC 8739 (Gram negative) were used as controls.

To determine whether the isolated strains produce a capsule, Maneval's capsule staining procedure was used (Maneval, 1941). Two stains were prepared, 1% *w/v* Congo red which stains the background and Maneval's solution which stain the vegetative cells. Maneval's solution was prepared by mixing 0.05 g acid fuchsin, 3.0 g ferric chloride, 5 mL glacial acetic acid and 3.9 mL liquefied phenol in 95 mL of distilled water. A drop of Congo red was placed on a slide and mixed with the culture, after the smear was air dried the slide was flooded with Maneval's solution and after 5 minutes the slide was rinsed off with water, dried and examined by bright field

microscopy using oil immersion. Congo red solution does not penetrate the capsule; therefore it provides a coloured background. When Maneval's solution is added to the slide, the acetic acid lowers the pH thereby changing Congo red background from red to blue. The Maneval's stain does not adhere to the capsule as it is non-ionic and is washed off by water, as a result the capsule remains clear while the vegetative cells are stained red (Plante, 2000). *E. coli* ATCC 8739 which produces capsule was used as a positive control.

Transmission Electron Microscopy

500 µL of a 24 hour culture grown in nutrient broth was centrifuged at 10 000x *g* for 10 minutes; the pellet was washed twice in 1mL of distilled water and then re-suspended in 500 µL of distilled water. The bacterial suspension was negatively stained by the flotation method whereby a copper grid coated in formvar is placed in the suspension for 4 to 5 minutes, gently dried using whatman's filter paper and then placed in 0.5% uranyl acetate for 30 seconds. The grid was then air dried and examined using a Philips CM120 Biotwin Transmission Electron Microscope.

Scanning Electron Microscopy

Sample for critical point drying were fixed in 2% gluteraldehyde in Cacodylate buffer (0.1 M, pH 7.4) for 2 hours. The samples were then transferred to a filter disk with a pore size of 0.2 µm, washed three times with the same buffer and then dehydrated successively in an ethanol series (10, 50, 70 and 100%) for 10 minutes. After the samples were dried using critical point drying, they were mounted on aluminum stubs and coated using tungsten and then examined by a Zeiss EVOLS15 Scanning Electron Microscope.

2.2.3.2. Biochemical characterization

Microbiological standard techniques were employed to determine the ability to utilize or catabolize various chemical substances as outlined by Holt *et al.* (1994).

The following biochemical characteristics were tested:

- Starch hydrolysis
- Citrate utilization
- Gelatin hydrolysis
- Catalase activity
- Oxidase activity
- Methyl red test
- Voges Proskeaur test
- Indole production from tryptophan
- Urease production
- Casein hydrolysis
- Triple sugar iron test
- O/F test
- Carbohydrate utilization (maltose, glucose, fructose, xylose, galactose, mannitol, sucrose and lactose).

For the preparation of the media, refer to Appendix A.

After inoculation, the media was incubated in the incubator in the dark at 25°C and the results were observed after 48 hours. As controls, the following type strains were used: *Bacillus cereus* ATCC 31293, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeroginosa* ATCC 9027 and *Salmonella* Typhimurium ATCC 14028.

2.2.3.3. Analysis of the 16S rRNA gene sequence

- DNA extraction

Genomic DNA of the bacterial isolates was extracted from a single colony of the overnight cultures growing on nutrient agar. The colony biomass was suspended in sterile 100 µL H_2O, heated for 10 minutes at 95°C followed by freezing for 10 minutes in liquid nitrogen. After thawing, the suspension was centrifuged at 12 000x *g* for 5 minutes. The DNA containing supernatant was immediately stored at -20°C.

- Amplification of the 16S rRNA gene

A set of established primers designed for amplifying bacterial 16S rRNA genes was used (Weisburg *et al*., 1991). These primers were purchased from Inqaba Biotechnical industries, South Africa.

Forward primer fD1 - 5’ -AGAGTTTGATCCTGGCTCAG- 3’
Reverse primer rP2 - 5’-ACGGCTACCTTGTTACGACTT- 3’

Table 2.1. Composition of the Master Mix used to amplify the 16S rRNA gene

Reagent	Per reaction (µL)
$MgCl_2$ (25mM) Kapa	2.5
dNTPs (10mM) Kapa	0.5
Buffer 5x Kapa	5.0
Primer forward – fD1 (10µM)	0.6
Primer reverse – rP1(10µM)	0.6
Taq (5U/µL) Kapa Hot Start	0.2
Template DNA	1.0
Milliq water	14.6
Total volume	25.0

The following PCR parameters were employed: Initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. The final extension was for 7 minutes at 72°C and the holding temperature at 4°C indefinitely.

- Analysis of PCR products

To confirm the size of the amplification products, these products were analyzed on 0.8% (*w/v*) agarose gel dissolved in 1X TAE buffer at 100V for 45 minutes. Gels were stained by SYBR safe DNA gel stain (Promega). Prior to loading of the gel, 2 µL PCR products were mixed with 5 µL blue orange loading dye (6x, Promega). For comparison, a size marker was used (1Kb DNA ladder, Promega).

- Sequencing

Amplified products were sent for direct sequencing to Inqaba Biotechnologies Industries (Pty) Ltd and Centre for Analytical Facility Stellenbosch University (South Africa). The resulting sequences obtained were then compared with those published in GenBank using the BLAST search for National Centre Biotechnology Information (www.ncbi.nim.nih.gov).

- Phylogenetic analysis

The 16S rRNA gene sequences of the isolated strains were aligned with the 16S rRNA gene sequences of the type strains obtained from the Ribosomal Database Project (http://rdp.cme.msu.edu/). The sequence alignment was done using ClustalW; phylogenetic trees were then generated using the neighbour joining algorithm with resampling for 1000 replicates. This was conducted using MEGA (Molecular Evolutionary Genetic Analysis) version 5.2 (Tamura *et al.,* 2011). The 16S rRNA gene sequence of *E. coli* ATCC 11775T X80725 was used as out-group. The tree topology was verified by Maximum Likelihood phylogeny.

2.2.3.4. MALDI-TOF mass spectrometry

The isolated strains were further characterized using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS, Bruker Daltonics, Bremen, Germany). Bacterial strains were analyzed following standardized MALDI Biotyper methods (Barbuddhe *et al.,* 2008).

- Sample preparation

Whole bacterial cells

Single overnight colonies of the isolated strains grown on nutrient agar were spotted on a steel target plate with a sterilized toothpick and left to air dry at ambient temperature. 1 µL of the matrix solution of HCCA (alpha-cyano-4-hydroxy cinnamic acid in 50% acetonitrile, 2.5% trifluoro acetic acid) was overlaid on each sample and was allowed to air dry before analysis.

Protein extract from bacterial cells

The rapid organic extraction procedure - Single overnight colonies of the isolated strains grown on nutrient agar were transferred to an Eppendorf tube with 300 µL sterile distilled water, mixed thoroughly and 900 µL of absolute ethanol was added. The mixture was centrifuged at maximum speed (14 000x *g*) for 2 minutes and after the pellet was air dried at room temperature, it was mixed thoroughly with 50 µL of formic acid (70% *v/v*); subsequently 50 µL acetonitrile (100% *v/v*) was added. The mixture was centrifuged again at 14 000x *g* for 2 minutes.

1 µL of the resulting supernatant was placed onto a spot of the target plate and air-dried at room temperature. Each sample was then overlaid with 1 µL of saturated matrix solution of HCCA (alpha-cyano-4-hydroxy cinnamic acid in 50% acetonitrile, 2.5% trifluoro acetic acid) and air dried.

- Data Analysis

The analysis were performed using a Microflex MALDI-TOF MS spectrometer (Bruker Daltonics, Bremen, Germany) at 337nm with flex control software (Bruker Daltonics) and the mean spectra were acquired as the average of 240 laser shots in positive linear mode at 60Hz. The spectra were analysed within a m/z range of 2 to 20 kDa.

The mass spectra acquired using Flex Control were analyzed with Flex Analysis software 2.4 (Bruker Daltonics). The mass spectra were compared with the database using Biotyper software version 3.0 with the reference library 1.0 version 3.1.2 (2011) containing 3,995 entries. To validate the analysis using MALDI Biotyper software, identification of bacteria test standard (*Escherichia coli* DH5α protein extract; Bruker Daltonics) was used as a positive control. The identification of the positive control was required to be *E. coli* with an identification score of more than 2.300 (highly probable species identification).

2.2.4. Effect of salt concentration on the growth of isolated stains

The effect of salinity on the growth of the isolated strains was determined in medium prepared by adding 5 g peptone powder and 2.5 g yeast extract to 1 L distilled water (pH adjusted to 7.4) in the presence of various NaCl concentrations (0% to 10%). A culture pre-grown in MSM with 2 mM salicylic acid, 2.2 mM benzyl salicylate or 0.5 g/L phenyl salicylate was centrifuged at 10 000x *g* for 10 minutes, washed twice with phosphate buffer and the pellet was re-suspended in the same buffer and then used to inoculate 25 mL peptone broth to an optical density at 600nm (OD_{600}) of 0.1. The OD_{600} was measured immediately after inoculation and then every 12 hours for 48 hours. The flasks were incubated on a rotary shaker in the dark at 25°C and 200 rpm. *E. coli* ATCC 8739 pre-grown in MSM with 2mM glucose was used as a control.

2.2.5. Effect of different concentrations of salicylic acid and phenyl salicylate on the growth of the strain S19 and S21

The isolated strains S19 and S21 were grown in MSM at different concentrations of the substrates. A 24 hour culture pre-grown in MSM with 2 mM salicylic acid and 0.5 g/L phenyl salicylate was centrifuged at 10 000x *g* for 10 minutes, washed twice with phosphate buffer (20 mM, pH 7.4) and the pellet was re-suspended in the same buffer. The suspension was then used to inoculate 25 mL fresh MSM to an OD_{600} of 0.1. The initial OD_{600} was measured immediately after inoculation and then measured at regular time intervals. The inoculated flasks were incubated in rotary shaker at 25°C at 200 rpm.

The range of salicylic acid concentrations tested for strain S19 was from 0 mM to 50 mM while the concentrations tested for strain S21 were between 0 mM and 100 mM. The growth was monitored every 24 hours by measuring OD_{600} for period of 72 hours.

The phenyl salicylate concentration tested for both isolated strains ranged from 0 g/L to 2.0 g/L. The growth was monitored every 24 hours by measuring the OD_{600} over a period of 192 hours.

2.2.6. Growth of the isolated strains and utilization of the target compounds

2.2.6.1. Growth of strain S19 and S21 with salicylic acid and phenyl salicylate

- Growth with salicylic acid

The growth of strain S19 and S21 was observed in MSM with 2 mM salicylic acid as sole substrate over a period of 48 hours at 25°C and 200 rpm. A 24 hour pre-grown culture grown in MSM with 2 mM salicylic acid was centrifuged at 10 000x *g* for 10 minutes, washed twice and the re-suspended pellet was used to inoculate 25 mL medium to an OD_{600} of 0.1. Two sets of controls were employed; control 1 was medium with carbon source but not inoculated and control 2 was medium without carbon source which was inoculated. The OD_{600} readings were taken every 3 hours. At the same time 1 mL samples including controls were centrifuged at 10 000x *g* for 10 minutes, the supernatant and the pellet were separated and stored at -20°C for further analysis (i.e. substrate and protein analysis).

- Growth with phenyl salicylate

The growth of strain S21 and S19 was studied in MSM with 0.5 g/L phenyl salicylate as growth substrate over 192 hours. 24 hour cultures pre-grown in MSM with the target compound were centrifuged at 10 000x *g* for 10 minutes, washed twice and the pellet was used to inoculate 50 mL medium to an OD_{600} of 0.1. The control employed was MSM without carbon source but inoculated. The OD_{600} readings were taken every 12 hours. 1 mL of culture was collected at time points and centrifuged at 10 000x *g* for 10 minutes; the supernatant and the pellet were separated and stored at -20°C for further analysis (i.e. substrate and protein analysis).

The culture was also examined via phase contrast microscope to determine whether cells of both strains attach the solid substrate crystals.

Growth measurements for all growth experiments:

Growth was monitored by measuring the optical density, establishing total cell counts and by measuring protein concentration.

- **Turbidity** - The optical density of 1 mL samples of cultures was measured at 600nm using a UV-VIS spectrophotometer. Non-inoculated medium was used as blank.
- **Cell concentration** - Total cell counts of cultures were carried out using a bacterial counting chamber (Helber type Hawksley, UK).
- **Analysis of protein concentration** - The protein from 1 mL of cell samples obtained during the growth experiment was quantified using the Bradford assay (Spector, 1978).

Alkaline cell lysis -The cell pellet was re-suspended in 1 mL of 0.15N NaOH and was heated to 95°C for 10 minutes. The samples where then cooled in ice bath and centrifuged at maximum speed (16 000x *g*) for 5 seconds.

Measuring the absorbance of protein -The supernatant (100 µL) or a dilution thereof in 0.15N NaOH was mixed with 1 mL coomassie blue reagent (prepared by dissolving 50 mg of coomassie blue G250 in 25 mL of 95% ethanol, then adding 50 mL of 85%

phosphoric acid followed by adding distilled water to a final volume of 500 mL). The absorbance was then measured at 595nm after 10 minutes of incubation in the dark. The blank used was 100 µL of 0.15N NaOH mixed with 1 mL coomassie blue reagent. The concentration of protein was quantified from a calibration curve using Bovine Serum Albumin as protein standard.

Calibration curve with Bovine Serum Albumin (BSA) - Appropriate volume of 0.15N NaOH was mixed with BSA stock solution of 0.5 mg/mL in 0.15N NaOH to obtain final concentrations of 0.5, 0.25, 0.15, 0.125, 0.1, 0.075, 0.05, 0.025, 0.01 and 0.005 mg/mL. 100 µL of these solutions were mixed with 1 mL of coomassie blue and the absorbance was measured at 595nm after 10 minutes incubation in the dark. 100 µL of 0.15N NaOH added to coomassie blue was used as a blank (Figure B1).

2.2.6.2. Analysis of salicylic acid and phenyl salicylate utilization

2.2.6.2.1. UV-VIS Spectroscopy analysis of salicylic acid

The cell free supernatants (thawed and then centrifuged at 10 000x *g* for 30 minutes) obtained during the growth experiments were used to evaluate the utilization of salicylic acid during growth of the isolated strains in MSM. Each sample was diluted 5 fold (200 µL of sample and 800 µL of distilled water) and the absorbance readings were measured at 300nm. The control samples (MSM with carbon source but not inoculated) were also evaluated. The blank used was MSM also diluted 5 fold. The salicylic acid concentration was extrapolated from the calibration curve of salicylic acid in methanol, concentrations used were from 0mM to 0.5 mM (Figure B2).

- UV-VIS Spectral analysis

The cell free supernatants collected at the start of the incubation and after 24 hours of incubation were subjected to spectral scan between the wavelength of 200nm and 400nm. Both the experiment and control samples were diluted 5 fold. The blank used was medium diluted as the sample. To determine the absorbance maxima of salicylic acid, 0.5 mM of salicylic acid in methanol was measured between 200nm and 400nm against methanol (Figure B2).

2.2.6.2.2. High Performance Liquid Chromatography

- Salicylic acid

Cell free supernatants (centrifuged at 10 000x *g* for 30 minutes after thawing) obtained during growth of the strains in MSM with 2 mM salicylic acid were analyzed by reversed phase HPLC (Varian Pro-Star) using a Microsorb 100 – 5 C18 (150 x 4.6 mm) column with UV detection at 210nm and 270nm. The mobile phase used was methanol and water (60:40% *v/v*) acidified with 0.5 g/L phosphoric acid, the flow rate used was 0.5 mL/min. 1 mL of cell free supernatant collected during the growth of cultures was added into the HPLC vials and the samples were analyzed isocratically for 10 minutes. The standards for salicylic acid were prepared by dissolving 0.0863 g in 25 mL of methanol to make a stock of 25 mM. Standard concentrations prepared were10, 50, 100, 250, 500, 750 and 1000 µM in methanol (Figure B5).

- Phenyl salicylate

Phenyl salicylate did not quantitatively dissolve in the aqueous medium (solubility 0.025 g/L). Therefore, phenyl salicylate was extracted from the medium to monitor the decrease in concentration during growth of the isolated strains with this compound as a growth substrate.

Extraction of phenyl salicylate - A 24 hour culture pre-grown in MSM with 2 mM salicylic acid was centrifuged at 10 000x *g* for 10 minutes, washed twice and re-suspended in the same buffer. The suspension was then used to inoculate 75 mL MSM containing 0.5 g/L (2.33 mM) to an OD_{600} of 0.1. The replicate flasks were incubated on a rotary shaker in the dark at 25°C at 200 rpm. The medium containing carbon source which was not inoculated served as abiotic control. The samples were collected after 0, 84, 108, 132 and 192 hours incubation for strain S19 and for S21 the sampling point were at 0, 72, 96, 152 and 192 hours. At sampling, the medium was acidified to pH 4.5 by phosphoric acid and 25 g of NaCl was added.

Phenyl salicylate in the medium was extracted 3 times with 25 mL chilled n-hexane using separating funnel. The organic phase was collected and was then dried with anhydrous magnesium sulphate. Prior to extraction, 1-naphthol was added to a final concentration of 1 mM. The solvent was removed under vacuum at 40°C and the residue was dissolved in 10 mL methanol.

The extracts were then analyzed by reversed phase HPLC, the mobile phase used was methanol and water (85:15% *v/v*) acidified with 0.5 g/L phosphoric acid and the flow rate was 0.5 mL/min. Only extract with more than 90% recovery based on the standard of 1-naphthol were recorded. Standard concentrations prepared for phenyl salicylate were 100, 250, 500, 750, 1000, 1250 and 1500 µM (Figure B6).

2.2.6.3. Growth of strain B12 with benzyl salicylate

2.2.6.3.1. Growth with 2.2 mM benzyl salicylate

The growth of the isolated strain B12 with 2.2 mM (0.5 g/L) benzyl salicylate was examined in minerals salts medium. A culture pre-grown in ASW was centrifuged at 10 000x *g* for 10 minutes, washed twice and the pellet was re-suspended in buffer to inoculate fresh mineral salt medium to an OD_{600} of 0.1. The initial OD_{600} was measured immediately and then every 12 hours for period of a 120 hours. At the same time, 1 mL of samples were centrifuged at 10 000x *g* for 10 minutes, the supernatant and the pellet were separated and stored at -20°C for further analysis. The growth was monitored by measuring OD_{600}, determining total cell counts and the protein concentrations.

2.2.6.3.2. The growth of strain B12 on possible intermediates of benzyl salicylate

The growth of the isolate was observed in mineral salts medium with possible intermediates as formed from hydrolysis of benzyl salicylate, i.e. benzyl alcohol, benzoate and salicylate serving as the sole carbon and energy source at a concentration of 2.2 mM. The fresh medium was inoculated with washed pellet of pre-grown culture to OD_{600} of 0.1. The growth was monitored every 24 hours by measuring turbidity at 600nm over a period of 120 hours.

2.2.6.3.3. Toxicity of possible intermediate salicylic acid on the growth of strain B12

The isolate was grown in mineral salts medium with salicylic acid at varying concentrations and 2 mM benzoic acid as an alternate carbon source. The concentrations of salicylic acid tested ranged from 0 to 3 mM. Fresh MSM was inoculated with the culture pre-grown in seawater based medium to an OD_{600} of 0.1. The initial OD_{600} was measured immediately after inoculation and then after 24 and 48 hours of incubation.

2.2.6.3.4. Enhancement of benzyl salicylate utilization

- Mechanical enhancement

The growth of strain B12 was monitored in mineral salts medium with 2.2 mM benzyl salicylate in 100 mL baffled Erlenmeyer flasks and normal Erlenmeyer flasks. Fresh medium was inoculated to an OD_{600} of 0.1 with the cells from culture pre-grown in seawater based medium, washed twice with phosphate buffer and re-suspended in the same buffer. The initial reading was measured immediately after inoculation and then every 24 hours for period of 120 hours. The growth was monitored by measuring OD_{600} and microscopic total cell counts.

- Chemical enhancement

The growth of *s*train B12 was monitored in mineral salts medium at different concentrations of benzyl salicylate with the addition of 50 µL (0.2% *v/v*) synthetic surfactant (Tween 80). Fresh medium was inoculated to an OD_{600} of 0.1 with the cells from culture pre-grown in seawater based medium, washed twice with phosphate buffer and re-suspended in the same buffer. The initial OD_{600} was measured immediately after inoculation and then every 24 hours for period of 120 hours. The flask where incubated in a rotary shake in the dark at 25°C and 200 rpm The concentrations of benzyl salicylate tested ranged from 0mM to 8.8 mM. A flask containing 50 µL Tween 80 in MSM and inoculated was employed as a control.

2.2.7. Dissolution kinetics of benzyl salicylate and phenyl salicylate

The solubility of benzyl salicylate and phenyl salicylate was studied in mineral salts medium and in distilled water. 500 µL of benzyl salicylate and 0.5 g of phenyl salicylate (pure compounds) were added into 250 mL of MSM and distilled H_2O. The flasks were incubated in a rotary shaker at 25°C and 200 rpm. The absorbance was measured every 30 minutes at 310nm (maximum absorbance of both compounds at 310nm). Concentrations of the substrate were derived from standard curve of benzyl salicylate and phenyl salicylate (Figure B3 and B4).

2.2.8. Utilization of other aromatic compounds

Various aromatic compounds were tested to determine whether the isolate had the ability to utilize other aromatic compounds. Compound tested include benzoic acid, phenol, salicylic acid, benzyl alcohol, 4-methyl phenol, phenyl salicylate, benzyl salicylate, toluene, 4-hydroxybenzoic acid, 4-methylbenzoic acid, 3,4-dimethyl phenol, 3,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 2-methoxybenzoic acid, 3,5-dinitrosalicylic acid, 2,4-dimethylphenol, 4-nitrophenol, 4-hydroxycatechol and hexadecane.

Overnight culture of the isolated strains were centrifuged at 10 000x *g* for 10 minutes, washed twice with phosphate buffer and then re-suspended in 1 mL phosphate buffer. 100 µL of the re-suspended culture was used to inoculate MSM agar plates by the spread plate technique. The substrate crystals (~5 mg) were then placed at the centre of the plate. For the control, no carbon source was provided. Plates were incubated at room temperature and examined for the presence and absence of visible growth.

For liquid compounds, 25 mL of fresh MSM was inoculated by washed culture to an OD_{600} of 0.1. The flasks were incubated in a rotary shaker in the dark at 25°C and 200 rpm. OD_{600} was measured immediately after inoculation and then every 3 hours for 48 hours. Concentration of substrate tested was 2 mM. Control employed was inoculated medium without the substrate.

2.3. Results

2.3.1. Characterization of the isolated strains

2.3.1.1. Phenotypic characteristics

A preliminary characterization of the isolates was performed, based on colony morphology after growth on solid medium and cell morphology determined by microscopy (Gram reaction, cell size and shape).

Three bacterial strains able to utilize salicylic acid, phenyl salicylate or benzyl salicylate as sole source of carbon and energy were isolated from estuarine water samples collected from Durban Harbour (KwaZulu-Natal, South Africa).

The first isolate, utilizing salicylic acid and phenyl salicylate designated S19 was a Gram-negative rod shaped bacterium (1.2 x 0.8 µm), motile by a monotrichous flagellum (Figure 2.1). The isolate produced a capsule but did not produce endospores. The colonies on ASW agar plates were light brown, circular, smooth and raised.

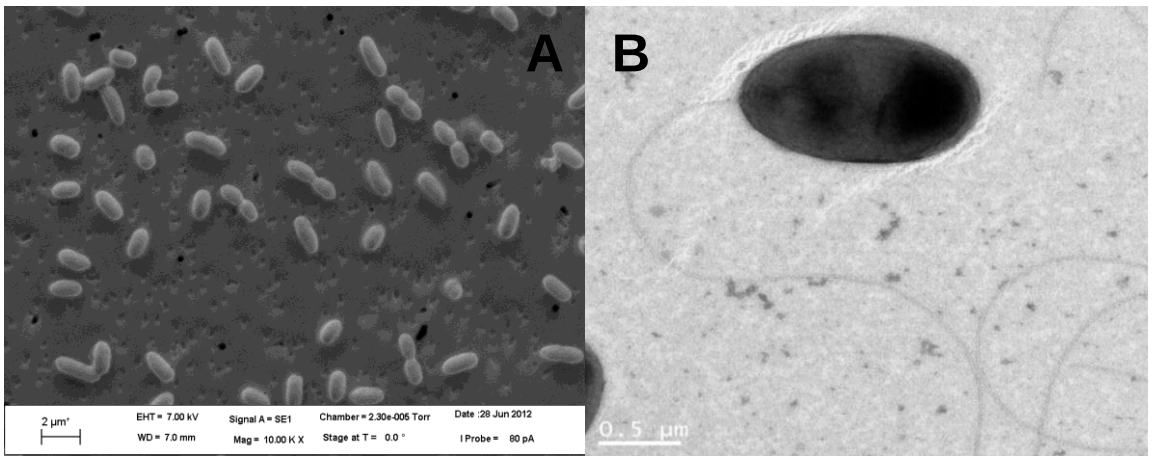

Figure 2.1. Scanning electron micrograph (A) and Transmission electron micrograph (B) of the rod shaped cells of the bacterial strain S19 with a single polar flagellum.

The second isolate able to utilize salicylic acid and phenyl salicylate designated S21 was Gram-negative and formed coccus to fat short rod shaped cells (1.2 x 1 µm) (Figure 2.2). The isolate was non-motile and did not produce a capsule or endospores. The colonies on the ASW agar plate were cream to white, raised and circular with a smooth texture.

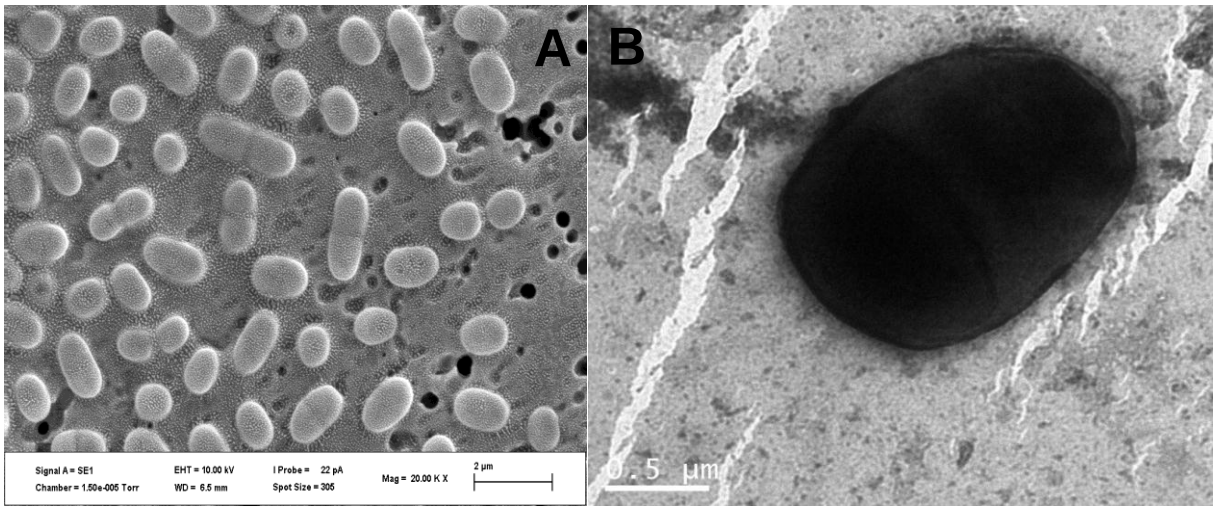

Figure 2.2. Scanning electron micrograph (A) and Transmission electron micrograph (B) of the coccus to plum rod shaped cells of the bacterial strain S21.

The third isolate, able to utilize benzyl salicylate, designated B12 was a Gram-negative rod shaped bacterium (1.2 x 0.4 µm) motile by a monotrichous flagellum (Figure 2.3). The isolate produced neither spores nor capsule. The colonies growing on ASW agar plates were brown, circular, smooth and raised.

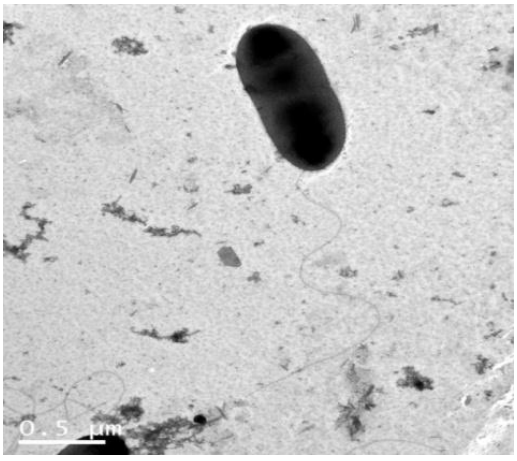

Figure 2.3. Transmission electron micrograph of a rod shaped cells of the bacterial strain B12 with single polar flagellum.

2.3.1.2. Biochemical characteristics

For further characterization, a number of diagnostic biochemical tests were performed as shown in Table 2.2.

Table 2.2. Biochemical characteristics of the three isolated strains.

Test	S19	S21	B12
Oxidase	+	-	+
Catalase	+	+	+
Starch hydrolysis	-	-	-
Citrate utilization	-	-	+
Gelatin hydrolysis	-	-	-
Methyl red test	-	-	-
Voges Proskauer test	-	-	-
Triple Iron Sugar			
- Butt	Red	Red	Red
- Slant	Red	Red	Red
- Gas production	-	-	-
- H_2S production	-	-	-
Oxidation/ fermentation			
- Open – oxidation	-	-	+
- Closed - fermentation	+	-	+
Indole production	-	-	-
Urease production	-	-	-
Casein hydrolysis	+	+	+
Carbohydrate utilization			
- Maltose	-	-	-
- Glucose	-	-	-
- Fructose	-	-	-
- Mannitol	-	-	-
- Sucrose	-	-	-
- Lactose	-	-	-
- Galactose	-	-	-
- Xylose	-	-	-

Key: (+) positive reaction (-) negative reaction

All three strains were catalase positive, hydrolyzed casein and did not produce acid and gas from the sugars. Strain S19 and B12 were oxidase positive while strain S21 was oxidase negative.

2.3.1.3. Analysis of the 16S rRNA gene sequence

The 16S rRNA gene is universal in bacteria and highly conserved between different species of bacteria. It is composed of both conserved and variable region and the sequence of variable region can be used to determine the phylogenetic relatedness of bacterial species. To determine the phylogenetic relation of the isolates, the 16S rRNA gene of the isolates were analyzed.

The amplification resulted in products of ~1.4 kb for all strains used including a control strain of *E. coli* ATCC 8739.

The resulting sequences obtained were then compared with those published in GenBank using the Basic Local Alignment Search Tool (BLAST, www.ncbi.nim.nih.gov). The best matches based on the sequences and the E-value from the database are listed in Table 2.3 to 2.5.

Table 2.3. Best matching sequences for strain S19 obtained from NCBI blast accessed on 25 November 2014

Accession	Description	Max score	E value	Identity
NR_114185.1	*Oceanimonas doudoroffii* strain NBRC 103032 16S ribosomal RNA gene, partial sequence	1602	0.0	98%
JX501723.1	*Oceanimonas doudoroffii* strain V9SED4 16S ribosomal RNA gene, partial sequence	1592	0.0	97%
JQ045791.1	*Oceanimonas doudoroffii* strain SDT4S6 16S ribosomal RNA gene, partial sequence	1592	0.0	97%
NR_027198.1	*Oceanimonas doudoroffii* strain MBIC1298 16S ribosomal RNA gene, partial sequence	1592	0.0	97%
NR_114475.1	*Oceanimonas doudoroffii* strain ATCC 27123 16S ribosomal RNA gene, partial sequence	1589	0.0	97%

Table 2.4. Best matching sequences for strain S21 obtained from NCBI blast accessed on 25 November 2014

Accession	Description	Max score	E value	Identity
KJ573528.1	*Acinetobacter junii* strain 16-6 16S ribosomal RNA gene, partial sequence	2549	0.0	99%
AB745654.1	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: PW89	2549	0.0	99%
JF901932.1	*Acinetobacter* sp. HS-B1 16S ribosomal RNA gene, partial sequence	2549	0.0	99%
FJ544395.1	*Acinetobacter junii* strain tu13 16S ribosomal RNA gene, partial sequence	2549	0.0	99%
FJ544392.1	*Acinetobacter junii* strain tu7-2 16S ribosomal RNA gene, partial sequence	2549	0.0	99%

Table 2.5. Best matching sequences for strain B12 obtained from NCBI blast accessed on 25 November 2014

Accession	Description	Max score	E value	Identity
JF681286.1	*Pseudomonas monteilii strain* EU45 16S ribosomal RNA gene, partial sequence	2582	0.0	99%
HQ840771.1	*Pseudomonas* sp. N1(2011) 16S ribosomal RNA gene, partial sequence	2582	0.0	99%
KF896124.1	*Pseudomonas sp*. SR18 16S ribosomal RNA gene, partial sequence	2579	0.0	99%
KF741207.1	*Pseudomonas* sp. Ph6 16S ribosomal RNA gene, partial sequence	2579	0.0	99%
KF263566.1	*Pseudomonas monteilii* strain JC2 16S ribosomal RNA gene, partial sequence	2579	0.0	99%

Comparison of the 16S rRNA gene sequence of the isolated strains to the sequences contained in GenBank gave a similarity level of more than 98% for all three strains; this is a similarity level regarded as sufficient to assign isolates to genus level. Accordingly, isolate S21 can be assigned to the genus *Acinetobacter*, B12 can be

assigned to genus *Pseudomonas* and isolate S19 can be assigned to the genus *Oceanimonas.*

Phylogenetic trees which incorporate the sequences of the three bacterial isolates and the 16S rRNA sequences for type strains obtained from the Ribosomal Database were constructed using MEGA version 5.2. The phylogenetic relatedness of the isolated strains with the type strains is demonstrated in Figure 2.4 to 2.6.

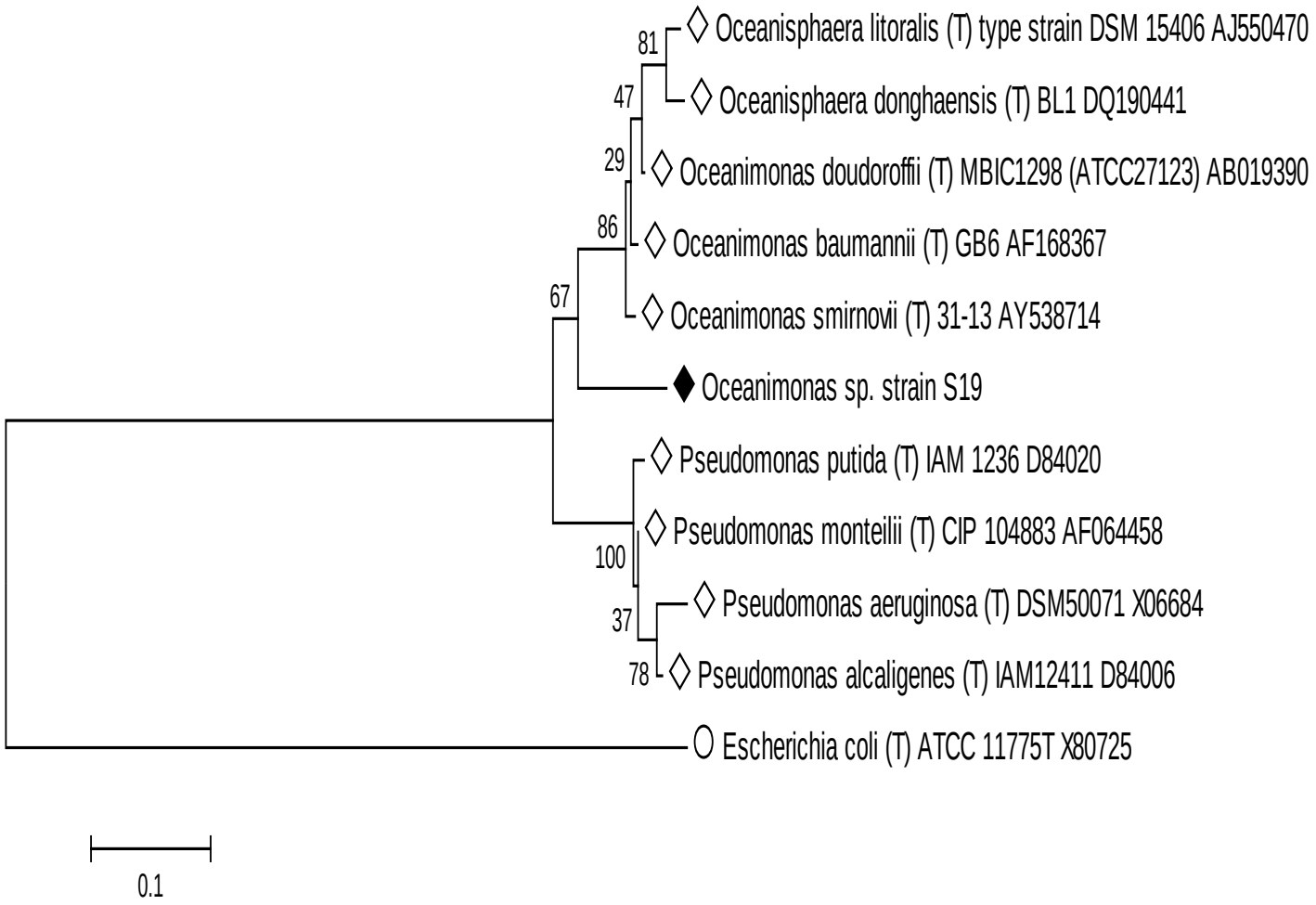

Figure 2.4. Phylogenetic assignment of strain S19 (♦) using the neighbour-joining model based on its 16S rRNA gene sequence in comparison with 16S rRNA gene sequences of selected type strains of *Oceanimonas*, *Oceanisphaera* and *Pseudomonas* (◊). The 16S rRNA gene sequence of *E. coli* (○) was used as out-group. The scale bar represents 10 estimated changes per 100 nucleotides.

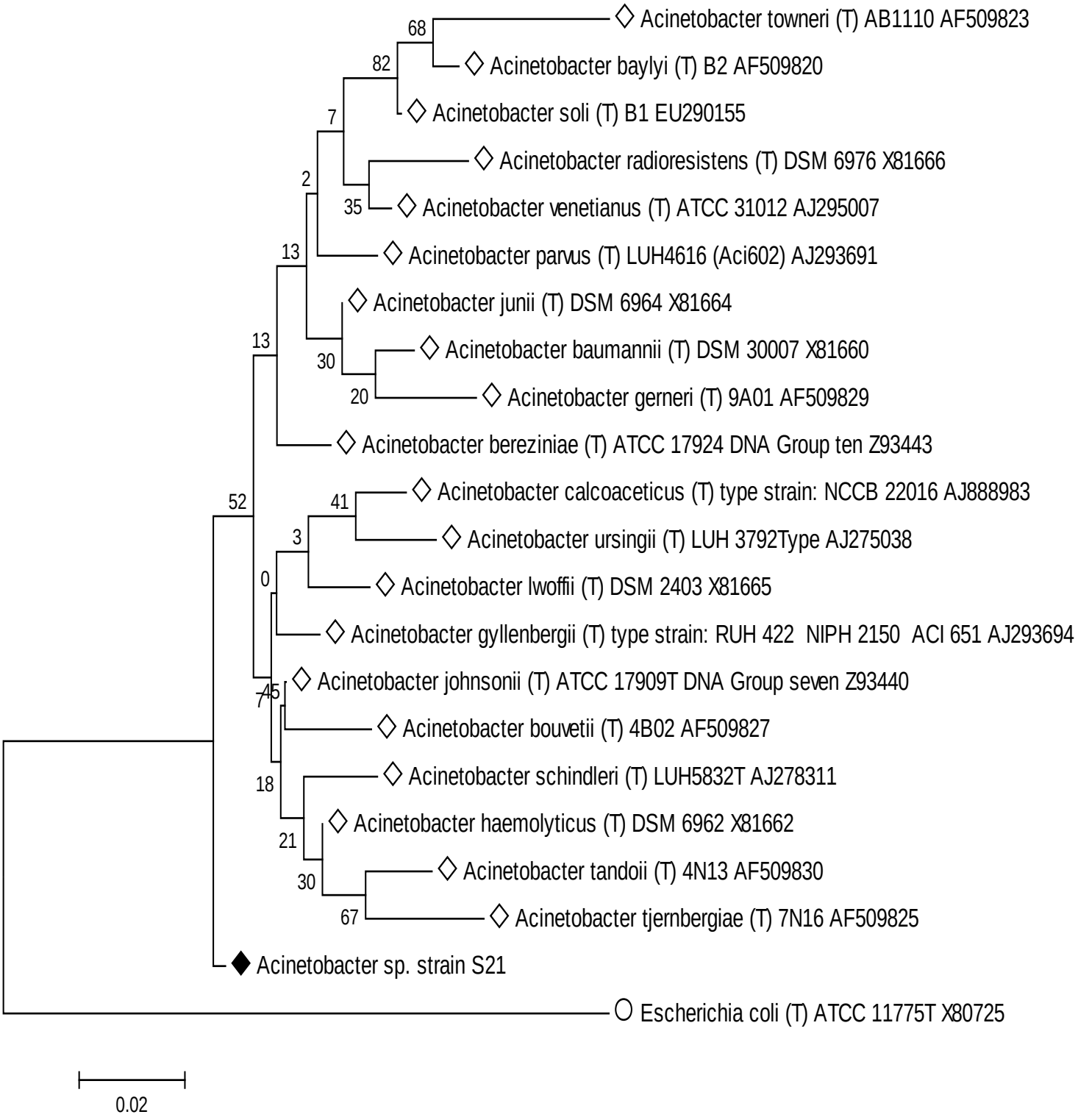

Figure 2.5. Phylogenetic assignment of strain S21 (♦) using the neighbour-joining model based on its 16S rRNA gene sequence in comparison with 16S rRNA selected type strains of *Acinetobacter* (◊). The 16S rRNA gene sequence of *E. coli* (○) was used as out-group. The scale bar represents 2estimated changes per 100 nucleotides.

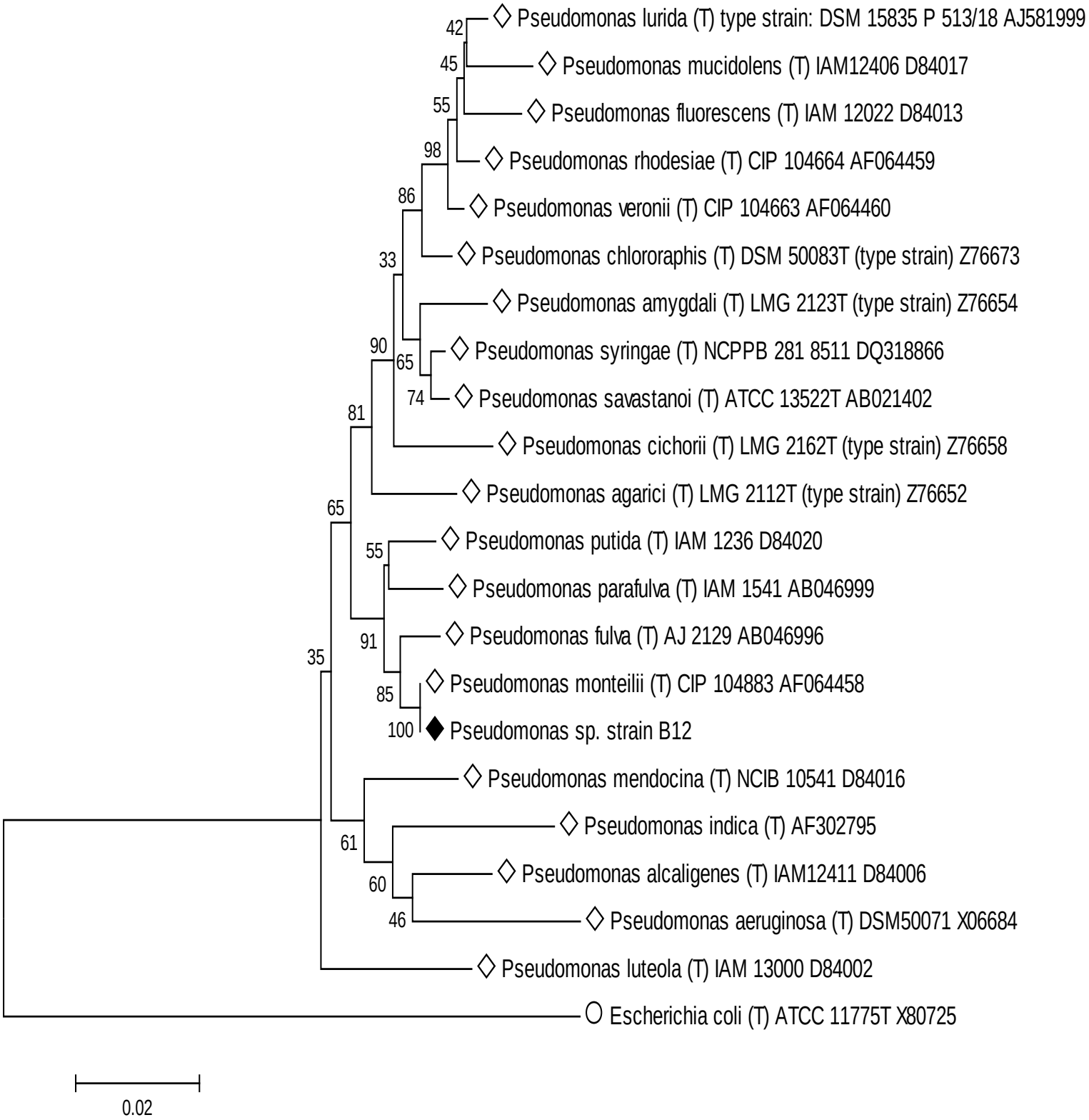

Figure 2.6. Phylogenetic assignment of strain B12 (♦) using the neighbour-joining model based on its 16S rRNA gene sequence in comparison with 16S rRNA selected type strains of *Pseudomonas* (◊). The 16S rRNA gene sequence of *E. coli* (○) was used as out-group. The scale bar represents 2 estimated changes per 100 nucleotides.

The phylogenetic trees illustrate the relationship of the isolated strains with selected type strains (Figure 2.4 to 2.6). The phylogenetic analysis revealed that strain S19 was closely related to the species of the genus *Oceanimonas*. Strain S21 was classified as a member of the genus *Acinetobacter;* however it could not be assigned to an existing species within this genus. Strain B12 was closely related with the species of *Pseudomonas* and forms a clade with *P. monteilii*. All three strains are members of the class *Gamma-Proteobacteria*.

2.3.1.4. Identification by MALDI- TOF Mass Spectroscopy

The classification of the isolated strains was further verified by the use of MALDI-TOF MS which detects highly abundant ribosomal proteins. The mass spectra generated were compared to the spectral database containing the characteristic spectral information of various species. A matching score is generated based on the identified masses and their intensity. The identification of isolates and the scores generated from the whole cell samples and protein extracts are listed in Table 2.6 and 2.7.

Table 2.6. MALDI-TOF MS scores obtained for the whole cell samples of strain S19, S21 and B12.

Analyte Name	Organism (best match)	Score Value	Organism (second best match)	Score Value
S19	Not reliable identification	1.664	Not reliable identification	1.512
S19	Not reliable identification	1.551	Not reliable identification	1.547
S21	*Acinetobacter tandoii*	1.798	Not reliable identification	1.612
S21	*Acinetobacter tandoii*	1.739	Not reliable identification	1.561
B12	Not reliable identification	1.875	Not reliable identification	1.292
B12	Not reliable identification	1.321	Not reliable identification	1.392
BTS	*Escherichia coli*	2.324	*Escherichia coli*	2.354
BTS	*Escherichia coli*	2.395	*Escherichia coli*	2.250

Table 2.7. MALDI-TOF MS scores obtained for protein extracts from strain S19, S21 and B12.

Analyte Name	Organism (best match)	Score Value	Organism (second best match)	Score Value
S19	*Pseudomonas monteilii*	2.308	*Pseudomonas putida*	2.074
S19	*Pseudomonas monteilii*	2.395	*Pseudomonas fulva*	2.032
S21	*Acinetobacter tandoii*	1.953	no reliable identification	1.662
S21	*Acinetobacter johnsonii*	1.739	no reliable identification	1.636
B12	*Pseudomonas monteilii*	2.445	*Pseudomonas fulva*	2.067
B12	*Pseudomonas monteilii*	2.462	*Pseudomonas putida*	2.094
BTS	*Escherichia coli*	2.365	*Escherichia coli*	2.354
BTS	*Escherichia coli*	2.299	*Escherichia coli*	2.250

A MALDI BioTyper score of $\geq$ 2.3 indicates highly probable species identification, a score of 2.000 to 2.299 indicates identification reliable at genus level and probable at species level and a score of 1.700 to 1.999 is indicative of probable genus identification.

MALDI scores obtained from the whole cell samples for all isolates were below 1.999. However, for S21 indicated assignment to the genus *Acinetobacter*. The similarities scores obtained from protein extracts for strain S19 and B12 were above 2.3 indicating reliable identification at species level and reliable assignment to the genus *Pseudomonas*. The results for strain S21 gave a reliable assignment to the genus *Acinetobacter* with a similarity score of 1.953. The control for both whole cell and protein extracts samples was reliably identified as *E. coli* with highly probable species identification.

MALDI-TOF MS confirmed the identity of strain S21 which was assigned to genus *Acinetobacter* and strain B12 which was assigned to genus *Pseudomonas*. Based on the analysis by MALDI-TOF MS, *Oceanimonas* is related to *Pseudomonas*.

2.3.2. Effect of salt concentration on the growth of isolated bacteria

Salinity in estuarine environments fluctuates as continuous mixing of seawater with freshwater take place; consequently, the inhabitants must continuously be adapted to varying salt concentrations. The effect that salt has on the growth of bacteria depends on the osmotic balance required for such growth. Therefore, the effect of salt on the growth of the isolated strains was determined in peptone broth at different NaCl concentration.

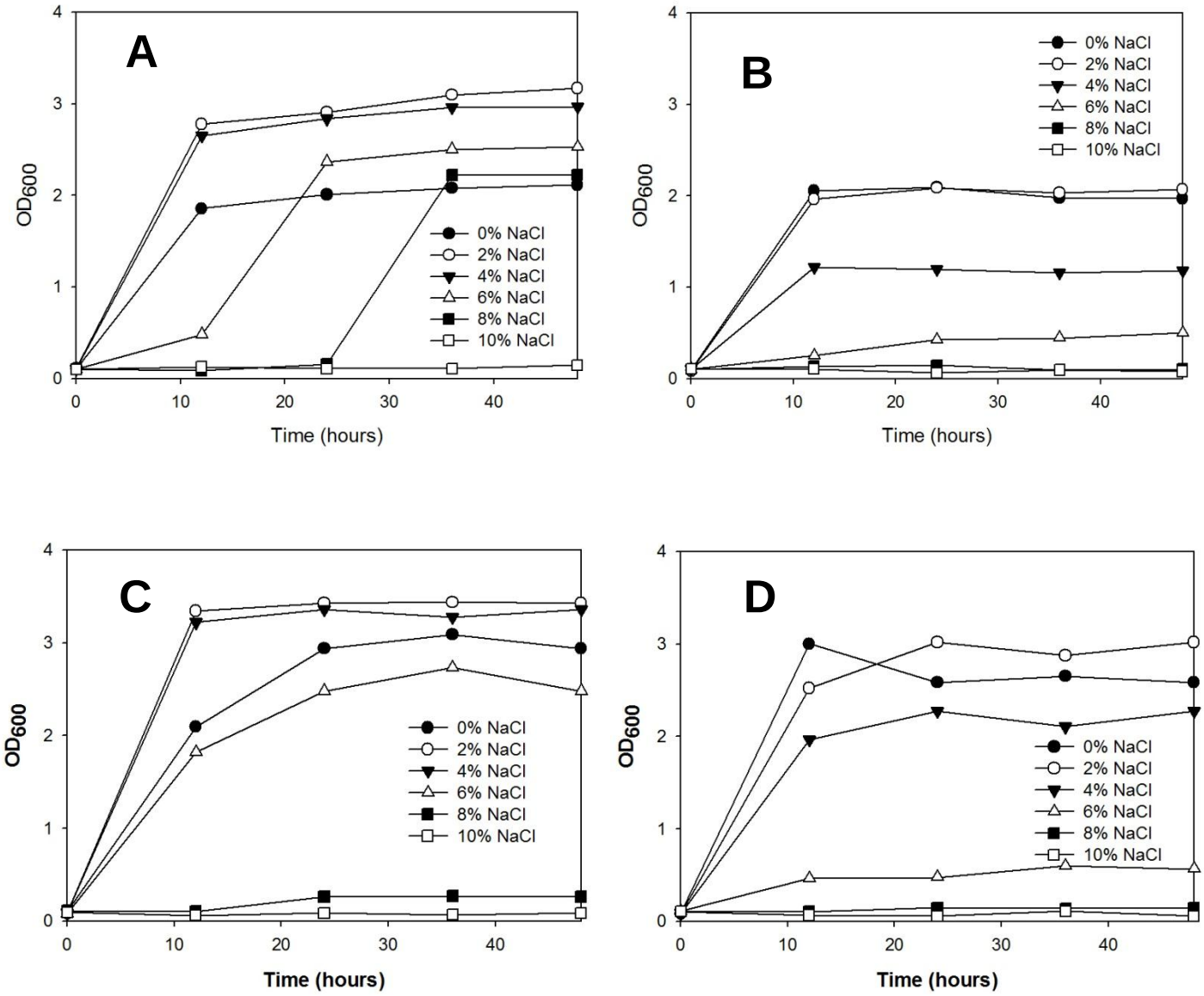

Figure 2.7. Growth of *Oceanimonas* sp. strain S19 (A), *Acinetobacter* sp. strain S21 (B), *Pseudomonas* sp. strain B12 (C) and *E. coli* ATCC 8739 (D) in peptone broth at various NaCl concentrations at 25°C and 200 rpm. The values are means of two independently performed experiments.

The three isolated strains did not require NaCl for growth; optimum growth was nevertheless observed in the presence of 2% and 4% NaCl for *Oceanimonas* sp. strains S19 and *Pseudomonas* sp. strain B12. Strain S19 grew up to 8%, S21 grew up to 6% while B12 grew up to 6% NaCl with little biomass formed at 8% NaCl. No growth was observed at 10% for all strains tested. *E. coli* which was used as a control grew up to 6% NaCl, no growth was observed above 8% NaCl (Figure 2.7).

2.3.3. Growth of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 and utilization of salicylic acid

2.3.3.1. Effect of salicylic acid on the growth of two isolated strains

Microorganisms do not grow sufficiently when the concentration of the growth substrate is lower than their required threshold concentration while higher concentrations might exhibit toxic effects inhibiting the growth of the microorganism. The effect of varying salicylic acid concentrations on the growth of the two strains S19 and S21 was therefore determined.

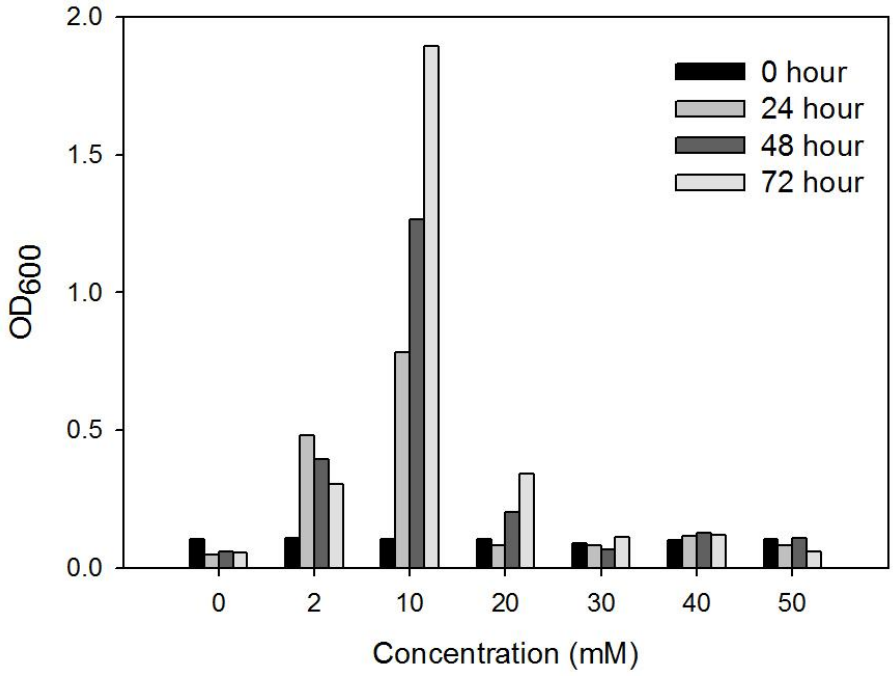

Figure 2.8. Growth of *Oceanimonas* sp. strain S19 in MSM at different concentrations of salicylic acid after different times of incubation at 25°C and 200 rpm. The values are means of two independently performed experiments.

The highest OD_{600} of strain S19 was observed at 10 mM while only slight growth was detected at 20 mM after 48 and 72 hours. At concentration above 20 mM salicylate, hardly any growth was detectable (Figure 2.8).

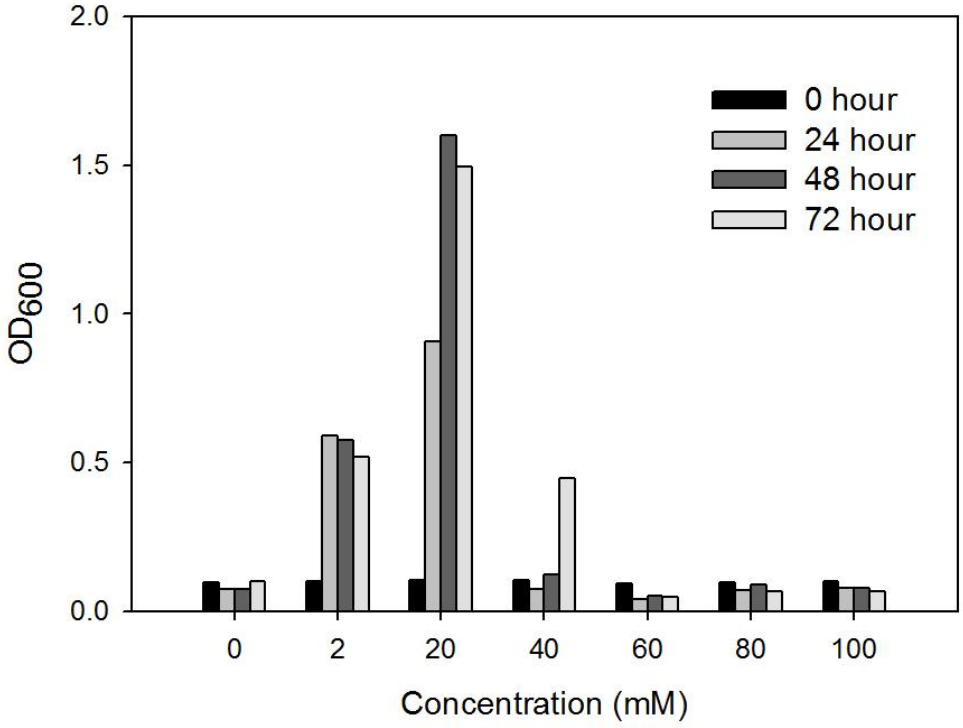

Figure 2.9. Growth of *Acinetobacter* sp. strain S21 at different concentrations of salicylic acid after different times of incubation at 25°C and 200 rpm. The values are means of two independently performed experiments.

For strain S21, the highest OD_{600} was observed at 20 mM salicylic acid. Growth was only evident at 40 mM after 72 hours while no growth was observed at concentrations above 40 mM (Figure 2.9).

2.3.3.2. Growth of *Oceanimonas* sp. strain S19 with 2 mM salicylic acid

To determine whether salicylic acid served as a carbon and energy source for growth of the isolated strains, initially isolate S19 was grown with 2 mM salicylic acid as a carbon source. The growth was analyzed over time by measuring the OD_{600} and the substrate utilization was analyzed by UV spectroscopy and verified by HPLC.

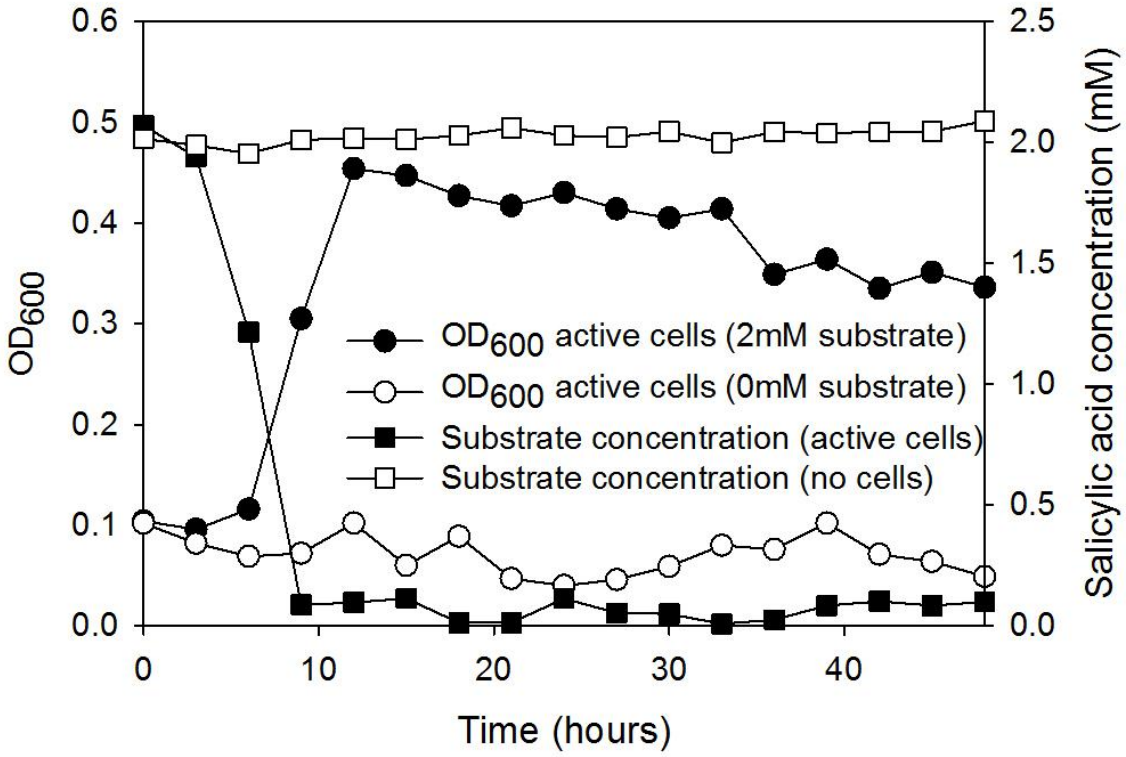

Figure 2.10. Growth of *Oceanimonas* sp. strain S19 with salicylic acid as the sole carbon source in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

Three phases were observed over the period of incubation (Figure 2.10), lag phase for 6 hours, exponential phase lasting for about 6 hours and stationary phase after 12 hours of incubation with slow decline. No growth was observed in the absence of substrate. With increasing biomass (OD_{600}), the substrate concentration decreased with 95% consumed within 9 hours of incubation. No change of substrate concentration was observed in the absence of cells. Analysis by HPLC revealed that salicylic acid concentration decreased over time and after 9 hours of incubation salicylic acid was not detected.

In addition to using OD_{600}, growth of strain S19 in MSM with 2 mM salicylic acid was also monitored by determining total microscopic cell counts for growth kinetics determination as well as protein concentrations over time.

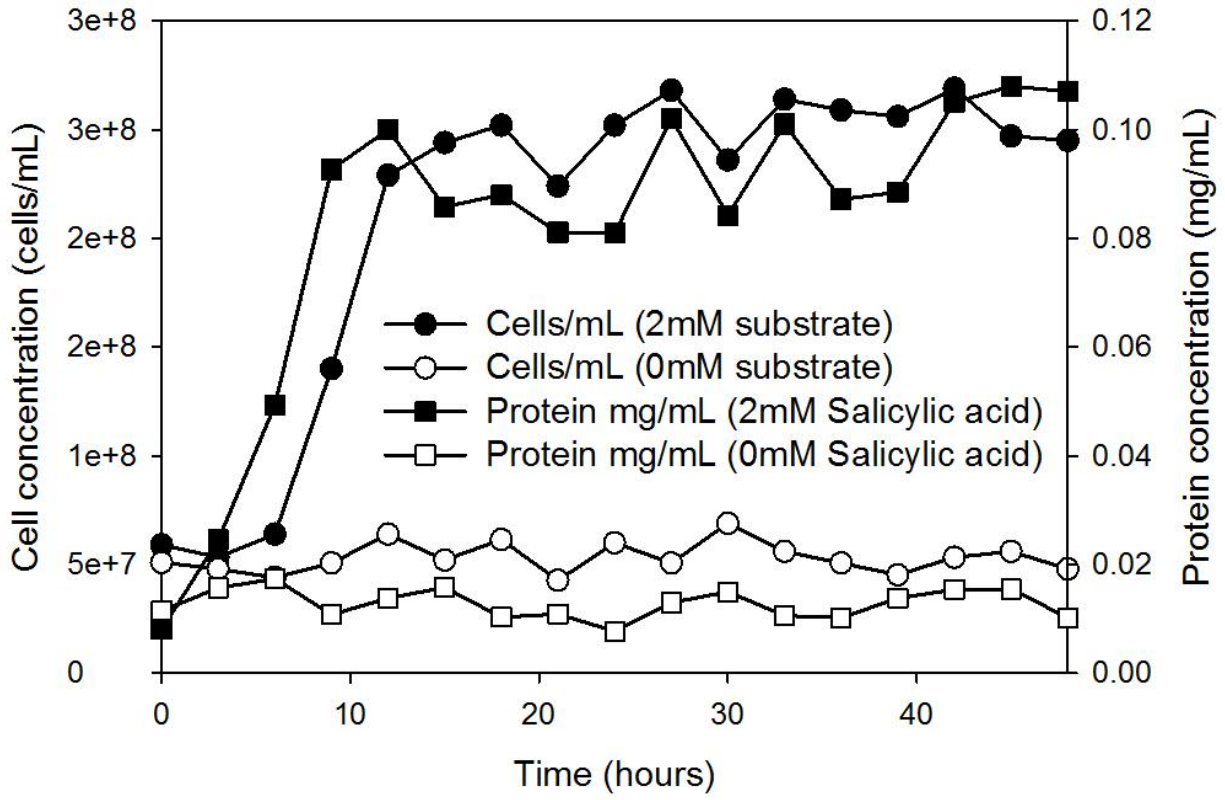

Figure 2.11. Protein concentration and cell concentration of *Oceanimonas* sp. strain S19 growing with salicylic acid as growth substrate in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

As shown in Figure 2.11, the protein concentrations increase with the increase in number of cells in the presence of salicylic acid as sole carbon source. The graph shows a typical growth curve with a lag phase for 6 hours, followed by an exponential phase and stationary phase. As expected, controls without substrate did not show any increase in protein concentration or cell concentration.

The growth kinetics of *Oceanimonas* sp. strain S19 growing with 2 mM salicylic acid in MSM were determined from these experiments. The strain had growth rate (μ) of 0.212 cells/mL per hour, and 0.218 mg/mL protein per hour, doubling time (t_d) of 3.06 hours (from OD_{600}) and 3.125 hours (from protein concentrations) and generation time (G) of 3.26 hours (from cell concentrations) with generation number (n) of 2.

In addition to microbial catabolism, the elimination of salicylic acid in the environment may be to some degree due to abiotic processes (i.e. photodegradation). To confirm that isolate S19 was responsible for the elimination of the aromatic substrate salicylic acid, UV-VIS spectroscopy was employed, scanning samples taken after 0 and 24 hour incubation in a range of 200nm to 400nm.

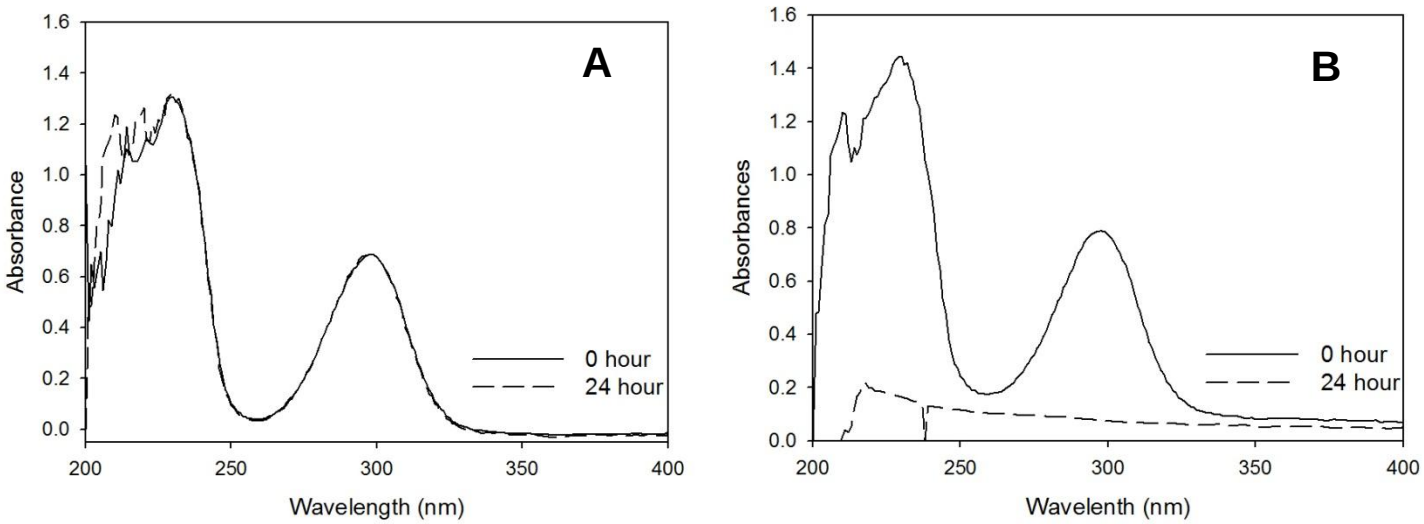

Figure 2.12. The UV-VIS spectral analysis of MSM with 2 mM salicylic acid in the absence (A) and presence (B) of *Oceanimonas* sp. strain S19 after 0 and 24 hours incubation at 25°C and 200 rpm.

No change in the spectrum of salicylic acid was observed in the absence of bacterial cells indicating that the aromatic compound remained intact throughout the incubation period. In the presence of the isolate, after 24 hours of incubation the spectrum clearly showed that the aromatic substrate had been catabolized by the cells of strain S19 (Figure 2.12). This indicates that the microbial activity was solely responsible for the disappearance of salicylic acid in the medium.

2.3.3.3. Growth of *Acinetobacter* sp. strain S21 with 2 mM salicylic acid

The growth of isolate S21 was studied in MSM with 2 mM salicylic acid to determine whether the target compound served as growth substrate. The growth was monitored by measuring OD_{600} and salicylic acid concentrations were analyzed by UV spectroscopy. The utilization of salicylic acid was also confirmed by HPLC analysis.

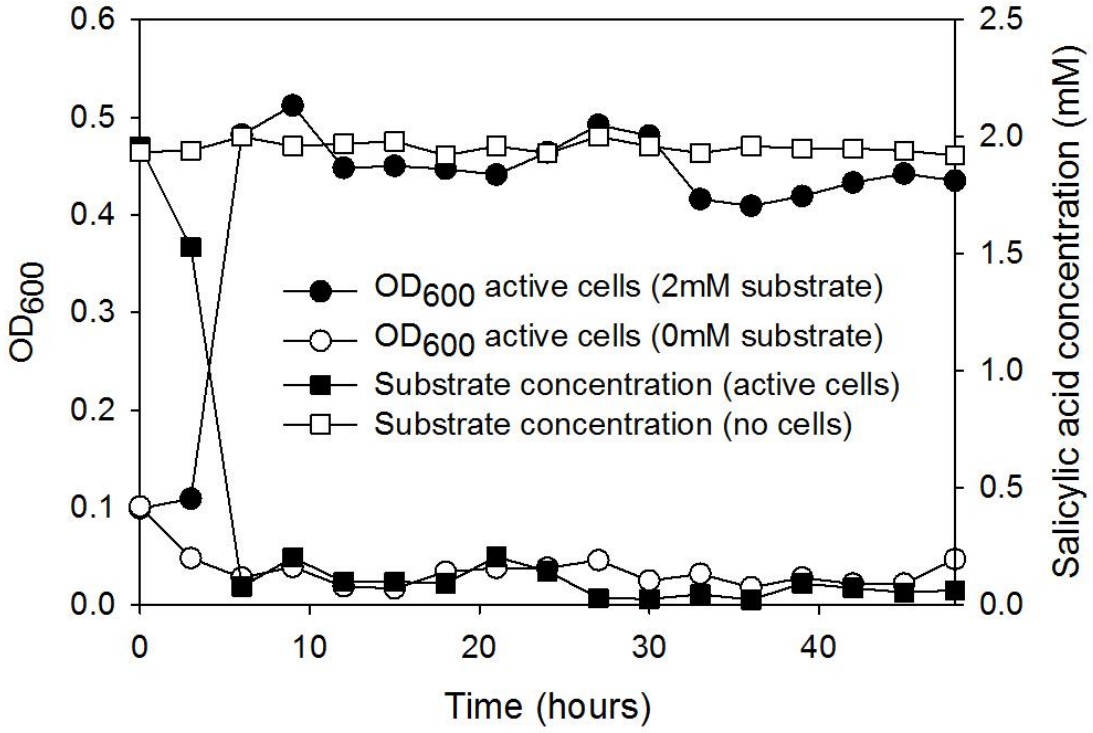

Figure 2.13.Growth of *Acinetobacter* sp. strain S21 with salicylic acid as the sole carbon source in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

The growth of isolate S21 in MSM with 2 mM salicylic acid shows a typical growth curve, a lag phase for about 3 hours, an exponential phase of about 3 hours and then stationary phase starting after 6 hours. No increase in biomass was observed when the isolate was incubated in the absence of substrate. Biomass formation increase correlated with substrate decrease with no change in the concentration without cells present (Figure 2.13). More than 95% the substrate was utilized after 6 hours of incubation and could not be detected anymore in medium by HPLC.

In addition, growth kinetics were monitored in MSM with 2 mM salicylic acid by establishing total microscopic cell counts and protein concentrations of isolate S21 over time.

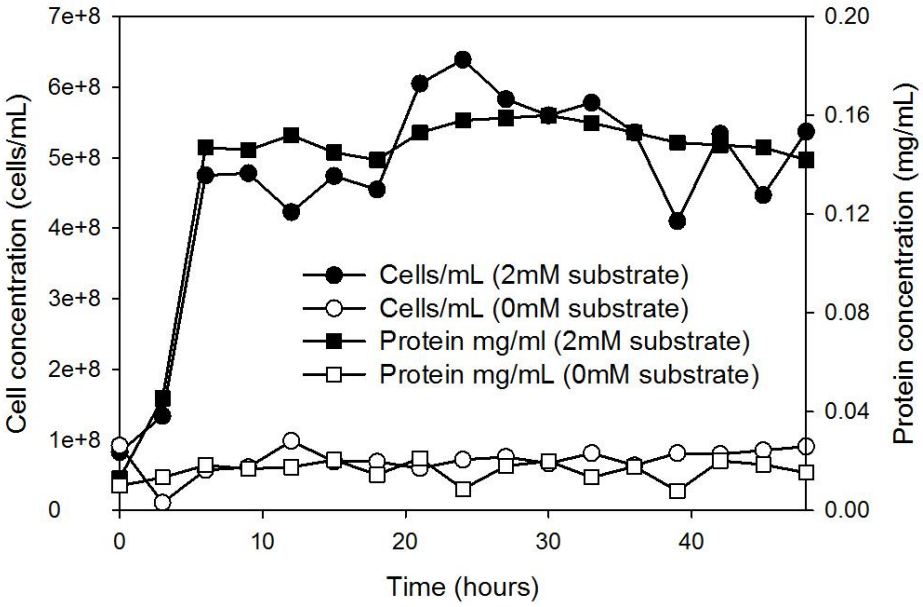

Figure 2.14. Protein concentration and cell concentration of *Acinetobacter* sp. strain S21 growing with salicylic acid as growth substrate in MSM overtime at 25°C and at 200 rpm. The values are means of two independently performed experiments.

As demonstrated in Figure 2.14, the graph shows a typical growth curve with a lag phase for 3 hours, followed by an exponential phase for about 3 hours and stationary phase after 6 hours. Again, protein concentrations increase with the increase in cell concentrations over time in the presence of salicylic acid. As expected, controls without substrate did not show any growth.

The growth kinetics of *Acinetobacter* sp. strain S21 growing with 2 mM salicylic acid in MSM were determined. The strain had a growth rate (μ) of 0.422 cells/mL per hour and 0.391 mg/mL protein per hour, doubling (t_d) of 1.4 hours (from OD_{600}) and 1.5 hours (from protein concentrations) and a generation time (G) of 1.6 hours (from cell concentrations) with generation number (n) of 2.

To verify that the isolate strain S21 was responsible for the disappearance of salicylic acid in the medium, samples were collect at 0 and 24 hours of incubation and were analyzed by UV-VIS spectroscopy over a range 200nm to 400nm.

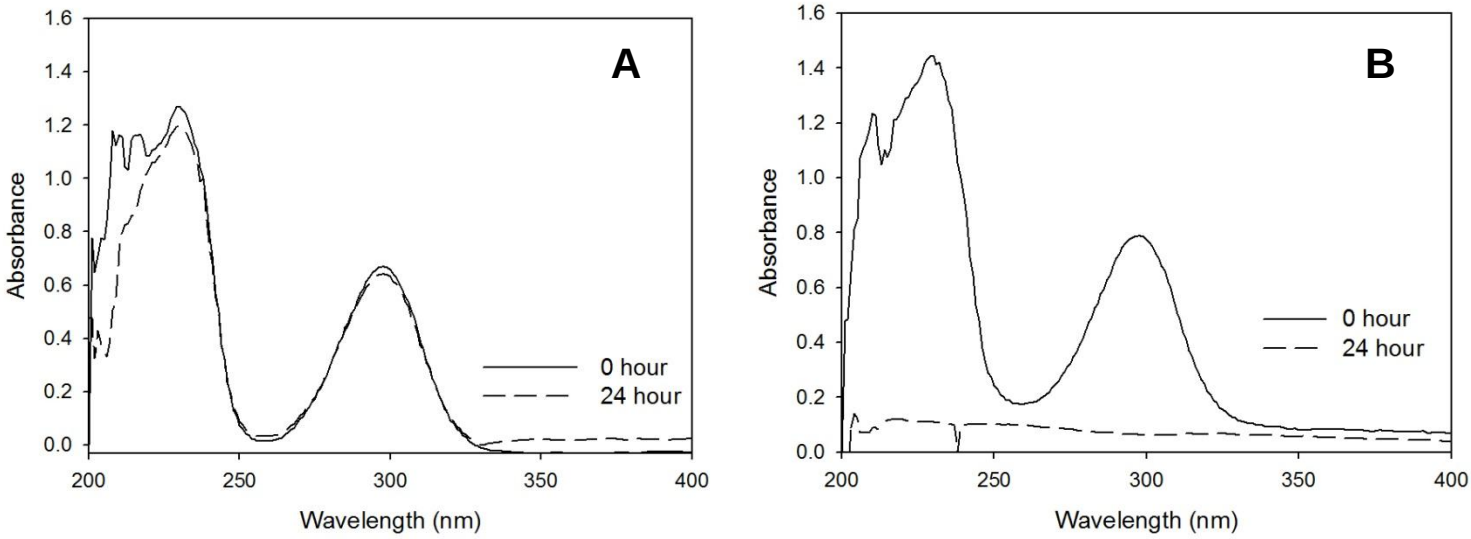

Figure 2.15. The UV-VIS spectral analysis of MSM with 2 mM salicylic acid in the absence (A) and presence (B) of *Acinetobacter* sp. strain S21 after 0 and 24 hours incubation at 25°C and 200 rpm.

No change in the spectrum of salicylic acid was observed in the absence of bacterial cells indicating that the aromatic compound remained intact throughout the incubation period. In the presence of the isolate, the scan shows that the aromatic substrate had been catabolized by the cells of strain S21 (Figure 2.15). Therefore the microbial activities were solely responsible for disappearance of salicylic acid in the medium.

2.3.4. Growth of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain and utilization of phenyl salicylate

2.3.4.1. Effect of phenyl salicylate on the growth of the two isolated strains

The effect of phenyl salicylate on the growth of strain S19 was determined in mineral salts medium at different concentrations of phenyl salicylate as sole carbon source.

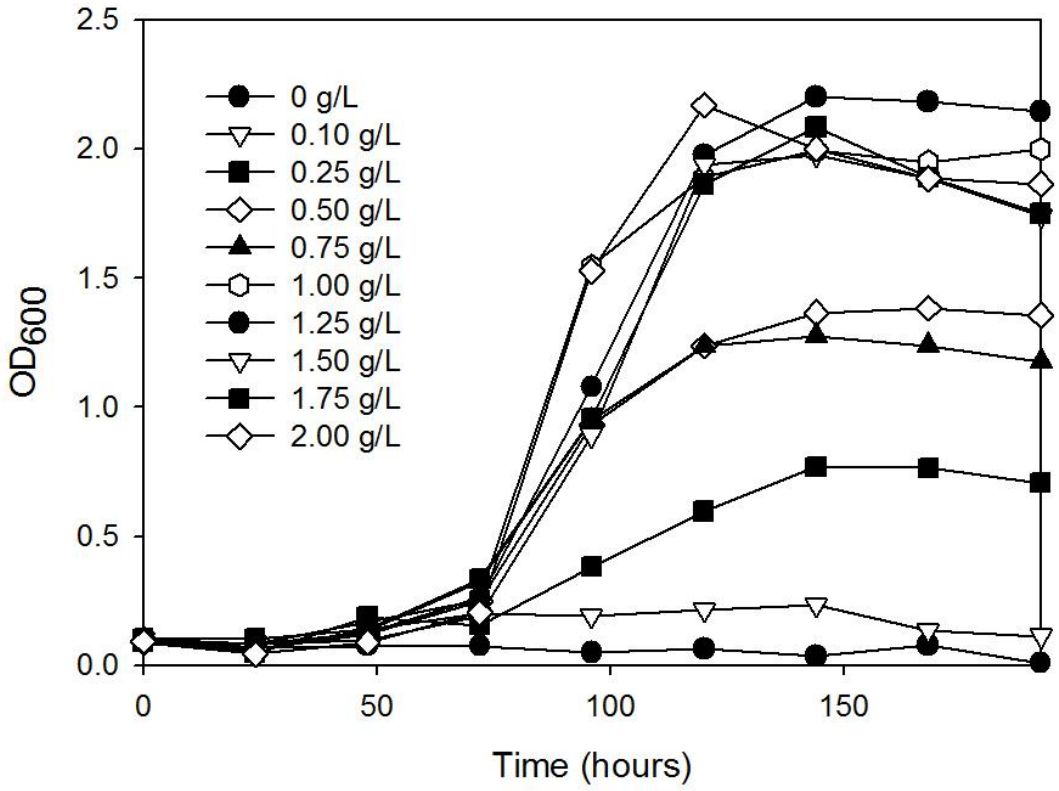

Figure 2.16. Growth of *Oceanimonas* sp. strain S19 in MSM with phenyl salicylate at different concentrations at 25°C and 200 rpm. The values are means of two independently performed experiments.

As demonstrated in Figure 2.16, phenyl salicylate did not inhibit the growth of strain S19 at the concentrations tested but the biomass formation increased with the increase in substrate concentration of up to 2 g/L. Only minute growth was observed at 0.1 g/L and no growth in the absence of substrate.

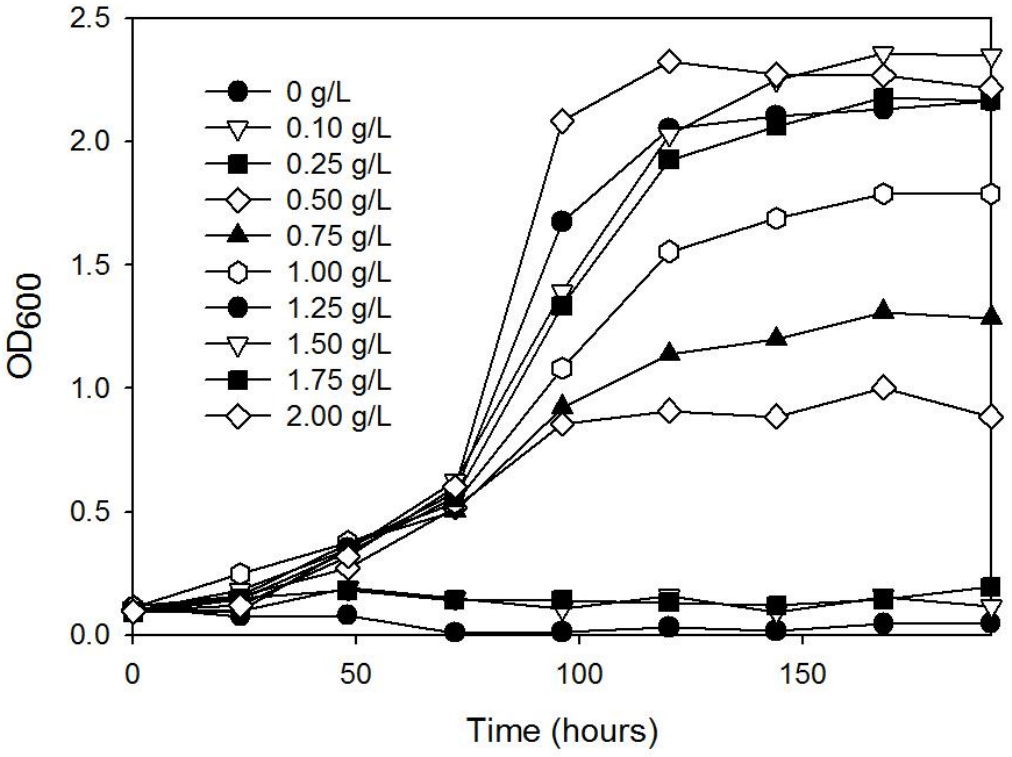

Figure 2.17. Growth of *Acinetobacter* sp. strain S21 in MSM with varying concentration of phenyl salicylate at 25°C and 200 rpm. The values are means of two independently performed experiments.

Phenyl salicylate did not inhibit the growth of strain S21 but the biomass formation increased with the increase in substrate concentration up to concentration of 2 g/L. Essentially no growth was observed in the absence of substrate as well as at 0.10 g/L and 0.25 g/L but became prominent from 0.5 g/L to 2.0 g/L (Figure 2.17).

2.3.4.2. Growth of *Oceanimonas* sp. strain S19 with phenyl salicylate

To determine the phenyl salicylate utilization over time, the growth of the isolated strain S19 was monitored in mineral salts medium with 0.5 g/L of phenyl salicylate as sole carbon source. The growth was monitored by determining the OD_{600} and the substrate concentration was analyzed by HPLC.

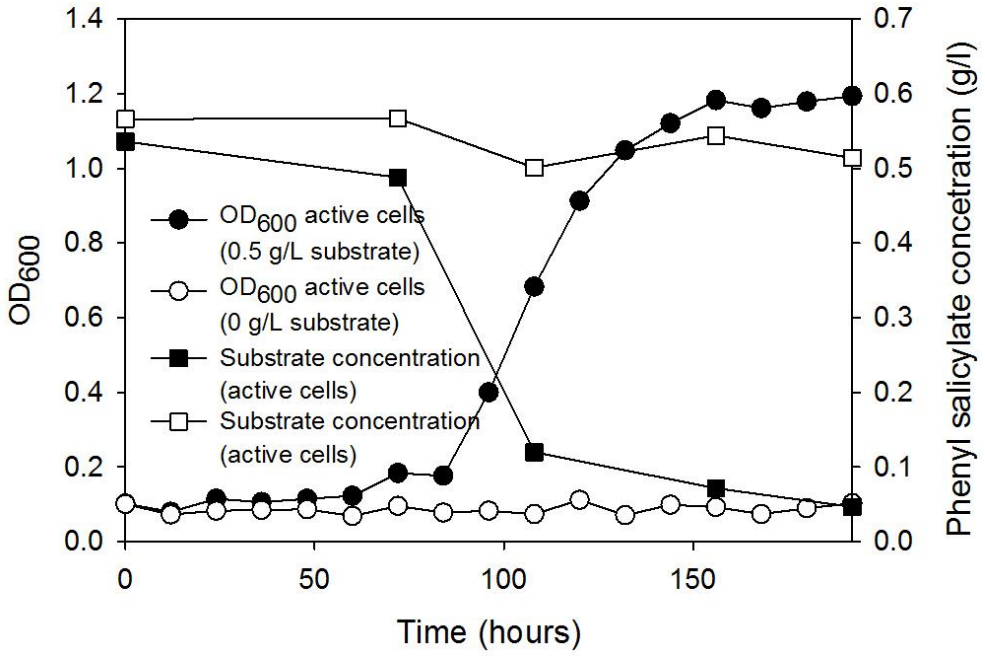

Figure 2.18. Growth of *Oceanimonas* sp. strain S19 with phenyl salicylate as the sole carbon source in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

The growth of isolate S19 with phenyl salicylate represents a typical growth curve, long lag phase that occurred for about 84 hours, an exponential phase of about 60 hours and then stationary phase starting at about 132 hours (Figure 2.18). No growth occurred in the absence of phenyl salicylate but the biomass increased with the decrease in substrate concentration with more than 90% utilized within 192 hours. This indicates that the growth of S19 was dependent on phenyl salicylate as growth substrate.

The growth kinetics of *Oceanimonas* sp. strain S19 with 0.5 g/L of phenyl salicylate was also monitored by determining the cell concentrations and the protein concentration over time.

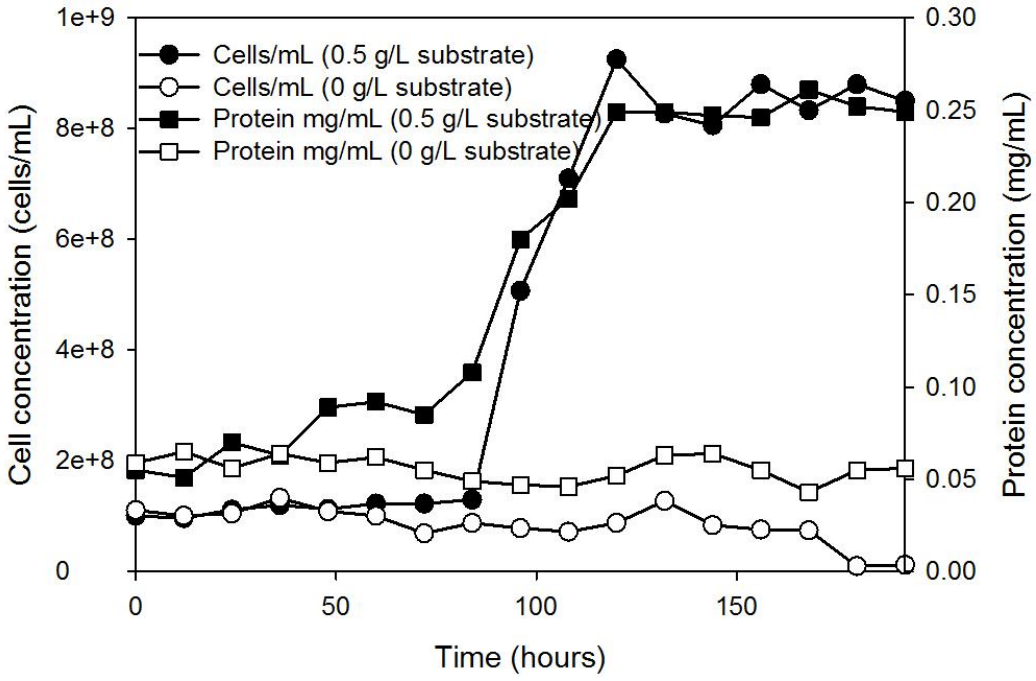

Figure 2.19. Protein concentration and cell concentration of *Oceanimonas* sp. strain S19 growing with phenyl salicylate as growth substrate in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

As shown in Figure 2.19, the protein concentration increases with the increase in cell concentration over time in the presence of phenyl salicylate. The growth shows a typical growth curve with lag phase for about 84 hours, an exponential phase which lasted for about 60 hours and stationary phase after 132 hours.

The growth kinetics of *Oceanimonas* sp. strain S19 growing with 0.5 g/L phenyl salicylate in MSM was determined. The strain had growth rate (μ) of 0.0234 cells/mL per hour and 0.0251 mg/mL protein per hour, doubling time (td) of 31.30 hours (from OD_{600}) and 34.05 hours (from protein concentrations), generation time (G) of 30.59 hours (from cell concentrations) with generation number (n) of 3.

Phenyl salicylate has a low solubility in aqueous medium (refer to section 2.3.6). For the utilization of hydrophobic compounds to occur, bacteria either have to attach to the substrate such in a fashion of biofilm to enable substrate or synthesize chemicals that facilitate dissolution. *Oceanimonas* sp. strain S19 was examined by phase contrast microscopy to determine whether the cells of the strain adhere to the crystals of phenyl salicylate.

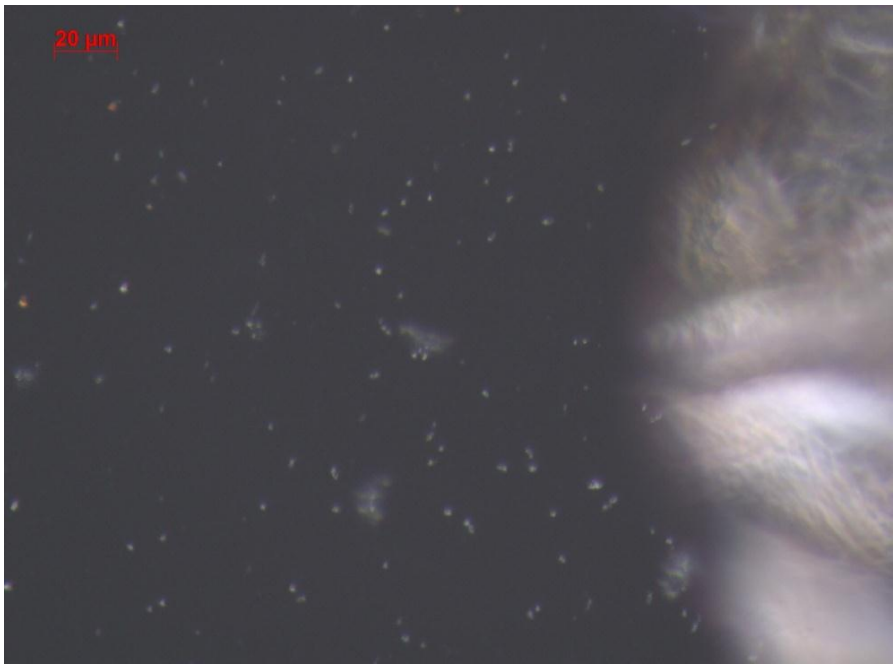

Figure 2.20. Phase contrast microscopy of samples during exponential growth of *Oceanimonas* sp. strain S19 in MSM with 0.5 g/L phenyl salicylate at 25°C and 200 rpm.

According to the microscopic analysis (Figure 2.20), the cells of strain S19 did not attached to the substrate crystal surface but move freely. This would indicate that they most likely synthesize a compound that solubilizes the substrate for catabolism or use only low amounts dissolved therefore slower growth.

2.3.4.3. Growth of *Acinetobacter* sp. strain S21 with phenyl salicylate

The growth of *Acinetobacter* sp. strain S21 in mineral salt medium with 0.5 g/L phenyl salicylate was analyzed to determine whether the strain utilized the compound as carbon and energy source. The growth was monitored every 12 hours by measuring the optical density at 600nm and the substrate utilization was analyzed by HPLC.

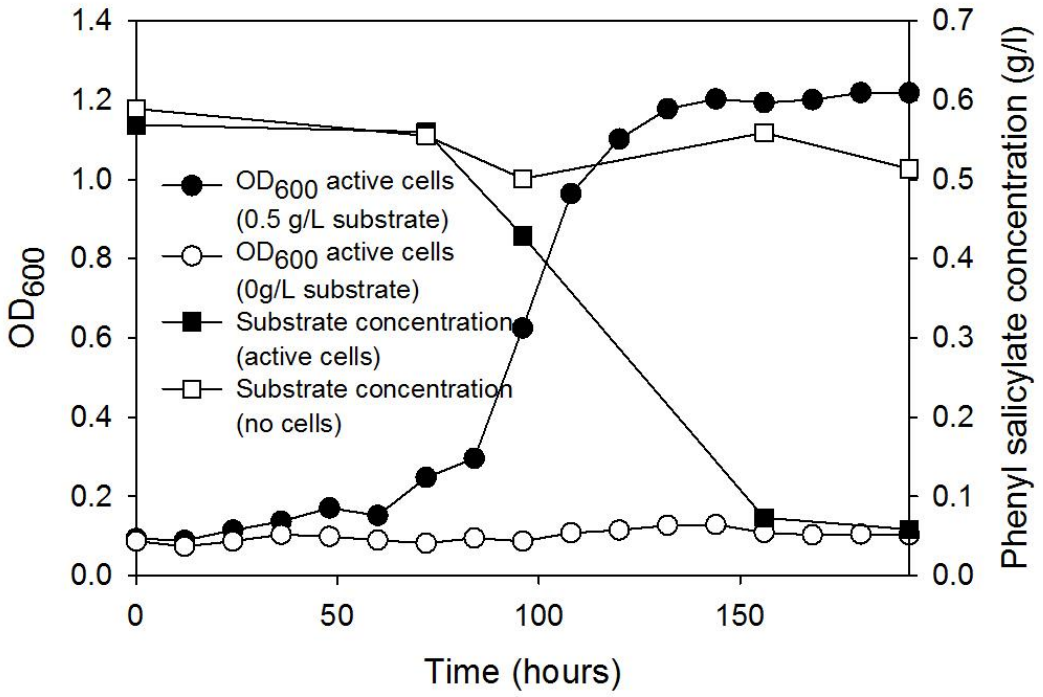

Figure 2.21. Growth of *Acinetobacter* sp. strain S21 with phenyl salicylate as the sole carbon source in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

The growth of *Acinetobacter* sp. strain S21 with phenyl salicylate follows a pattern expected for a typical growth curve, an extended lag phase that occurred for about 60 hours, an exponential phase lasting for about 72 hours and then stationery phase after 132 hours and no growth occurred in the absence of phenyl salicylate (Figure 2.21). Biomass increased with the decrease in concentration of the substrate with more than 90% utilized within the period of incubation. No growth was detected in the absence of substrate. Thus phenyl salicylate served as a carbon source for growth of *Acinetobacter* sp. strain S21.

The growth of *Acinetobacter* sp. strain S21 with 0.5 g/L phenyl salicylate was also monitored over time by determining the cell concentration and protein concentration.

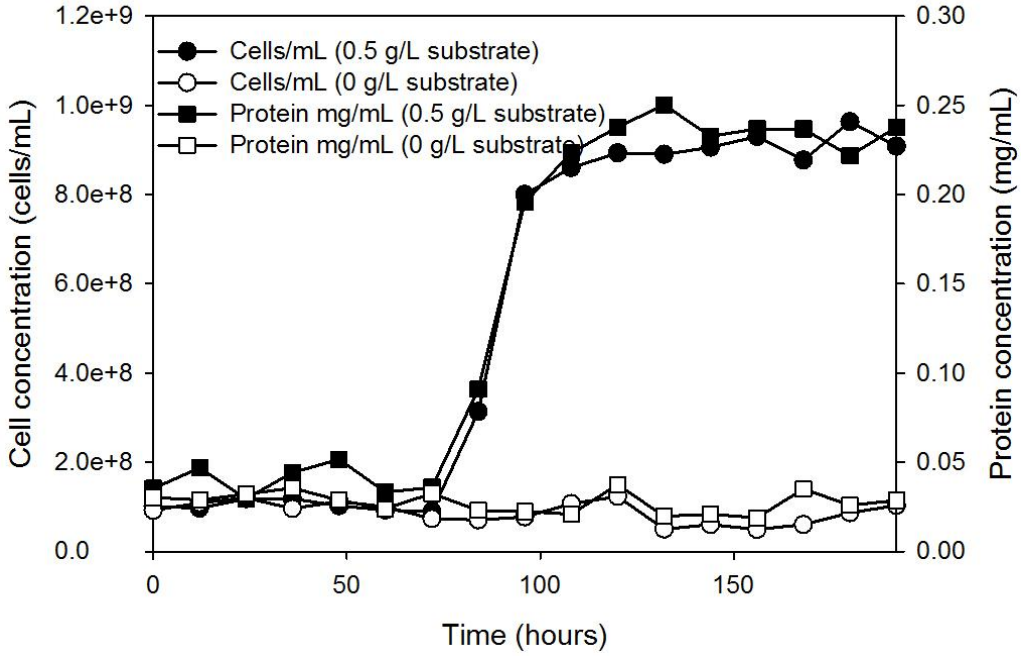

Figure 2.22. Protein concentration and cell concentration of *Acinetobacter* sp. strain S21 growing with phenyl salicylate as growth substrate in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

There was concomitant increase in protein concentration with an increase in cell concentration overtime in the presence of phenyl salicylate showing a typical growth curve lag phase for about 60 hours followed by exponential phase that lasted for about 60 hours and then stationery phase after 120 hours. No biomass was detected in the absence of substrate (Figure 2.22).

The growth kinetics of *Acinetobacter* sp. strain S21 with 0.5 g/L phenyl salicylate in MSM were determined. The strain had a growth rate (μ) of 0.0302 cells/mL per hour and 0.0281 mg/mL protein per hour, doubling time (t_d) of 24.37 hours (from OD_{600}) and 24.71 hours (from protein concentrations) and generation time (G) of 23.06 hours (from cell concentrations) with generation number (n) of 3.

As phenyl salicylate has low water solubility, the culture samples of *Acinetobacter* sp. strain S21 were examined by phase contrast microscopy to determine whether the cells attach to the substrate crystal surface.

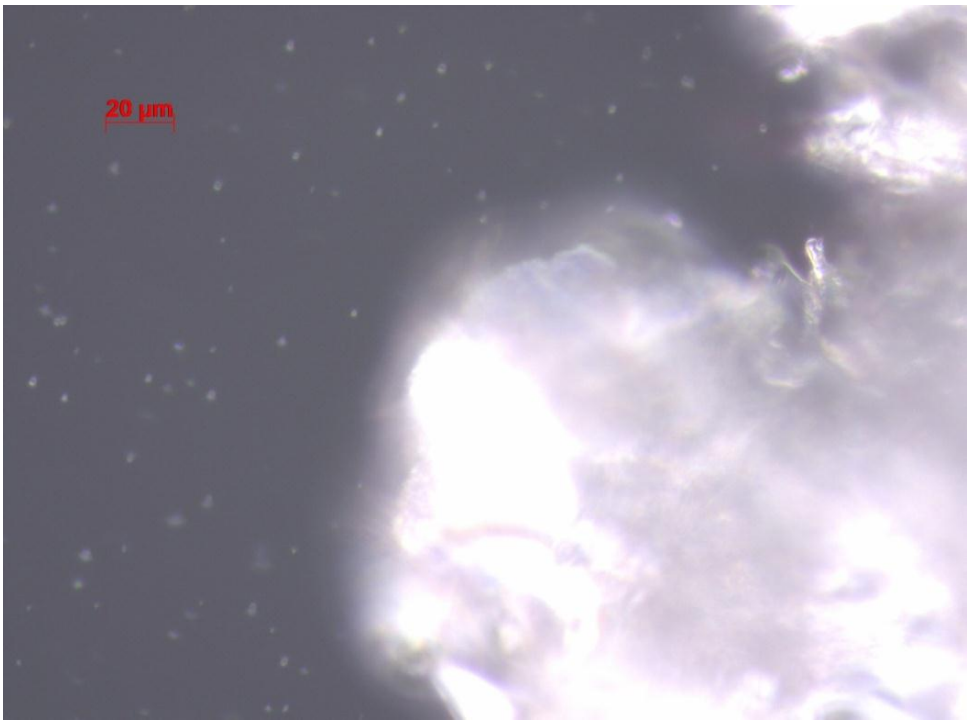

Figure 2.23. Phase contrast microscopy of samples during exponential growth of *Acinetobacter* sp. strain S21 in MSM with 0.5 g/L phenyl salicylate at 25°C and 200 rpm.

Similarly to *Oceanimonas* sp. strain S19, the cells of *Acinetobacter* sp. strain S21 did not attach to the substrate surface but move freely, thus the strain most likely synthesize a compound that facilitated the solubility of phenyl salicylate for catabolism or only use low amount of substrate dissolved and therefore slower growth.

2.3.5. Growth of *Pseudomonas* sp. strain B12 with benzyl salicylate

2.3.5.1. The growth *Pseudomonas* sp. strain B12 with 2.2 mM benzyl salicylate

To determine whether the isolated strain was able to utilize benzyl salicylate as sole carbon and energy source, the strain was grown in mineral salts medium with 2 mM added benzyl salicylate. The growth was monitored every 12 hours by determining the OD_{600}.

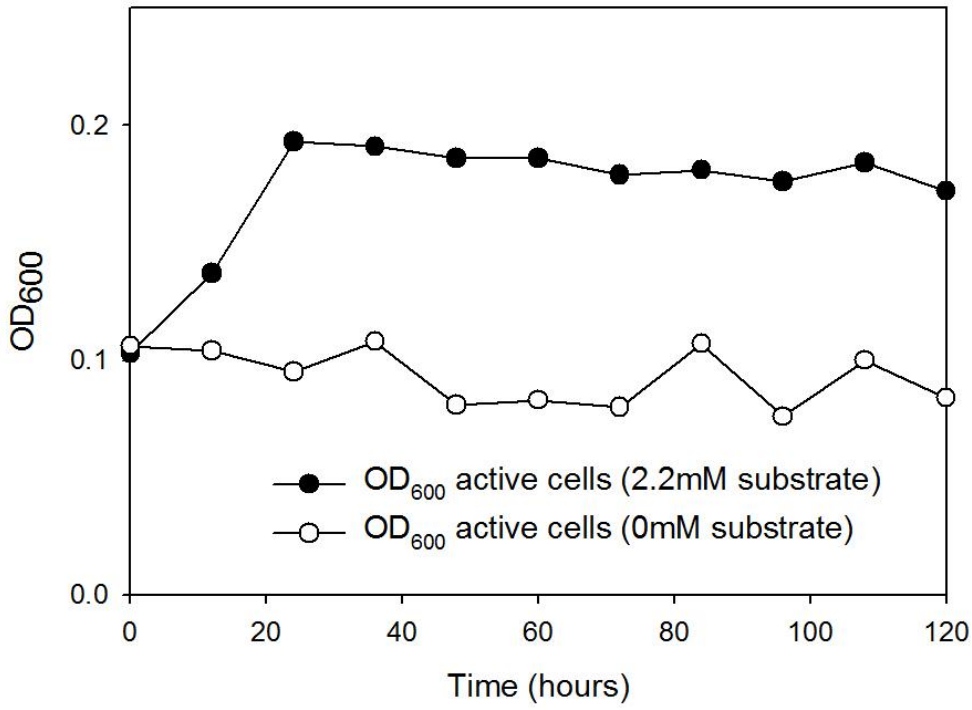

Figure 2.24. Growth of *Pseudomonas* sp. strain B12 in MSM with 2.2 mM benzyl salicylate at 25°C and 200 rpm. The values are means of two independently performed experiments.

Based on the OD_{600} measurements (Figure 2.24), no growth was observed in the absence of the substrate. However, biomass was formed in the presence of the substrate though in limited quantities. Stationary phase was reached after 24 hours of incubation and no lag phase was observed.

To verify the results shown in Figure 2.24, in addition to OD_{600} the growth of *Pseudomonas* sp. strain B12 was also monitored by determining protein and cell concentration over time.

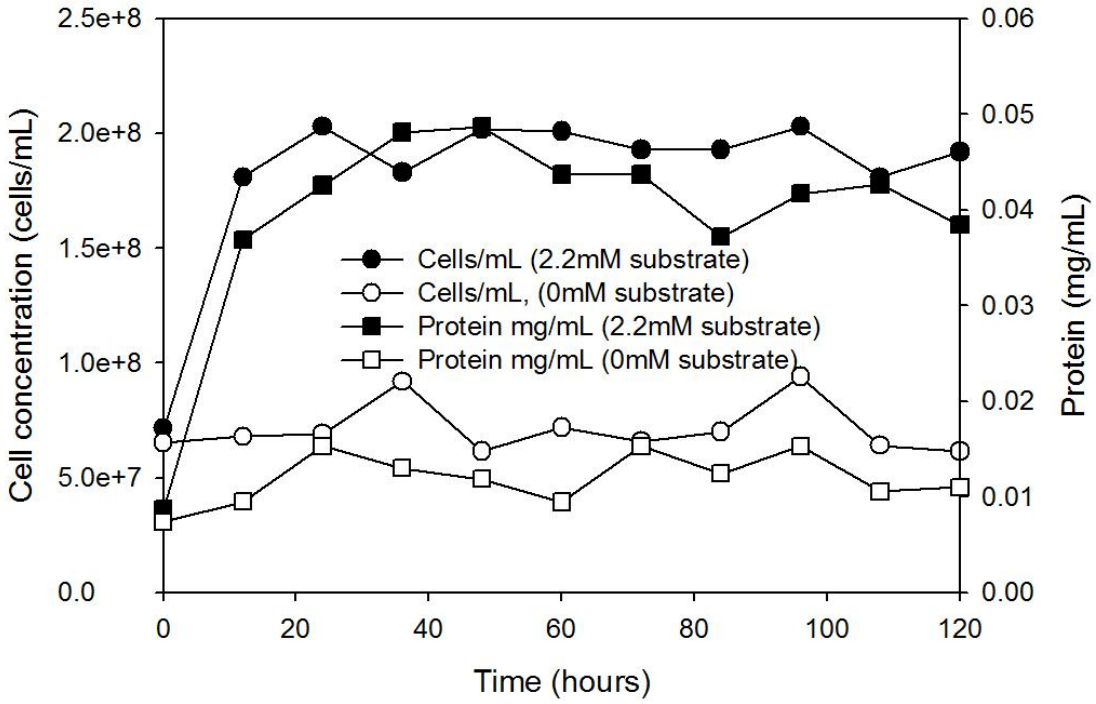

Figure 2.25. Protein concentration and cell concentration of *Pseudomonas* sp. strain B12 growing with 2.2 mM benzyl salicylate as growth substrate in MSM over time at 25°C and 200 rpm. The values are means of two independently performed experiments.

This experiment confirmed that in the presence of benzyl salicylate an increase in both cell concentration and protein concentration took place in comparison to controls without benzyl salicylate (Figure 2.25). The growth kinetics of *Pseudomonas* sp. strain B12 with 2.2 mM benzyl salicylate in MSM were determined. The strain had a growth rate (μ) of 0.0409 cells/mL per hour and 0.0549 mg/mL protein per hour, doubling time (td) of 20.14 hours (from OD600) and 18.71 hours (from protein concentrations) and generation time (G) of 18.67 hours (from cell concentrations) with generation number (n) of 1.

Benzyl salicylate has a limited aqueous solubility (refer to section 2.3.6.). To determine whether the cells attach to the substrate surface, the cell of strain B12 were examined by phase contrast microscopy.

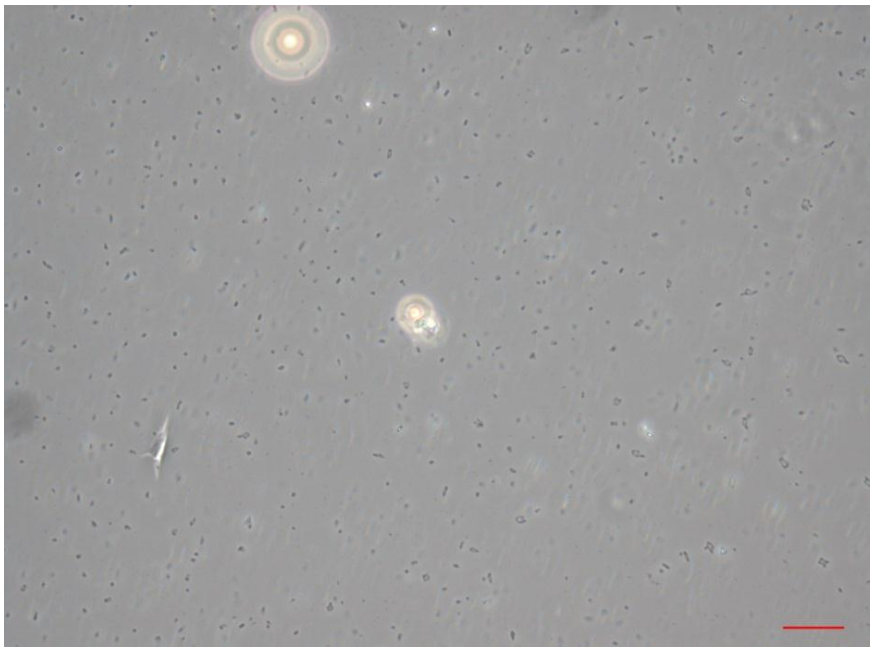

Figure 2.26. Phase contrast microscopy of samples during exponential growth of *Pseudomonas* sp. strain B12 in MSM with 2.2 mM benzyl salicylate at 25°C and 200 rpm.

As demonstrated by the phase contrast microscopy (Figure 2.26), the cells of *Pseudomonas* sp. strain B12 did not attach the substrate surface but move freely.

2.3.5.2. Growth of *Pseudomonas* sp. strain B12 with possible intermediates

To determine whether possible intermediates formed by hydrolysis of the ester bond during and subsequent oxidation of the benzyl alcohol to benzoate during utilization of benzyl salicylate were utilized by *Pseudomonas* sp. strain B12, it was grown in mineral salts medium with 2.2 mM of either benzyl alcohol, benzoic acid or salicylic acid. The growth was monitored by determining the OD_{600} every 24 hours.

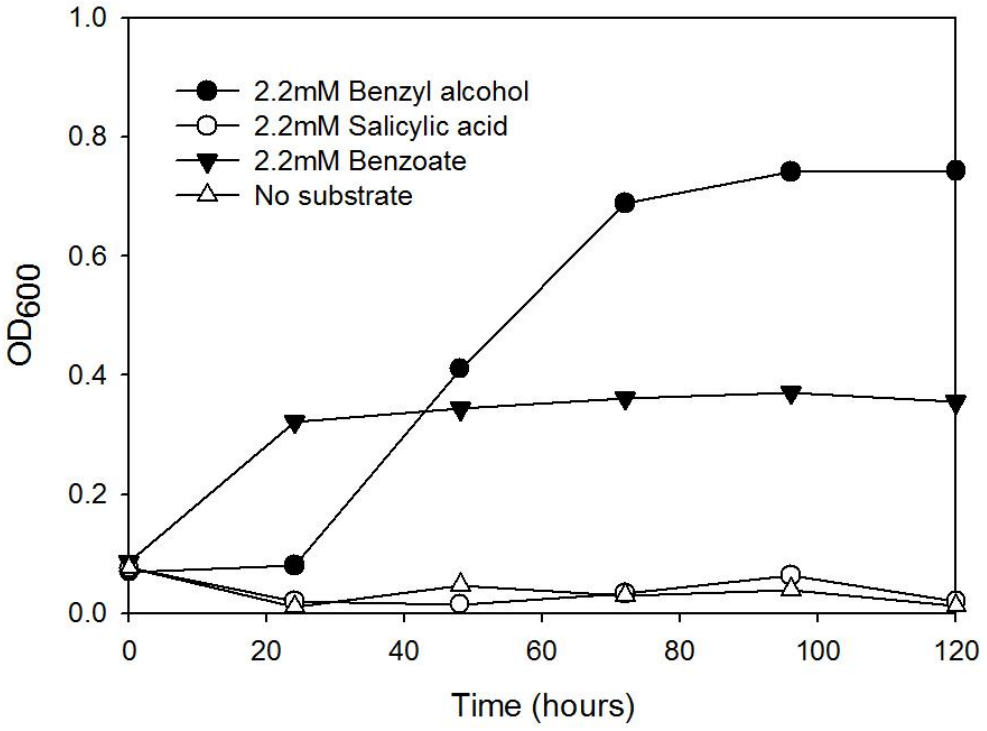

Figure 2.27. Growth of *Pseudomonas* sp. strain B12 with 2.2 mM benzyl alcohol, benzoic acid and salicylic acid as sole carbon and energy source in MSM at 25°C and 200 rpm.

Growth of the strain occurred in the presence of growth substrate benzyl alcohol and benzoic acid but no biomass was formed with salicylic acid. However, growth was higher with benzyl alcohol after 48 hours than with benzoate, a lag of 24 hours was evident in case of benzyl alcohol (Figure 2.27).

2.3.5.3. Toxicity of salicylic acid on the growth of *Pseudomonas* sp. strain B12

Strain B12 did not utilize salicylic acid as a growth substrate when present at 2.2 mM; this might be due to either the toxicity of salicylic acid or the lack of suitable enzymes to further metabolize this compound. To determine whether salicylic acid had a toxic effect, the isolate was grown in mineral salts medium with salicylic acid at varying concentration and 2 mM benzoic acid as an alternative carbon source. The growth was monitored every 24 hours by measuring OD_{600}.

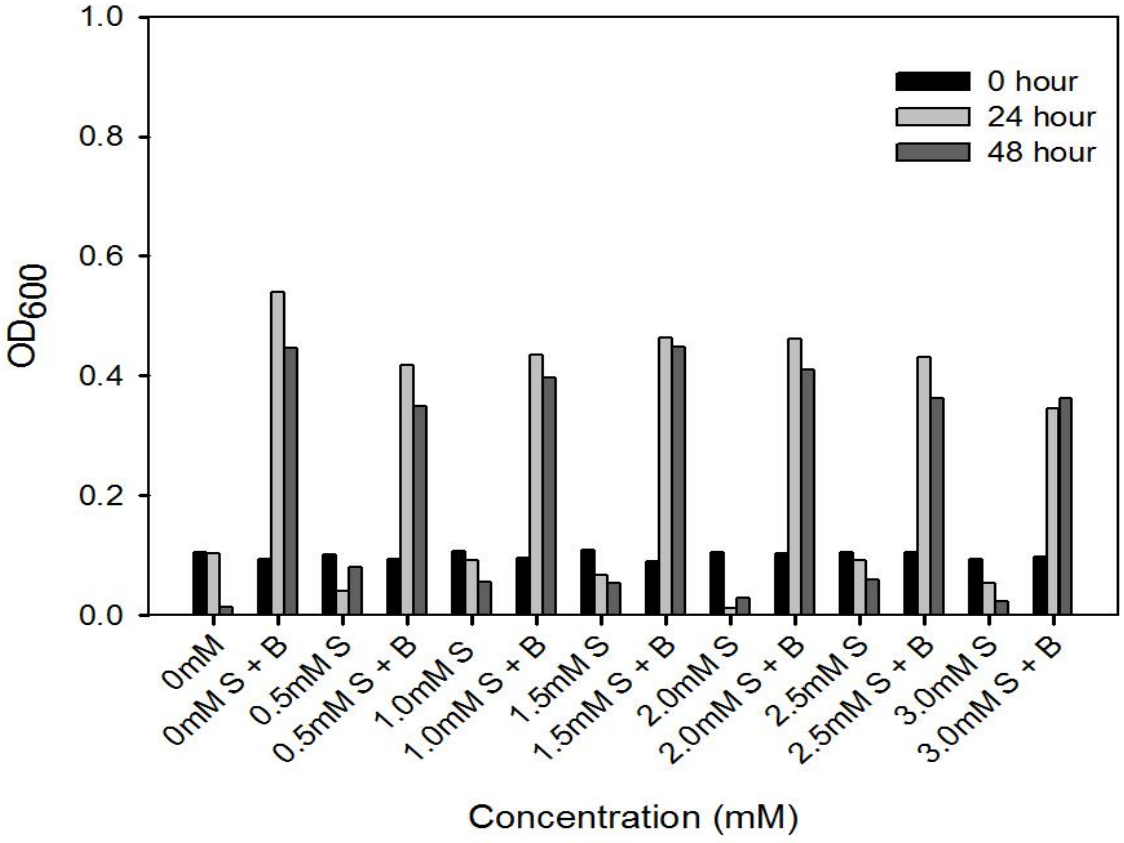

Figure 2.28. Growth of *Pseudomonas* sp. strain B12 with 2 mM benzoic acid (denoted as B) in the presence of varying concentrations of salicylic acid (denoted as S) in MSM at 25°C and 200 rpm.

Salicylic acid was not used as sole carbon source at the concentration tested. Growth was slightly reduced by salicylic acid up to the concentration of 3 mM in the presence of 2 mM benzoic acid. The biomass formed with 2 mM benzoic acid in the presence of 0.5 mM salicylic acid was reduced by about 20% compared to growth with 2 mM benzoic acid as sole carbon source.

2.3.5.4. Enhancement of benzyl salicylate utilization by *Pseudomonas* sp. strain B12

Benzyl salicylate has a low water solubility (refer to section 2.3.6) thus the bioavailability is limited. Therefore both physical methods (i.e. baffled flasks) and chemical methods (addition of surfactants) were tested to determine whether biodegradation of benzyl salicylate can be enhanced in mineral salts medium.

- Physical enhancement

To determine whether the use of baffled flasks can increase growth with this substrate by increasing the surface area for bacterial contact, *Pseudomonas* sp. strain B12 was grown in mineral salts medium with 2.2 mM benzyl salicylate in baffled Erlenmeyer flasks and normal Erlenmeyer flasks. The growth was determined every 12 hours by determining OD_{600} and total microscopic cell counts.

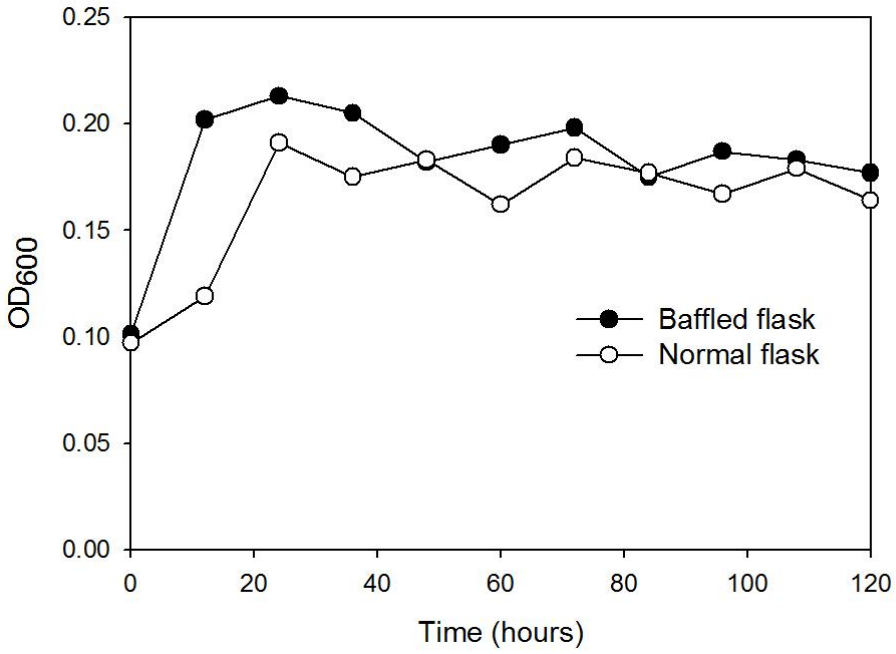

Figure 2.29. Comparison of the growth of *Pseudomonas* sp. strain B12 with 2.2 mM benzyl salicylate in normal Erlenmeyer flasks and baffled Erlenmeyer flasks in MSM at 25°C and 200 rpm. The values are means of two independently performed experiments.

The onset of growth in baffled Erlenmeyer flask was faster than growth in normal Erlenmeyer flask growth in normal flask. The growth rates were determined for growth both in non-baffled and baffled flask.

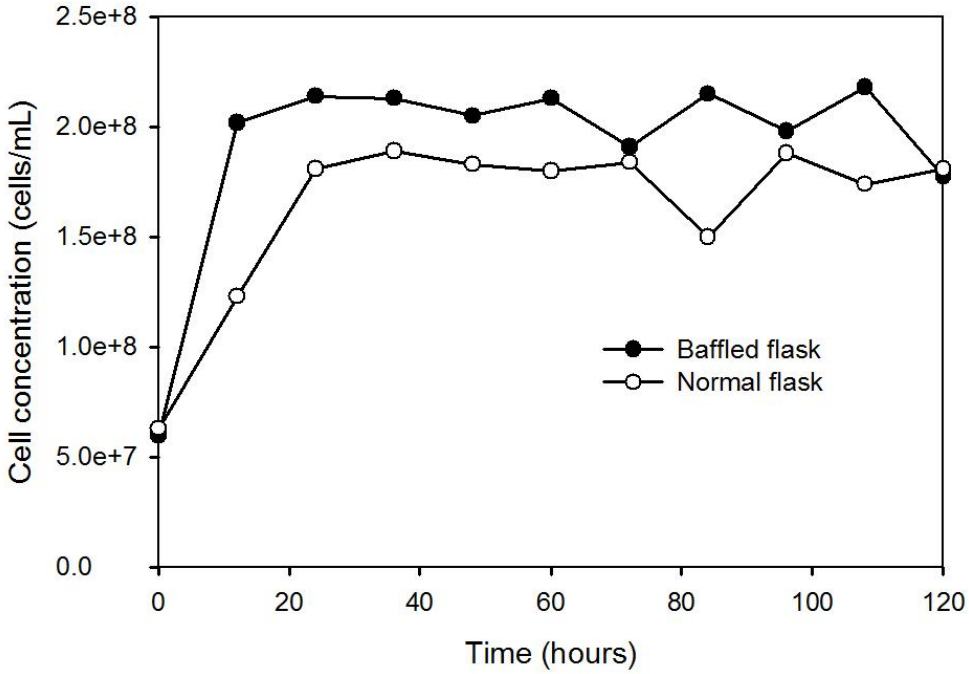

Figure 2.30. Increase in cell concentration of *Pseudomonas* sp. strain B12 in MSM with 2.2 mM benzyl salicylate in normal Erlenmeyer flask and baffled Erlenmeyer flask at 25°C and 200 rpm. The values are means of two independently performed experiments.

The growth of *Pseudomonas* sp. strain B12 was slightly higher in baffled flasks than in non-baffled flask (Figure 2.30). The growth kinetics of *Pseudomonas* sp. strain B21 with 2.2 mM benzyl salicylate in MSM were determined. In baffled Erlenmeyer flask, the strain had a growth rate (μ) of 0.0904 cells/mL per hour, doubling time (t_d) of 12.55 hours and generation time of 10.45 hours with generation number (n) of 2. Whereas in normal Erlenmeyer flask, the strain had a growth rate (μ) was 0.026 cells/mL per hour, doubling time (t_d) of 28.59 hours and generation time (G) of 26.72 hours with generation number (n) of 2.

- Chemical enhancement

The dissolution of the chemical for biodegradation can be increased by the use of biologically produced or synthetic surfactants. Strain B12 was grown in mineral salts medium at different concentrations of benzyl salicylate with the addition of Tween 80 as synthetic surfactant to determine whether this can enhance biodegradation.

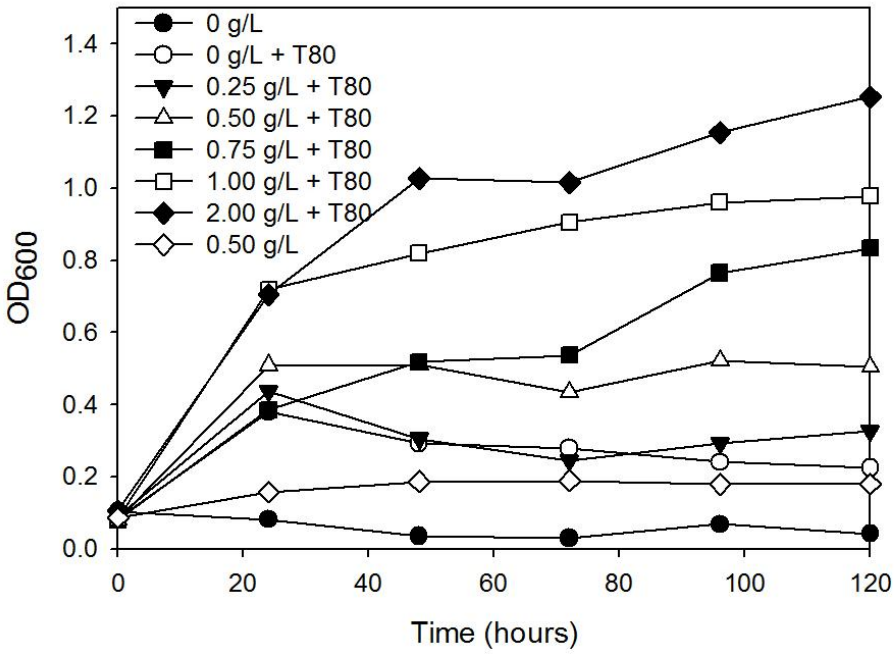

Figure 2.31. Growth of *Pseudomonas* sp. strain B12 with varying concentrations of benzyl salicylate and addition of 0.2% *v/v* Tween 80 (T80) in mineral salt medium at 25°C and 200 rpm.

In the presence of the surfactants, the growth of the isolate was clearly increased as the concentration of benzyl salicylate increases. However, the isolate also utilized Tween 80 as a growth substrate (Figure 2.31).

2.3.6. Dissolution kinetics of phenyl salicylate and benzyl salicylate

Phenyl salicylate and benzyl salicylate are both hydrophobic compounds with very low aqueous solubility. The dissolution and highest aqueous concentration of phenyl salicylate and benzyl salicylate were determined in mineral salts medium and distilled water.

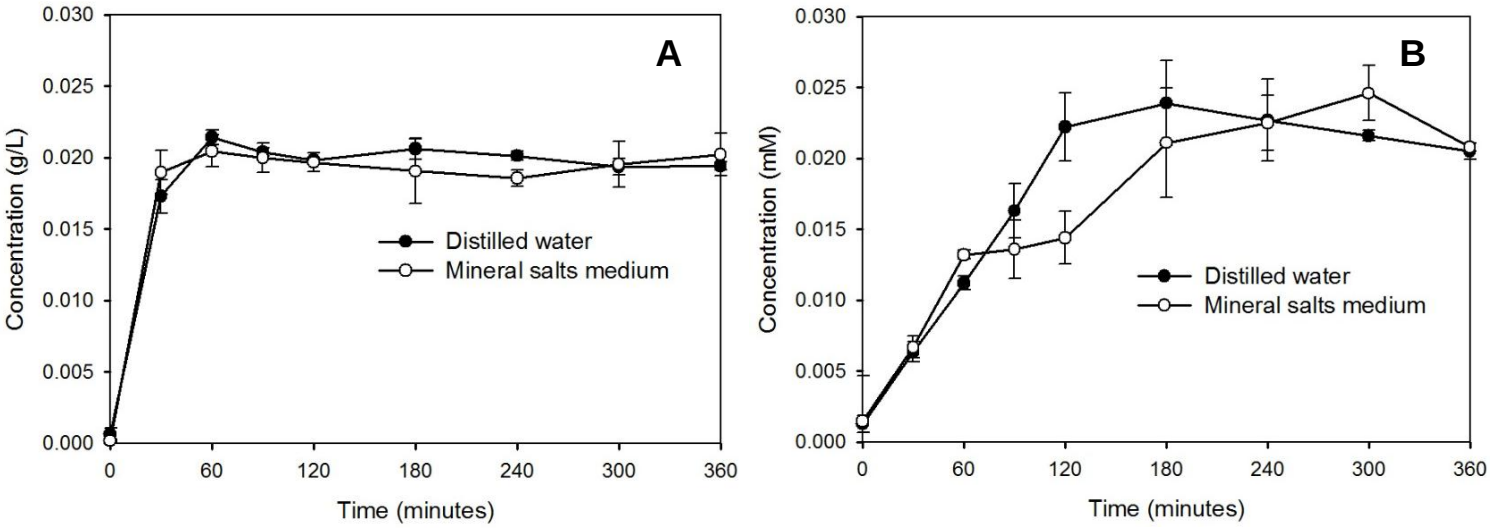

Figure 2.32.Dissolution of phenyl salicylate (A) and benzyl salicylate (B) in mineral salts medium and distilled water overtime at 25°C and 200 rpm. The values are means of three independently performed experiments.

The maximum saturation of phenyl salicylate was faster established than that of benzyl salicylate. The solubility of both benzyl salicylate and phenyl salicylate in mineral salt medium and distilled water is relatively the same. The solubility of phenyl salicylate was ~0.0205 g/L (0.10 mM) in MSM and ~0.0214 g/L (0.12 mM) in distilled water. The solubility of benzyl salicylate was 0.024 mM (~5.45 mg/L) in mineral salts medium and 0.025 mM (~5.61 mg/L) in distilled water.

2.3.7. Utilization of other aromatic compounds

All three isolates were tested for their ability to use other aromatic compounds as sole source of carbon.

Table 2.8. Growth of the isolated strains on mineral salts agar with various carbon sources after 48 hours incubation at 25°C.

Compound tested	*Oceanimonas* sp. strainS19	*Acinetobacter* sp. strainS21	*Pseudomonas* sp. strain B12
No carbon source (control)	-	-	-
Benzoic acid	+	+	+
Salicylic acid	+	+	-
Benzyl alcohol	+	+	+
Phenol	+	+	+
4-methyl phenol	+	-	+
Phenyl salicylate	+	+	-
Benzyl salicylate	-	-	+
Toluene	-	-	-
4-Hydroxybenzoic acid	+	-	+
4-Methylbenzoic acid	-	-	-
3,4-Dimethylphenol	-	-	-
3,4-Dihydroxybenzoic acid	-	+	+
2,6-Dihydroxybenzoic acid	-	-	-
2-Methoxybenzoic acid	-	-	-
3,5-Dinitrosalicylic acid	-	-	-
2,4-Dimethyl phenol	-	-	-
4-Nitro phenol	-	-	-
4-Hydroxycatechol	-	-	-
Hexadecane	-	-	-

Key: (+) – Visible growth (ring) around the crystals (-) – No visible growth

As expected, no visible growth was observed in the absence of substrate. *Oceanimonas* sp. strain S19 utilized 7 compounds while *Acinetobacter* sp. strain S21 utilized 6 compounds and *Pseudomonas* sp. strain B12 utilized 7 of the tested compounds (Table 2.8). All three strains showed visible growth around the crystals of benzoic acid, benzyl alcohol and phenol.

2.4. Discussion

Isolation and characterization

Aromatic compounds degrading bacteria have been previously isolated from coastal and marine environments (Dyksterhouse *et al.,* 1995; Geiselbrecht *et al.,* 1996; Kostka *et al.,* 2011; Pinyakong *et al.,* 2012). However, the majority of the isolated bacteria are related to terrestrial and freshwater microbes; this is probably due to the run-off from the land and influx from rivers into the estuaries. Only few of these are truly marine bacteria most commonly belonging to the genera of *Vibrio*, *Marinobacter* and *Cycloclasticus* (Hedlund *et al.,* 1999; Chung and King, 2001; Martins dos Santos *et al.,* 2008), therefore efforts were undertaken in recent years to isolate microbes native to marine and estuarine environments (Dìaz, 2004).

Artificial seawater based medium was used for initial enrichment, its ionic composition is similar to that of natural seawater and hence it allows for the preparation of medium appropriate for marine organisms. The pH of the medium was adjusted to 7.8 as it falls within the typical pH range of seawater of 7.5 to 8.3. (Berges *et al.,* 2001). The medium was later replaced by well-established mineral salts medium for analytical purposes as the artificial seawater based medium used contained organic compounds (i.e. Tris-HCl) that can interfered with the analysis. Tris-HCl is an organic buffer compound added to seawater based medium to stabilize the pH of the medium. It has been previously reported to serve as an organic substrate for bacterial growth (Fabregas *et al.,* 1993).

Isolation of pure cultures from mixed populations is a necessity in the study of hydrocarbon degradation as only this allows for the proper physiological analysis of the organisms (Holt *et al.,* 1994). Several pure cultures were obtained by successive sub-culturing and the selected isolates were characterized based on their cell morphology, biochemical reactions and both the analysis of 16S rRNA gene sequence and MALDI-TOF MS. Based on the results obtained, strain S19 could be assigned to the genus to *Oceanimonas*, strain S21 to the genus *Acinetobacter* and strain B12 was assigned the genus *Pseudomonas* (Table 2.3 – 2.5). The selected biochemical characteristics of the isolated strains (Table 2.2) matched those specified in Bergey's manual for these genera of *Acinetobacter* and

Pseudomonas(Holt *et al.,* 1994) and those reported in literature for *Oceanimonas* (Ivanova *et al.,* 2005).

Members of the genus *Oceanimonas* and *Pseudomonas* are Gram negative rod-shaped bacteria, motile by one or more polar flagella and are both oxidase and catalase positive. Both are unable to utilize starch and liquefy gelatin, however, *Pseudomonas* can utilize citrate (Lysenko, 1961; Park, 1962; Franzetti and Scarpellini, 2007; Yeganeh *et al.,* 2012). Members of the genus *Acinetobacter* are Gram negative cocci to plump rods that are non-motile. They are catalase positive but oxidase negative and do not produce gas and acid from glucose (Moore *et al.,* 2006; Göttsching and Schmidt, 2007; Vaz-Moreira *et al.,* 2011). Some species of *Acinetobacter* and *Pseudomonas* are known to produce a capsule (Holt *et al*., 1994) while *Oceanimonas* species have not been reported to produce capsule (Ivanova *et al.,* 2005).

16S rRNA gene sequencing has become the reference method for bacterial classification as it can provide confirmatory identification at genus and even species level (Clarridge, 2004). The 16S rRNA gene sequence providing genus identification is typically assumed at a sequence similarity level of more than 97%, while similarity scores of more than 99% are considered as good indicators for species level assignment. Scores of less than 97% represent a new species (Janda and Abbott, 2007). All three isolates had sequence similarity scores between 97 and 99% when compared to sequences deposited in GenBank which enabled assignment at genus level for all isolates (Table 2.3 – 2.5). Strain S19 had a similarity score of 98% to the genus *Oceanimonas* (*O. doudoroffii*), strain S21 and B12 had a similarity value of 99% to genus *Acinetobacter* (*A. junii*) and *Pseudomonas* (*P. monteilii*) respectively.

The phylogenetic trees based on the 16S rRNA gene sequences show the relationship between the strains isolated and selected representatives of the genus and closely related genus is shown in Figure 2.4 - 2.6. It is evident from the phylogenetic analysis that strain S21 cluster with the genus *Acinetobacter* though it could not be grouped with any of the selected type strains. Strain B12 was found to be closely related to *Pseudomonas monteilii* and the phylogenetic tree supports the assumption that the strain B12 belongs to the genus *Pseudomonas* (Hedegaard *et al.,* 1999). Strain S19 forms a cluster with species within the genus *Oceanimonas*. However, it did not group closely with any of the type strain sequences (Figure 2.4).

Members of the genus *Oceanimonas* were initially described as belonging to the genus *Pseudomonas* as they exhibit similar phenotypic characteristics (Brown *et al.,* 2001; Hamana *et al.,* 2003). It was later discovered that the species of *Oceanimonas* located at the lower boarder of the rRNA branch of the *Aeromonadaceae* and therefore cannot belong to the same lineage as *Pseudomonas* (De Vos *et al.,* 1989). The genus *Oceanimonas* and *Oceanisphaera* comprise *Alteromonas*-like bacteria as they exhibit some characteristics typical for *Alteromonas* such as they are native marine bacteria found in the open ocean or in coastal waters, with rod to curved rod shaped cells and a single polar flagellum (Ivanova *et al.,* 2004). *Oceanimonas* and *Oceanisphaera* are genera within the family *Aeromonadaceae* and according to a study by Saha and Chakrabarti (2006), the species of these genera formed robust cluster and had common signature nucleotides (Kumar *et al.,* 2009). Thus the sequences of type strains of these two genera were used for the construction and comparison of the phylogenetic tree for strain S19.

Additional experiments were carried out to verify the identification of the isolated strains. MALDI-TOF mass spectrometry detects the abundant ribosomal proteins which are conserved and specific to individual species and are thus suitable as biomarkers (Uhlik *et al.,* 2011). The identity of the isolated strains was verified with highly probable species identification obtained for isolate S19 and B12 and the score for isolate S21 gave probable genus identification (Table 2.6 and 2.7). The results obtained by MALDI-TOF MS mostly correspond with the identity obtained by analysis of the 16S rRNA gene sequences; isolate S21 belonged to the genus *Acinetobacter* and B12 belonged to the genus *Pseudomonas*. However, there were discrepancies between the taxonomic affiliation based on the 16S rRNA gene sequence analysis of strain S19 and the MALDI-TOF MS results; the sequence similarity of S19 was 98% to species within the genus *Oceanimonas* while the matching score generated by MALDI-TOF MS gave highly reliable species identification for the genus *Pseudomonas.* The similarity of strain S19 to the genus *Pseudomonas* determined by MALDI-TOF MS can be accounted to the fact that *Oceanimonas* species are related to the genus *Pseudomonas* (Brown *et al.,* 2001). *Oceanimonas* species were only recently classified as a genus of their own with only three valid species described so far (Ivanova *et al.,* 2005; Garrity *et al.,* 2007). Consequently, the Bruker spectral database does not contain spectra of these environmental species. One of the major limitations for bacterial identification using MALDI-TOF MS is the limited

information contained in commercial reference libraries which do not include spectra for many environmental bacterial species but are mostly focusing on the clinically important bacterial species associated with human disease (Bessède *et al.,* 2010; Emami *et al.,* 2012).

Species of the genus *Pseudomonas* and *Acinetobacter* are frequently detected in various marine and coastal environments (Austin *et al.,* 1979; Maeda *et al.,* 2010), whereas *Oceanimonas* species have only been isolated from marine environments (Brown *et al,* 2001; Ivanova *et al.,* 2005). The presence of this heterotrophic, metabolically versatile *gamma-Proteobacteria* in water sample from Durban Harbour therefore confirms the presence of such species in South African marine environments.

Effect of salinity

The effect of salinity on the growth of bacteria depends on the osmotic balance required for the particular bacterium. While some bacteria are inhibited in the presence of salt, some essentially require salt for their growth. Salt can cause cell lysis when the concentration of solute in the cell is higher than in the surrounding environment as water moves into the cell and causes the cell to expand. When the solutes concentration in the cell is lower than in the surrounding environment, the water moves out of the cell causing shrinkage (Leahy and Colwell, 1990; Ventosa *et al.,* 1998). The salinity in estuaries varies as there is constant mixing of seawater with freshwater, thus the bacteria inhibiting these ecosystem must be well adapted to fluctuations of salinity (Roberts, 2005).

Marine bacteria are distinguished from terrestrial and freshwater bacteria by having an obligate sodium requirement for growth, while terrestrial and freshwater bacteria do not essentially require significant amount of sodium ions (Baumann *et al.,* 1972; Reichelt and Baumann, 1974). *Oceanimonas* species are native to marine environments; they are classified as halotolerant as they can tolerate NaCl concentrations of up to 12% (Brown and Cumming, 2001). Of the three species that have been described so far (Saha and Chakrabarti, 2006), *Oceanimonas smirnovii* had no obligate requirement for salt (Ivanova *et al.,* 2005; Lin and Shieh, 2006). Likewise, *Oceanimonas* sp. strain S19 did not require salt for growth as there was

growth in the absence NaCl. However, biomass formation was higher in the presence of 2 and 4% NaCl. At 6 and 8% NaCl, growth started slower (longer lag phase) (Figure 2.7A). *Acinetobacter* and *Pseudomonas* are not typical marine genera but their species are commonly found in marine and coastal environments (Austin *et al.,* 1979; Maeda *et al.,* 2010; Satpute *et al.,* 2010). *Acinetobacter* species have been found to adapt to increasing salinity up to 900mM (~5% NaCl) (Sand *et al.,* 2011) while *Pseudomonas* species have been reported to grow up to 20% NaCl (Khan *et al.,* 2010). Growth of *Acinetobacter* sp. strains S21 and *Pseudomonas* sp. strain B12 was reduced as the concentration of NaCl increased; strain S21 was inhibited at above 6% salt concentration (Figure 2.7B) while strain B12 had higher growth at 2 and 4% of NaCl and was inhibited at concentration of 8 and 10% (Figure 2.7C). They are therefore slightly halo-tolerant with no obligate requirement for NaCl as also described in literature for members of these genera (Oren 2006; Oren, 2008; Vaz-Moreira *et al.,* 2011). These three strains are well adapted to this environment as they can tolerate salinity within the range typically found in estuaries (i.e. salt concentration of 0 to 3%).

Effect of substrate concentration

The initial substrate concentration has an impact on the susceptibility of substrate to microbial attack. Little or no biodegradation may occur when the concentration of the substrate is below the required threshold as the microorganism cannot obtain enough energy from the oxidation of the chemical to meet the energy demands of the initial population and are thus unable to reach cell densities sufficient to cause substantial mineralization (Boethling and Alexander, 1976). At elevated concentrations the substrate might pose a toxic effect, consequently inhibiting growth of microorganisms. Toxicity may affect the normal functioning of the cell and this varies with the type and concentration of the toxicant (Sikkema *et al.,* 1995; El-Naas *et al.,* 2009).

Oceanimonas sp. strain S19 and *Acinetobacter* sp. strain S21 were found to tolerate high levels of salicylic acid when compared to other Gram negative bacteria that have been previously studied. For example, the EC_{50} of *Phytobacterium* for salicylic acid is ~0.12 g/L, strain S19 however grew up to 20 mM with optimum growth at 10

mM (~1.3 g/L) while strain S21 grew up to 40 mM with optimum growth at 20 mM (~2.6 g/L) (Figure 2.8 and 2.9). Salicylic acid is a well-known bacteriostatic which interferes with enzymatic processes such as production of bacterial proteins or DNA replication, thus prevent cell replication and consequently limit the growth of bacteria (Block, 2001).

However, growth of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 was not inhibited by phenyl salicylate, the growth increased instead with the increase in substrate concentration (Figure 2.16 and 2.17). Phenyl salicylate is a bactericidal compound and its antimicrobial activity is dependent on the hydrolysis with the resultant intermediates (phenol and salicylic acid) being more toxic than the parent compound (Inqbal and Chaudhry, 2008). For the microbial cell to be susceptible to impact of substrate toxicity, the compound must dissolve in aqueous solution. Phenyl salicylate is a hydrophobic compound with very low aqueous solubility thus the mass transfer of the molecule to cell is limited and the interaction between the molecule and the cell is limited as well (Boopathy, 2000). Ecotoxicological studies of compounds with limited solubility attempts to increase the solubility of the chemical tested typically by addition of a solvent in order to fully evaluate the impact of the compound on the survival of the microorganisms (OECD, 2000).

Utilization of the substrates

An increase in optical density correlated with a decrease in the salicylic acid concentration was observed with 2 mM salicylic acid and no growth of strain S19 and S21 was observed in the absence of salicylic acid (Figure 2.10 and 2.13). This indicated that *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 utilized salicylic acid as sole carbon and energy source and the substrate was utilized to completion within 12 hours.

Elimination of aromatic compounds in the environment is due to biotic and abiotic factors. To demonstrate that microbial activity was responsible for the elimination of salicylic acid, UV-VIS spectroscopy was employed. Intact aromatic compounds absorb light strongly in the UV region due to their delocalized pi (π) electrons. This is caused by the excitation of the π electrons on the unsaturated bonds of aromatic compound at a spectrum between 200nm and 700nm (Watson *et al.,* 2001). The

absorbance spectrum of salicylic acid in ethanol exhibit two maximum peaks at 271nm and 301nm (Ahmad and Vaid, 2009). This is the same with the spectrum obtained in this study; peaks obtained at 270nm and 300nm (Figure B2). No change in absorbance was observed in the absence of the cells of strain S19 and strain S21 isolates indicating that the salicylic remained intact. After 24 hours incubation in the presence of cells of strain S19 and strain S21 only baseline absorbance was observed as salicylic acid had been catabolized (Figure 2.12 and 2.15). Disappearance of salicylic acid in the medium was due to the activity of the isolates *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21.

Phenyl salicylate was also utilized as a sole carbon and energy source for growth by strain S19 and strain S21; this was demonstrated by growth experiments (Figure 2.18 and 2.21) where the growth of the isolated strain progressed with the decrease in concentration of phenyl salicylate. Over 90% of the substrate was utilized within 192 hours of incubation when the cells were present in the medium and no change in concentration of substrate occurred in their absence; therefore the *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 were responsible for the elimination of phenyl salicylate.

For bacteria to utilize an organic compound, the cell or the cellular enzymes must have physical contact with the molecule. To attain this contact, the compound must be available to the microbial cell in aqueous solution so that it can diffuse directly into the cell (Ortega-Calvo *et al.,* 1995). The rate at which microbial cells convert organic compounds depends on the rate of uptake and metabolism and the rate of molecule transfer to the cell, i.e. the mass transfer (Bosma *et al.,* 1997; Simoni *et al.,* 2001). Many compounds are poorly soluble in water and therefore exist in non-aqueous phase (hydrophobic compounds) in the environment (Boopathy, 2000), therefore the rate of mass transfer of these compounds is limited; as a result, the rate of mineralization is slow (Law and Aitken, 2003; Tang *et al.,* 2005). Microorganisms that degrade hydrophobic compounds aid the transport of molecules across the cell membrane by either of the two following mechanisms; direct contact of the cell with the compound where the bacterial cells move towards the substrate by chemotaxis or attach to the surface of the compound. Some microorganisms have been reported to produce enzymes bound to the outer surface of the cell wall that mediate the uptake of the compound (Hintner *et al.,* 2005). The second mechanism is to increase the availability of the compound by synthesizing extracellular surface active

compounds that solubilize the hydrocarbon (Stelmack *et al.,* 1999; Pandey and Jian, 2002; Law and Aitken, 2003). Certain microorganisms are known to synthesize extracellular surface active compounds to degrade poorly soluble substrates. Members of the genus *Acinetobacter* are known to produce emulsifiers (Bakay *et al.,* 1999; Satpute *et al.,* 2010) while *Oceanimonas* species have not been reported to utilize hydrophobic compounds. Phenyl salicylate is a hydrophobic compound with low aqueous solubility of ~0.0214 g/L (0.12 mM) (Figure 2.32A), thus the mass transfer to the microbe is low resulting in longer lag phase observed during growth of strain S19 and strain S21 with phenyl salicylate. Aromatic compounds are good chemoattractants and some strains of *Pseudomonas* and *Acinetobacter* have been reported to possess chemotaxis ability towards aromatic acids (Harwood and Parales, 1996). The cells of strain S19 and S21 did not adhere to the crystal surface (Figure 2.20 and 2.23), indicating that they most probably produce an active compound that enhanced bioavailability of phenyl salicylate for microbial uptake.

The moderate growth of *Pseudomonas* sp. strain B12 with benzyl salicylate as a growth substrate (Figure 2.24) may be due to a number of factors. The bioavailability of benzyl salicylate to the cells was limited as this compound has a low aqueous solubility of only ~5.45 mg/L (0.024 mM) (Figure 2.32B). Hence, the substrate bioavailability limited further growth of the culture. This compound is also bactericidal and its mode of action is due to the intermediates of the hydrolysis of the parent compound to salicylic acid and benzyl alcohol (Radulović *et al.,* 2011; Sulaiman *et al.,* 2008). The metabolites of benzyl salicylate are potentially more bioavailable and could be more toxic than the benzyl salicylate. The strain either lacked suitable enzymes to further metabolize salicylic acid, one of the intermediates of benzyl salicylate hydrolysis, as no biomass was formed with salicylic acid but the strain grew with benzyl alcohol (Figure 2.27). Or salicylic acid reduced or prevented further growth as shown in Figure 2.28. Salicylic acid did not completely inhibit the growth but rather reduced the growth even at low concentrations. *Pseudomonas* species are known to produce extracellular substances that facilitate the uptake of hydrophobic compounds into the cell (Deziel *et al.,* 1996; Satpute *et al.,* 2010). The cells of strain B12 did not adhere to the droplets of the substrate but moved about freely (Figure 2.26). The strain either lacked the ability to synthesize substances that can increase the bioavailability of the substrate for cell uptake. The physicochemical properties of

the contaminant play a major role in determining the extent of bacterial breakdown of the target compound in the environment (Leahy and Colwell, 1990).

Applying the criteria specified in the OECD guidelines for the testing chemicals for biodegradability, both salicylic acid and phenyl salicylate can be classified probably as ready biodegradable as the compounds were utilized to completion within the 10 days window period of biodegradation whereas benzyl salicylate can be classified as non-biodegradable as the substrate was still present in the medium at the end of the incubation period (OECD, 1992).

Enhancement of biodegradation of hydrophobic compound

The growth efficiency of microorganisms utilizing hydrophobic substance can be limited due to the low mass transfer of the contaminant to the cell (Shin *et al.,* 2008; Tang *et al.,* 2005). An approach to enhance biodegradation of such organic compounds often attempts to increase the apparent solubility of the compounds. This can be achieved by treatments that promote partitioning of the hydrophobic compound such as vigorous shaking and addition of surfactants (Barkay *et al.,* 1999; Garcia-Junco, 2003).

Tween 80 (synonym Polysorbate 80) is a non-toxic synthetic surfactant; it has been used in number of studies and was found to improve biodegradation of hydrophobic contaminants such as phenanthrene by *Sphingomonas* sp. 2MPII (DSMZ 11572) (Cuny *et al.,* 1999) and polyaromatic hydrocarbons in the soil slurry (Kim *et al.,* 2001). Surfactants are amphiphillic molecules containing distinct hydrophobic and hydrophilic domains. They increase availability of the compound by reducing the interfacial tension between the aqueous and the non-aqueous phase of the hydrocarbon thereby increasing the contact area. Alternatively, they increase solubility by forming micelles where the hydrophobic end aligns with the hydrocarbon and the hydrophilic end aligns with the water thereby solubilizing by micro-emulsion (Zhang and Miller, 1994; Makkar and Rockne, 2003; Shin *et al.,* 2008). With the addition of Tween 80, growth of *Pseudomonas* sp. strain B12 increased with increasing concentrations of benzyl salicylate (Figure 2.31). This demonstrates that growth was due to benzyl salicylate used as a substrate and that Tween 80 enhanced its utilization. In addition, the isolate also utilized Tween 80 as a carbon

source. The disadvantage of using other chemical surfactants in biodegradation is that they have a potential to be toxic to microorganisms and are commonly used as antimicrobial agent, thus preventing growth and mineralization of the contaminants (Singh *et al.,* 2007). Triton X- series are surfactants that are toxic to bacteria, they inhibit cell proliferation by solubilizing the membrane lipid bilayer and thus reducing efficiency of bacteria to degrade pollutants (Li and Chen, 2009; Ivanković and Hrenović, 2010).Tween 80 has been previously reported to be utilized by microbial populations as a growth substrate resulting in increased microbial biomass to promote a comparative increase in contaminant removal (Cuny *et al.,* 1999). Strain B12 also grew with Tween 80 as a carbon and energy source demonstrating that the surfactant did not have any toxic effect on the strain of B12 (Figure 2.31).

The use of baffled flasks to increase the bioavailability of organic compounds with low solubility was reported (Göttsching and Schmidt, 2007); it was found that the growth of *Acinetobacter* sp. AG1 with benzyl benzoate was higher in baffled flask whereas in normal flask the growth was much slower and almost linear with a 10-fold increase of doubling time. The baffles in Erlenmeyer flasks increase the surface area of attachment for the cells by breaking the substrate droplets into smaller droplets. In addition, aeration rates and mass transfer in baffled flasks is higher than in non-baffled flask (Duetz *et al.,* 2000). The use of baffle flasks (mechanical treatment) did not have any impact on the utilization of benzyl salicylate by *Pseudomonas* sp. strain B12 but the growth rate of strain B12 was slightly higher and the stationary phase was reached faster in baffled Erlenmeyer flasks than in normal flasks (Figure 2.31).

Utilization of other aromatic compounds

There is a specificity of the utilization of individual hydrocarbons by different microorganisms. This depends upon the enzyme range of the organism that are involved and the molecular conjugation of the hydrocarbon. Microorganisms possess the ability to use a wide array of pollutants as carbon and energy source as they can express different catabolic pathways. This ability is related to the fact that these compounds are present in the environment (Zobell, 1946; Smith, 1990; Dìaz, 2004; Satpute *et al.,* 2010). Members of the genus *Acinetobacter* and *Pseudomonas* are well known to utilize various organic compounds as growth substrate and they play

an important role in the degradation of pollutants in the environment (Stanier, 1948; Fischer, 2008) while *Oceanimonas* has so far only been reported to utilize phenol (Brown *et al.,* 2001). The isolated strains utilized other simple aromatic hydrocarbons such as benzoic acid, benzyl alcohol, and phenol (Table 2.8).

2.5. Reference

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CHAPTER 3

Catabolism of salicylic acid and phenyl salicylate by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

3.1. Introduction

The diverse metabolic routes for the aerobic catabolism of aromatic compounds by bacteria are fairly well characterized (Cain *et al.,* 1968; Harwood and Parales, 1996). In order to utilize aromatic compounds as growth substrate, the bacteria must have the ability to convert the aromatic substrate into intermediates that can be channelled into central metabolic pathways (Gibson, 1968; Dìaz, 2004; Smith, 1990). In aerobic catabolism, this is achieved by monooxygenases and dioxygenases which catalyze hydroxylation reactions activating the stable aromatic ring leading to the formation of common central intermediates such as catechol, gentisate and protocatechuate (Dìaz *et al.,* 2013). The activated aromatic substrate is further cleaved by dioxygenases via ortho- or meta-cleavage, subsequent reactions then yields products that enter the tricarboxylic acid cycle (Harwood and Parales, 1996).

The catabolic pathway and the enzymes involved in the aerobic bacterial metabolism of salicylic acid have been well characterized in terrestrial and freshwater isolates. Salicylic acid is usually converted either to catechol by salicylate-1-hydroxylase or to gentisic acid by salicylate-5-hydroxylase (Ohmoto *et al.,* 1991; Karegoudar and Kim, 2000; Ishiyama *et al.,* 2004). Even a direct ring cleavage of salicylate has been reported. *Pseudaminobacter salicylatoxidans* oxidized salicylate to 2-oxohepta-3,5-dienedioic acid catalyzed by salicylate-1,2-dioxygenase (Hintner *et al.,* 2001). So far, no productive aerobic catabolic pathway for phenyl salicylate has been described. However, the microbial catabolism of structurally similar diaryl ester compounds has been reported. Degradation was found to commonly occur by initial hydrolysis of the ester bond resulting in two separate mono-aromatic compounds which can further be utilized to central intermediates (Reich *et al.,* 1999; Göttsching and Schmidt 2007; Schmidt, 2002).

Therefore, the aim of this study was to identify the pathway of the aerobic catabolism of salicylic acid and phenyl salicylate by two isolates - *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21- obtained from the estuarine environment.

3.2. Materials and methods

3.2.1. Analysis of metabolites produced by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 during growth with target substrates

The cell free supernatant obtained from the growth experiments of both strains with salicylic acid in section 2.2.6.1 (Chapter 2) and the extracts of phenyl salicylate in methanol obtained in section 2.2.6.2.1 (Chapter 2) were further analyzed by reversed phase HPLC following the method described in 2.2.6.2.1 (Chapter 2). The concentrations of metabolites were extrapolated from the calibration curves of authentic compounds in methanol (Appendix C). For salicylic acid, the standard concentrations of authentic catechol and gentisic acid ranged from 10 to 1000 µM. For phenyl salicylate, the authentic standard concentrations of phenol, salicylic acid, catechol and gentisic acid ranged from 100 to 1500 µM (mobile phase used was methanol: water 60:40% (unless stated otherwise), acidified with 0.5 g/L phosphoric acid).

3.2.2. Turn-over experiment for *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

- Salicylic acid

Exponentially growing cells of strain S19 and S21 in mineral salts medium (MSM) with 2 mM salicylic acid were harvested by centrifugation (10 000x *g* for 10 minutes at 4°C), washed twice with 20 mM phosphate buffer (pH 7.4) and the pellet re-suspended in the same buffer was used to inoculate fresh MSM (25 mL) with 5 mM salicylic acid to an OD_{600} of 1.5 – 2.0 to safeguard sufficient activity. The flasks were incubated in a rotary shaker in the dark at 25°C and 200 rpm and the growth was monitored every 30 minutes for 6 hours. 1 mL culture samples were centrifuged and the supernatant was used for analysis of substrate and metabolites by reversed phase HPLC as previously described (Chapter 2, section 2.2.6.1.2). The initial protein concentration as analysed by the Bradford assay was 0.206 mg/mL for strain S19 and 0.372 mg/mL for strain S21.

- Phenyl salicylate

Exponentially growing cultures of strain S19 and S21 in MSM with 0.5 g/L of phenyl salicylate were harvested by centrifugation (10 000x *g* at 4°C for 10 minutes), washed twice with phosphate buffer (20 mM, pH 7.4) and re-suspended in the same buffer. The cell suspension was used to inoculate 25 mL of fresh MSM with 4 g/L phenyl salicylate to an OD_{600} of 1.5 – 2.0 to safeguard sufficient activity and the flasks were incubated in rotary shaker in the dark at 25°C and 200 rpm. The growth was monitored every 6 hours for a period of 72 hours. 1 mL samples were centrifuged at time points and the supernatant was analyzed by reversed phase HPLC as described in section 2.2.6.1.2 (Chapter 2). The initial protein concentration as analyzed by the Bradford assay was 0.326 mg/mL for both strain (S19 and S21).

3.2.3. Determination of the rate of substrate dependent oxygen consumption

Oxygen uptake assay

The net oxygen consumption of resting cells was measured in a Clark-type oxygen electrode (Hansatech Oxytherm). A pre-culture grown in 25 mL MSM with the target compound as substrate was centrifuged at 10 000x *g* at 4°C for 10 minutes, washed twice with phosphate buffer (20 mM, pH 7.4) and the pellet was used to inoculate a 250 mL Erlenmeyer flask with 50 mL fresh MSM supplemented with the target compound (2 mM salicylic acid or 0.5 g/L phenyl salicylate) as growth substrate. Non-induced cells were produced by using 50 mL MSM containing succinic acid (2 mM) inoculated to an initial OD_{600} of 0.1. The flasks were incubated in a rotary shaker in the dark at 25°C and 200 rpm. The cells were harvested at the late exponential phase, washed twice with sodium phosphate buffer (20 mM, pH 7.4) and then re-suspended in the same buffer to an optical density at 600nm of 0.8 – 1.0. 1 mL of the sample was centrifuged and the pellet was used for quantification of protein by the Bradford assay.

To determine the specific oxygen uptake rate at 25°C, 998 µL of resting cell suspension were placed in the electrode chamber with a magnetic stirrer bar adjusted to 70% rpm. The endogenous oxygen uptake rate was measured for 3 minutes prior to adding 2 µL of the selected carbon source dissolved in DMSO via the use of a Hamilton syringe to give a final concentration of 1 mM.

Respiratory substrates tested

Salicylic acid - For salicylic acid grown cells, the substrates tested were salicylic acid, catechol, gentisic acid, 4-hydroxybenzoic acid, phenol, benzoic acid, 3,4-dihydroxybenzoic acid and toluene. For non-induced cells grown with succinate, the substrates tested were succinic acid and salicylic acid.

Phenyl salicylate - For phenyl salicylate grown cells, the substrates tested were phenyl salicylate, salicylic acid, phenol, catechol, gentisic acid, benzoic acid, 3,4-dihydroxybenzoic acid and toluene. For non-induced cells grown with succinate, phenyl salicylate and succinic acid were tested.

All aromatic compounds were dissolved in 1 mL DMSO to a concentration of 500 mM. All measurements were done in triplicate. The net oxygen consumption was calculated by subtracting the endogenous oxygen uptake rate from the rate after addition of test substrate and the specific oxygen uptake was expressed as nmol O_2 x min^{-1} x mg^{-1} protein.

3.2.4. Enzyme assays

Preparation of cell free extract

Batch cultures of the two strains were carried out in 1 L Erlenmeyer flasks with 400 mL working volume of MSM supplemented with 2 mM salicylic acid or 0.5 g/L phenyl salicylate. A pre-culture grown in 100 mL MSM with the target compound as a substrate was centrifuged at 10 000x *g* at 4°C for 10 minutes, washed twice with phosphate buffer (20 mM, pH 7.4) and then used to inoculate 400 mL MSM medium to an OD_{600} of 0.1. The flasks were incubated in a rotary shaker in the dark at 25°C and 200 rpm. For non-induced cells, MSM with 2 mM succinate was inoculated with a pre-grown culture grown in MSM with 2 mM succinate and incubated at 25°C and 200 rpm. Exponentially growing cells were then harvested by centrifugation at 10 000x *g* at 4°C for 10 minutes, washed three times with 0.85% saline solution and the pellet was drained well from the liquid. The crude extracts were prepared according to the method described by McIlwain (1948); an equivalent amount of powdered alumina was added to the cell pellet together with an equal volume of sodium phosphate buffer (20 mM, pH 7.4) and then transferred to a cooled mortar.

Liquid nitrogen was added and the biomass treated for about 10 minutes with a cooled pestle. The solution obtained was then centrifuged for 10 minutes at 4°C at 10 000x *g*, the pellet was separated from the supernatant and the supernatant was centrifuged at 20 000x *g* for 60 minutes at 4°C. The clear supernatant was used for all enzyme assays. The protein concentration was determined by the Bradford assay.

Enzyme assays

Specific enzyme activities relating to the catabolism of the aromatic compounds were measured in micro quartz cuvettes (500 µL capacity, 1-cm light path) at 25°C using a recording spectrophotometer with integrated CPS-temperature controller (UV-1800 Shimadzu). All assays were performed in triplicate. Specific enzyme activities were expressed as nmol per minute per mg protein at 25°C.

The activity of catechol-1,2-dioxygenase was measured by monitoring the increase in absorbance at 260nm due to accumulation of *cis*,*cis*-muconate. The molar extinction coefficients used for muconic acids were as reported. Muconic acid from catechol, the extinction coefficient was 16 800 L x mol^{-1} x cm^{-1}, extinction coefficient of methyl muconic acid from methylated catechol was taken as 13 900 L x mol^{-1} x cm^{-1}(Dorn and Knackmuss, 1978aand Dorn and Knackmuss, 1978b). Catechol-2,3-dioxygenase activity was determined by monitoring the increase in absorbance at 375nm due to the formation of 2-hydroxymuconic semialdehyde (Nozaki *et al.,* 1970), the extinction coefficient was taken as 33 000 L x mol^{-1} x cm^{-1} (Kaschabek *et al.,* 1998). Gentisate-1,2-dioxygenase activity was measured according to Lack (1959) by monitoring the increase of absorption at 330nm due to the formation of maleyl pyruvate and the extinction coefficient was taken as 10 800 L x mol^{-1}x cm^{-1} (Liu and Zhou, 2012).

The assay system contained 450 µL of 20 mM phosphate buffer (pH 7.4), 5 – 10 µL enzyme extract and the reaction was initiated by addition of substrate of 2.5 mM to a final concentration of 20 µM and final volume of 500 µL. For the reference cuvette, the substrate was omitted from the reaction mix. Substrates tested were catechol, 3-methylcatechol, 4-methylcatechol and 4-bromocatechol dissolved in ethanol. In addition, the reaction mixture was subjected to spectral scanning between 200nm

and 400nm to determine the turnover of catechol to a cleavage product by monitoring the change in absorbance.

The activity of hydroxylases for salicylic acid and phenol was determined according to Fuji and Kaneda (1985) by measuring the decrease in absorbance at 340nm due to oxidation of NADH + H^+, the molar extinction coefficient used was taken as 6 200 L x mol^{-1} x cm^{-1} (Jouanneau *et al.,* 2007).The assay mixture contained 450 µL phosphate buffer (20 mM, pH 7.4), 5 – 10 µL enzyme extract, 20 µM final substrate concentration and the reaction was initiated by the addition of 100 µM NADH + H^+ to a final volume of 500 µL.

General esterase activity was measured by monitoring the absorption increase at 405nm due to the production of *p*-nitrophenol from *p*-nitrophenylacetate. Extinction coefficient of *p*-nitrophenol was taken as 16 300 L x mol^{-1} x cm^{-1} (Clauβen and Schmidt, 1999). The assay mixture contained 450 µL phosphate buffer (20 mM, pH 7.4), 5 µL enzyme extract and 2.5 µM *p*-nitrophenylacetate in a final volume of 500 µL.

The enzymatic hydrolysis of phenyl salicylate was assayed by reversed phase HPLC using a reaction mixture containing 5 mL phosphate buffer (20 mM, pH 7.4), 100 µL enzyme extract (S19 = 0.338 mg/L and S21 = 0.381 mg/L protein from phenyl salicylate grown cells) and the reaction was started by addition of 0.1 g/L phenyl salicylate. The flasks were incubated at 25°C and 200 rpm. The samples were drawn every 30 minutes for 2 hours and the reaction was stopped immediately by freezing in liquid nitrogen. After thawing, the samples were centrifuged (10 000x *g* for 30 minutes) and the supernatant was analyzed by reversed phase HPLC (mobile phase methanol: water 60:40% *v/v* acidified with 0.5 g/L phosphoric acid).

3.2.5. Detection of β-ketoadipate formation from catechol

The ability of the strains to form β-ketoadipate from catechol was determined using a modified Rothera reaction (Rothera, 1908). To 3 mL of 20 mM phosphate buffer (pH 7.4), 1 mL of enzyme extract (S19 = 0.122 mg/L and S21 = 0.279 mg/L protein from salicylic acid grown cells, S19 = 0.338 mg/L and S21 = 0.381 mg/L protein from phenyl salicylate grown cells) was added together with 50 µL of 100 mM catechol. The flasks were incubated in a rotary shaker at 30°C and 200 rpm for 30 minutes.

After incubation, 500 µL of the reaction mixture was transferred to a test tube and 1 g of ammonium sulphate, 1.5 mL of 25% ammonia solution and 50 µL sodium nitroprusside (5% w/v) were added to the test tube. Development of purple to red color indicates the presence of β-ketoadipate. *Marinobacter* sp. KM2 grown with 2 mM phenol and *Vibrio* sp. KM1 grown with 5 mM benzoate were used as negative (meta cleavage) and positive control (ortho cleavage) respectively (Moxley and Schmidt, 2010; Moxley and Schmidt, 2012).

3.2.6. Utilization of possible intermediates by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

- Salicylic acid

1 mL of a 24 hour culture grown in MSM with 2 mM salicylic acid was centrifuged at 10 000x *g* for 10 minutes, the pellet was washed twice with sterile phosphate buffer (20 mM, pH 7.4) and then re-suspended in 1 mL sterile MSM. 100 µL of the cell suspension was used to inoculate mineral salts agar plates by spread plating. Crystals of catechol and gentisic acid (~5 mg) were placed at the centre of the plate and growth was observed. Succinic acid was used as a positive control and plates with no substrate added served as negative control.

- Phenyl salicylate

The ability of both strains to grow with possible intermediates of phenyl salicylate catabolism was determined by monitoring growth in MSM for phenol and salicylic acid and by using spread plate assay for catechol and gentisic acid. A pre-culture grown in MSM with 0.5 g/L phenyl salicylate was centrifuged at 10 000x *g* for 10 minutes, washed twice with 20 mM phosphate buffer (pH 7.4) and the pellet was used to inoculate fresh MSM with 2 mM substrate (phenol or salicylic acid) to an optical density at 600nm of 0.1. For controls, no carbon source was added to the medium. The growth was monitored by measuring the OD_{600} every 6 hours for a period of 48 hours. MSM agar was inoculated by spread plating 100 µL of cell suspension (washed and re-suspended in medium). Crystals of catechol and gentisic acid (~5 mg) were placed at the centre of the plates. For controls, succinic acid was used as a positive control and no substrate added as negative control.

3.3. Results

3.3.1. Determination of metabolites formed from salicylic acid and phenyl salicylate by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

3.3.1.1. Salicylic acid

Salicylic acid can be aerobically catabolized to central intermediates via two commonly encountered routes, the catechol pathway or the gentisate pathway. HPLC analysis of supernatant samples collected during growth of both strains with 2 mM salicylic acid did not detect any metabolites.

Therefore, both strains were grown in the presence of higher concentrations of substrate (5 mM). Supernatant samples collected at regular time intervals were analyzed by reversed phase HPLC and showed the presence of metabolites for *Oceanimonas* sp. strain S19 but not for *Acinetobacter* sp. strain S21. The results obtained for strain S19 are presented in Figure 3.1 and 3.2.

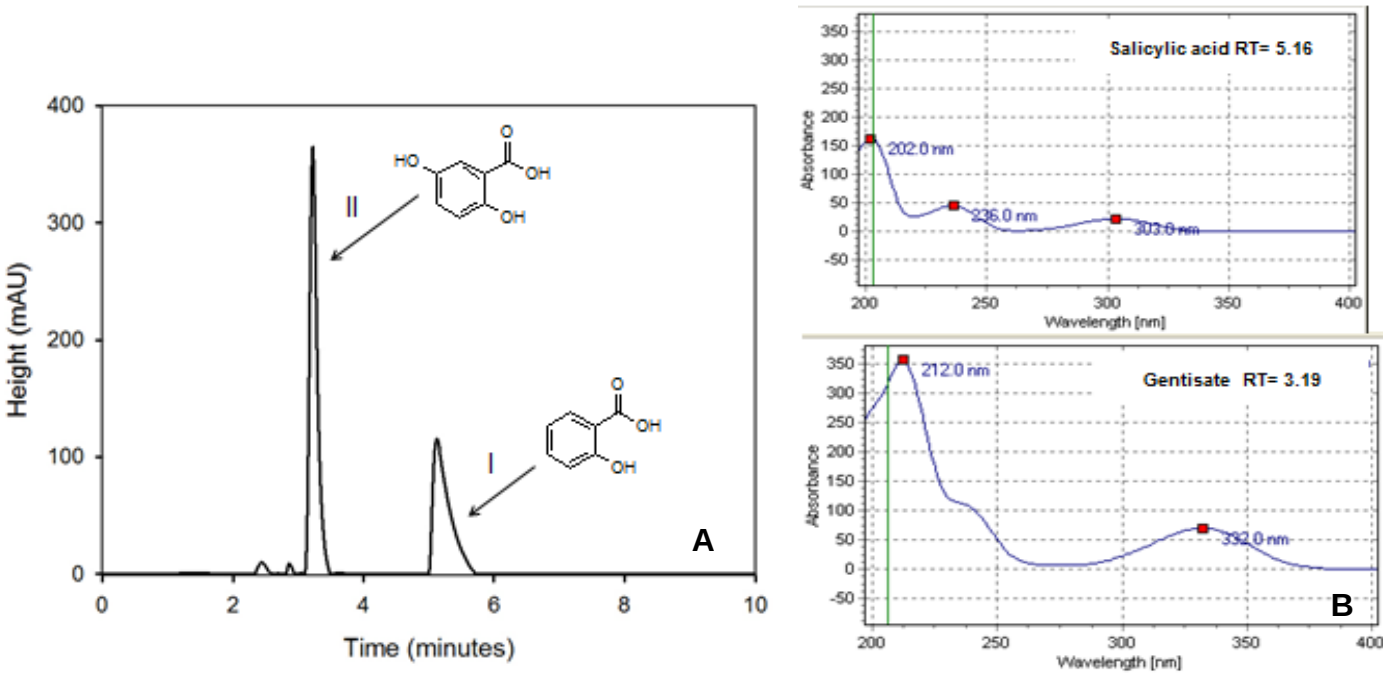

Figure 3.1. HPLC chromatogram (A) of supernatant samples with salicylic acid (I) and its metabolite gentisate (II) detected at 210nm. The in situ spectral scan (B) of the substrate (I) and the metabolite (II) after 120 minutes of incubation of *Oceanimonas* sp. strain S19 in MSM with 5 mM salicylic acid at 25°C and 200 rpm.

After 120 minutes of incubation, gentisate was detected as a metabolite of salicylic acid during growth of *Oceanimonas* sp. strain S19. The in situ spectra of the metabolite matched that of the authentic standard of gentisic acid (maxima at 212nm and 334nm).

Culture samples of *Oceanimonas* sp. strain S19 growing with 5 mM of salicylic acid collected at regular time intervals were analyzed by HPLC to verify the transformation of salicylic acid to gentisate.

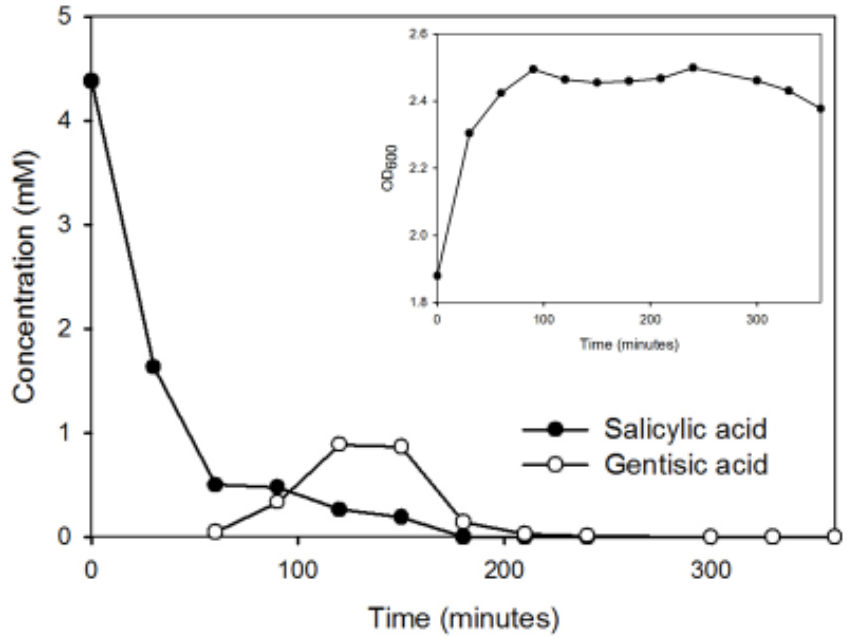

Figure 3.2. Transient accumulation of gentisate from salicylic acid by *Oceanimonas* sp. strain S19 during growth with salicylic acid (as shown in the inset) in MSM at 25°C and 200 rpm.

As shown in Figure 3.2., transient accumulation of the metabolite gentisate took place when about 90% of salicylate had been consumed. The maximum concentration of gentisate detected was 1 mM after 120 minutes of incubation. After 150 minutes of incubation, gentisate was utilized to completion by *Oceanimonas* sp. strain S19.

3.3.1.2. Phenyl salicylate

Diaryl ester compounds are typically hydrolyzed at the ester linkage resulting in the mono-aromatic carboxylic acid and the corresponding alcohol. In the case of phenyl salicylate, the resulting metabolites can be further metabolized to central intermediates either via the catechol or the gentisate pathway. To determine the metabolites produced by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 from phenyl salicylate, the strains were grown with an excessive concentration of phenyl salicylate (4.0 g/L). The supernatant samples collected at regular time intervals were analyzed by HPLC and the obtained results are presented in Figure 3.3 to 3.6.

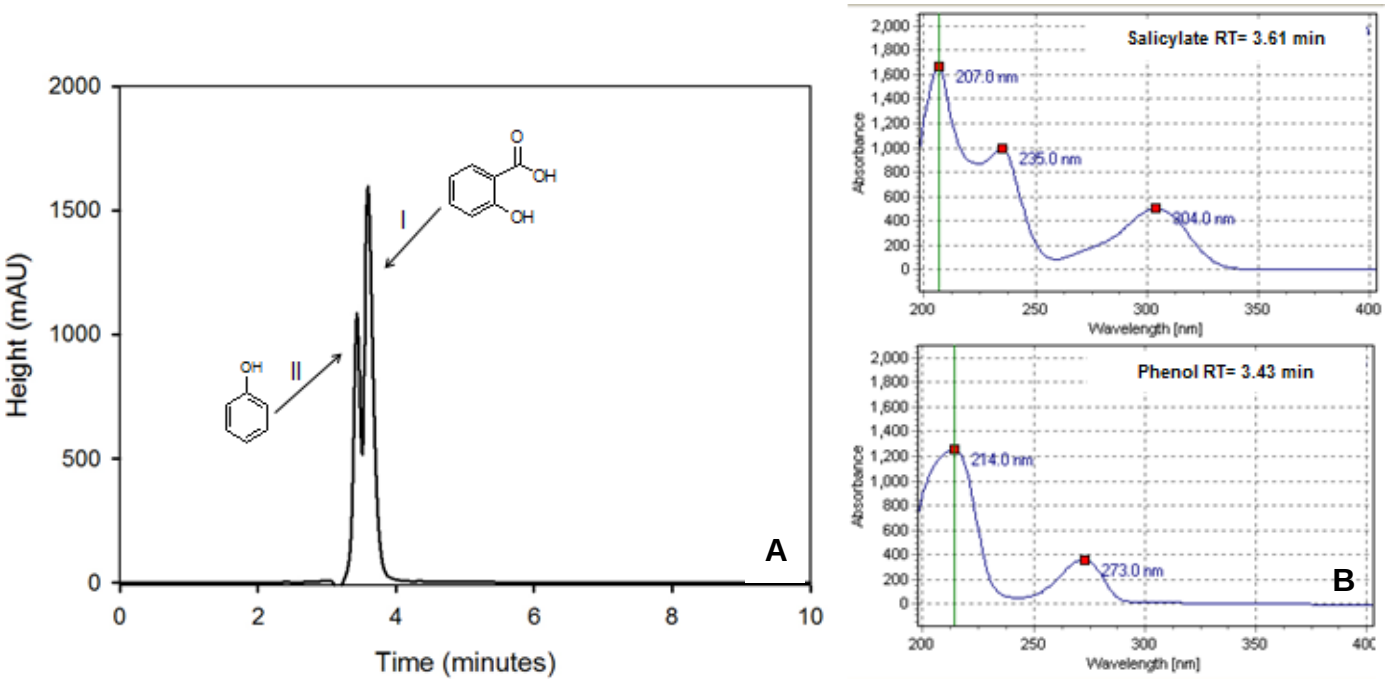

Figure 3.3. HPLC chromatogram (A) of culture supernatant with salicylic acid (I) and phenol (II) detected at 270nm. In situ spectral scans (B) of metabolites (I) and (II) after 42 hours incubation of *Oceanimonas* sp. strain S19 in MSM with 4 g/L phenyl salicylate at 25°C and 200 rpm. (Mobile phase: 85% methanol).

Phenol and salicylate were confirmed as metabolites of phenyl salicylate after 42 hours of incubation during growth with 4 g/L of substrate. The UV-VIS spectral scan of the metabolites matched that of the authentic standard of salicylic acid (maxima at 208nm, 235nm and 303nm) and phenol (maxima 215nm and 273nm), thus indicating

that the initial step of phenyl salicylate catabolism was the hydrolysis of the ester linkage.

The supernatant samples collected at regular time intervals were analyzed by reversed phase HPLC to monitor the accumulation of phenyl salicylate metabolites by *Oceanimonas* sp. strain S19 over time.

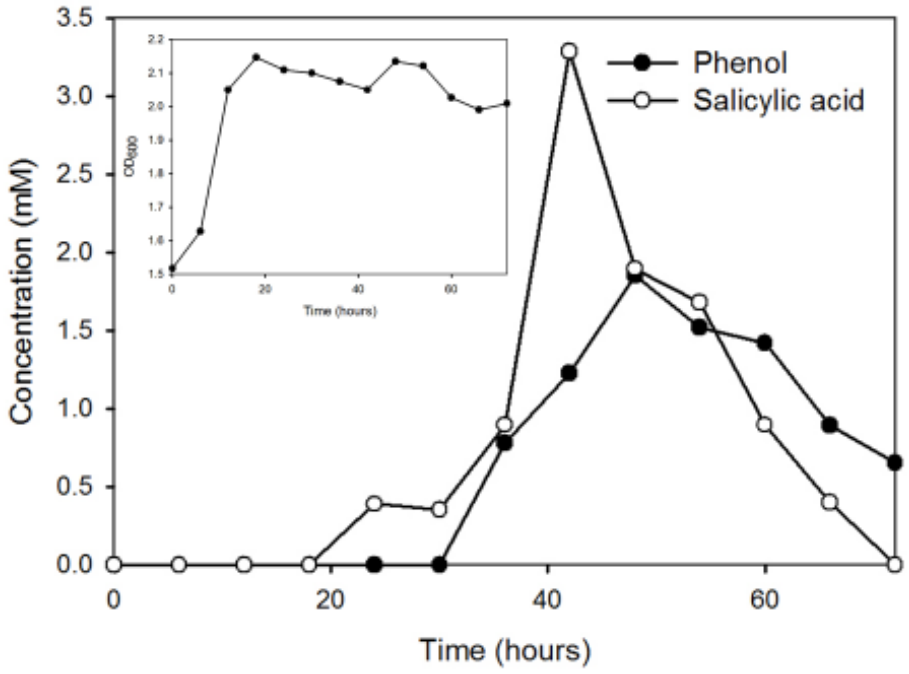

Figure 3.4. Formation and utilization of salicylate and phenol from phenyl salicylate by *Oceanimonas* sp. strain S19 during growth with 4.0 g/L phenyl salicylate (as shown in the inset) in MSM at 25°C and 200 rpm.

Two mono-aromatic metabolites which were identified as phenol and salicylate accumulated transiently during growth of *Oceanimonas* sp. strain S19 with phenyl salicylate. The maximum concentration of salicylate detected was about 3.3 mM and the maximum concentration of phenol was about 1.8 mM. The metabolites were further utilized upon further incubation (Figure 3.4).

As for *Oceanimonas* sp. strain S19, *Acinetobacter* sp. strain S21 was grown with 4 g/L of phenyl salicylate in MSM; cell free supernatants were sampled at regular time intervals and were analyzed by reversed phase HPLC.

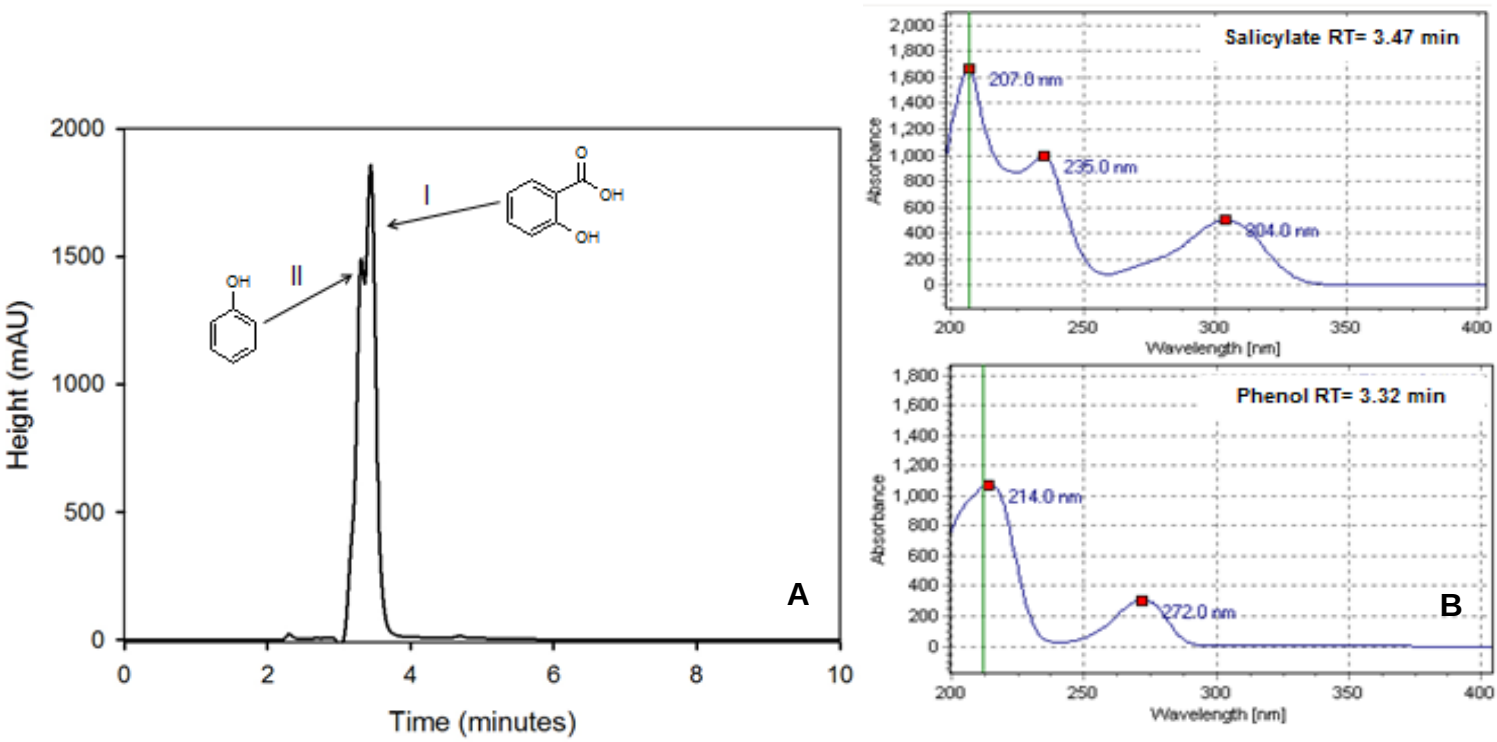

Figure 3.5. HPLC chromatogram (A) of culture supernatant with salicylate (I) and phenol (II) detected at 270nm. In situ spectral scans of metabolites (I) and (II) after 36 hours incubation of *Acinetobacter* sp. strain S21 in MSM with 4.0 g/L phenyl salicylate at 25°C and 200 rpm. (Mobile phase: 85% methanol).

As demonstrated in Figure 3.5, the peaks of salicylate and phenol were not sufficiently separated during HPLC analysis. However, the UV-VIS spectra of the detected metabolites matched those of the authentic standards, salicylic acid (207nm, 235nm and 304nm) and phenol (208nm and 273nm). Thus phenol and salicylate were detected as metabolites of phenyl salicylate after 36 hours of incubation indicating the catabolism of phenyl salicylate was initiated by hydrolysis of the ester linkage.

The cell free supernatant samples collected at regular time intervals were analyzed by reversed phase HPLC to monitor the accumulation of phenyl salicylate metabolites over time.

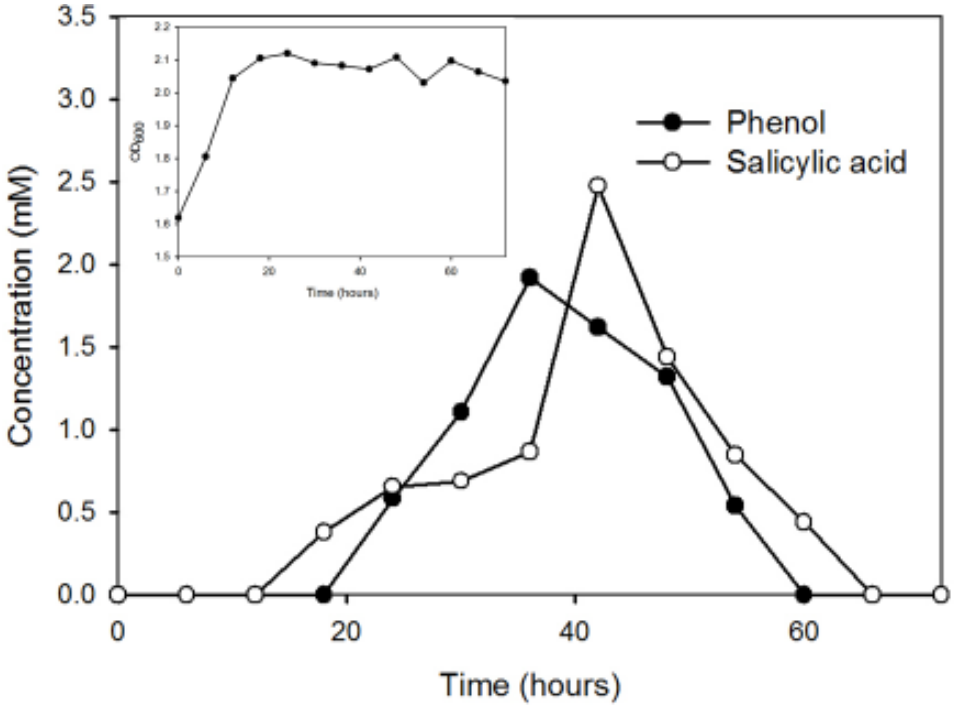

Figure 3.6. Formation and utilization of salicylate and phenol from phenyl salicylate by *Acinetobacter* sp. strain S21 during growth with 4.0 g/L phenyl salicylate in MSM at 25°C and 200 rpm.

Two metabolites, identified via their spectral characteristics and retention times as phenol and salicylate accumulated during growth of *Acinetobacter* sp. strain S21 with phenyl salicylate as a growth substrate. The maximum concentration of phenol detected was about 2 mM after 42 hours and that of salicylate was about 2.5 mM detected after 36 hours incubation. Both metabolites were further metabolized and not detected after 66 hours of incubation (Figure 3.6).

3.3.2. Specific oxygen consumption rates for various substrates by resting cells of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

Bacterial breakdown of organic contaminants under aerobic conditions leads to consumption of oxygen as it serves as a co-substrate for oxygenases and electron acceptor during metabolism of aromatic compounds. The rate of substrate specific oxygen consumption with salicylic acid and its potential metabolites and selected simple aromatic compounds was studied using an oxygen electrode and resting cells.

Table 3.1. Substrate specific oxygen uptake rates obtained for resting cells of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

Substrate	*Oceanimonas* sp. strain S19 Specific O_2 rate after growth with:		*Acinetobacter* sp. strain S21 Specific O_2 rate after growth with:	
	Salicylic acid	Succinate	Salicylic acid	Succinate
Salicylate	157	8	292	19
Succinate	163	213	112	243
Catechol	495	-	343	-
Gentisic acid	30	-	5	-
4-Hydroxybenzoic acid	7	-	13	-
Phenol	15	-	7	-
Benzoic acid	326	-	300	-
3,4-Dihydroxybenzoic acid	12	-	14	-
Toluene	20	-	7	-

Specific oxygen uptake rates shown are the average of three independently performed experiments. The rates are given as nmol per O_2 consumed per minute per mg protein and are corrected for endogenous respiration and solvent effects (i.e. DMSO). (-) indicates that the specific oxygen uptake was no measured.

Growth with salicylic acid induced the activity required for the oxidation of this substrate and its potential metabolites whilst growth with succinate did not induce high activity for salicylic acid. Cells of both strains showed high oxygen uptake rates for catechol with about 3 times the rate for strain S19 and 1.5 times the rate for strain S21 compared to the rate of salicylic acid but only minute activity for gentisate.

To evaluate the rate of aerobic catabolism of phenyl salicylate by both strains, the specific oxygen consumption in the presence of phenyl salicylate, its potential metabolites and other selected aromatic compounds was studied using an oxygen electrode and resting cells.

Table 3.2. Substrate specific oxygen uptake rates obtained for resting cells of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

Substrate	*Oceanimonas* sp. strain S19 Specific O_2rate after growth with:		*Acinetobacter* sp. strain S21 Specific O_2 rate after growth with:	
	Phenyl salicylate	Succinate	Phenyl salicylate	Succinate
Phenyl salicylate	95	100	89	34
Succinic acid	340	480	418	417
Salicylic acid	166	-	134	-
Phenol	109	-	99	-
Catechol	583	-	616	-
Gentisic acid	108	-	31	-
Benzoate	212	-	289	-
4-Hydroxybenzoic acid	50	-	51	-
3,4-Dihydroxybenzoic acid	93	-	116	-
Toluene	36	-	142	-

Specific oxygen uptakes rates shown are the average of three independently performed experiments. The rates are given as nmol per O_2 consumed per minute per mg protein and are corrected for endogenous respiration and solvent effects (i.e. DMSO). (-) indicates that the specific oxygen uptake was no measured.

Cells of *Acinetobacter* sp. strain S21 grown with phenyl salicylate showed high oxidizing activity for the expected intermediates such as salicylate, phenol and catechol while growth with succinate did induce oxidizing activity for phenyl salicylate in case of *Acinetobacter* sp. strain S21. Similarly, *Oceanimonas* sp. strain S19 showed high activity for salicylate, phenol and catechol after growth with phenyl salicylate but similar activity for phenyl salicylate. Both strains showed almost similar specific activity with phenol and salicylic acid with 6 times fold higher activity for catechol compared to the rate of phenyl salicylate. Strain S19 also showed elevated activity for gentisate in comparison to strain S21.

3.3.3. Analysis of specific enzyme activity of the strains *Oceanimonas* sp. S19 and *Acinetobacter* sp. S21

The growth substrate can influence the type of enzyme activities induced by the organism. The catabolic properties of these enzymes determine the productivity of the catabolic pathway for the substrate. Therefore, the assays for selected key enzymes required for the aerobic catabolism of salicylic acid and its potential metabolites were performed to determine whether their activities were induced and therefore involved in metabolism of this substrate.

Table 3.3. Specific activities of catabolic enzymes in crude extract of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 after growth with salicylic acid and succinate

Enzyme / substrate	*Oceanimonas* sp. strain S19 Specific activity after growth with:		*Acinetobacter* sp. strain S21 Specific activity after growth with:	
	Salicylic acid	Succinate	Salicylic acid	Succinate
Salicylate Hydroxylase				
NADH + H^+	13	<5	5	<5
Salicylic acid	<5	<5	<5	<5
NADH + H^+ + salicylic acid	35	8	397	<5
Catechol-1,2-dioxygenase				
Catechol	876	<5	1292	8
3-Methylcatechol	85	<5	129	6
4-Methylcatechol	63	13	114	<5
4-Bromocatechol	34	<5	23	<5
Catechol-2,3-dioxygenase				
Catechol	<5	<5	<5	<5
3-Methylcatechol	<5	<5	<5	<5
4-Methylcatechol	<5	<5	<5	<5
4-Bromocatechol*	<5	<5	<5	<5
Gentisate-1,2-dioxygenase				
Gentisic acid	<5	<5	<5	<5

All the data shown are means of three independently performed experiments and the activity was expressed as nmol x min^{-1}x mg^{-1} protein. *As the molar extinction coefficient for bromomuconic acid was not available, the ε for *cis,cis*-muconate was used instead.

Salicylic acid grown cells of both strains showed high specific activity of the catechol-1,2-dioxygenase for catechol and to a lesser degree for 3-methylcatechol, 4-methylcatechol and 4-bromocatechol. No activity was detected for catechol-2,3-dioxygenase and gentisate-1,2-dioxygenase. Growth with salicylic acid induced NADH + H^+ dependent salicylate hydroxylase activity whilst growth with succinate did not cause elevated activity of any of the enzymes tested. *Acinetobacter* sp. strain S21 had the highest specific activity of salicylate hydroxylase and catechol-1,2-dioxygenase.

For confirmation, crude extract obtained from *Oceanimonas* sp. strain S19 after growth with phenyl salicylate was used to follow the fate of this expected key intermediate catechol via UV-VIS analysis between wavelength of 200nm and 400nm to observe the change in absorbance due to oxygenolytic cleavage of catechol and the corresponding accumulation of *cis*,*cis*-muconate.

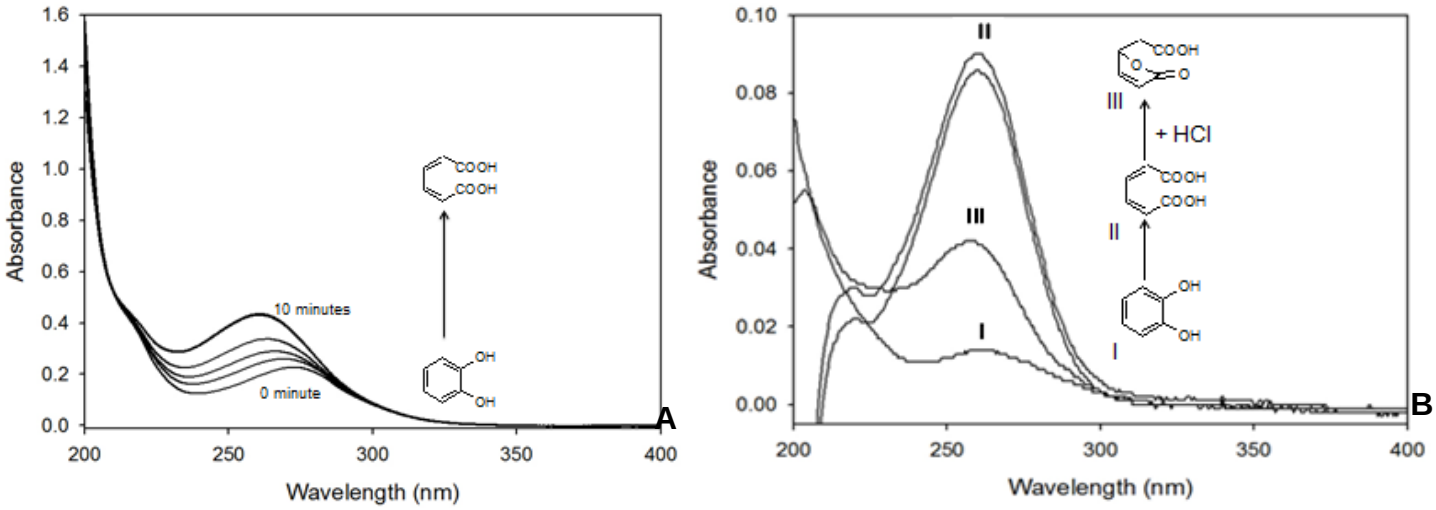

Figure 3.7. Enzymic turn-over of catechol to *cis*,*cis*-muconic acid (A) and the change in absorbance from catechol (I) to *cis*,*cis*-muconate (II) and acid catalyzed formation of the butenolide tautomer (III) in aqueous solution in the presence of HCl (pH 2) by crude extract of *Oceanimonas* sp. strain S19 (0.122 mg/mL protein) after growth with salicylic acid.

The spectra show that the absorbance increased at 260nm indicating accumulation of *cis*,*cis*-muconate from catechol. Upon addition of HCl to pH 2, the maximum absorbance formed at 215nm is probably due to the acid catalyzed formation of butenolide from *cis*,*cis*-muconate.

The enzyme assay for catechol-1,2-dioxygenase was used to verify the enzymic turn-over of salicylic acid by crude extract of *Acinetobacter* sp. strain S21. UV-VIS analysis from 200nm to 400nm over time was used to observe the change in absorbance due to the accumulation of *cis*,*cis*-muconate from catechol.

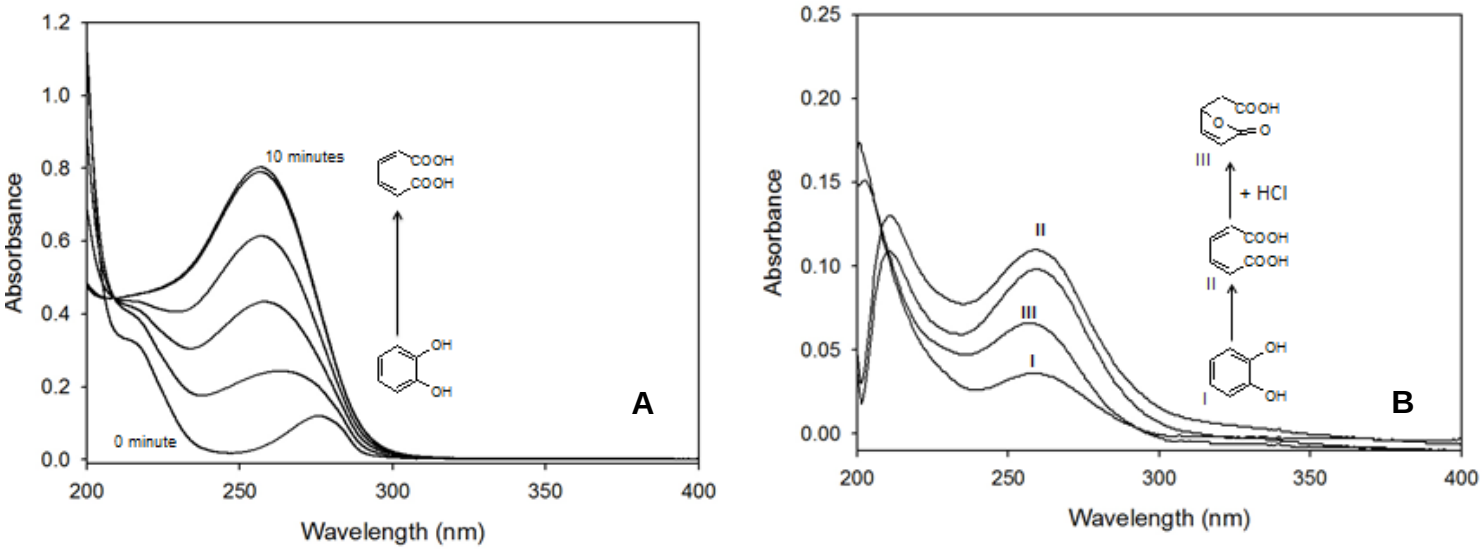

Figure 3.8. Enzymic turn-over of catechol to *cis*,*cis*-muconic acid (A) and the change in absorbance from catechol (I) to *cis*,*cis*-muconate (II) and acid catalyzed formation of the butenolide tautomer (III)in aqueous solution in the presence of HCl (pH 2) (B) by the crude extract of *Acinetobacter* sp. strain S21 (0.279 mg/mL protein) after growth with salicylic acid.

The spectral analysis shows that the absorbance increased at 260nm indicating accumulation of *cis*,*cis*-muconate from catechol. Again, addition of HCl to adjust the pH to 2 increased the absorbance at about 210nm probably due to the acid catalyzed formation of the butenolide from *cis*,*cis*-muconate.

The specific activities of enzymes required for the aerobic utilization of phenyl salicylate and its metabolites were assayed to determine whether these enzymes were inducible.

Table 3.4. Specific activities of catabolic enzymes in crude extract of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 after growth with phenyl salicylate and succinate

Enzyme / substrate	*Oceanimonas* sp. strain S19 Specific activity after growth with		*Acinetobacter* sp. strain S21 Specific activity after growth with	
	Phenyl salicylate	Succinate	Phenyl salicylate	Succinate
Esterase				
p-Nitrophenylacetate	19	<5	352	<5
Phenyl salicylate	17	<5	19	<5
Salicylate hydroxylase				
NADH + H^+	7	<5	42	<5
Salicylic acid	<5	<5	<5	<5
NADH + H^+ + salicylic acid	93	6	169	10
Phenol hydroxylase				
NADH + H^+	7	<5	42	<5
Phenol	<5	<5	<5	<5
NADH + H^+ + phenol	78	8	132	<5
Catechol 1,2-dioxygenase				
Catechol	162	11	1494	10
3-Methylcatechol	25	<5	105	6
4-Methylcatechol	18	<5	290	7
4-Bromocatechol	8	<5	25	<5
Catechol-2,3-dioxygenase				
Catechol	<5	<5	<5	<5
3-Methylcatechol	<5	<5	<5	<5
4-Methylcatechol	<5	<5	<5	<5
4-Bromocatechol	<5	<5	<5	<5
Gentisate-1,2-dioxygenase				
Gentisic acid	<5	<5	<5	<5

All data shown are the average of three independently performed experiments and the specific activities were expressed as nmol x min^{-1}x mg^{-1} protein. The specific activity for phenyl salicylate was determined by reversed phase HPLC.

The crude extract of phenyl salicylate grown cells of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 showed inducible, high specific activity of catechol-1,2-dioxygenase. Catechol-1,2-dioxygenase of *Oceanimonas* sp. strain S19 showed lower activity on 3- and 4-methylcatechol and 4-bromocatechol than for catechol whilst the specific activity of catechol-1,2-dioxygenase in *Acinetobacter* sp. strain S21 was generally higher than that found in *Oceanimonas* sp. strain S19 for all catechols tested. Although the esterase activity for strain S19 was lower than that of strain S21 for *p*-nitrophenylacetate, it was clearly induced. The activity of phenol hydroxylase and salicylate hydroxylase of both strains was induced by growth with phenyl salicylate while growth with succinate did not induce the activity of these two enzymes. No activity of catechol-2,3-dioxygenase and gentisate-1,2-dioxygease was detected.

The crude extract obtained from *Oceanimonas* sp. strain S19 after growth with phenyl salicylate was used to follow the fate of the expected intermediate catechol via UV-VIS spectral analysis between 200nm and 400nm to observe the change in absorbance due to oxygenolytic cleavage of catechol by catechol-1,2-dioxygenase to *cis*,*cis*-muconate.

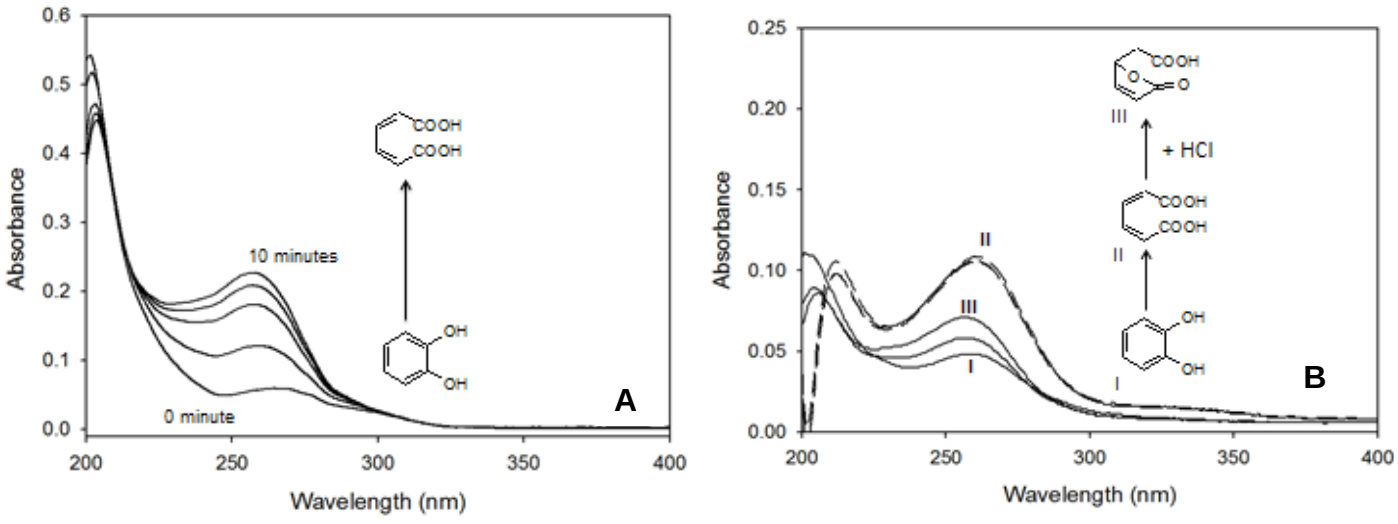

Figure 3.9. Enzymic turn-over of catechol to *cis*,*cis*-muconic acid (A) and the change in absorbance from catechol (I) to *cis*,*cis*-muconate (II) and acid catalyzed formation of the butenolide tautomer (III)in aqueous solution in the presence of HCl (pH 2) (B) by the crude extract of *Oceanimonas* sp. strain S19(0.338 mg/mL protein) after growth with phenyl salicylate.

The spectral analysis shows that the absorbance increased at 260nm indicating accumulation of *cis*,*cis*-muconate from catechol. Addition of HCl to adjust the pH to 2 increased the absorbance at about 210nm probably due to the acid catalyzed formation of the butenolide from *cis*,*cis*-muconate.

As for *Oceanimonas* sp. strain S19, the enzyme assay for catechol-1,2-dioxygenase was used to verify the enzymic turn-over of catechol by crude extract of *Acinetobacter* sp. strain S21.

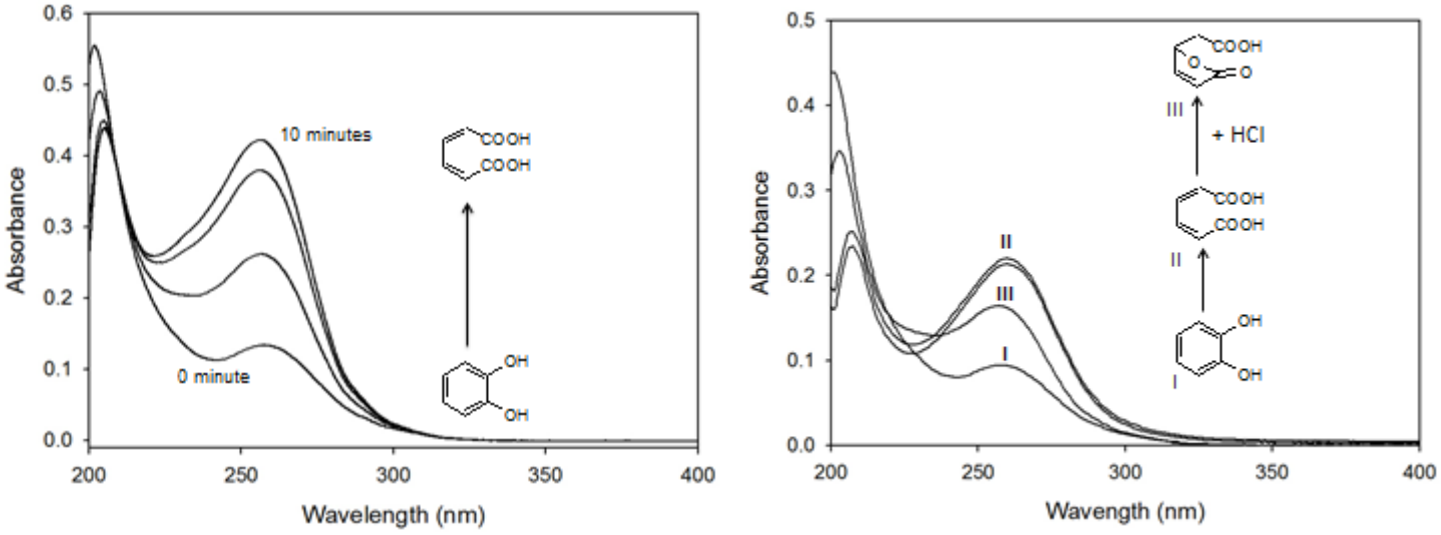

Figure 3.10. Enzymic turn-over of catechol to *cis*,*cis*-muconic acid (A) and the change in absorbance from catechol (I) to *cis*,*cis*-muconate (II) and acid catalyzed formation of the butenolide tautomer (III)in aqueous solution in the presence of HCl (pH 2) (B) by the crude extract of *Acinetobacter* sp. strain S21 (0.381 mg/mL protein) after growth with phenyl salicylate.

As before, the spectral analysis confirmed the enzymic activity of catechol-1,2-dioxygase producing *cis*,*cis*-muconic acid. Upon addition of HCl to adjust the pH to 2 increased the absorbance at 210nm which is probably due to the acid catalyzed formation of butenolide from *cis*,*cis*-muconate.

The enzymatic hydrolysis of phenyl salicylate to the expected products by crude extract of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 was assayed by reversed phase HPLC to determine the stoichmetric ratio of the metabolites formed from phenyl salicylate, i.e. phenol and salicylic acid.

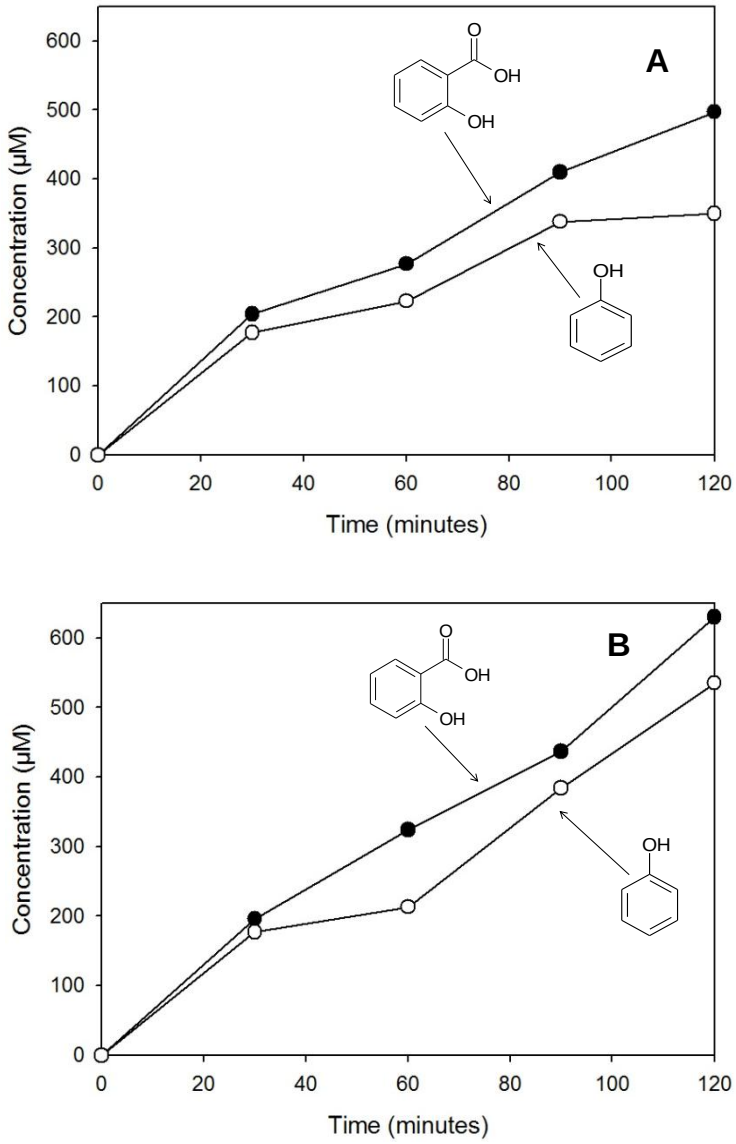

Figure 3.11. Hydrolysis of phenyl salicylate by crude extract obtained from phenyl salicylate grown cells of *Oceanimonas* sp. strain S19 (A) and *Acinetobacter* sp. strain S21 (B). Data shown are the average of two independently performed experiments.

Phenyl salicylate was thus hydrolyzed at the ester bond yielding two mono-aromatic metabolites; the crude extract catalyzed an almost stoichiometric release of phenol and salicylate from phenyl salicylate. Thus the initial step of phenyl salicylate catabolism in both strains is hydrolysis of the ester bond.

3.3.4. Determination of the type of oxygenolytic cleavage

Catechol either undergoes intradiol (ortho) or extradiol (meta) cleavage. Intradiol cleavage of catechol is catalyzed by catechol-1,2-dioxygenase to *cis*,*cis*-muconate which is sequentially transformed to β-ketoadipate. As the enzyme assays indicated no meta cleavage activity but high catechol-1,2-dioxygenase activity was detected (Table 3.3 and 3.4). Therefore, an additional assay to detect the formation of β-ketoadipate from catechol was employed.

The Rothera reaction is a colorimetric test which detects the presence of β-ketoadipate whereby nitroprusside reacts with the ketone forming a colored complex. A positive Rothera reaction is indicated by the development of a red to purple color.

The assay was positive for *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 grown with salicylic acid and phenyl salicylate. This indicates that β-ketoadipate, was formed from catechol when catabolized via the ortho cleavage and subsequently channelled into the β-ketoadipate pathway. As expected, the positive control *Vibrio* sp. KM1 which utilizes benzoic acid via the ortho pathway gave a positive Rothera reaction while *Marinobacter* sp. KM1 which utilizes phenol via the meta cleavage pathway gave a negative Rothera reaction (no formation of the color complex of β-ketoadipate with nitroprusside).

3.3.5. Growth with possible intermediates of salicylic acid and phenyl salicylate

Metabolites produced during utilization of aromatic compounds can have a potential to cause toxic effects on the organism, especially if the organism lacks suitable enzymes to further metabolize such compounds thereby causing their accumulation to critical levels.

- Salicylic acid grown cells of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

Salicylic acid is generally metabolized aerobically via two routes, the catechol pathway or the gentisate pathway. Therefore, both strains were grown with two authentic metabolites of salicylic acid, catechol and gentisic acid, to determine whether they can utilize these substrates.

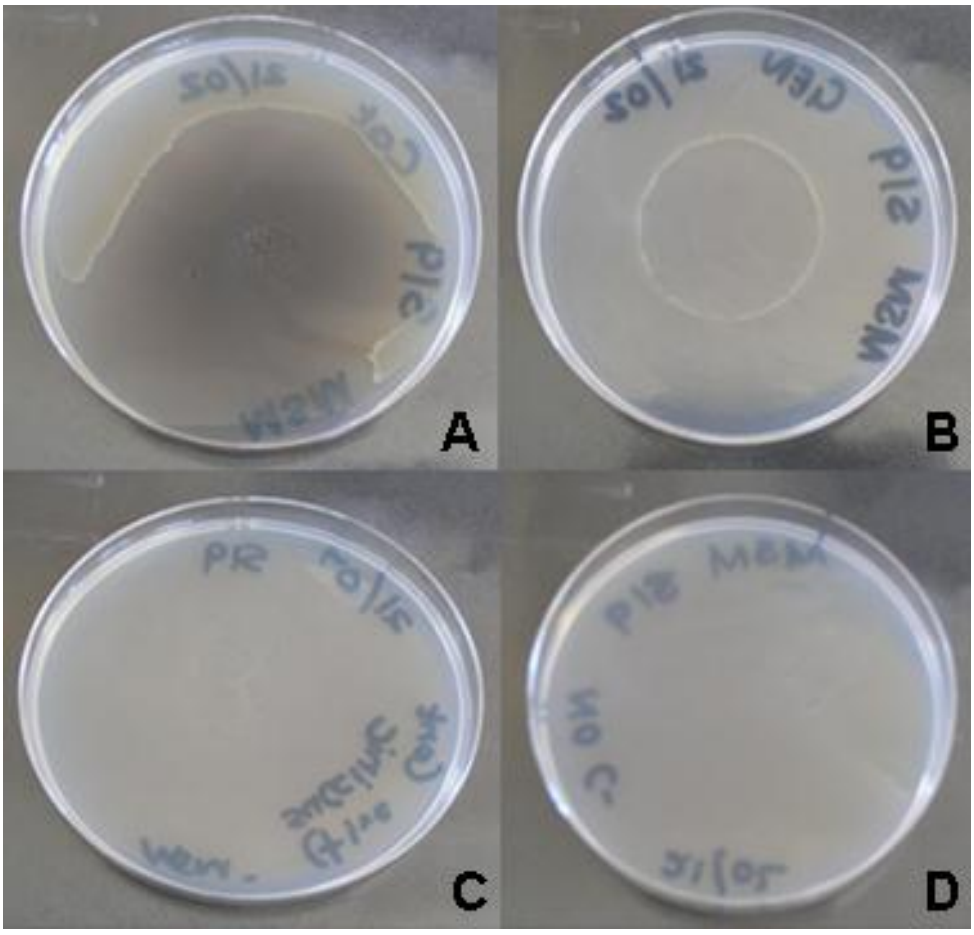

Figure 3.12. Growth of *Oceanimonas* sp. strain S19 with authentic catechol (A) and gentisate (B), the positive control succinate (C) and the negative control (no carbon source, D).

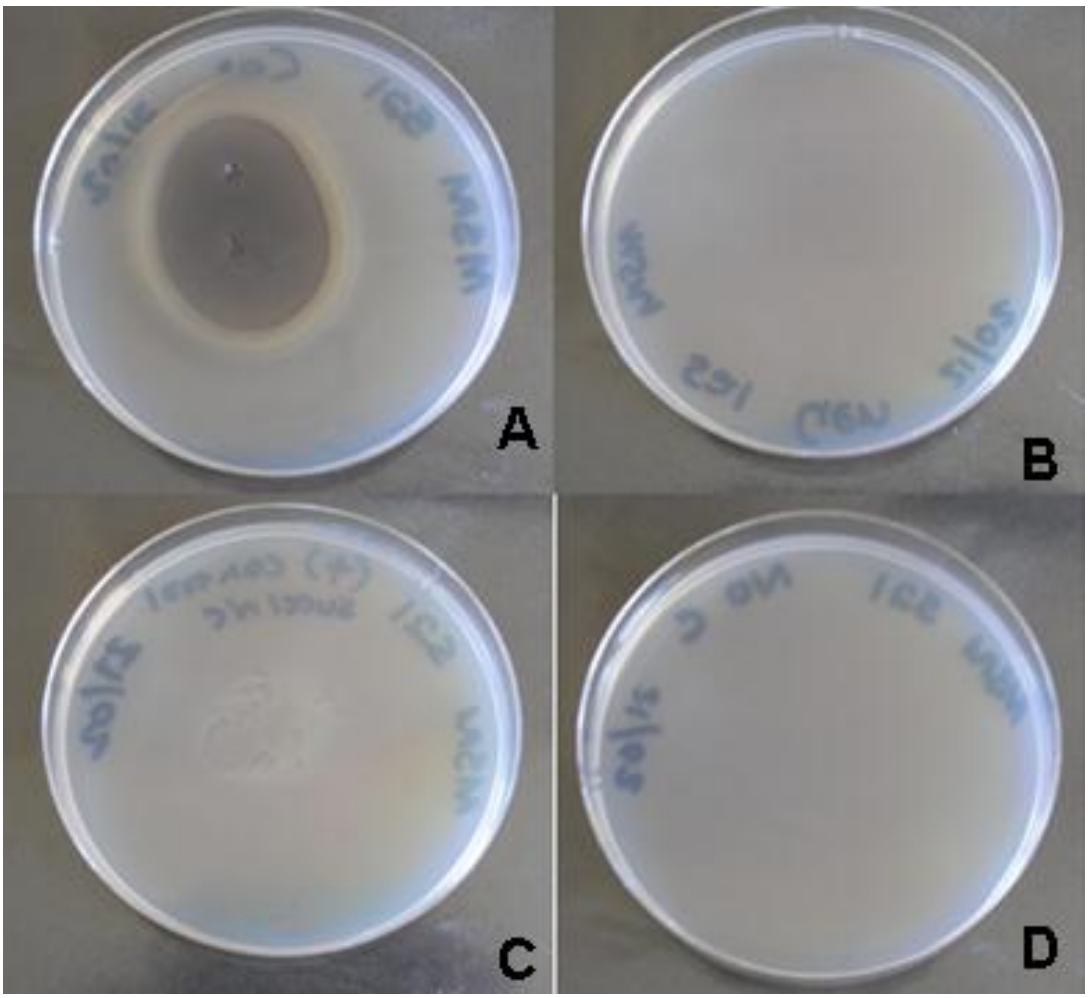

Figure 3.13. Growth of *Acinetobacter* sp. strain S21 with authentic catechol (A) and gentisate (B) the positive control succinate (C) and negative control (no carbon source, D).

While *Oceanimonas* sp. strain S19 showed growth with catechol and gentisate, *Acinetobacter* sp. strain S21 grew only with catechol. As expected, growth was observed for both strains with succinic acid and no growth was detected without the addition of carbon source.

- Phenyl salicylate grown cell of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21

The initial step of phenyl salicylate utilization by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 is the hydrolysis of the ester bond by esterase activity leading to the mono-aromatic products salicylic acid and phenol.

Therefore, to determine whether *Oceanimonas* sp. strain S19 can utilize salicylic acid and phenol as a carbon and energy source, cells of the strains were grown with authentic mono-aromatic metabolites of phenyl salicylate (i.e. salicylic acid and phenol) in MSM.

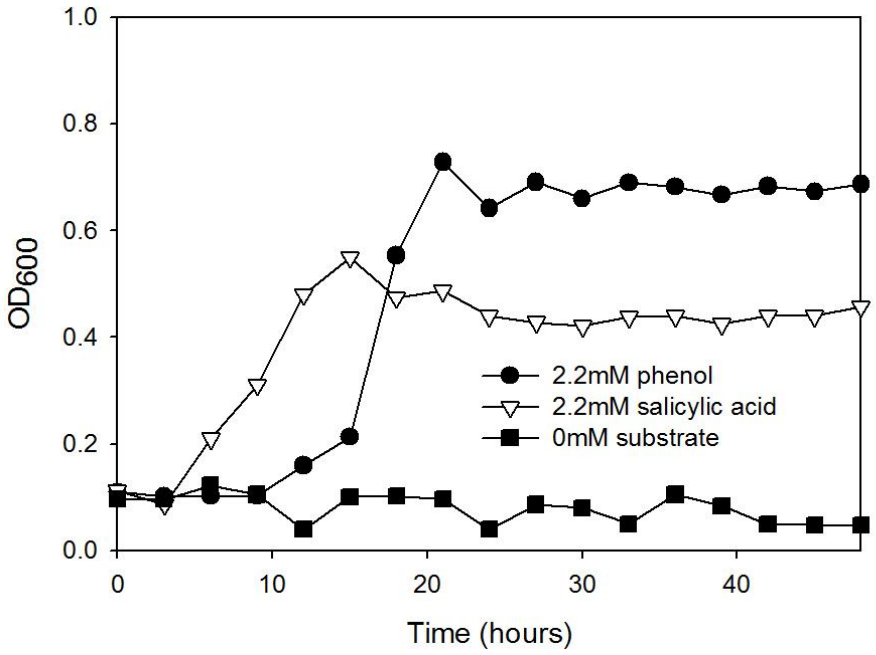

Figure 3.14. Growth of *Oceanimonas* sp. strain S19 with authentic salicylate and phenol as substrate in MSM at 25°C and 200 rpm.

The mono-aromatic metabolites formed from phenyl salicylate can be further utilized via products that are channelled into the TCA cycle. To determine whether *Oceanimonas* sp. strain S19 can utilize the two expected intermediates, catechol (from phenol and salicylate) and gentisate (from salicylate), their utilization was tested as before using MSM agar with authentic catechol and gentisic acid present as crystals.

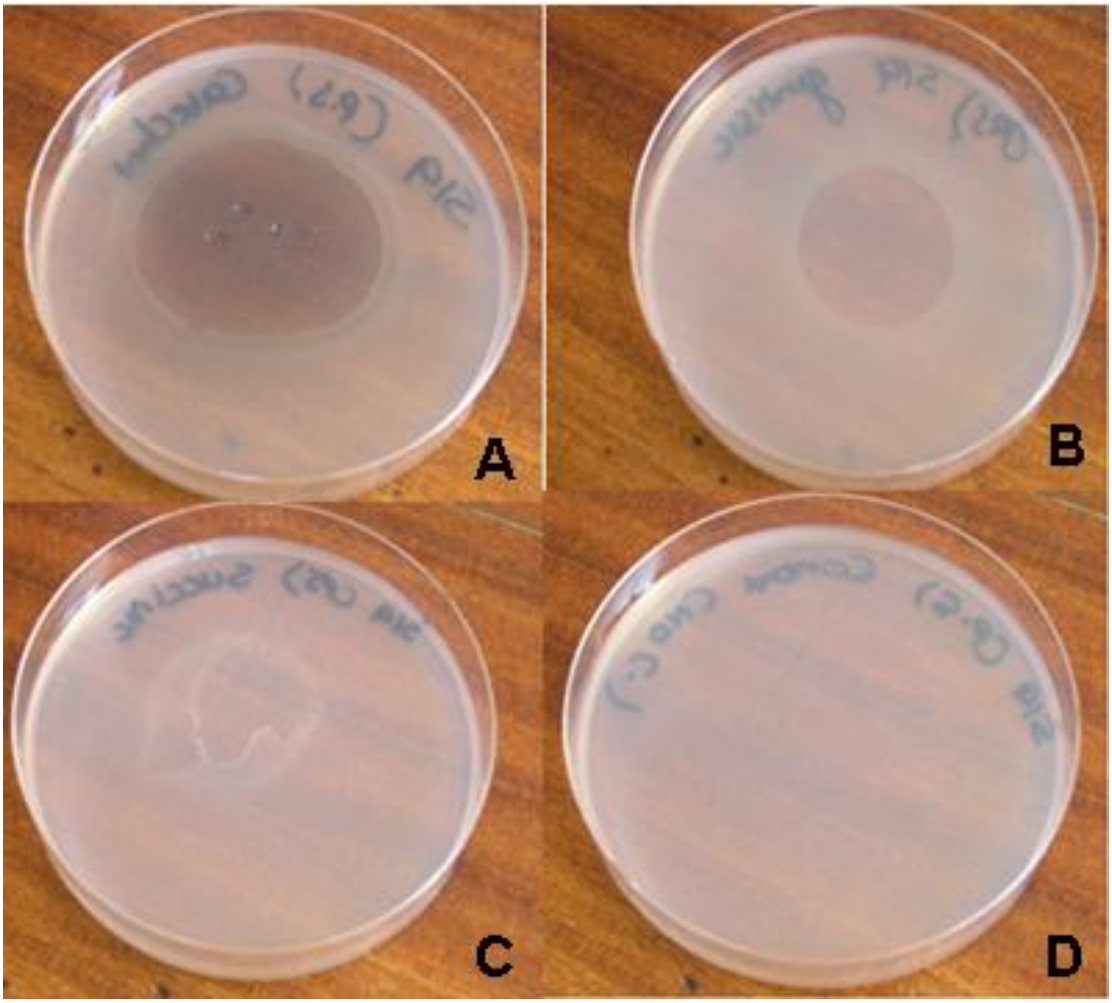

Figure 3.15. Growth of *Oceanimonas* sp. strain S19 with authentic catechol (A) and gentisate (B), the positive control succinate (C) and the negative control (no carbon source, D).

Oceanimonas sp. strain S19 was able to utilize the two mono-aromatic substrates (phenol and salicylate, Figure 3.14) formed upon hydrolysis of phenyl salicylate and the two potential intermediates catechol and gentisate (Figure 3.15) as growth substrate and as expected, the strain grew with succinic acid and no growth was detected without carbon source.

To verify if *Acinetobacter* sp. strain S21 can grow with mono-aromatic metabolites generated of phenyl salicylate, the strain was grown with authentic salicylic acid and phenol in MSM to determine whether it can utilize these two substrates.

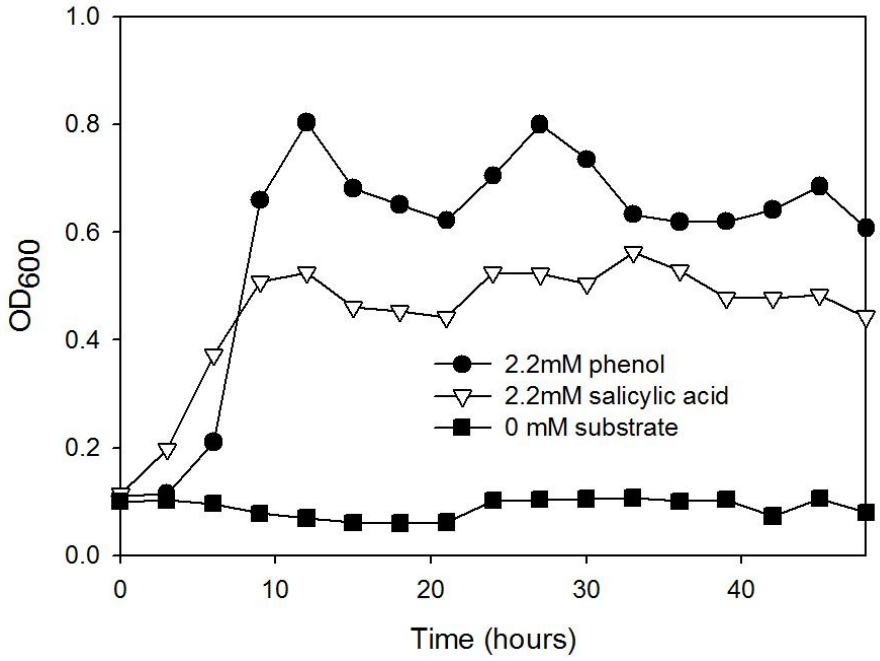

Figure 3.16. Growth of *Acinetobacter* sp. strain S21 with authentic salicylate and phenol as substrate in MSM at 25°C and 200 rpm.

The utilization of catechol and gentisate by *Acinetobacter* sp. strain S21 was determines via MSM agar.

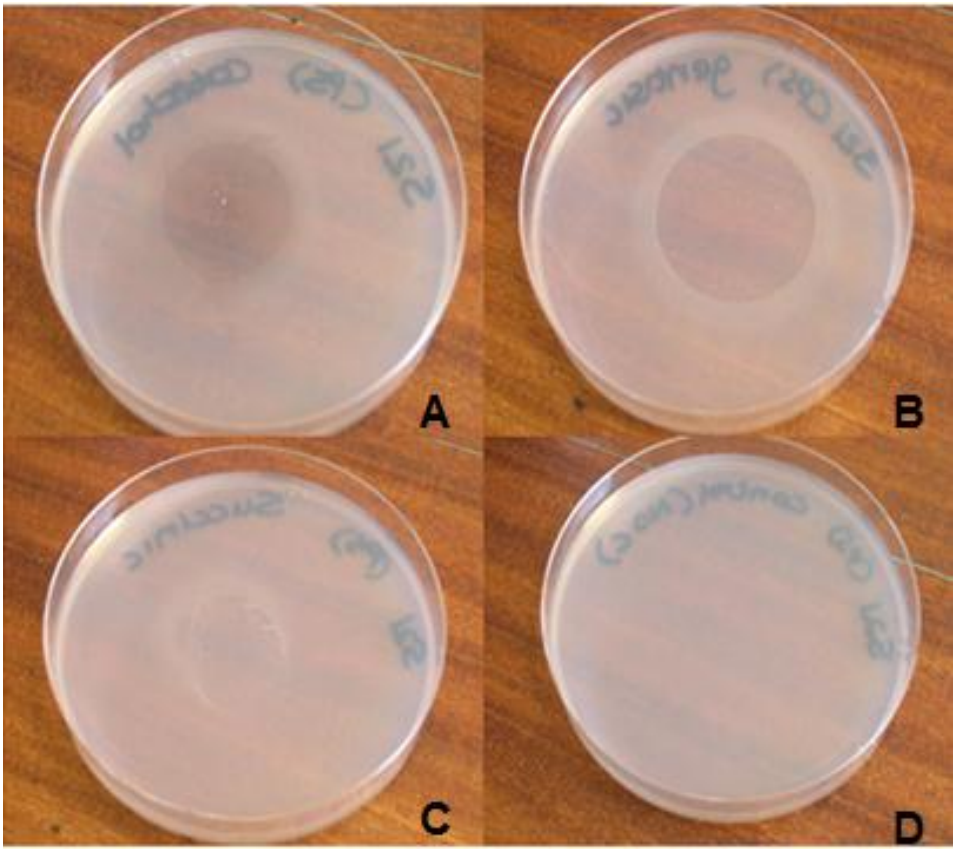

Figure 3.17. Growth of *Acinetobacter* sp. strain S21 with authentic catechol (A) and gentisate (B), the positive control succinate (C) and the negative control (no carbon source, D).

Acinetobacter sp. strain S21 was able to utilize the two mono-aromatic substrates phenol and salicylate (Figure 3.16) formed upon hydrolysis of phenyl salicylate. In addition, the two intermediates catechol and gentisate were apparently used as carbon and energy source. The strain grew with succinic acid while no growth without carbon source was detected (Figure 3.17).

3.4. Discussion

The metabolism of aromatic compounds by bacteria usually requires the isolation and characterization of metabolites and identification of enzymes involved in metabolism (Gibson, 1968). Assaying the probable enzymes involved and demonstrating the sequential reactions of enzymes oxidizing specific substrates and potential intermediates provides an understanding of the catabolic pathway employed for the utilization of aromatic compounds (Karegoudar and Kim, 2000; Dìaz, 2004).

Catabolic pathways for the aerobic utilization of salicylic acid and phenyl salicylate by the estuarine isolates *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 are proposed based on the enzymatic and oxygen uptake studies and HPLC analysis. Both salicylic acid and phenyl salicylate are catabolized via the catechol branch of the β-ketoadipate pathway catalyzed by a series of specific enzymes (Figure 3.18). Salicylic acid is oxidatively decarboxylated to catechol by salicylate-1-hydroxylase. Phenyl salicylate metabolism is initiated by esterase activity which hydrolyses the substrate at the ester linkage to transiently yield two mono-aromatic metabolites, phenol and salicylate (Figure 3.3 - 3.6). This was verified when the crude extracts of phenyl salicylate grown cells of both strains was incubated with the diaryl ester, the substrate was hydrolyzed at the ester bond and the stoichiometric relationship of phenol and salicylate was 1:1.5 for strain S19 and 1:1.21 for strain S21 (Figure 3.11). Phenol is further metabolized to catechol by phenol hydroxylase while salicylate is metabolized to catechol catalyzed by salicylate-1-hydroxylase. Salicylate hydroxylase and phenol hydroxylase are flavoprotein monooxygenases which are dependent on coenzymes as primary electron donor (Jensen *et al.,* 2013), their activity on phenol and salicylic acid was only detected in the presence of NADH + H^+ (Table 3.3 - 3.4). Catechol, which is a key metabolite formed from both substrates was cleaved via ortho-cleavage catalyzed by catechol-1,2-dioxygenase to produce *cis*,*cis*-muconic acid which was detected at 260nm (Figure 3.7 - 3.10).

The intradiol cleavage product, *cis*,*cis*-muconic acid is converted to muconolactone which is a *cis*,*cis*-muconic acid tautomer. The cycloisomerization of *cis*,*cis*-muconic acid to muconolactone catalyzed by muconate lactonizing enzymes involves the anti-closure and opening of the lactone ring (Ornston, 1966; Ngai and Kallen, 1983; Cain *et al.,* 1996; Wells and Ragauskas, 2012). This reaction similarly is catalyzed by acid

and the muconolactone exist in cyclic form in acid solution (Cain *et al.,* 1997; Pollman *et al.,* 2002). Butenolides, *cis*,*cis*-muconic acid tautomers, have been identified and characterized after acidification followed by extraction and the typical spectra of pure butenolides showed maxima in a wavelength range of 200 to 220nm (Cain *et al.,* 1996; Cain *et al.,* 1997; Schmidt and Kirby 2001). Upon addition of hydrochloric acid to the enzyme assay reaction system, butenolide formed which caused the increase in absorbance at a wavelength between 200nm and 220nm (Figure 3.7 - 3.10).

Muconolactone formed from cycloisomerization is subsequently converted to β-ketoadipate enol-lactone and then to β-ketoadipate catalyzed by β-ketoadipate enol-lactone hydrolase. This funnels the aromatic breakdown products into the TCA cycle intermediates, i.e. succinyl-coA and acetyl-coA (Ornston, 1966; Cain *et al.,* 1968; Harwood and Parales, 1996; Wells and Ragauskas, 2012). The presence of a functional ortho-cleavage pathway for catechol in the two estuarine isolates was further confirmed by a positive Rothera reaction which detects the presence of β-ketoadipate metabolite formed after ortho-cleavage.

The type of growth substrate and its metabolites can influence the type of enzymes involved in productive catabolism via induction. The enzymes involved in catabolism of aromatic compounds are typically inducible (Ornston and Stanier, 1966; Meagher and Ornston, 1973; Kurane *et al.,* 1980; Pèrez-Pantoja *et al.,* 2008). Catabolic enzymes involved in utilization of salicylic acid and phenyl salicylate were induced in both strains after growth with salicylic acid and phenyl salicylate (Table 3.3 and 3.4). The crude enzyme extract of cells after growth with succinate showed low to no activity indicating that this substrate did not induce these catabolic enzymes. Catechol-1,2-dioxygenase is classified into two classes, type I which catalyzes the oxygenolytic cleavage of catechol and has low or no activity for halogenated catechols and type II, which includes enzymes with high activity for halogenated catechols (Reineke *et al.,* 1978; Murakami *et al.,* 1997; Caposio *et al.,* 2002; Dìaz *et al.,* 2013). The enzyme of both strains showed high activity for catechol, moderate activity with methylated catechol and very low activity for 4-bromocatechol indicating that they had type I catechol-1,2-dioxygenase.

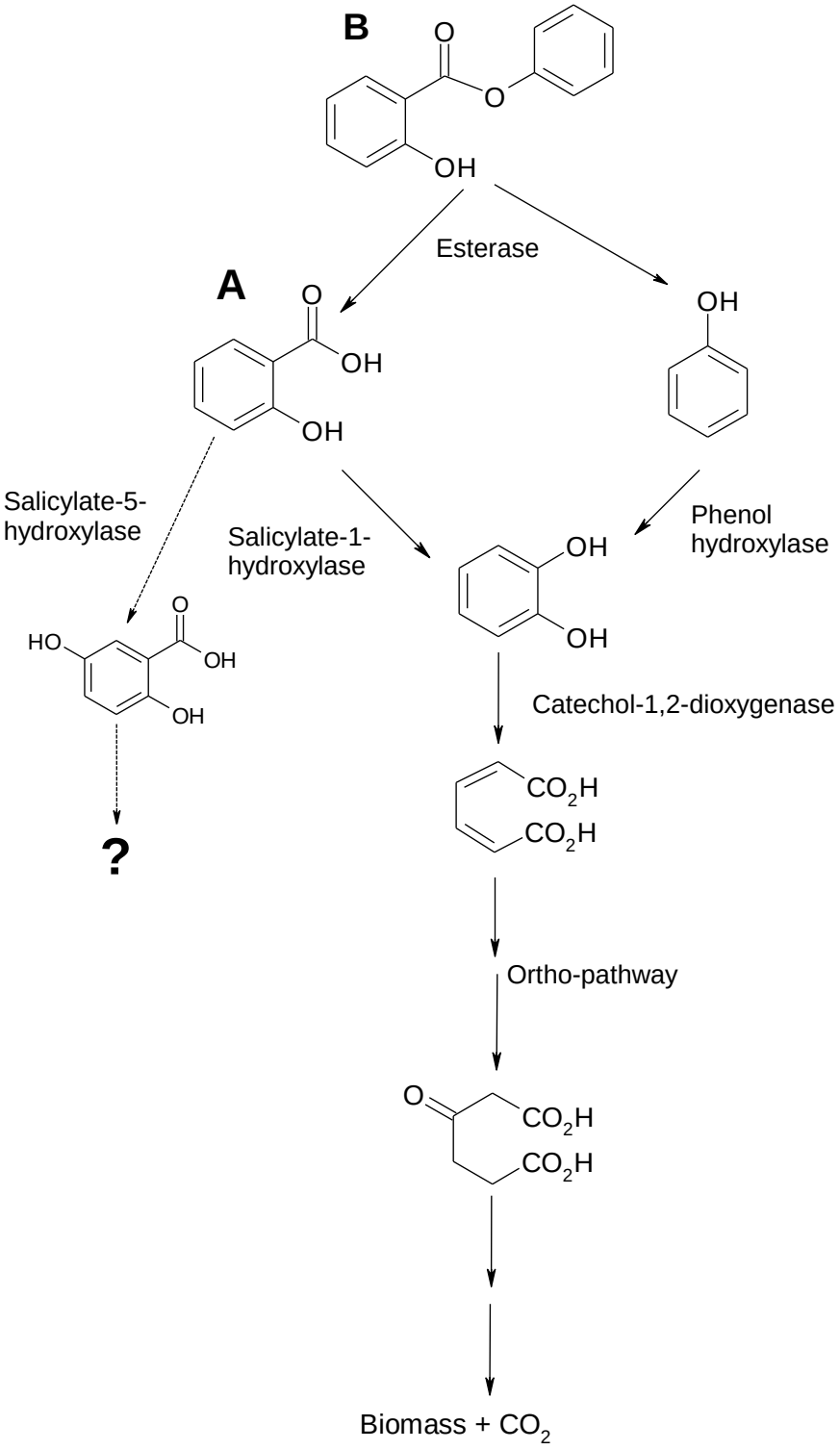

Figure 3.18. Proposed catabolism for salicylic acid (A) and phenyl salicylate (B) by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21.

The diaryl ester phenyl salicylate is hydrolytically cleaved to yield salicylate and phenol. Salicylic acid and phenol are then channelled via the central pathway intermediate catechol into the ortho pathway. Salicylic acid was also oxidized to gentisate by *Oceanimonas* sp. strain S19 but only at high concentration.

During aerobic growth of strains with only 2 mM of the target compounds present, metabolites were not detectable by HPLC. The intermediates of metabolic pathways do not normally accumulate inside or outside the cell in significant amounts as they are transformed serially and rapidly as they are formed to avoid accumulation of reactive intermediates that might cause toxic effects. Nonetheless, a metabolite accumulated transiently when cells of *Oceanimonas* sp. strain S19 were grown in the presence of salicylic acid at higher concentration of 5mM which was identified as gentisate (Figure 3.1). The oxygen uptake of salicylic acid and phenyl salicylate grown cells showed only moderate activity with gentisate (Table 3.1). The strain also grew with both catechol and gentisate as carbon and energy source (Figure 3.12 and 3.15). This was not in accordance with the enzyme assay of strain S19 which showed no activity for gentisate-1,2-dioxygenase which cleaves gentisate to maleyl pyruvate (Figure 3.3 and Table 3.3). Whilst growing with 2 mM salicylic acid, gentisate was not produced in cultures to induce the activity of gentisate-1,2-dioxygenase which is usually an inducible enzyme (Zhou *et al.,* 2001).

Although the presence of more than one catabolic pathway of aromatic compounds in one species has been previously reported (Nakazawa and Yokota, 1973), there are only few reports on different catabolic pathways occurring concurrently. The ability of bacteria to simultaneously express two pathways for aromatic compounds is determined by the chemical nature of the aromatic substrate with which the organism is grown (Feist and Hegeman, 1969; Williams *et al.,* 1975). However, the use of two different cleavage pathways simultaneously was demonstrated for *Pseudomonas cepacia*, both the ortho- and meta cleavage pathway were induced during utilization of benzoate while utilization of salicylic acid only induced the ortho-cleavage pathway (Hamzah and Al-Baharna, 1994). Even though *Oceanimonas* sp. strain S19 expressed both pathways of salicylic acid utilization based on the data of transiently formed gentisate in the presence of high salicylic acid concentration, the catechol pathway was the dominant pathway of salicylic acid metabolism based on the high activity of catechol-1,2-dioxygenase for catechol. The gentisate pathway was therefore probably expressed as a possible side reaction of salicylic acid catabolism at high concentrations. Salicylic acid is in fact most frequently metabolized through catechol which is the most common intermediate in bacterial metabolism of many aromatic compounds (Henderson, 1961; Hamzah and Al-Baharna, 1994; Hintner *et al.,* 2001; Ishiyama *et al.,* 2004).

Catechol did not accumulate in detectable amounts as it is the key intermediate of salicylic acid by strain S19. Due to the high activity of catechol-1,2-dioxygenase, it is transformed rapidly to succeeding metabolites channelled into the TCA cycle. The gentisate pathway, which occurs when ring cleavage is taking place between a hydroxyl group and a carboxyl group, is a less common route for the aromatic catabolism of aromatic compounds (Zhou *et al.,* 2001). The meta-cleavage pathway by catechol-2,3-dioxygenase was not detected (Table 3.3 and 3.4) and no yellow color due to the production of the meta product was formed in oxygen uptake experiments when catechol was used as a substrate. Ortho cleavage of catechol is generally a preferred pathway by bacteria as it is more efficient in conversion of substrate carbon to cell biomass than the meta-cleavage (Pèrez-Pantoja *et al.,* 2008). Utilization of aromatic substrates via the ortho- or gentisate pathway does not yield any colored intermediates that can be visually detected (Harwood and Parales, 1996).

As reported in the literature, the hydrolysis of the ester linkage of diaryl ester compounds and the ortho-cleavage of mono-aromatic compounds is the most common route of metabolism of such compounds (Zhou *et al.,* 2001; Schmidt, 2002). Similarly, this was evidently the pathway identified during phenyl salicylate catabolism. As there is only limited information on bacterial assimilation of salicylic acid and phenyl salicylate in marine environments; the results obtained in this study provide some insight on the ability of marine bacteria to aerobically degrade such compounds and show that they may play a key role in elimination of aromatic contaminants in such environments.

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CHAPTER 4

Conclusion

Three gram negative bacteria able to utilize salicylic acid, phenyl salicylate and benzyl salicylate were isolated by selective enrichment from estuarine water samples. This indicates the presence and the potential role that such organisms can play in the elimination of organic pollutants in the estuarine environments. Based on the morphological, biochemical and molecular characteristics, strain S19, a rod shaped motile bacterium was assigned to the genus *Oceanimonas*, strain S21, a coccus to rod shaped bacterium was assigned to the genus *Acinetobacter* and strain B12, a rod shaped motile bacterium was assigned to the genus *Pseudomonas. Pseudomonas* and *Acinetobacter* species have been previously isolated from various environments including marine and estuarine environments and are well known to utilize a wide range of aromatic compounds (Maeda *et al.,* 2010). However, *Oceanimonas* species have only been isolated from marine environments (Ivanova *et al.,* 2005) and have only been previously reported to utilize phenol (Brown *et al.,* 2001).

The three isolates demonstrated to be able to catabolize selected PPCPs and might have the potential for microbial elimination of such organic pollutants. *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 utilized salicylic acid and phenyl salicylate as sole source of carbon and energy to almost completion. However, growth with phenyl salicylate was slow as the substrate has only limited aqueous solubility (0.0205 g/L). Benzyl salicylate, which has even a lower aqueous solubility, was only utilized by *Pseudomonas* sp. strain B12 in the presence of synthetic surfactants which enhanced bioavailability of the substrate. Initial concentrations of substrate and oxygen play a vital role in determining the rate of aerobic degradation. The physicochemical properties of the contaminant such as aqueous solubility play a major role in determining the extent of bacterial breakdown of the target compound.

Of the PPCPs used in this study, salicylic acid was catabolized via the catechol pathway and phenyl salicylate was hydrolyzed at the ester linkage to yield phenol and salicylate which are further metabolized to catechol. Catechol then undergoes intradiol (ortho) cleavage catalyzed by catechol-1,2-dioxygenase, the key enzyme of the β-ketoadipate pathway. Enzymes involved in catabolism of the target compounds

were induced by the substrate and their metabolites and both strains contained type I catechol-1,2-dioxygenase. Studies on the aerobic catabolism of pollutants provide information on the potential fate of such contaminants in the environment. This study provided some insight on the degradation of salicylic acid and selected diaryl ester compounds by marine bacteria and the pathway involved in metabolism of the compounds by such bacteria.

The metabolism of benzyl salicylate could not be studied any further as this compound was only utilized considerably in the presence of another organic compound enhancing utilization. As the strain also grew with the surfactant Tween 80 as a carbon source, it was not possible in this study to quantify metabolites produced and determine whether the enzyme induction was due to growth with benzyl salicylate or the added surfactant. Considering the good growth of *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 with phenyl salicylate as carbon source albeit having only limited aqueous solubility, these bacteria have obviously developed mechanisms to overcome some factors that may inhibit or reduce the rate of organic hydrocarbon transformation. Potential mechanisms to overcome the limited solubility of the organic substrate and its bioavailability have been previously described (Stelmack *et al.,* 1999; Pandey and Jian, 2002; Hintner *et al.,* 2005). *Acinetobacter* and *Pseudomonas* are some of the bacterial genera reported to possess such ability to increase the solubility of hydrophobic compounds (Bakay *et al.,* 1999; Satpute *et al.,* 2010).

Although there are other processes involved in the elimination of pollutants, bacteria play a foremost role in the elimination of toxic organic pollutants in aquatic environments thereby reducing the exposure of other organisms. Isolation of these bacteria from the estuarine environment indicates that typical marine bacteria and salt tolerant fresh water related bacteria are present in marine and estuarine environments in KwaZulu-Natal that can eliminate organic contaminants in such environments.

There is a growing number of chemicals being produced worldwide; thus the number and amount of chemicals being discharged and accumulating in aquatic environments is escalating (Gomez *et al.,* 2007). Because of the potential toxic impact that these chemicals have on the ecosystem, their removal from the environment is crucial. In South Africa, there is only limited information available

concerning marine and estuarine pollution. Only a few chemicals have been targeted and subsequently been detected in South African marine environments; this includes phenol, DDT, PAH and HCH (Brown, 1987; Moloney *et al.,* 2013). However, this is just a miniscule amount of chemicals that are expected to be present in the marine and estuarine environments because in KwaZulu-Natal, a pollutant burden of rivers is ultimately deposited in the receiving waters (CSIR, 2010). Consequently, bacterial degradation in these environments should be studied more extensively. In fact, marine bacteria utilizing aromatic compounds are present in South African marine and estuarine environments (Moxley and Schmidt, 2010; Moxley and Schmidt, 2012).

The results obtained in this study indicate that the following would be worth pursuing:

- Mechanisms used by *Oceanimonas* sp. strain S19 and *Acinetobacter* sp. strain S21 to increase bioavailability of phenyl salicylate.

Phenyl salicylate has only limited aqueous solubility, thus bioavailability for cell uptake and utilization is limited. However, the two strains utilized this hydrophobic compound regardless of its limited bioavailability. The strains therefore had a mechanism to overcome this limitation which warrants further analysis.

- Assaying the role of the gentisate pathway as a potential side reaction at high concentration of salicylic acid.

Gentisate was detected when *Oceanimonas* sp. strain S19 was grown with 5 mM salicylic acid. To determine the underlying regulations controlling the expression of both catechol and gentisic pathway would be of great interest.

- Determining the catabolic pathway of benzyl salicylate and the probable enzymes involved.

To isolate an estuarine strain that can utilize benzyl salicylate without the addition of synthetic surfactants that enhance bioavailability of this substrate to assay catabolic sequence and the key enzymes involved in the utilization of this substrate.

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APPENDIX A

- Preparation of media for biochemical tests

1. Starch hydrolysis

Composition	g/L
Soluble starch	12.0
Beef extract	10.0
Agar	15.0

Final pH 7.5 at 25°C

After incubation, Lugol's iodine is added to the starch agar plates, microorganisms that are amylase positive give a clear zone around the colonies indicating starch utilization.

2. Citrate utilization

Composition	g/L
NaCl	5.0
Sodium citrate	2.0
K_2HPO_4	1.0
$(NH_4)H_2PO_4$	1.0
$MgSO_4.7H_2O$	0.2
Bromothymol blue	0.08
Agar	15.0

Final pH 6.8 at 25°C

Bacteria that utilize citrate as a sole carbon source turn the medium from green to blue.

3. Gelatin hydrolysis

Composition	g/L
Gelatin powder	120.0
Beef extracts	3.0
Peptone	5.0

Final pH 7.8 at 25°C

After incubation, the medium is transferred into the fridge for 30 minutes and if the medium remains liquid, then the microorganism is positive for gelatin hydrolysis.

4. Catalase activity

A loopful of culture is mixed with 3% v/v freshly prepared hydrogen peroxide solution on a slide. Catalase positive bacteria cause a rapid bubbling of the solution due to the evolution of oxygen.

5. Oxidase activity

A drop of 1% w/v Tetramethylphenylene diamine dihydrochloride in distilled water is mixed with culture material using a tooth pick on a filter paper. Development of purple color indicates oxidase activity.

6. Methyl red test / Voges Proskauer test

Composition	g/L
Glucose	5.0
KH_2PO_4	5.0
Pancreatic digest of casein	3.5
Peptic digest of animal tissue	3.5

After incubation, 5 drops of methyl red indicator is added to the test tube and the production of acid is indicated by a colour change to cherry red. For the Voges Proskauer test 15 drops of 5% naphthol (synonymn, hydroxynaphthalene) and 5 drops of 40% KOH are added to the tubes and the colour change to pink burgundy is positive for acetyl methyl carbinol (acetoin) production.

7. Indole production from tryptophan

Composition	g/L
Tryptone	10.0
NaCl	5.0

Final pH 7.0 at 25°C

After incubation, Kovac's reagent is added and the formation of a red ring on the surface of the medium indicates for Indole production.

8. Urease production

Composition	g/L
Urea	20.0
Na_2HPO_4	9.5
KH_2PO_4	9.1
Yeast extracts	0.1
Phenol red	0.01

Final pH 6.8 at 25°C, the medium was filter sterilized and then distribute into test tubes.

Change of medium colour from red to deep pink demonstrates production of urease which catalyzes the breakdown of urea to ammonia and carbon dioxide.

9. Casein hydrolysis

Composition	g/L
Casein	10.0
Meat extracts	3.0
NaCl	5.0
Na_2HPO_4	2.0
Bromothymol blue	0.05
Agar	15.0

Casein hydrolysis is indicated by the change of the medium to midnight blue due to hydrolysis of casein.

10. Triple sugar iron test

Composition	g/L
Enzymatic Digest of Casein	5.0
Enzymatic Digest of Animal Tissue	5.0
Yeast Enriched Peptone	10.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferric Ammonium Citrate	0.2
Sodium Chloride	5.0
Sodium Thiosulfate	0.3
Phenol Red	0.025
Agar	13.5

Final pH 7.3 at 25°C

The change of the medium to yellow is due to acid production indicating fermentation of dextrose, lactose and sucrose. Non-fermentation of sugars is indicated by the medium remaining red. Fermentation of dextrose is indicated by the change in medium to yellow at the bottom (butt) of the text tube due to production of acid and the top part (slant) of the test tube remain red due to alkaline. Cracks and bubbles in

the medium are due to gas production. Production of H_2S changes the medium to black.

11. Oxidation /Fermentation test

Composition	g/L
Dextrose	3.0
Peptone	10.0
NaCl	2.0
K_2HPO_4	5.0
Bromothymol blue	0.3
Agar	0.08

Final pH 7.2 at 25°C

Oxidation test (open tube) – mineral oil was not added to cover the medium

Fermentation (closed tube) – mineral oil was added to 1cm to cover the medium

Oxidation of glucose is indicated by the change of medium of the open tube to yellow due to acid production by oxidation while fermentation is indicated by the colour change of medium to yellow in the closed tube due to acid production from glucose by fermentation.

12. Carbohydrate catabolism (maltose, glucose, fructose, xylose, galactose, mannitol, sucrose and lactose)

Composition	g/L
Peptone	10.0
NaCl	5.0
Phenol red	0.018
Carbohydrate solution (20mM)	0.5 mL

Final pH 7.4 at 25°C

Sugar solutions (stock concentration of 20mM, sterile filtered) were added after autoclaving to the medium. Transformation of the tested sugars to acid and gas production is indicated by the change in medium from red to yellow and gas collection in Durham tube respectively.

APPENDIX B

- Calibration curves

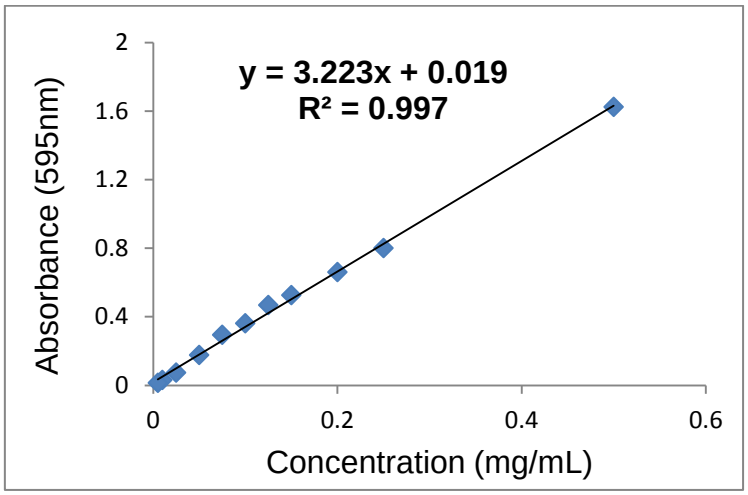

Figure B1. Standard curve of Bovine Serum Albumin in 0.15N NaOH at 595nm.

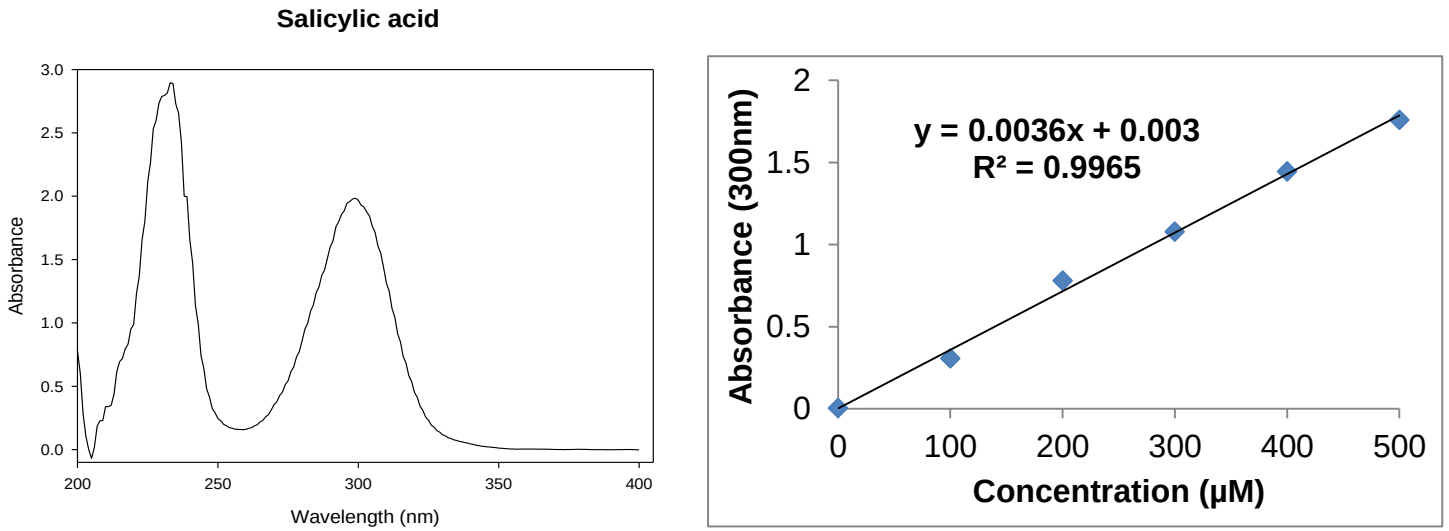

Figure B2. Spectral scan of 0.5 mM salicylic acid between 200nm and 400nm (A) and the standard curve of salicylic acid at 300nm (B) in methanol.

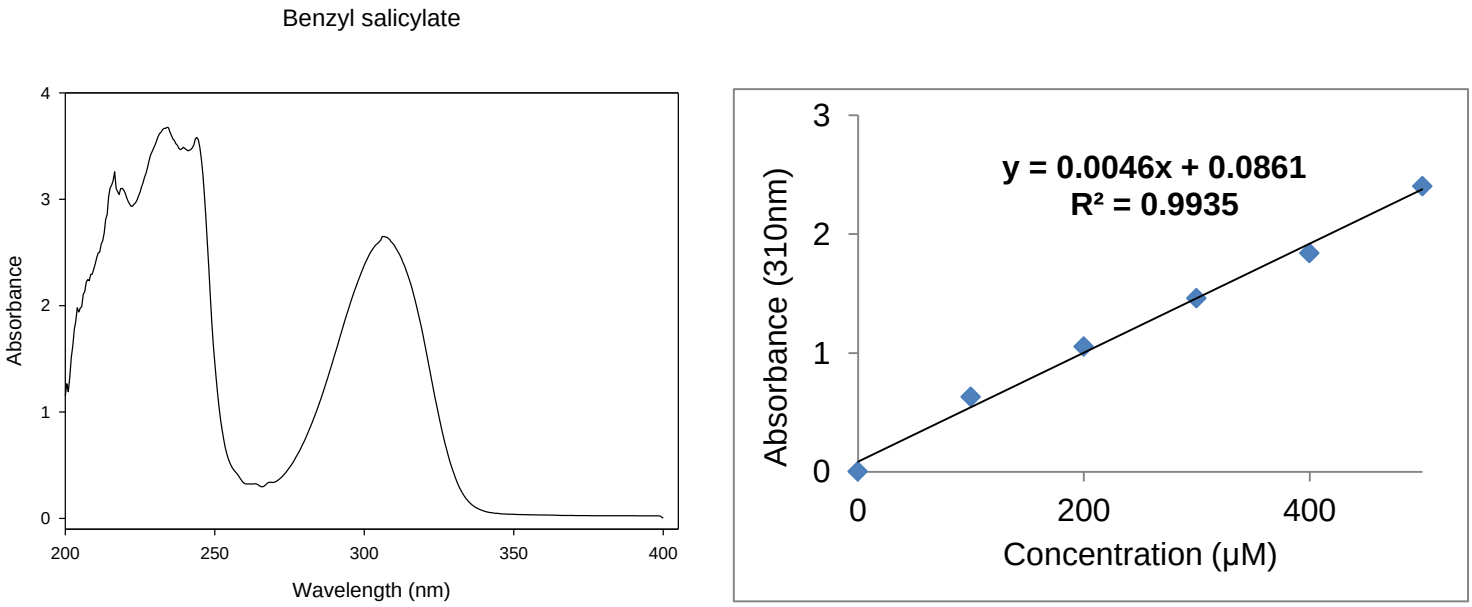

Figure B3. Spectral scan of 0.5 mM benzyl salicylate between 200nm and 400nm (A) and the standard curve of salicylic acid at 310nm (B) in methanol.

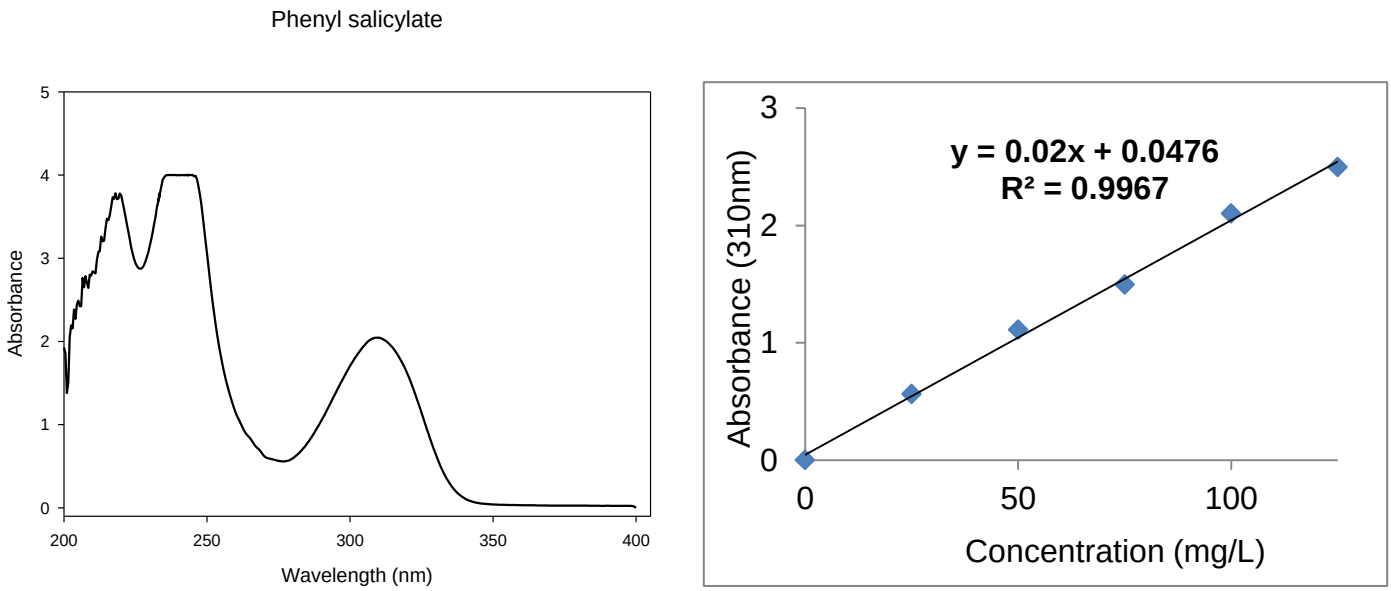

Figure B4. Spectral scan of 0.5 mM salicylic acid between 200nm and 400nm (A) and the standard curve of phenyl salicylate at 310nm (B) in methanol.

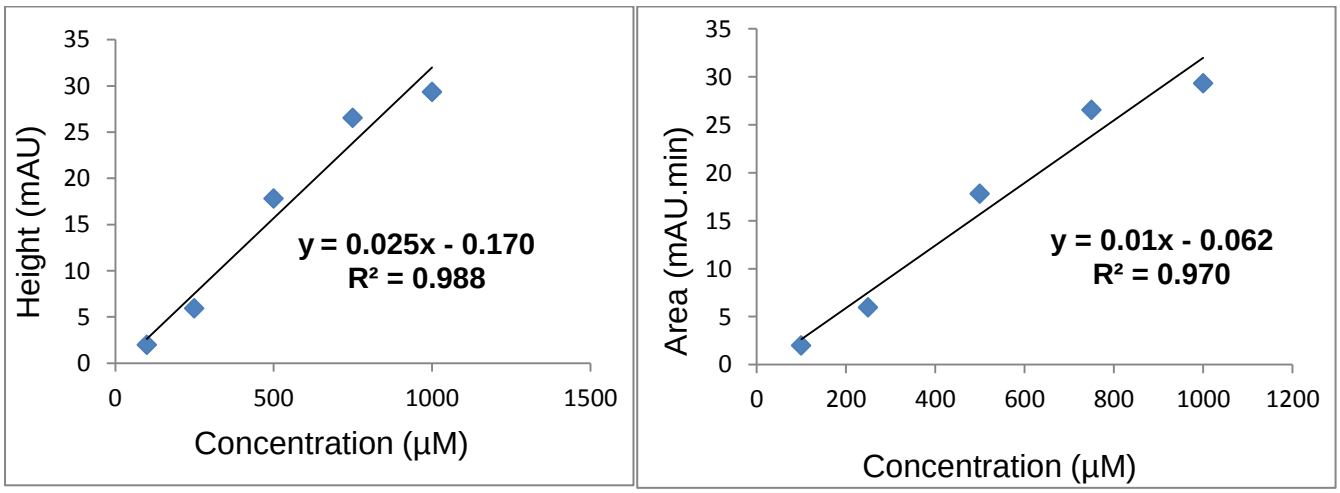

Figure B5. Standard curve of salicylic acid in methanol (60% *v/v*) determined by HPLC at 270 nm.

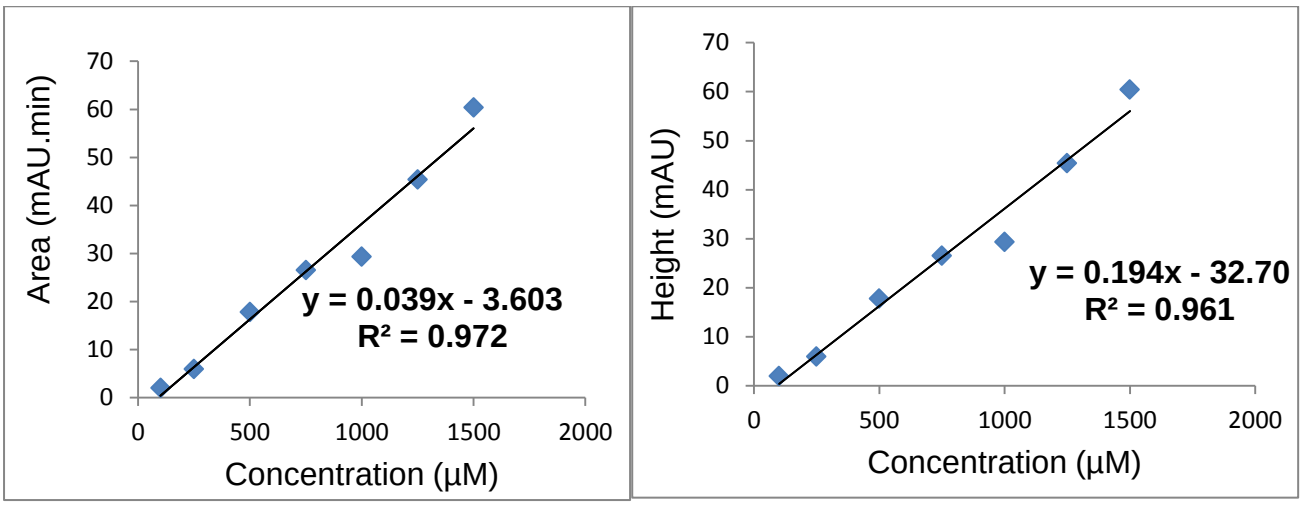

Figure B6. Standard curve of phenyl salicylate in methanol (85% *v/v* mobile phase) determined by HPLC at 270 nm.

Appendix C

Standard curves of salicylic acid and phenyl salicylate authentic metabolites

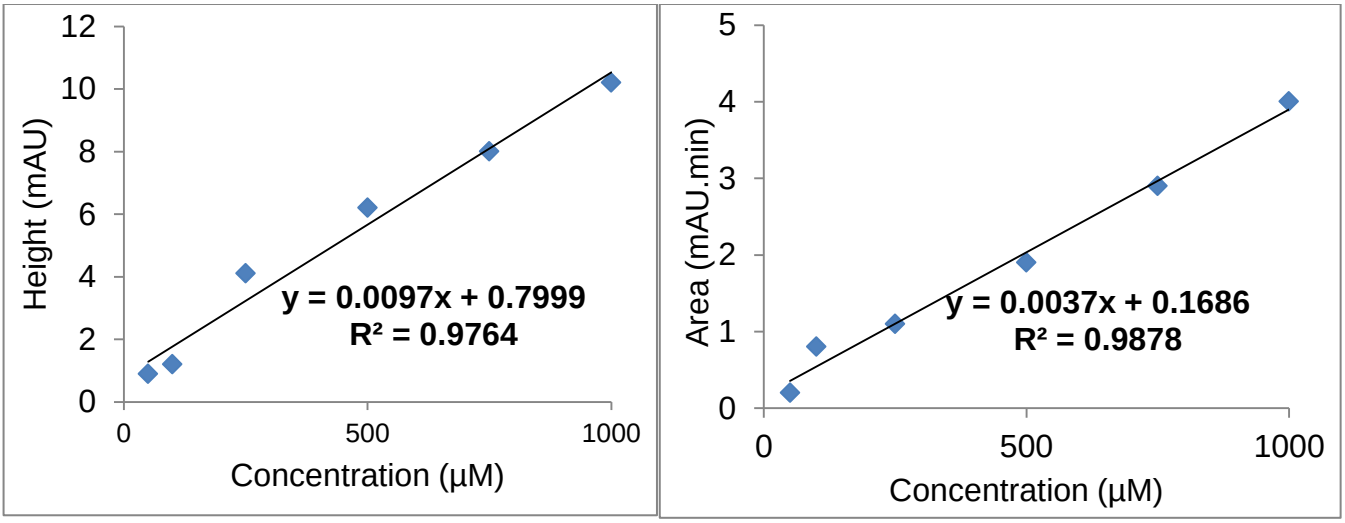

Figure C1. Standard curve of gentisic acid in methanol (60% *v/v* mobile phase) detected at 270nm

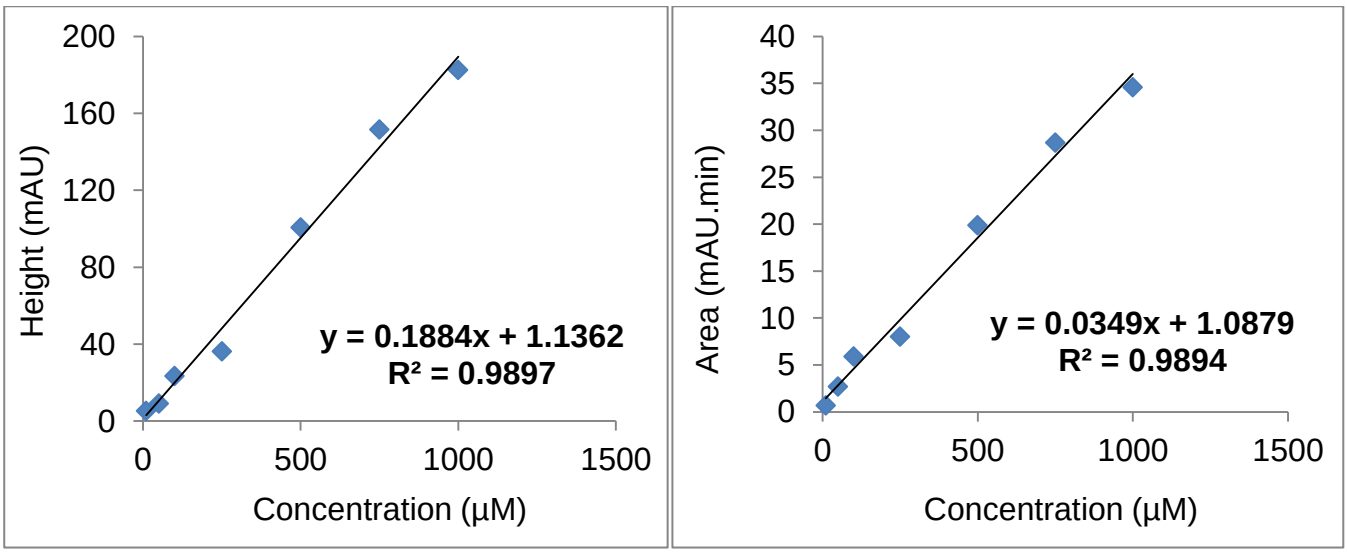

Figure C2. Standard curve of catechol in methanol (60% *v/v* mobile phase) detected at 270nm.

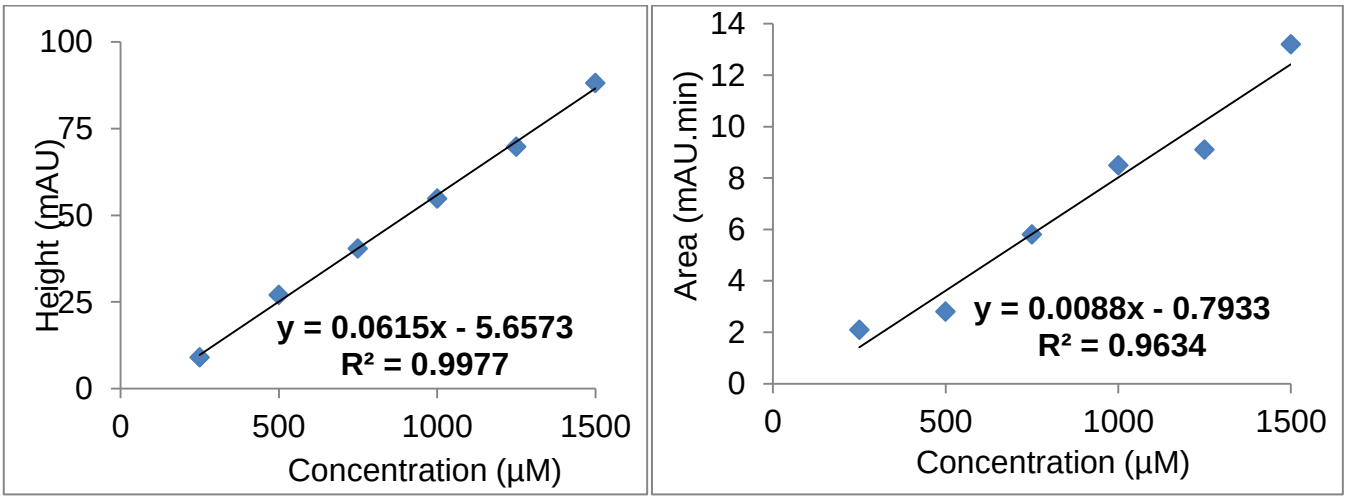

Figure C3. Standard curve of salicylic acid in methanol (85% *v/v* mobile phase) detected at 270nm.

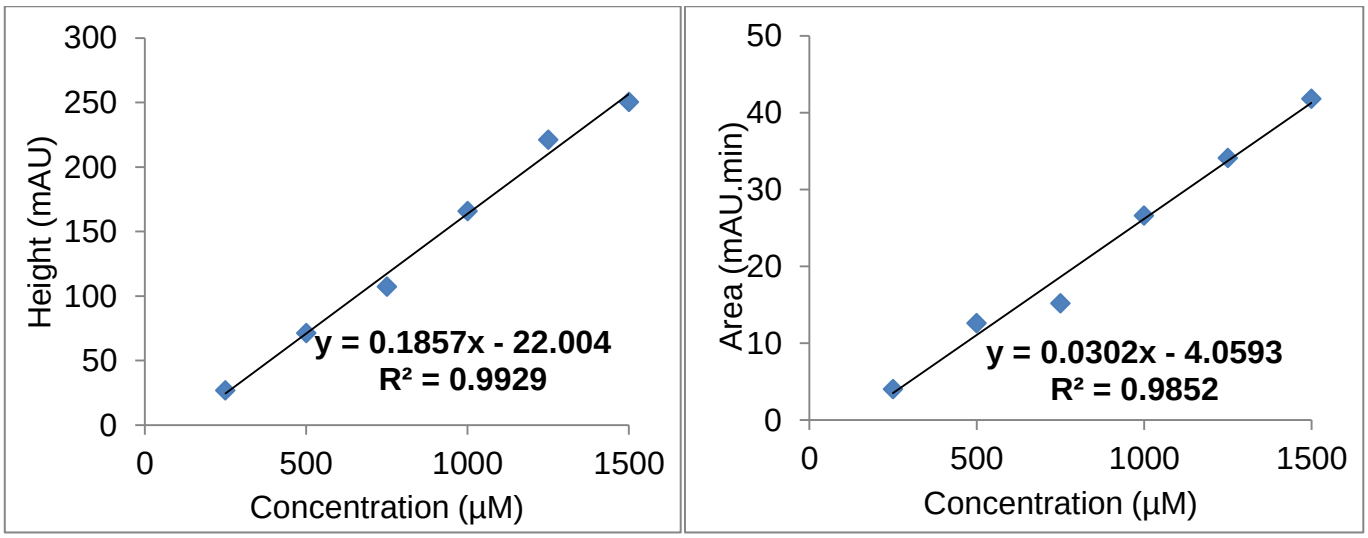

Figure C4. Standard curve of phenol in methanol (85%*v/v* mobile phase) methanol detected at 270nm.