# Bioremediation of Phenol in Water using Polymer-supported Bacteria

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# Abstract

The immobilisation of bacteria onto substrates is a common method for using bacteria as biocatalysts via using range of polymers and different strategies that have been used for biotechnological, medical and environmental applications. The research presented here uses this technique to produce a novel macroporous cryogel with sponge-like properties (‘*bacterial sponge*‘) utilising commercial strains (*Pseudomonas mendocina* and *Rhodococcus koreensis*) and indigenous bacteria (*Acinetobacter radioresistens* isolated from oil contaminated soil) to remedy toxic phenol in aquatic environment. Four different types of crosslinking systems at different concentration were used, i. classic systems: GA 0.5% and PVA 1% + GA 0.5%, ii. modified systems: PVA-al 1% + PEI-al 0.25% and PVA-al 0.5% + PEI-al 0.6%. The CCC samples were able to degrade 50 mg/L of phenol in batch cultures. The CCC samples exhibited good mechanical stability and cell viability that enabled repeated use for phenol degradation for up to five weeks under various conditions. The use of a novel crosslinking system results in a highly permeable and stable bacterial 3D porous structure with pore sizes in the range 10-100 µm, exhibiting low diffusion constraints and a high bacterial content (more than 98% to total polymer content), which led to create monolithic units of macroporous bacterial sponge termed as crosslinked cells-cryogels (CCC). Then, CCC produced from modified polymers were developed for use as a bioreactor for water treatment under shaking conditions via placing them into Kaldnes carriers (CCC-KC). An assessment of phenol-degrading efficiency by CCC-KC using environmental and tap water samples spiked with 50 mg/L of phenol was also performed and the results were promising. The novelty of the approach is that a high ratio of bacterial cells to polymer was used to produce 3D bio-cryogel structure. The results of this research suggest that this procedure is a promising tool for developing novel, environmentally-friendly bioremediation materials based on active bacterial cells.

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# Abbreviations

ANOVA - analysis of variance
ARDRA - amplified ribosomal DNA restriction analysis
ATSDR - Agency of Toxic Substances and Disease Registry
BDD - boron doped diamond anode
CCCs - crosslinked cells cryogel samples
CCCs-KC – crosslinked cells cryogel samples- Kaldnes carrier
CLSM - confocal laser scan microscopy
$CO_2$ – carbon dioxide
COD - chemical oxygen demand
COP - catalytic ozonation process
DMSO - dimethyl sulfoxide2,
DNPH – di nitro phenyl hydrazine
G' - storage (elastic) modulus
G'' - loss (viscous) modulus
GA – glutaraldehyde
GC-MS - gas chromatography–mass spectrometry
$H_2O$- dihydrogen oxide
HPLC – high performance liquid chromatography
IC – ion chromatography
MBBR - moving bed bioreactor
MFCs - microbial fuels cells
MMSM - minimal mineral salts medium
MSM - mineral salt medium
MTT - (3-(4, 5-dimethylethioazol-2-yl)-2, 5-diphenyle tetrazolium bromide
NCIMB - National Collection of Industrial food and Marine Bacteria
NDIR - non-dispersive infrared detector)
NPL - National Priority List
PAHs - polycyclic aromatic hydrocarbons
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PEI-al - polyethyleneimine - aldehyde

PEO – polyethylene oxide

Poly (AAm-AGE) - poly (crylamide-allyl glycidyl ether)

POPs - persistent organic pollutants

PUF – polyurethane foam

PVA - polyvinyl alcohol

PVA-al - polyvinyl alcohol - aldehyde

RFLP - restriction fragment length polymorphism)

ROS - reactive oxygen species

Ru-MMO - ruthenium mixed metal oxide

SEM - scanning electron microscopy

TAE - Tris-Acetate-EDTA

TBE buffer - Tris89mM-Boric acid 89mM–EDTA 2mM

TE buffer – Tris-EDTA buffer

TOC - total organic carbon

TOC - total organic carbon

TSA - tryptone soya agar

TSB - tryptone soya broth

US, UV- ultrasound, ultraviolet

USEPA - United States Environmental Protection Agency

WHO - World Health Organization

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# Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

**Signed**

**Dated**

# Chapter 1 : General Introduction and research structure

## 1.1 Introduction

A major problem facing countries around the world is environmental contamination caused by the release of toxic organic compounds such as phenols. These compounds can contaminate different sectors of the environment such as atmosphere, water and soil. Some toxic organic compounds are easily degraded after entering the environment, whilst others may persist in the environment and not decompose into simpler units (Potters, 2013). Most toxic organic pollutants enter the environment through the disposal of organic compounds, mainly from human activity via the scenario of industrial development (Simoneit, 2004; Hossain *et al.,* 2012), although the decomposition of plant matter also makes a contribution (Perez-Jimenez *et al.,* 2010; Ferreira *et al.,* 2015).

The removal of toxic contaminants from the environment has been broadly investigated. Potters (2013) classified contamination events depending on the physiochemical components of the materials. Pollutants were divided into inorganic contaminants such as NO, $NO_2$, $SO_2$ and CO, gases, or metallic ions, and organic contaminants such as wastewater effluents from industry or oil refineries. The impact of contamination by organic compounds has caused concern because they are broadly distributed into the environment, especially in surface and ground waters, and soils. Moreover, the adverse effects on human and environmental health and the ability for their long term persistence in the environment as Persistent Organic Pollutants (POPs) makes the efficient removal of these particular compounds an important priority (Ritter *et al.,* 1995; Pal, 2010; Jurado *et al.,* 2012; Gami, 2014).

Aromatic hydrocarbons are large groups of organic compounds which can persist in the environment. Structurally they comprise of one or more benzene rings bonded with other chemicals to form various structures. They are classified

into three large main categories: substituted aromatics compounds, polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic compounds (Seo *et al.,* 2009; McQuarrie *et al.,* 2011; Kim *et al.,* 2013).

Due to the wide distribution of aromatic compounds such as phenols in the environment as a result of discharges from sources such as natural degradation of plant materials, and industrial discharges from the petrochemical, oil, coal and textile industries. These compounds are regarded as haemo-toxic and hepato-toxic, may also initiate mutagenesis and carcinogenesis in humans and other living organisms (Straif *et al.,* 2005; Michałowicz and Duda, 2007; Lu *et al.*, 2008; Duan *et al.,* 2017). As a consequence, such compounds are included in the priority list of hazardous substances identified by ATSDR (Agency of Toxic Substances and Disease Registry) (ATSDR, 2007) as part of National priority list (NPL) of toxic substances (Smith *et al.,* 2009; Gami, 2014).

For this reason, removal of phenolic compounds from the environment is a crucial strategy to maintain human and environmental health, and different strategies have been explored to reduce and eliminate the release of phenolic compounds. Various physical, chemical and biological methods have been employed to reduce toxic levels of these compounds, with a particular emphasis on phenol compounds, in wastewater effluents prior to their discharge into the environment (Kulkarni and Kaware, 2013). Despite the success of some treatments methods, some of the disadvantages of these methods were high costs, and uncompleted chemical reactions leading to the production of by-products potentially more toxic than the original pollutants (Olaniran and Igbinosa, 2011; Dubey and Hussain, 2013). Consequently, low cost and effective alternative solutions are necessary.

Bioremediation strategies were advanced in the last three decades, using microbial species as degrading agents as a more efficient means to remove these pollutants from the environment (O'Sullivan, 1998; Nair *et al*., 2008; Adams *et al*., 2015), in this research, phenol was used as a model compound

for removal by microorganisms. Table 1.1 shows some microorganisms used by other authors for phenol degradation.

Table 1.1: Phenol- degrading microorganisms

| Microorganisms | References |
|---|---|
| **Bacteria**<br>*Acientobacter sp.strain AQ5NOL 1* | Ahmad *et al.,* (2017) |
| *Alcaligenes sp. ATHE8* | Hoodaji *et al.,* (2013) |
| *Bacillus amyloliquefaciens strain WJDB-1* | Lu *et al.,* (2011) |
| *Pseudomonas aeruginosa MTCC4997* | Kotresha and Vidyasagar (2014) |
| *Pseudomonas ap.AS01* | Shourian *et al.,* (2008) |
| *Pseudomonas fluorescens (strain Yas2),*<br>*Klebsiella penumoniae (strain Yas1)* | Ruiz *et al.,* (2013) |
| *Pseudomonas sp.* | Saravanan *et al.,* (2008) |
| *Pseudomonas sp. NBM11* | Mohanty and Jena (2017) |
| *Pseudomonas sp.JS150* | Morzik *et al.,* (2011) |
| *Rhodococcus UKMP-5M* | Suhaila *et al.,* (2013) |
| *Streptococcus epidermis coded as (OCS-B)* | Mohite *et al.,* (2010) |
| **Yeast**<br>*Candida tropicalis strain K1& K11 ,*<br>*Pichia guilliermondii strain K2,*<br>*Meyerozyma guilliermondii strain 7* | Karimi & Hassanshahian (2016) |
| **Fungi**<br>*Fusarium flocciferum* | Mendonca *et al.,* (2004) |

However, classical bioremediation techniques using microorganisms to degrade the pollutants were often ineffective, because of the lethal effect of the compounds and the inhibition of metabolic activity of the microbial cells (Boopathy, 2000; Krastanov, *et al.,* 2013). Immobilisation of microbial cells capable of degrading phenols a possible way to enhance and increase the efficiency of the bioremediation process through the physical isolation of the microbial cells from the external medium and loading them in a large quantity. Furthermore, immobilising the cells make them easy to control and also minimises losses during washing out (Sheehan, 1997; Wang, 2002; Li *et al.,* 2017; Stepanov and Efremenko, 2018). Such immobilisation systems have been widely investigated for medical, biological and biotechnological applications (Prieto *et al.,* 2002; Zajkoska *et al.,* 2013; Kotresha and Vidyasagar, 2014).

The immobilisation of bacterial cells within cryogel materials is a novel approach for biotechnological applications (Philp *et al.,* 2003; Lozinsky, 2004). Cryogels have desirable characteristics compared to other macro porous hydrogels, such as high mechanical strength during twisting, elongating and squeezing which minimises damage to the structure, and the formation of a macro porous structure as a result of the freeze-thaw production strategy. The technique also benefits from its simplicity of synthesis. The monomers and polymer precursor are water-soluble and combine in the unfrozen reaction to form a thin layer around the template of ice crystals. The templates are easy to remove from the reacted cryogel materials by defrosting them at a temperature greater than the temperature of synthesis (Okay and Lozinsky, 2014).

Crosslinking polymers are usually used to create bridges that link two or more molecules. Also, they may have specific functional groups which could react with proteins or other molecules to promote linking between reacted materials. The characteristics of the polymer, their concentration and their behaviour before or during the reaction and beyond the application process have to be kept in mind before selecting the type of polymer (Gutiérrez *et al.*, 2007). In this research, different crosslinking systems have been used such as glutaraldehyde (GA) and polymers such as polyvinyl alcohol (PVA) and polyethyleneimine (PEI).

The unique features of these cryogels is that they function as a carrier and scaffold to protect and enhance microbial activity; this makes them a promising tool to remove specific contaminants from industrial wastewater and mitigate environmental pollution (Plieva *et al.,* 2008b; Önnby, 2016). However, these cryogels have some drawbacks related to the properties and structure of cryogels; for instance, longevity and ways of disposable could lead to more issues. Moreover, the pores sizes ranging from 10-100µm within the cryogel structure could have a negative effect on the immobilised cells by washing them out during long utilization periods (Plieva *et al.,* 2008b).

Therefore, in this thesis a novel structure to immobilise bacterial cells as crosslinked cells-cryogels is presented and applied to degrade phenol as a tool for bioremediation. Furthermore, a phenol concentration of 50 mg/L was selected as the maximum real-world concentration detected in the Shatt Al-Arab river in Basrah city, Iraq or from industrial effluents in that city.

## 1.2 Research structure

The research in this study was divided into four stages: The first stage was concentrated on choosing and selecting bacterial strains that are known as degraders of phenol and its derivatives. Two strains, *Pseudomonas mendocina* and *Rhodococcus koreensis*, were chosen depending based on the literature review. The bacterial cells were prepared for the immobilisation in using four different polymer combinations; pelleted cells were mixed with small volumes and differing concentrations of various polymers to synthesise cryogels with novel structures formed from the crosslinked bacterial cells.

The ability of crosslinked cells cryogel samples (CCCs) to degrade phenol at a concentration of 50 mg/L was examined under optimum conditions of 30$^{o}$C and pH 7. Furthermore, the characteristic structure, organisation and topography of crosslinked cells cryogels using various crosslinking systems were investigated by scanning electron microscopy (SEM). The mechanical strength of the crosslinked cell cryogel samples using standard rheology tests and assessment

of viability of cells by confocal laser scan microscopy (CLSM) and MTT assay were also applied.

The second stage of this research was focused on the isolation of indigenous autochthonous bacterial strains from oil contaminated soil from Iraq that show the ability to tolerate and degrade elevated concentrations of phenol. Identification of one isolated strain showing good phenol-degrading potential was applied via standard biochemical tests. Molecular methods such as the polymerase chain reaction (PCR), 16S rDNA typing and amplified restriction DNA analysis (ARDRA) were also applied to confirm the genus and species identity. The third stage of this study was dedicated to the synthesis of the isolated phenol-degrading strain as crosslinked cell cryogels and to examine them under optimum and variable laboratory conditions. Additionally, an assessment was made of the efficiency of the cell-cryogels to degrade phenol using fresh and freeze-dried samples.

Finally, the phenol-degrading efficacy of the crosslinked cell cryogels comprising the isolated strain supported in plastic Kaldnes carriers (CCCs-KC) was determined. Water samples collected from various sources in the environment were spiked with phenol and exposed to the CCS-KC for possible use in a moving bed bioreactor (MBBR) to degrade phenol in water.

# Chapter 2 : Literature review

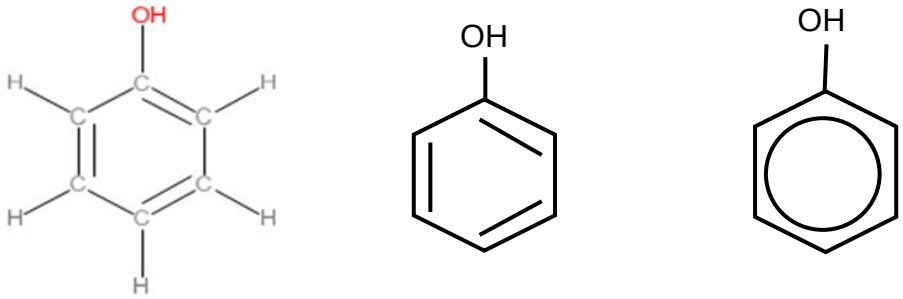
## 2.1 Introduction to phenol

Phenol (originally termed ‘carbolic acid’) is a simple form of aromatic compound (a chemical compound that contains containing conjugated planar ring systems) discovered in 1834 by Runge, who extracted it (in impure form) from coal tar. Its structure was not recognized until 1841 (Basha *et al.,* 2010). Initially, phenol was used as a constituent of creosote paint to prevent the weathering of railway connections and ship timbers (Selvi *et al.,* 2015). It was also used to reduce sewage odours and in carbolic disinfectant soap due to his distinct odours that make it sickeningly sweet and tarry (Berry *et al.,* 1956). Since 1867 it was exploited as an antimicrobial agent and used in hospitals as a sterilising agent and disinfectant and in wound dressings (Goddard and McCue, 2001).

Phenol can be tested at a level that is not associated with harmful levels (ATSDR, 2011). Nowadays, phenolic compounds are common in products used daily. For instance, many cosmetic products and salon preparations contain one or more phenol derivatives, furthermore, certain medicinal and pharmaceutical drugs such as ointments contain phenol and its derivatives, breath freshener and sore throat lozenges and for the treatment of Alzheimer’s disease (Huang *et al.,* 2010; ATSDR, 2011; Kovacic and Weston, 2017).

### 2.1.1 Physical and chemical characteristic

Phenol is a benzene ring ($C_6H_6$) bonded to a hydroxyl group (−OH), as shown in figure 2.1, and it is mildly acidic and requires careful handling due to its propensity to cause chemical burns (McQuarrie *et al.,* 2011).




**Phenol ($C_6H_5OH$), carbolic acid, hydroxyl benzene**

Figure 2.1: Chemical structure of phenol

Phenol has a molecular weight of 94.11, a boiling point of 182$^{o}$C, and a density of 1.072. Manufactured phenol can be either in a solid or liquid state and colourless crystals of phenol in a pure condition turn a red or pink colour when in direct contact with air. Phenol can be recognised by its sweet characteristic odour (Weber *et al.,* 2000; ATSDR, 2008) . It is appreciably soluble in water, with about 84.2 g dissolving in 1000 mL (0.88 M); it is also very hydroscopic. It is also more soluble in organic solvents such as petroleum compounds, glycerol and alcohol than in water (Luttrell, 2003; Michałowicz and Duda, 2007; Nair *et al.,* 2008; Basha *et al.*, 2010; Dubey and Hussain , 2013).

## 2.1.2 Sources of phenol contamination

Phenol is found naturally in food as free or bounded compound in red grapes, cocoa and tomato (Barlow *et al.*, 2007) and is released into the environment from two main sources, natural and anthropogenic. Natural sources are mostly decomposing plant material, especially herbs, vegetables and dietary plants (Ferreira *et al.*, 2015; Kulkarni, 2017). At the end of their life cycle these phenolic compounds are released to the environment because of the degradation process, and other natural sources of phenol is the breakdown of organic materials, animal waste, the decay of dead organisms, and the combustion of wood and coal. Moreover, phenol is considered one of the main metabolites of benzene which is a pollutant of concern in the environment (Smith *et al.*, 2009;

Basha *et al.*, 2010). The second major source of phenol contamination in the environment is industrial sources related to human activity. Research has highlighted that phenolic compounds are discharged into the environment through the release of insecticides, herbicides, effluents from oil refineries, and from the petrochemical, textile, pharmaceutical and mill pulp industries (Kuyukina *et al.*, 2009; Kulkarni and Kaware, 2013; Kim *et al.*, 2013; Selvi *et al.,* 2015).

### 2.1.3 Uses and production

Phenolic compounds such as phenolic acids and flavonoids are normally taken by human being as a daily food (fruits and vegetables) due to their availability as natural compounds in these plants (Ferreira *et al.*, 2015). They are mainly considered as a natural source of antioxidant and an essential key for plant growth and reproduction. In addition, they are produced in plants as a natural response to injury or to defend against various infections (Karakaya, 2004). Recent research has focussed on plants that contain elevated concentrations of phenolic compounds as natural antioxidant materials (Dimitrios, 2006) , to treat different diseases and cancer (Huang *et al.*, 2009;Perez-Jimenez *et al.*, 2010). Therefore, they are widely used in medical manufacturing and dietary products.

Although pure phenol is used less nowadays in the production of detergents and sterile products, many of its derivatives have industrial and commercial uses Dubey and Hussain (2013) reported that various derivatives of phenol such as bisphenol, carprolactam, phenyl amine, alkyl phenols, chlorophenols and salicylic acid, are used in the manufacture of products such as pesticides, dyes, nylon and plastics, chemical agricultures, and medical and pharmaceuticals products.

Phenol can be synthesised through different strategies. The most common one is the cumene process. This approach uses benzene as a precursor which is reacted with propane to produce 1 methyl ethyl benzene (cumene) followed by an oxidation process to produce cumene hydroperoxide. Phenol is then

produced following the decomposition of cumene hydroperoxide (kujawski *et al.,* 2004). Approximately 7 million tonnes of phenol are produced annually worldwide (Weber *et al.*, 2004) and this amount was increased to reach approximately 10 million tonnes in 2015, mainly for production of bisphenol A (Plotkin, 2016), as shown in figure 2.2.

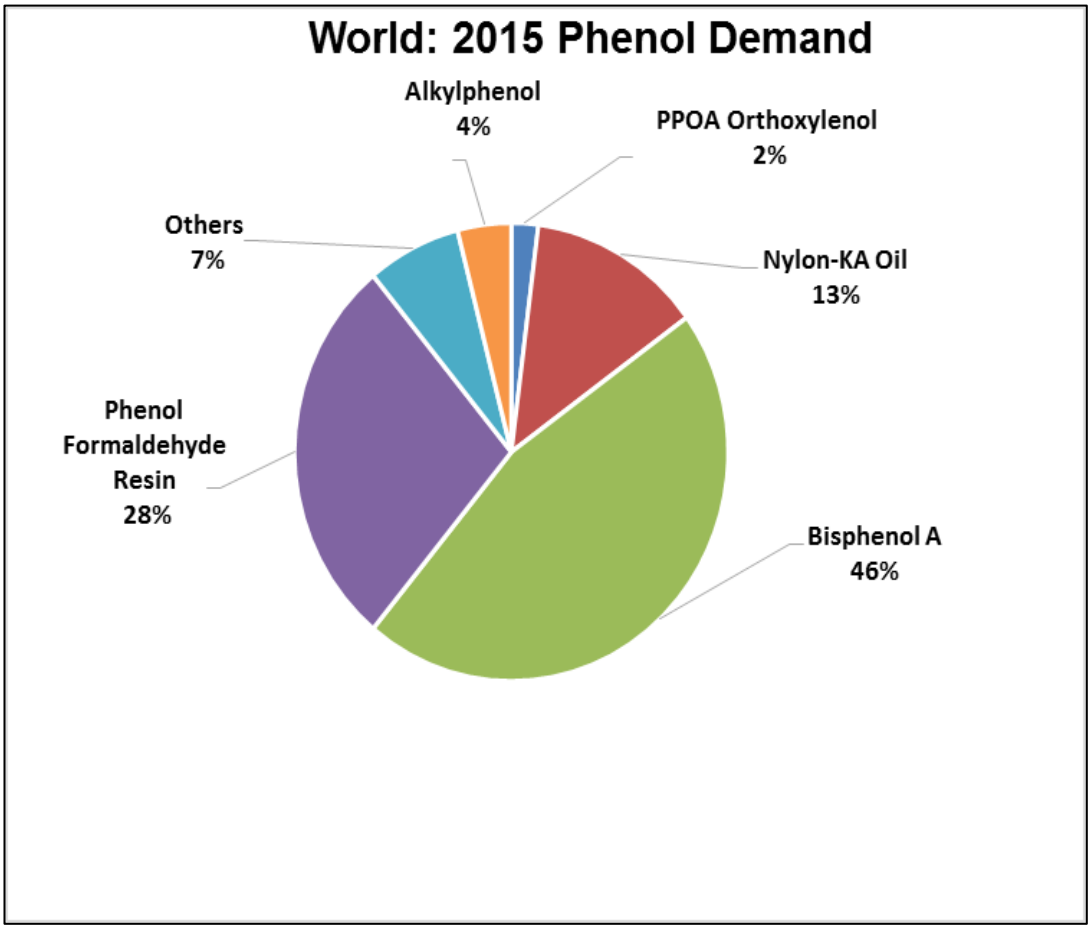



Figure 2.2: The percentage production of phenol in 2015 (Plotkin, 2016)

### 2.1.4 Toxicity

Phenol and phenol-based compounds are considered toxic according to risk evaluations; therefore, they are included in the Priority List of Hazardous Materials of the United States Environmental Protection Agency (USEPA) (Shah, 2014). Due to its distribution in the environment, especially in water and wastewaters, there is a high risk of negative impacts on the health of humans and other organisms (*Lu et al.,* 2008; Basha *et al.,* 2010). Phenol enters the human body via two pathways, either via the skin through direct contact and by the lungs through inhalation. It enters the blood circulation via skin adsorption or breathing air containing phenol. It is metabolised through conjugation with glucuronic acid and sulphate in the liver, lungs, digestive tract and kidneys. The majority is excreted via the urine as free phenols or one of its metabolites after approximately 24 hours (Luttrell, 2003). The ATSDR (2008) states that phenol can also enter living organisms via ingestion.

Michałowicz and Duda (2007) highlighted that phenol and catechol (a benzenediol breakdown product of phenol) are lethal and their toxicity can increase when reacted or substituted with other chemical groups, such as chlorine or nitrate, methyl or alkyl molecules. Such compounds are considered to be haemato-toxic, hepato-toxic, mutagenic and carcinogenic owing to the possession of high per oxidative efficiency towards living organisms. For that reason, research has been carried out to clarify the mechanisms and long-term effects of phenol toxicity. Likewise, Gad and Saad (2008) investigated the toxicity and the long-term effects of phenol on Oreochromis niloticus (Nile tilapia) after exposing them to phenol for 16 weeks.

The authors also studied the physiological behaviour of the fish by exposing them to three potentially lethal concentrations of phenol (0.7, 1.4, 2.8 mg/L), which resulted in a decrease of hormone production and growth. The percentage accumulation of phenol in the tissues, serum total cholesterol and lipid was increased, in addition to an increased probability of genotoxicity, as observed by an increase in the number of micronuclei produced.

Duan *et al.,* (2017) highlighted that phenol toxicity on four marine microalgae depends on several biotic factors such as ability of cells to degrade phenol, size of cells, and accumulation of reactive oxygen species (ROS). However, further data are needed to assess and clarify the mechanism of phenol toxicity because most toxicity experiments have been performed only on animals (Smith *et al.,* 2009).

Mechanisms of phenol toxicity on microbial cells occurs via its effects on the permeability of the cell membrane which leads to a loss of cellular integrity and functional disturbances of the metabolism that may cause cell death (Wasi *et al.,* 2013). Further research about the mechanisms of toxicity was published by Gami (2014) who suggested that two mechanisms are associated with this effect: the formation of organic and free radical species, and hydrophobicity. In addition, the structure of phenol itself is highly reactive, which can lead to its persistence in the environment in its native or altered form.

### 2.1.5 Legislation and limit concentrations

Research into the impact of phenolic compounds even at low concentrations on the environment and human health was initiated by the World Health Organization (WHO) in their second meeting in China in 1994 (WHO,1995). The priority of chemical pollutant characteristics classified according to WHO are: toxicity, resistance to environmental degradation, mobility into the environment, and bioaccumulation (Panagos *et al.,* 2013). Rocha-Guzman *et al.,* (2007) stated that ingestion of 1 g of phenol per kg of body weight represented a hazardous amount for human health and other organisms. Kulkarni and Kaware (2013) found that toxic concentrations for human consumption is approximately 10-24 mg/L, and 9-24 mg/L for fish. The lethal blood concentration for human is approximately 150 mg/100mL.

Consequently, a concentration limit for phenol in the aquatic environments was determined to be no greater than 1 mg/L. For example, the concentration value for surface water is 0.5 mg/L and 1 mg/L for sewage, and no greater than 1 µg/L

for drinking water (Vasudevan, 2014). Whilst, the European Council Directive set a limit concentration of 0.5 µg/L to control phenol concentration in drinking water (Gami, 2014), the concentration limits for phenol vary depending on each county's legislation. Table 2.1 shows the concentrations of phenol in discharges from various industrial sources.

Table 2.1: EU limit and maximum concentration limits for phenol discharges from industrial sources (Kulkarni and Kaware, 2013)

| **Industrial sources** | **Phenol concentration (g/l)** |
|---|---|
| European Water Framework Directive (2000/60/EC); European Council Directive 2006/61/EC; | 0.0005 (0.5 µg/L) |
| Oil refinery | 2-20 |
| Coal mining | 1-2 |
| Lignite transformation | 10-15 |
| Gas production | 4 |
| Petrochemicals | 0.05 -0.07 |
| Pharmaceuticals | 1 |
| Discharge limits | 0.001(for surface water)<br>0.005 (for sewerage and oceans) |

## 2.2 Remediation strategies

Various techniques have been used to remove phenol from the environment, and these will be discussed below:

### 2.2.1 Physical and Chemical processes

Phenols are widely distributed in the environment and because of their toxicity, sustainable methods to remove them from industrial effluents before discharge to the environment are a necessary requirement for the safety of the environment and organisms within it.

Adsorption is one of the physical processes that are widely used to remove contaminants from wastewater. For example, Bohdziewicz, *et al.* (2012) tested the ability of adsorption to exclude phenols from urban wastewater influent and effluent by using activated carbon AKPA-22 as the sorbent. At initial phenol concentrations of 0.54 mg/L, and 0.27 mg/L for influent and effluents, respectively, at pH 7.5, there was a 49-94% reduction in phenol in the effluents. On the other hand, Dakhil (2013) showed that sawdust was a more suitable absorbent to remove phenol at an initial concentration of 130 mg/L at pH 6.7 with 120 minutes contact time. The reduction in phenol concentration increased in tandem with the quantity of adsorption material. Similarly, El-Naas *et al.* (2010) confirmed this finding when they used activated carbon derived from date-pits to reduce the concentration of phenol in refinery wastewater.

Electrocoagulation is another method used to treat pollution caused by poly aromatic hydrocarbons (Nassef, 2014). A new combined technique called the ‘’peroxi-electrocoagulation method’’ was examined, utilising a mild steel anode and a graphite cathode to remove phenol from wastewaters at an initial concentration range of 0.5-5 mg/L. Other parameters were studied such as pH, temperature, density of ions in the solution and initial phenol concentration, and the highest removal efficiency was 92% at pH 2.0 and a current density of 0.10 A/dm2 which removed 2.5 mg/L of phenol (Vasudevan, 2014). However,

Abdelwahab *et al.* (2009) announced that sweep-electro-chemical-coagulation and adsorption method was more efficient, with a reduction in phenol concentration from 13 mg/L to 1 mg/L in 2 hours at an optimum pH of 7.0.

Wu *et al.* (2001) studied of using the photosonochemical combination strategy (a combination of ultrasound and photochemistry) to remove phenol from water. They showed a 99% decrease in phenol concentration rates with the combined effect of ultrasound (US) and ultraviolet (UV) together, as compared to individually. They also demonstrated that reducing the pH and raising the amount of dissolved oxygen could enhance phenol degradation. Furthermore, the presence of ferrous iron ($Fe^{2+}$) in the phenol solution improved the elimination of total organic carbon (TOC). However, uncompleted reactions produced intermediate compounds such as hydroquinone, catechol, benzoquinone, and resorcin, which revealed that formation of an OH radical played an important role in phenol degradation efficiency (Vasudevan, 2014). Another photosonochemical study by Mahvi and Maleki (2010) found that the degradation of phenol obeyed first-order kinetics i.e. the first-order decay constant increased with decreased solute concentration.

Shahamat *et al.* (2014) investigated a combination of an ozonation method with biodegradability of the effluents by synthesizing a magnetic carbon nano-Fe3O4 composite as a catalyst, then exploited it in “the catalytic ozonation process” (COP). The results showed a removal rate for phenol of 98.5% and 69.8% for chemical oxygen demand (COD) after 5 minutes reaction time. The concentration of phenol in the effluents from this process was acceptable for post-treatment in a sequencing batch reactor (SBR) to reduce and remove the remaining pollutants completely. It was emphasized that the optimum conditions were 2 g/L of the catalyst at pH 7.0 and that the combined system was more efficient in comparison to the individual systems.

Other process have also been investigated; for instance Yavuz *et al*. (2010) studied the electrochemical oxidation process in four different systems: (i) direct and indirect electrochemical oxidation using a boron doped diamond

anode (BDD), (ii) direct electrochemical oxidation using a ruthenium mixed metal oxide (Ru-MMO) electrode, (iii) the electro-Fenton ($Fe^{2+}/H_2O_2$) process comprising a coagulation–flocculation processes with a simultaneous advanced oxidation stage and (iv) electrocoagulation using iron electrode. The results showed that the highest efficiency removal of phenol was the electro-Fenton process followed by direct and indirect electrochemical oxidation by using (BDD) anodes, whilst the electrocoagulation system was inefficient to treat wastewater effluents.

Several researchers have examined the development of classic methods of remediation and the combination of two or more techniques to enhance the reduction of phenol contamination. Nevertheless, the high costs involved, the problem of inefficient removal of pollutants and the production of intermediate compounds as a result of incomplete reactions may led to the production of secondary pollutants. For example, the potential formation of organohalides materials, notably chloroform, can occur during the chlorination process (Bellar *et al.*, 1974; Rengaraj *et al.*, 2002). Therefore, rather than using physical and chemical processes, biological methods such as microbial and enzymatic systems could be a promising approach to manage various contaminants spreading in the environment, especially in wastewater (Pradeep *et al.,* 2015).

### 2.2.2 Biological processes

Employing microorganisms such bacteria, fungi and algae to detoxify contaminated soil, water and sediments, is called as bioremediation. Economical and environmentally friendly, bioremediation processes are considered a robust strategies for bioremediation because it results in ‘’biologically catalysed reduction in chemicals’’(Fritsche and Hofrichter, 2000) to produce harmless products or by transforming into sub-products, that are less toxic, soluble and transportable in biodegradation process (Alexander, 1999; Atlas and Philp, 2005; Fritsche and Hofrichter, 2000; Luka *et al.* , 2018).

Biological methods have attracted the attention of many researchers (Kobayashi and Rittmann, 1982; Kavita and Geeta, 2014), and there are several advantages

in using biological waste treatment methods. These include lower set-up and running costs; remediation can be completed in one location and *in situ*; the pollutants are degraded into simple, non-toxic constituents and produces fewer adverse environmental impacts; and they can be combined with physical and chemical remediation processes (Kumar *et al.* , 2011; Fulekar, 2012) or using Kaldnes carriers to increase the efficiency of activated sludge process throughout providing additional spaces and hard place for cells to stick and build a biofilm termed as moving bed bioreactor (Rusten *et al.*, 2006; Ayati *et al.*, 2007; Accinelli *et al.*, 2012; Borkar *et al.,* 2013) or fluidised bed biofilm reactor (Mownica and Manimekalai, 2017) However, there are concerns that traditional biological methods may not degrade specific materials such as inorganic pollutants or not be efficient at elevated pollution concentrations (Lee and Speight, 2000).

Certain native bacteria ( Krastanov *et al.*, 2013), adapted bacteria ( Reshma *et al.*, 2014), fungi (Mendonça *et al.*, 2004), yeast (Karimi and Hassanshahian, 2016) and algae (Semple *et al.*, 1999) are considered key components for bioremediation since they possess enzymes and metabolic pathways able to convert complex organic compounds into simple breakdown products such as $CO_2$, $H_2O$ and inorganic substances (Dix, 1981). Since phenols are a major hazard in the environment, researchers have isolated and identified specific microorganisms with tolerance to high concentrations of phenol (Singh, 2006). According to Boopathy (2000) successful bioremediation depends on a combination of three main factors: a suitable microbe, optimum conditions and the right location.

#### 2.2.2.1 Phenol degrading bacteria (individual and consortia)

The isolation and identification of specific bacterial strains able to degrade toxic organic pollutants has been achieved by culturing oil contaminated soil, and petrochemical and industrial wastewater. Such isolates, frequently autochthonous, have adapted to elevated concentrations of pollutants and thus

have the potential to degrade phenols via the bioremediation process (Saravanan *et al.,* 2008; Kotresha and Vidyasagar, 2014; Reshma *et al.,* 2014). Singh *et al.* (2009) investigated of degradation of phenol using pure and mixed cell of *Paenibacillus sp*. AY952466 and *Bacillus cereus* DQ002384, which were isolated from the effluents and sludge of a paper pulp mill. The results indicated that degradation of phenol by *Bacillus cereus* was effectively higher compared with *Paenibacillus sp.* and the mixed cells at a concentration of 500mg/L in an incubation period of 168 hours.

Meanwhile, Shourian *et al.* (2009) reported that a newly isolated bacterial strain identified as *Pseudomonas sp.* SA01 isolated from pharmaceutical wastewaters was able to degrade up to 1 g/L of phenol completely within 30 hours. Concentrations of phenol greater than this inhibited cell growth, and moreover, showed a long lag phase at an initial phenol concentration 0.7 g/L. Banerjee and Ghoshal (2010) isolated two strains of *Bacillus cereus* from oil-contaminated wastewater; their capacity to degrade phenol was studied at a number of initial concentrations ranging from 100-2000 mg/L. Strain AKG1 was able to degrade phenol at initial concentrations of 800 mg/L, and strain AKG2 at 200 mg/L. The authors proposed that a meta-cleavage pathway was the route to degrade phenol via the synthesis of 2-hydroxymuconic semi-aldehyde as an intermediate product.

Another study showed that a bacterium isolated from oil contaminated soil and identified as *Streptococcus epiderms* coded as (OCS-B) tolerated and removed phenol at a concentration of 200 mg/L (Mohite *et al.,* 2010). Meanwhile, Chandra *et al.* (2011) screened the ability of seven aerobic bacterial strains to degrade phenol at different concentrations. Two strains, which they identified as *Paenibacillus thiaminolyticus* (DQ435022) and *Bacillus cereus* (DQ435023) showed an ability to degrade phenol at a concentration of 700 ppm but only in the presence of 1 % glucose. Ahmad *et al*. (2011) investigated *Acinetobacter sp.* strain AQ5NOL 1 which was able to degrade up to 1500 mg/L of phenol under optimum conditions. Mrozik *et al*. (2011) used a genetically modified

bacterium, *Pseudomonas sp*.JS150, that has, tested them to enhance the soil bioaugmentation activity towards phenol degradation in soil and the results were significant.

Other bacterial strains isolated by Ruiz *et al.* (2013) and identified as *Pseudomonas fluoresens* strain Yas 2 and *Klebsiella pneumoniae* strain Yas1 were able to break down 65% polyethoxylated nonylphenols with an incubation time of 54.5 and 134 hours for both strains, respectively. Suhaila *et al.* (2013) stated that *Rhodococcus* UKMP-5M was able to utilise up to 900 mg/L of phenol in medium supplemented with 2 g/L of glucose. Bacterial stains were isolated from oil contaminated soil by Rathore and Sinha, (2014) using enrichment techniques. The bacterial genera included *Staphylococcus, Bacillus, Pseudomonas, Micrococcus*, and *Streptococcus.* All the strains showed an ability to degrade phenol, but certain *Pseudomonas* species were identified as superior phenol degraders. One strain of *Pseudomonas* was able to degrade 60-70 mg/L of phenol after 24 hours and up to 90 mg/L in 48 hours.

Kotresha and Vidyasagar (2008) highlighted that *Pseudomonas aeruginosa* MTCC 4997, which was isolated from petrochemical industrial effluent, showed a strong ability to degrade phenol at a concentration of 1400 mg/L in 6 days under optimum conditions. Therefore, exploitation of natural microbes with high efficiencies for detoxification may be considered a powerful and feasible remediation approach to remediate pollutants in environment (Wasi *et al*., 2013). Li *et al.* (2017) isolated bacterial strains from sewage sludge and a strain with the highest phenol degradation ability was identified as a gram negative bacillus named as FB2. The cells were able to degrade phenol at a concentration of 100 mg/L. Increasing the concentration to 2200 mg/L reduced the growth rate of the cells to 20%.

A number of authors have recommended that a mixture of two or a consortia of bacterial strains with phenol-degrading abilities could be more effective and quicker for phenol remediation than using a single bacterial strain (Saravanan

*et al.,* 2008; Chandra *et al.,* 2011; Rathore and Sinha, 2014; Arunkumar and Anitha, 2014; Renukaprasad *et al.,* 2017; Hassan *et al.,* 2018).

Recently, Yang *et al.* (2018) confirmed that a mixed culture of microbial fuels cells (MFCs) is an Efficient process to remove phenol in the presence of low concentrations of oxygen. In return an electrical current is generated by the micro-oxygen bio-anode. However, Singh *et al.* (2009) stated that mixed bacterial cultures may led to antagonistic effects that could suppress the degradation process.

Generally, the first step for phenol oxidation by microorganisms is di-hydroxylation process via monooxygenase with co-operating of oxygen molecules. Following that reaction another hydroxyl react in ortho-position to convert to catechol that considered as the initial compound for two metabolism pathways: meta-cleavage and ortho-cleavage (m-cleavage, o-cleavage), as shown in figure 2-3 (Fritsche and Hofrichter, 2000). In ortho-cleavage, phenol is ultimately degraded to succinate and acetyl-coA, whereas meta-cleavage results
in the formation of acetaldehyde and pyruvate, both of which are transported inside the cells.

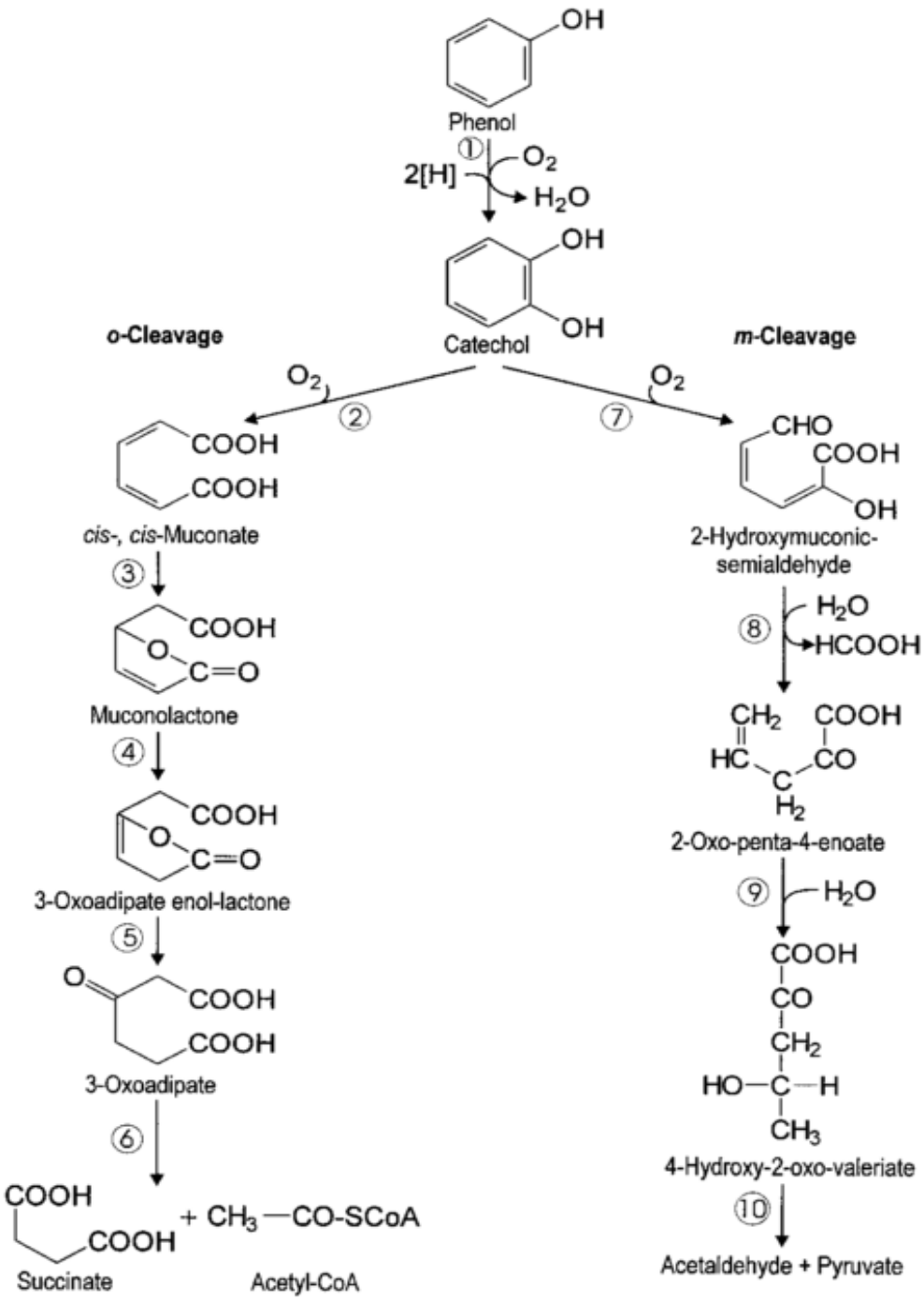



Figure 2.3: The two pathways to degrade phenol by microorganisms (Fritsche and Hofrichter, 2008).

#### 2.2.2.2 Limiting factors

Previous studies have concluded that many bacterial species have the physiological and metabolic ability to degrade and utilise hazardous materials as a source of energy. Nevertheless, the effectiveness of conventional biological remediation methods in managing the contamination of industrial wastewaters may be limited by a number of factors (Dubey and Hussain, 2013), these can be summarised as internal factors and external factors.

Internal factors relate to the microorganisms themselves. These include the ability of phenol-degrading bacteria to function in the presence of other bacterial consortia, and the possession and expression of enzymes and metabolic pathways to perform these reactions. External or environmental factors that can impact on the bioremediation process include temperature, pH and the availability of nutrients that could affect the metabolic activity of cells and utilisation of the compounds as an energy source. Moreover, the stability of some organic materials in the environment or the presence of toxic pollutants in elevated concentrations may inhibit the metabolic activity of microbial cells (Boopathy, 2000; Borden, 2017; Luka *et al.*, 2018).

Although bioremediation methods are considered environmentally friendly and cost effective when compared to other technologies, the limiting factors mentioned above can inhibit the bioremediation process. Therefore, research has been performed in order to decrease the impact of these factors to enhance the efficacy of biological remediation methods.

#### 2.2.2.3 Immobilisation of microbial cells

An immobilisation strategy can be described as a physical separation process of the bacterial cells or their enzymes and organelles from the peripheral medium. Many benefits are derived from this type of immobilisation, which appears to enhance the performance of, the bioremediation strategies (Sheehan, 1997). Immobilising the microorganisms or enzymes offers various advantages. For instance, an increased in stability of the bacterial cells, the

ability to reuse them on several occasions, the ability to store them until needed, an increase in biomass density, protection from highly toxic materials, and enhance their resistance efficiency. In other hand, the immobilised matrices must have special features to use them such as non-harmless or polluting and non-biodegradable to use and could expand for several uses. Immobilisation processes can be performed in different ways(i) cross-linking and covalent binding, (ii) entrapment and encapsulation and (iii) adsorption by natural attachment to the immobilisation substrate or synthetically using chemical linkages (Martins *et al.*, 2013; Abubakar and Shukor, 2017; Liu *et al.*, 2018).

It has been suggested that immobilising bacterial cells by joining them with polymers as a support materials enables their use in a semi-fluidised bio-reactor to treat phenolic compounds in watewater (Meikap and Roy, 1997). Further research supported this suggestion, for instance Wasi *et al.* (2013) recommended that the immobilisation of *Pseudomonas spp.* is more effective in reducing the levels of heavy metals, pesticides and phenols rather than the use of free planktonic cells. Kotresha and Vidyasagar (2014) used six different materials and different techniques to immobilise *Pseudomonas aeruginosa* (MTCC 4996) in polyurethane foam (PUF) by adsorption. The immobilised cells had the capability to utilize up to 1400 mg /L of phenol in 12.5 hours and complete degradation was achieved within 6 days under the optimum conditions of 37$^{o}$C and pH 7-7.5. It was also observed that using minimal mineral salts medium (MMSM) enhanced the phenol degradation rate, also the feasibility of using the cells for up to 150 days in a constant flow-rate packed-bed reactor.

Attachment techniques were applied to immobilise *Aspergillus awamori* (NRRL 3112) in a poly acrylonitrile membrane to degrade phenol at initial concentrations of 0.2-1.0 g/L (Yordanova *et al.*, 2009). The immobilised cells were most effective at concentrations of 0.5 g/L phenol when compared to free, planktonic cells. Furthermore, the recirculation of immobilised spores of *A. awamori* in spirally-wound membranes was also a successful strategy. In another approach, Ahmad *et al.* (2011) reported that *Acinetobacter sp*. strain AQ5NOL-1 immobilised and supported in gellan gum via encapsulation or

entrapment was able to degrade up to 1900 mg/L of phenol after 24 hours incubation whereas free cells degraded only 1100 mg/L of phenol within the same time. Moreover, the supported cells showed a constant ability to remove phenol efficiency for up to 45 cycles, after which they lost about 10-38% of their activity by 46- 47 cycles. Similar techniques have been used to immobilise *Pseudomonas sp*. SA01 in different type of hydrogels to enhance the removal of phenol (Shourian *et al.,* 2009).

A combination of polyvinyl alcohol (PVA) with alginate using a boric acid method has been used to prepare cell beads (Mollaei *et al.*, 2010) that showed strong phenol degrading activity. Further confirmation of the positive impact of immobilisation on the cells' efficiency to degrade phenol was achieved when *Pseudomonas sp.* NBM11, isolated from a site contaminated by medical wastes and wastewater, was immobilised in alginate beads. This system was able to consume up to 1 g/L of phenol and could be reused for seven cycles without reducing their efficiency (Mohanty and Jena, 2017). An entrapment strategy was also used to immobilise a strain of the fungus *Debaryomyces sp* into Ca-alginate beads with nanoscale $Fe_3O_4$ to degrade 0.9 g/L of phenol under harsh conditions of temperature and salinity (Jiang *et al.*, 2017). Assessment of the immobilisation of *Sphingomonas sp*.GY2B with polyvinyl alcohol (PVA)–sodium alginate–kaolin beads to degrade phenol at 300 mg/L under harsh incubation conditions of acidic pH equal to 1 and alkaline pH equal to 12, showed that immobilised cells can significantly enhance the degradation performance (Ruan *et al.*, 2018).

In summary, these investigations have demonstrated that the immobilisation of microbial cells in a suitable matrix is an efficient technique to remove phenol and other hazardous pollutants from industrial contaminated sites, and that the immobilised cells were more efficient in phenol removal than free planktonic cells.

## 2.2.3 Cryogels as immobilisation scaffolds

Cryogels are gel materials which have found use in medical, biological and biotechnological applications. The term cryogel was derived from Greek word (kryos) which means ice, and it was first mentioned in the early 1980s (Okay and Lozinsky, 2014). Although they were first produced more than thirty years ago, interest in utilising them as bioremediation scaffolds is a recent development (Lozinsky, 2014). Their unique characteristics have permitted to engage and contribute effectively in many different fields, including medical and pharmaceutical applications (La Spina *et al.,* 2014; Erturk and Mattiasson, 2014; Ingavle *et al.,* 2015; Kumari and Kumar, 2017), industrial and biotechnological applications ( Hyoung-Joon *et al.,* 2010; Oztoprak *et al.,* 2014; Savina *et al.,* 2016; Hixon *et al.,* 2017) and increasingly for industrial and municipal wastewater treatments (Kuyukina *et al.,* 2009; Jahangiri *et al.*, 2014; Önnby, 2016).

### 2.2.3.1 Outline of the cryogelation process

The cryogelation process has been defined as a crosslinking reaction between polymeric materials under freezing conditions. This is achieved by dissolving the monomers or polymer precursors in specific solvent, which is generally water (Okay and Lozinsky, 2014). The formation of ice crystals is the principle stage of the cryogelation process, where the initial monomers are dissolved in the solvent, and frozen at sub-zero conditions to cause crystallization within the solvent, followed by defrosting at room temperature.

The polymerisation or gel formation occurs in the frozen samples around the ice-crystals formed so that the ice-crystals perform as a porogen. The resultant cryogel is a macroporous polymer hydrogel which forms three dimensional structures with a well-developed system of channels (Plieva *et al.*, 2007; Gun'ko *et al.*, 2013), as shown in figure 2.4.

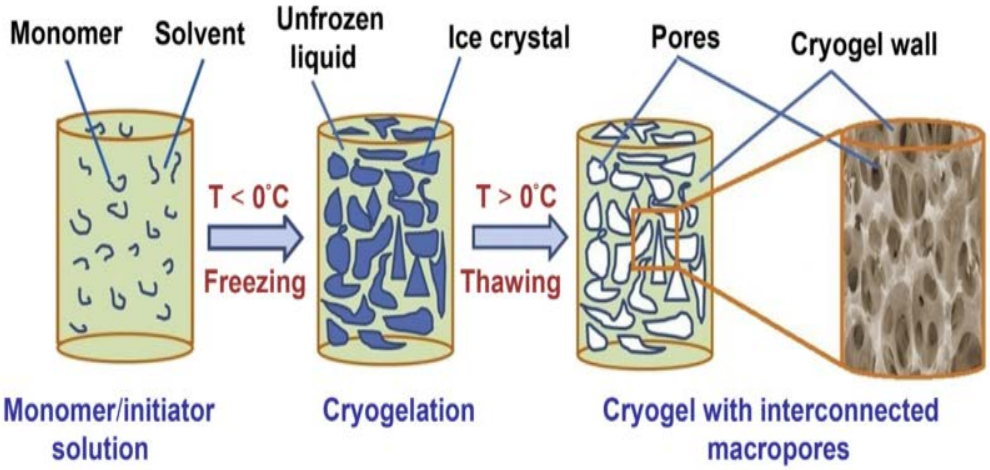



Figure 2.4: Diagram of cryogelation process (Ingavle *et al*., 2015)

### 2.2.3.2 Characteristics of cryogels

Cryogels have specific features that makes them desirable for biotechnological applications. Firstly, the synthesis of cryogels is much simpler than that for other freeze-dried gels which require reduced pressures to remove any remaining excess solvent. Due to the macroporous structure of these cryogels, they have been utilized as materials to separate and purify different products in biotechnological processes and in chromatographic columns (Erturk and Mattiasson, 2014). Secondly, cryogels can be synthesised with different morphologies such as blocks, tubes, discs and cylinders (Lozinsky, 2003), figure 2.5 b.

The interconnected pore size of the cryogels can be controlled during production and the formation of the pores depends on the properties of initial system and conditions of production. Cryogels with different pore sizes can be produced: macroporous cryogels with pores ranging from 0.1-10 micro meters, and super macroporous cryogels with pore sizes of up to 100 micrometres in size; the latter have a spongy morphology which helps facilitate the transport of solutes through the cryogels (Lozinsky, 2004; Hixon *et al.*, 2017). An appropriate mixture of biomass with polymers via the cryogelation process can produce novel forms of cryogels as shown in figure 2.5 a, (Plieva *et al.,* 2008).

The specific characteristics of cryogels such as macro porosity, mechanical stability, ease of synthesis using water as the solvent and the lack of toxicity after preparation make them suitable for the treatment of industrial effluents (Lozinsky, 2002; Plieva *et al.*, 2011). Bayram *et al.* (2007) investigated the use of super macroporous cryogels comprising poly (acrylamide-allyl glycidyl ether) [poly (AAm-AGE)] cryogel as carriers of Cibacron Blue F3GA dye by immobilising them using a covalent bonding technique to reduce phenol and chlorophenols in wastewater. The result showed the feasibility of using such cryogels to reduce phenols in aquatic solutions. Furthermore, they have been shown to be biocompatible, Cryogels also act as a barrier to protect and enhance the cells' efficiency against toxic concentrations of pollutants. Thus, it is apparent that cryogels combined with cells or enzymes are a promising bioremediation tool.

#### 2.2.3.3 Cryogels for the immobilisation of microbial cells

Cryogels have attracted attention because of their physiochemical properties which enable them to be used as scaffolds for immobilising bacterial cells. Immobilisation of the bacterial cells or extracted enzymes within cryogels can be achieved via two routes: direct attachment techniques (covalent or adsorption) to already prepared cryogels or by entrapment techniques via mixing the suspension cells with polymer solution before cryogelation process, as shown in figure 2.5 a (Plieva *et al.,* 2008a).

An attachment strategy used by Kuyukina *et al.* (2009) was the development of hydrophoboized polyacrylamide cryogel columns (C12-cryoPAAG) to immobilise hydrocarbon-oxidizing *Rhodococus ruber* cells by the adsorption technique. These were then tested against petroleum hydrocarbons treatments. The results indicated that the cells maintained their adhesions for three weeks and that the respiration activity of the immobilised cells was about 1.6 -1.8 times higher than that of the free cells.

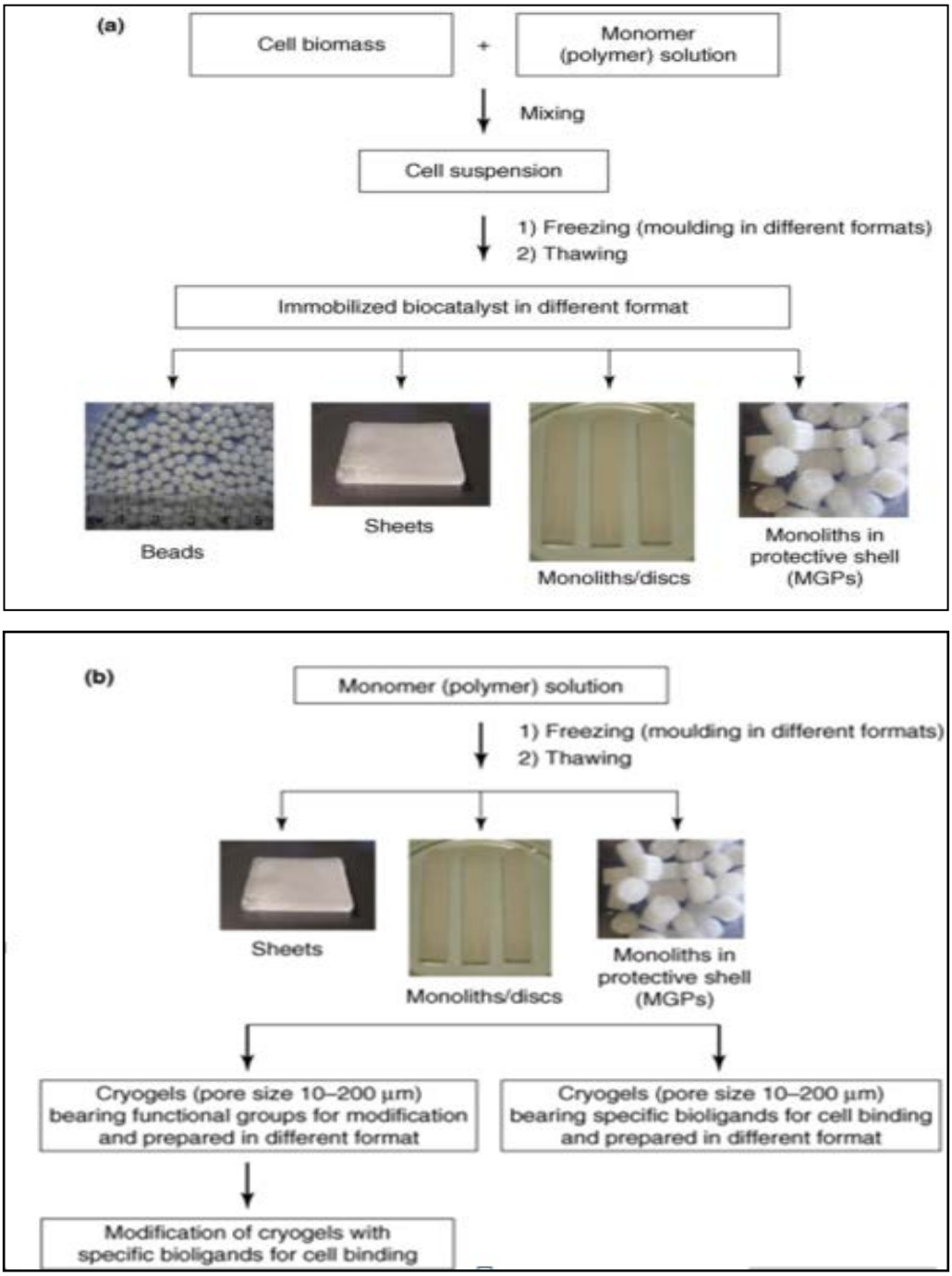



Figure 2.5: Schematic of cell immobilisation approaches in cryogels, (a) entrapment of cells into cryogels during preparation process, (b) attachment of cells into already prepared cryogels (Plieva *et al.,* 2008a).

Topalova *et al.* (2011) examined the activity of *Bacillus laterosporus* BT-271 immobilised in a polyethylene oxide cryogel (PEO). Different methods of seeding

PEO gels with biomass were studied. It was found that direct soaking of the gel in cell biomass was a more effective way of cell immobilisation, forming a stable biofilm. This approach achieved a 100% efficiency in phenol degradation, which was maintained for 15 days after the system was initiated.

Other researchers have applied entrapment strategies to produce functional cryogels. Philp *et al.* (2003) examined the immobilisation of genetically modified bioluminescent *Photobacterium (Vibrio) fischeri* and *Pseudomonous putida* into polyvinyl alcohol (PVA) as a system to treat phenol and 3-chlorophenol in wastewaters. In addition to that, enhance and protect the biological wastewater treatment plants from toxic materials, flexibility in recirculation tests, which means they were amenable to reuse. Similar cryogel materials and techniques were used by El-Naas *et al.* (2009) to immobilise *Pseudomonas putida* using the freeze–thaw process at -20$^{o}$ C, to produce pelleted cryogels that were used for the evaluation of phenol degradation rates in a bubble column bioreactor.

Liu *et al.* (2009) stated that using cryogels for immobilising cells of two novel species of *Acinetobacter* (XA05) and *Sphigomonas* (FG03) into polyvinyl alcohol (PVA) via the freeze-thaw technique were better than planktonic cells to degrade phenol at concentrations of up to 500 mg/L under optimum conditions of 30$^{o}$ C at a pH 7.2. It was determined that cell stability, pH, ambient temperature and the initial concentration of phenol can have a significant impact on the degradation process. Recent research has been performed using the same entrapment technique for immobilising various bacterial cells or enzymes into cryogels to remove phenol and its derivatives. The authors recommended their use for the treatment of industrial wastewater (Satchanska *et al.*, 2015; Hristov *et al.* , 2016). Therefore, it can be concluded that the application of cryogels as a scaffold material with attached bacterial strains delivered an elevated capacity to degrade aromatic compounds, in particular phenol, thus producing an effective tool to reduce and remove contaminants from the environment. However, all these advantages of cryogels that have been presented earlier about cryogels, some disadvantages were also noticed that could have drawback impact in the biotechnological applications. For instance;

to prepare cryogels with superior hydrodynamic performance (high porosity, low backpressure and less compressibility at high flow rate) this required increase in monomers concentrations and monomers /cross linker ratio (Plieva *et al.,* 2011). Moreover, may lead to other issues such as longevity of cryogels and long time to dispose them. Another feature of cryogels that could have opposite direction are size of pores, which is normally (10-100µm) and size of bacterial cells (e.g. *E.coli* 1x3µm) are smaller than these pores. So that, they will be washed out continuously in repeated use (Lozinsky *et al*., 2003; Henderson *et al.,* 2013 ) when they immobilised in attachment technique, and the only way to obtain such a small pore (1-2µm) is entrapment strategy (Plieva *et al.,* 2008). All advantages and disadvantages were summarised in table 2.2.

Table 2.2: Advantages and disadvantages of cryogels

| Advantages | Disadvantages |
|---|---|
| • Easy to synthesise, water is the main solvent.<br>• Spongy structure with various pore sizes ranging between 10-100µm.<br>• Different shapes such as discs, beads, sheets, cylinders and blocks can be produced.<br>• High mechanical strength and elasticity.<br>• Widely used in different fields such as biotechnological, industrial and medical applications. | • High sub-zero temperatures are required to produce them before gelation process.<br>• Increases in the sub-zero temperature results in increases in the size of pores.<br>• Elevated concentrations of monomers and the potentially toxic crosslinker may inhibit microbial activity.<br>• Loss of immobilised cells as a result of large connecting canals and pore sizes. |

## 2.3 Knowledge gap and research questions

Based on the evidence provided above, it can be observed that much of the literature is focused on the isolation and identification of new strains that could

be used in the phenol biodegradation process. In parallel with these studies, other researchers have concentrated on using various macro porous polymer constructs (natural and synthetic) as a tool to support microbial activity in the removal of specific pollutants from contaminated areas.

From reviewing the literatures, the following knowledge gaps and research questions have been identified:

Does a reduction in the amount of polymer used to immobilise the cell biomass improve the stability of the monolithic cryogel and enhance the degradation of phenol? (Chapter 4).

Do indigenous and autochthonous bacterial strains isolated from contaminated sites in the environment show increased tolerance to high concentrations of phenol? (Chapter 5)

Does variation in environmental factors have an effect on the performance of immobilised cells and what are the outcomes if these factors are changed? (Chapter 6)

Does the production of cryogels within Kaldnes carriers enhance the efficiency of the immobilised cells to degrade phenol in a real environmental water scenario? (Chapter 7)

## 2.4 Hypothesis

The immobilisation of a high biomass of indigenous bacterial strains from contaminated sites or strains known for their ability to degrade phenol within non-toxic polymers at low concentrations will produce flexible and reusable monolithic 3D macroporous cryogel structures, that can be used to remediate high concentrations of phenol and its derivatives in water or wastewater.

## 2.5 Aims of the research and objectives

The aims of this research are (i) to assess the feasibility of using bacteria isolated from oil contaminated soil immobilised in cryogels for the bioremediation of phenol contamination of water; (ii) to synthesis 3D macroporous cryogels with a high biomass of crosslinked bacterial cells and low concentrations of crosslinking polymers, for the remediation of phenolic compounds in water. To achieve these aims the following objectives will be followed:

(1) Screen, select and isolate bacteria that show the potential to degrade phenol. Their efficacy in degrading phenol under controlled optimum laboratory conditions will be examined.

(2) Produce crosslinked cell-cryogels using crosslinking polymers to form immobilisation platforms and growth support substrates for the bacterial species demonstrated to be robust phenol degraders under objective 1.

(3) Assess the performance of crosslinked cell-cryogels in phenol degradation at varying temperatures and pH for a set concentration of phenol.

(4) Assess the potential of the cell-cryogels containing phenol degrading bacteria within Kaldnes carriers as an effective bioremediation technique under realistic operating conditions in different water scenarios.

# Chapter 3 : Materials and Methodology

## 3.1 Chemicals and equipment

Chemicals, reagents and equipment used in this research were purchased from different companies and presented as follows, tables 3.1 and 3. 2.

Table 3.1: Chemical materials

| **Chemicals and reagents** | **Company** |
|---|---|
| Tryptone soya agar<br>Tryptone soya broth<br>Phosphate buffered saline | Oxoid Ltd |
| Sodium chloride.<br>Sodium hydrogen carbonate.<br>Glycerol.<br>Phenol.<br>Ammonium chloride.<br>Ethyl acetate.<br>Acetonitrile (HPLC grade).<br>MTT salt (Thiazolyl blue tetrazolium bromide) MTT 98% purity.<br>Dipotassium hydrogen phosphate ($K_2HPO_4$)<br>Potassium dihydrogen phosphate ($KH_2PO_4$) | Fisher Scientific Co.UK. |

| | |
|---|---|
| Sodium chloride (NaCl)<br>Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)<br>Manganese sulphate hydrate ($MnSO_4.H_2O$)<br>Ferrous (II) sulphate monohydrate (Fe ($SO_4$). $H_2O$)<br>Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)<br>Ammonium sulphate (($NH_4)_2$ $SO_4$ )<br>Glucose ($C_6H_{12}O_6$)<br>TE buffer<br>TBE buffer (Tris 89 mM -Boric acid 89 mM–EDTA 2 mM)<br>Glycerol ($92.09_{Mw}$),<br>Tetramethyle p- phenylene diamine (TMPD),<br>Phenylalanine Agar<br>Iron chloride ($FeCl_3$)<br>Agarose gel ($C_{12}H_{18}O_9$)<br>Orion Application solution (pH: 4.01, 7.01 and 10.02) | |
| Poly (vinyl alcohol) 40-80 (Mw 205,000)<br>Glutaraldehyde solution 50%<br>4-aminoantipyrine.<br>Glutaraldehyde solution (25% w/v in $H_2O$).<br>Polyethyleneimine, linear (250 mL) (typical Mn: 423) | Sigma-Aldrich |

| | |
|---|---|
| Isopropyl alcohol | |
| 2, 4-Dinitro-phenyle hydrazine. | May & Baker .Ltd |
| Potassium ferricyanide, 99+%, ACS reagent.<br>Ammonium hydroxide (28-30 wt. % solution of $NH_3$ in water). | Acros Organics |
| Universal primers 27F<br>5'-AGAGTTTGATCMTGGCTCAG-3'<br>and 1492R<br>5'-ACCTTGTTACGACTT-3'.<br>Universal primers<br>5'-TGGCTCAGATTGAACGCTGGCGGC-3'<br>(5'end of 16S) (24 length), and<br>5'-ACCTTGTTACGACTTCACCCCA-3'<br>(3' end of 16S) (23 length).<br>3. 1Kb Ladder (Gel Pilot® wide range ladder | Eurofins Scientific, UK |
| Mega Mix-Royal PCR buffer<br>MicroLysis DNA extraction solution<br>1 Kb DNA Ladder | Microzone limited, UK. |

| | |
|---|---|
| API 20E and API 20NE biochemical identification Kits | Biomerieux, UK. |
| Gel loading Dye x6 | Nalgene Co. |
| Bacterial strains identified as Acinetobacter spp. School of Pharmacy and Biomolecular Science) | University of Brighton |
| Restriction endonuclease enzymes (*Mbol, Rsal, Mspl, Cfol, Alul*) | Promega corporation, USA. |
| DNeasy TM Blood & Tissue DNA extraction kit | QIAGEN, Germany |

Table 3.2: Equipment

| **Equipment name** | **Supplier company** |
|---|---|
| Colony counter<br>Stuart SC6+ Orbital incubator (Stuart SI500) | Bibby Scientific Limited. |
| UV-spectrophotometer | HACH Lange DR,3900 |
| Centrifuge ( Megafuge 16R)<br>Blades single edge<br>Polyethylene tubes at 50 mL volume.<br>syringe-driven filter unit 0.22, | Thermo and Fisher Scientific. |

| | |
|---|---|
| Syringes at 5 mL and 10 mL volume (BD Plastipak ™) Vials 1.5 mL volume<br><br>Whiteman filter paper (70mm dimeter)<br><br>Vials and filter cap at 5mL size from Poly Vial Tm<br><br>pH meter (Metter Todedo AG 8603 Schwerzenbach, Switzerland)<br><br>Centrifuge (My spin 12): Heathrow scientific (model: Gusto, made in China) | |
| Pur-A-Lyzer ™ Mega 1000 Dialysis Kit (1 kDa) pore size<br><br>Dialysis bag (14 kDa pore size) | Sigma-Aldrich |
| Rheometer, type 379-0001 | Thermo-HAAKE Germany |
| Millex® syringe-drivenfilter unit (0.22µm) | Merck Millipore Ltd |
| Julabo cryostat bath | Julabo, F34 G13, Germany |
| Freeze-dryer | Christ ALPHA 2-4 |
| Platinum coater | Quorum (Q150TES) |
| SEM | Zeiss Sigma field emission gun (Zeiss NTS) |
| Flask shaker SF1 | Stuart Scientific, UK |

| | |
|---|---|
| G-Storm thermocycler model GS04822 (Manufactured in UK) | LabTech international Ltd., UK |
| Gel electrophoresis plate HU15 | SCIE-PLAS.LTD, UK |
| SynGene INGENIUS DNA visualisation camera. | Syngene, A Division of Synoptics Ltd., UK |
| Electrophoresis power supply, A.A Hoefer (PS300-B) | Hoefer, Inc. USA. |
| Nano Drop Lit nucleic acid quantification system (Manufacturer, ) | Thermo- Scientific, UK. |
| PCR tubes 0.2 mL volume | QIAGEN, UK |
| Vortex, TB 15012 Top mix. Manufactured in U.S.A. | Mo Bio laboratories, Inc. |
| Kaldnes carriers for moving bed bio-reactor (MBBR) (size: 10*7mm and model number is NX-FM) | Pingxiang Nanxiang Chemical Packing Co., Ltd. |
| Aurora 1030 wet oxidation analyser (TOC) | OIAnalytical, Xylem Inc. USA |

## 3.2 Production of crosslinked cells cryogels form commercial bacterial strains

### 3.2.1 Reactivation of bacterial strains

Two strains of known phenol-degrading bacteria, *Pseudomonas mendocina* (13264, strain 5) isolated from soil polluted with hydrocarbons and *Rhodococcus koreensis* (13709, strain DNP505) isolated from industrial wastewater were purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB). The lyophilised cultures were reactivated by following the instructions provided. Under aseptic conditions, each lyophilised strain was suspended in 0.5 mL of tryptone soya broth (TSB) in 1.5 mL vials. Aliquots were cultured on tryptone soya agar (TSA) and the remainder was inoculated into individual polyethylene tubes containing 10 mL of TSB and incubated at 30$^{o}$C for 24 hours.

After incubation, a single colony of each strain on the TSA was selected for further culture and 1 mL from each broth culture was transferred into individual tubes contained 10 mL of TSB and re-incubated overnight for further purification. Sterile glycerol was added to the broth cultures at a concentration 5% (v/v) as a cryo-protectant, then 1 mL of each culture was transferred into 1.5 mL vials which were stored at - 80$^{o}$C for future use.

### 3.2.2 Preparation of pelleted bacterial cells

Cultures of *P. mendocina* and *R. koreensis* were prepared for the crosslinking process by inoculating a single colony into individual tubes containing 10 mL TSB and incubating them at 30$^{o}$C for 24 hours to obtain fresh active cells. One mL of each culture was transferred into 500 mL of fresh TSB and incubated in an orbital incubator at 30$^{o}$C and 150 rpm until the cells reached the stationary phase (as determined by absorbance readings). The bacterial cells were harvested by centrifuging them at 10,000g for 10 minutes all supernatant discarded and pelleted cells washed with 20 mL of ice-cold 0.9% sodium chloride.

After discarding the supernatant of sodium chloride, pelleted cells were divided into two groups:

- Free cells: pelleted cells were incubated directly with phenol and buffer at concentration 50 mg/L to examine to determine their efficiency to degrade phenol comparative with the crosslinked cells.
- Crosslinked cells: pelleted cells kept at 4$^{o}$C approximately for subsequent mixing with a crosslinking solution to synthesis crosslinked cells cryogels by the freeze-thawing technique.

### 3.2.3 Synthesis of crosslinking solutions

Four different crosslinking solutions were used in this experiment, table 3.3.

Table 3.3: Types of crosslinking polymer solutions

| No. | cross-linkers and concentrations | Name |
|---|---|---|
| 1 | Glutaraldehyde (GA 0.5%) | CCC1 |
| 2 | Polyvinyl alcohol + Glutaraldehyde (PVA 1% + GTA 0.5%) | CCC2 |
| 3 | Polyvinyl alcohol - aldehyde + Polyethyleneimine - aldehyde (PVA-al 1% + PEI-al 0.25%) | CCC3 |
| 4 | Polyvinyl alcohol - aldehyde + Polyethyleneimine - aldehyde (PVA-al 0.5% + PEI-al 0.6%) | CCC4 |

➢ **Glutaraldehyde solution 50% (w/v)** was used at a final concentration of 0.5% (Kirsebom *et al.*, 2009).

➢ **Polyvinyl alcohol polymer (PVA)** was prepared by dissolving 0.6 g of PVA powder in 10 mL in warm distilled water with stirring.

➢ **Polyethyleneimine-aldehyde solution (PEI-al)** was prepared from a combination of polyethyleneimine polymer and glutaraldehyde: initially, 0.15 g of linear polyethyleneimine polymer was dissolved in 7.5 mL of distilled water and the pH was adjusted to 7.5 by adding 0.5 mL of (12M) HCl with stirring. Separately, a glutaraldehyde solution was prepared by adding 1.88 mL of GA (50%) stock solution to 7.5 mL of distilled water which was degassed for 5 minutes with stirring. The GA solution was slowly mixed with polyethyleneimine-aldehyde solution (PEI-al) drop by drop then degassed for 5 minutes. Finally, the mixture was stirred for an hour until an orange colour was formed, specifying the creation of Schiff's bases. The solution was transferred immediately into a spectropore dialysis bag with a 1 k Da pore size and dialysed against 5 litres of distilled water for 3 days, changing the water twice a day.

➢ **Polyvinyl alcohol- aldehyde (PVA-al)** was prepared by adding 0.5 g of PVA to 5 mL distilled water and stirred overnight at room temperature to dissolve all the polymer, followed by mixing with 0.3 mL of 50% glutaraldehyde solution. After one hour's incubation with stirring at room temperature the solution was dialysed against 5 L of distilled water for three days at 4$^{o}$C in a dialysis bag of 14 k Da pore size, the water was changed once a day (Zaushitsyna *et al.,* 2014).

### 3.2.4 Determination of aldehyde group in an activated crosslinking polymer

2, 4-dinitrophenylhydrazine (DNPH) solution (0.05 M) was prepared according to pevious published work (Mendoza *et al.*, 2011; Berillo *et al.,* 2012a). An equal

volume (1:1) of acetonitrile solution and hydrochloric acid at a concentration (2M) had been used as a solvent. 0.5 mL of DNPH was mixed with 4% of activated polymer solution (PVA-al) and (PEI-al) to form orange precipitate then, extracted with 0.5 mL of absolute ethyl acetate twice. Unreacted DNPH was calculated by measuring the optical density using spectrophotometer at 350 nm.

### 3.2.5 Production of crosslinked cells cryogels (CCC)

Two known phenol-degrading bacteria *Pseudomonas mendocina* and *Rhodococcus koreensis* and a mixture of both with four types of crosslinking polymer solutions were used to produce crosslinked cells cryogels as shown in figure 3.1.

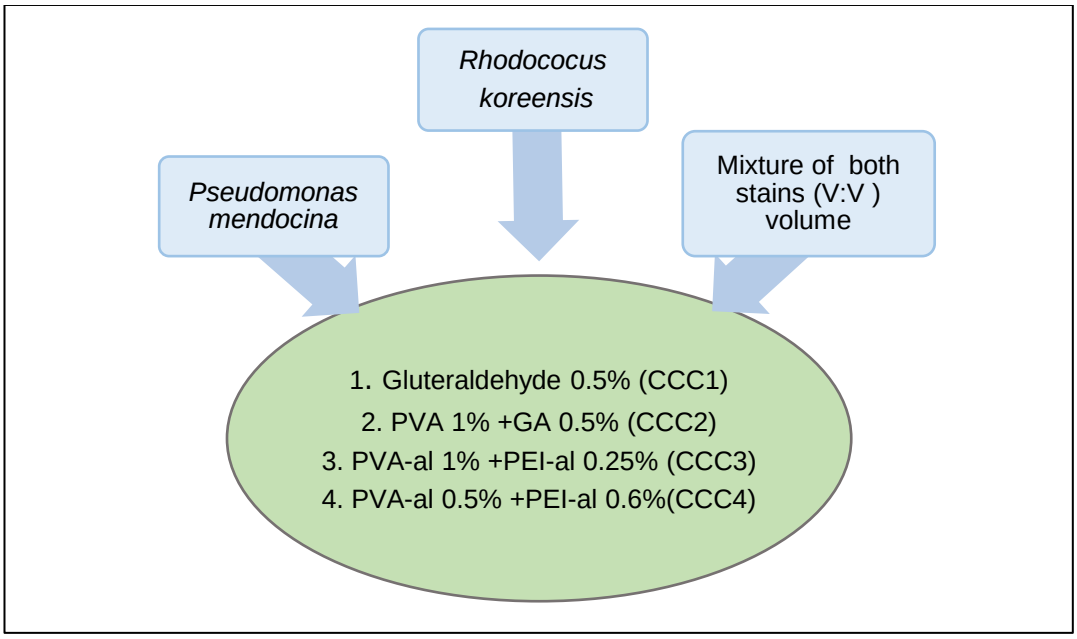



Figure 3.1: Bacterial strains and four cross-linking polymers solutions

0.15 g of the pelleted bacterial cells were mixed with 0.5 mL of each cross-linked polymer solution with gentle vortex to avoid bubble production. One mL of the bacteria-polymer suspension was transferred into a 9 mm diameter glass tube closed at one end with a rubber stopper in the bottom and frozen at -12ºC for at least 3 days in a Julabo cryostat (Aragão e*t al.*, 2014; Zaushitsyna *et al.*, 2014).

At the end of the cryogelation process the samples were thawed at room temperature and the viability, mechanical properties, and morphological structures of the cells were assessed as well as their efficiency to degrade phenol as the sole source of carbon.

## 3.2.6 Characterizations of CCC samples

### 3.2.6.1 Preparation for cells viability assessment

The viability of the crosslinked cells in the cryogel was assessed by using Live/Dead Bac Light kit (catalogue number: L7007) and MTT assay (Patel *et al.*, 2013). *P. mendocina* and *Rh. koreensis* and their mixture crosslinked with two combinations of crosslinking polymers: CCC3 (PVA-al 1% +PEI-al 0.25%) and CCC4 (PVA-al 0.5% + PEI-al 0.6%) were stained with the Live/Dead Bac Light kit and analysed using confocal laser scan microscopy (CLSM) and Leica TCS SP5 software. Preparation of the samples started with thawing of crosslinked cell-cryogels at room temperature and cutting them into thin slices approximately 1 mm thick using sterile scalpel blades.

The slices were washed with distilled water to remove unreacted cells and incubated with 5mL of TSB at 30$^{o}$C for 24 hours followed by discarding the broth and washing them with 0.9% NaCl buffer a twice to remove any remaining broth that could affect the staining results. Each slice was transported into Eppendorf tube 2 mL volume and incubated with 1 mL of NaCl buffer for staining with SYTO 9 stain (excitation/emission peak at 480/ 500 nm) and propidium iodide (excitation/emission peak at 490/635 nm) following the manufacture's protocol. After 15 minutes of staining at room temperature in dark conditions, the samples were transported into a 24-well plate for scanning by confocal microscopy using a Leica TCS SP5 and Leica advanced fluorescence (LAS AF) software.

The MTT assay is a tetrazolium salt stain [(3-(4,5-dimethylethioazol-2-yl)-2,5-diphenyle tetrazolium bromide] (MTT) used to quantify and assess the viability of bacterial cells (Wang *et al.,* 2010), fungal cells (Krishnan *et al.*, 2005; Patel *et al.*, 2013) and eukaryotic cells (Riss *et al.*, 2016). The viability is assessed by

monitoring the reduction of MTT salt because of chemical reactions with the oxidoreductase enzymes located into live cells (cytoplasm, mitochondria, and plasma membrane). These convert the yellow colour to purple indicating production of insoluble salts of MTT-formazan. The absorbance is determined using a spectrophotometer at wavelengths between 500-600 nm (Berridge *et al.,* 2005).

The viability of the cells was determined according to the protocol of Patel *et al.* (2013). MTT solution was prepared by dissolving 0.5 g of tetrazolium bromide into 100 mL of PB and sterilized by filtration using 0.22 µm Millex syringe-driven filter unit. This solution was stored in dark, cold conditions for further use because of its sensitivity to light. Free cells and crosslinked cells were prepared by centrifuging 1 mL of broth for free cells and one sample of crosslinked cells cryogel for 10 minutes. The supernatant was discarded and the sample was incubated with 900 µL of BPS and 100 µL of MTT solution in the dark at 30$^{o}$C and 150 rpm for 90 minutes. The samples were centrifuged to remove all buffer and the remaining stain, then mixed thoroughly with 800 µL of isopropyl alcohol acidified to 0.04M with HCl. Another centrifugation was done for 10 minutes before transferring 100 µL of supernatant into 96 well cell culture plate to measure the absorbance at a wavelength 590 nm using a microplate reader (BioTeck Instruments) and KC Junior software.

The viability of the free bacterial suspension and CCC samples was determined by a colour change of the reagent to purple indicative of the formation of MTT-formazan. Furthermore, the intensity of the purple colour indicated the percentage of cell viability; the free suspension cells appeared darker compared with crosslinked cell-cryogels, whereas samples after phenol degradation appeared as a light purple colour.

#### 3.2.6.2 Preparation for mechanical properties assessment

Rheology is a standard method for studying the mechanical behaviour and physical properties of soft cryogels, by combining the elastic, solid and viscous

fluid measurements of viscoelastic material (Franck, 2004). Samples were prepared by defrosting them at room temperature and cutting carefully into thin slices approximately 2mm thick with a sterile scalpel. Slices were stored at 4$^{o}$C in 5 ml of TSB to avoid dehydration until analysis with the rheometer (ThermoHaake, Type, 379-0001, Germany and ThermoHaake software) figure 3.2. The sinusoidal oscillating stress was set with a small amplitude to identify the linear viscoelastic region for all the crosslinked cell-cryogels. Samples were measured for amplitude stress sweep and the phase lag of the output strain was compared with the input stress under the following conditions: 25$^{o}$C, 0.1 Pa and 0.1-100.0 Hz to obtain the characteristic module such as Storage Modulus (a measure of dynamic elasticity) G', and the Loss Modulus (a measure of dynamic viscosity) G''. Following initial evaluation the oscillation frequency sweep was set at a 25$^{o}$C, 0.1 Pa with frequency range between (0.1-1 Hz) (Wettz *et al.,* 2007).


Figure 3.2: Rheometer equipment

#### 3.2.6.3 SEM Images for morphological structure of CCCs

Scanning electron microscopy (SEM) was used to demonstrate the macroporous structure of crosslinked cell-cryogel (CCC) samples. All samples for SEM analysis were prepared after completing the cryogelation process. A slice of crosslinked-cell cryogel, approximately 2 mm thick, was cut with a sterile scalpel and stored at -80$^{o}$C into a small petri dish until the analysis. Crosslinked cell-cryogel samples were defrosted at room temperature and washed with PBS and fixed with 5% (v/v) GA solution in PBS by incubating them for 24 hours at 4$^{o}$C. The PBS was replaced with a fresh solution of PBS for 30 minutes and changed three times. Slices were then soaked in fresh sterile deionised water for 60 minutes and the water was discarded, repeated twice with a fresh deionised water. The sample slices were frozen for an hour at -80$^{o}$C before transferring them to a Christ ALPHA 2-4 freeze-dryer for 24 hours to remove all the water from the cryogel matrix. Each freeze-dried sample was placed on the surface of a sample holder and coated with a layer of platinum using a Quorum (Q150TES) coater. The samples were scanned using Zeiss Sigma field emission gun SEM (Zeiss NTS).

### 3.2.7 Determination the efficiency of CCCs to degrade phenol

#### 3.2.7.1 Preparation of reagents

The following reagents were used to determine the absorbance of phenol using VIS-Spectrophotometry for each CCC sample. (i) a stock solution of 1 g/L phenol; (ii) ammonium buffer solution adjusted to pH 10;(iii) incubation buffer 2 g/L sodium hydrogen carbonate (NaHCO3); (iv) 2% 4-aminoantipyrine solution and (v) 8% potassium ferricyanide solution (EPA, 1978; Rice *et al.,* 2012). Finally, solutions of phenol at concentrations between 5-50 mg/L were prepared to derive a standard curve for phenol as described in appendix 1.

#### 3.2.7.2 Determination of phenol concentrations

The degradation of phenol by free cells and crosslinked cell-cryogels was studied. Free pelleted cells were prepared as described in section (3.2.2) and

crosslinked cell-cryogel samples were defrosted at room temperature and washed with distilled water to remove unreacted polymers and non-linked cells. CCC were placed in 50 mL polyethylene tubes, to which 40 mL of phenol at a concentration of 50 mg/L in sodium hydrogen carbonate buffer at 7.0 ± 0.01 pH was added and incubated at 30$^{o}$C in a static incubator for 7 days. Controls of phenol solution only and crosslinked polymer only in phenol solution were used.

The phenol concentration was measured as follows. 100 µL aliquots from each incubated sample were diluted to 1mL with distilled water. 50 µL of ammonium buffer was added to adjust the pH to 10.0 ± 0.3 followed by 25 µL of 4-aminoantipyrine solution. This solution was mixed thoroughly before adding 25 µL potassium ferricyanide solution to produce a reddish brown colour. The absorbance was measured by UV-spectrophotometry at 510 nm after 15 minutes incubation at room temperature (EPA, 1978; Rice *et al.,* 2012).

## 3.3 Isolation phenol-degrading bacteria

### 3.3.1 Preparation of soil and isolation of phenol-degrading bacteria

Soil samples from oil contaminated sites in Basra, Iraq were organised into four groups based on the locations they were collected from. Suspensions of the soil samples were prepared by placing 10 g of soil from each group into a 250 mL sterile bottle, mixed with 45 mL of sterile saline buffer (NaCl 0.9%) and 5 mL of glycerol. The bottles were shaken at 300 oscillations per minutes for 30 minutes at room temperature and left static until all heavy materials settled onto the bottom of the flasks, figure 3.3. One mL of supernatant from each flask was transferred into 1.5 mL Eppendorf tubes, which were stored at -80$^{o}$C for later use

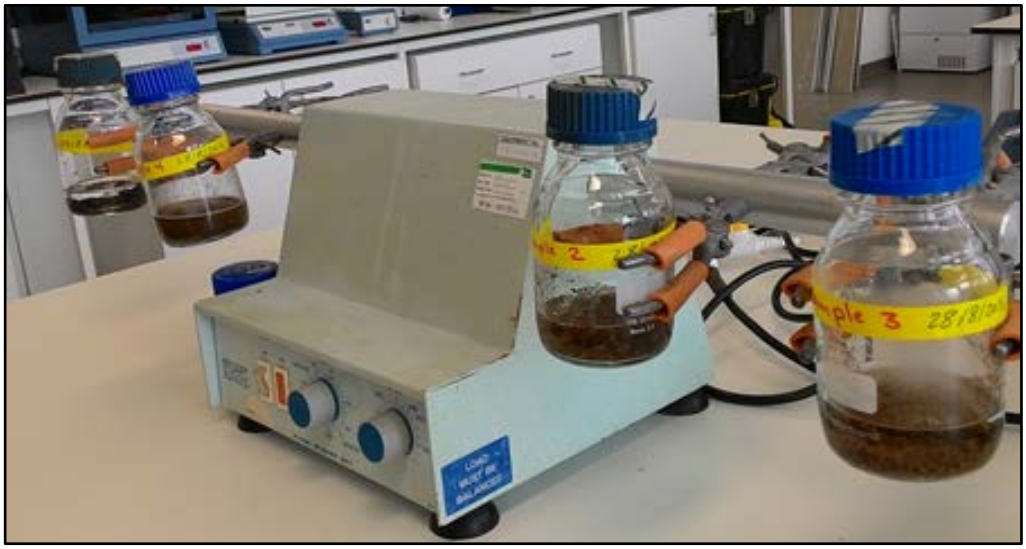


Figure 3.3: Preparation of soil samples

To culture the bacterial species from the soil samples, nutrient broth (NB) and mineral salt medium (MSM) were used. 10 mL of NB (prepared and described in appendix 2) was inoculated with 1 mL of soil suspension supernatant and incubated at 25$^{o}$C on an orbital incubator at 75 rpm for 24 hours. 5 mL of this broth were transferred to 100 mL of MSM (preparation described in in appendix 2), supplemented with 25 mg/L of phenol as the sole source of carbon and incubated for 120 hours at 37$^{o}$C in an orbital incubator at 75 rpm.

At the end of the incubation period, 1 mL of the culture was transferred to prepare serial dilutions in the range $1x10^{1}$ to $1x10^{7}$, and from each dilution 0.1 mL was spread onto two MSM agar plates, one containing phenol at a concentration of 25 mg/L, and one plate supplemented with phenol at the same concentration and 0.5% glucose. After 24 hours incubation at 37$^{o}$C, three different colonial morphologies were observed on only the plates supplemented with phenol and glucose. A single colony from each plate was picked and sub-cultured three times on MSM agar under the same conditions to obtain purified bacterial cultures.

### 3.3.2 Selection of robust phenol-degrading bacteria

A single colony of each purified strain isolated from the oil-contaminated soil samples was cultured on MSM agar supplemented with 100 mg/L phenol and 0.5% glucose; this experiment was repeated with agar containing phenol and without glucose to see if the presence of another source of carbon in the media influenced growth rates. To prepare fresh cultures, a single colony from each strain was inoculated into 10 mL of MSM broth supplemented with phenol at a concentration of 100 mg/L at 37$^{o}$C and 120 rpm for 24 hours. Following incubation, 1 mL of each culture was transferred to 250 mL sterile bottles containing 100 mL mineral salt medium (MSM) supplemented with phenol at 100 mg/L and incubated under the same conditions for five days.

Evaluation of phenol degrading activity was performed by daily absorbance measurements of phenol at a wavelength of 510 nm using the 4-aminoantipyrine method (EPA, 1978; Rice *et al.,* 2012), described in section 3.2.7.2. Strains with the ability to degrade 100 mg/L of phenol were selected to undergo tolerance tests at higher concentrations of phenol. Selected strains were re-cultured in 10 mL TSB for 24 hours at 37$^{o}$C, then transferred into 1.5 mL Eppendorf tubes and stored at -80$^{o}$ C for future use.

### 3.3.3 Identification of phenol-degrading bacteria

#### 3.3.3.1 Morphological identification

The colonial morphologies of phenol degrading bacteria can assist in provisional identification. A single colony was picked with a sterile toothpick and inoculated into 5 mL of nutrient broth and incubated at 37$^{o}$C and 150 rpm until cells were at the exponential phase. A loop of broth culture was streaked onto tryptone soya agar (TSA) and mineral salt medium (MSM) agar supplemented with 100 mg/L of phenol and 0.5 % glucose. Plates were incubated for 24 hours at 37$^{o}$C to note the size, shape, colour and surface structure of the colonies.

#### 3.3.3.2 Biochemical tests

A wide range of biochemical tests can be used for the identification of bacterial cells (Singleton, 2004). However, in this chapter only eight basic tests were employed for the bacteria that were isolated: Gram stain (Coico, 2005); growth on differential and enriched media such as MacConkey agar and blood agar (Singleton, 2004), the oxidase test, catalase test, motility test and phenylalanine deaminase test (Macfaddin, 2002). According to the results of the tests above, the API 20E and API 20NE biochemical identification kits were applied for fermenting and non-fermenting bacteria respectively (Biomeriexu, 2015). See appendix 2 for protocols for these methods.

#### 3.3.3.3 Genomic identification

Two gene-based identification methods were used for further identification of the isolated strains: the Polymerase Chain Reaction (PCR) with 16S rDNA sequencing and Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Dijkshoorn *et al.*, 1998).

##### 3.3.3.3.1 Polymerase Chain reactions method

**a. Primer preparation**

Biochemical tests suggested that the isolated bacteria were of the genus *Acinetobacter.* Universal primers 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-ACCTTGTTACGACTT-3) were used for DNA amplification (Weisburg *et al.*, 1991; Jiang *et al.*, 2013). Prior to suspension primers were centrifuged to settle the contents then suspended with appropriate volumes of TE buffer (10 mM of Tris, 0.1 mM of EDTA, pH 8.0).

To obtain 100µM concentrations of primer in TE the manufacturer's instructions were followed. From the stock primer solutions, each primer was distributed into micro-tubes, diluted with an appropriate volume of molecular grade sterile water

according to manufacturer's protocol, vortexed for one minute and stored at -20$^{o}$C for future use.

### b. DNA extraction

DNA extraction can be summarized in three steps: disruption or lysis of the cells, removal of protein and carbohydrate material, and recovery of the DNA. In this study, the DNeasy$^{TM}$ Blood & Tissue kit (Qiagen) was used following the manufacturer's instructions. Concentrations of the extracted DNA were determined by using Nano Drop Lite$^{TM}$. Neat and 1:10 extracts diluted with molecular biology grade water were stored in micro-tubes at -20$^{o}$C for future use.

### c. Preparation of samples for PCR process

The extracted DNA was added to MegaMix-Royal PCR buffer, which contains all the necessary components to amplify the DNA. MegaMix–Royal contains a stabilised solution of an optimum concentration of Taq polymerase, 2x enhancing buffer (6 mM $MgCl_2$) with 400 µM dNTPs and blue MiZN loading dye. 12.5 µL volume of that solution is considered as a desirable amount for each PCR tube after mixing it with an equal volume of DNA, primer and water to form a total volume of 25 µL. The protocol for the PCR is shown in table 3.4.

Table 3.4: Conditions of the polymerase chain reaction (PCR)

| Steps | Temperature (°C) | Time (min) | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95.0 | 5 | 1 |
| Denaturation | 95.0 | 1 | 30 |
| Annealing | 50.0 | 0.45 | |
| Extension | 72.0 | 1.30 | |
| Final extension | 72.0 | 10 | 1 |

### d. Electrophoresis of PCR amplicons

Separation and visualisation of the amplified DNA fragments was achieved through gel electrophoresis in a 1.5% agarose gel. 10 µL of each amplified DNA product from isolated strains was added to wells in the gel. For positive controls DNA from known culture collection strains of *Acinetobacter. lowffii* and *A. radioresistens* were used respectively. Biomolecular grade water was added to PCR mixture as a negative DNA control. 10 µL of a 1 Kb DNA ladder was used to estimate amplicon size.

### e. Electrophoresis

This process was carried out in 1X Tris-Acetate-EDTA (TAE) buffer for three hours at 100V in a horizontal gel tank. Gels were stained with 0.5% ethidium bromide for 15 minutes, followed by washing in water for 5 minutes (Sambrook *et al.*, 1989). The amplicons on the gel were imaged and documented by using the SynGene programme and SynGene INGENIUS UV imaging system.

### f. DNA Sequencing

Sequencing of the 16S rDNA amplicons (25 µl volumes) was performed by Eurofins Genomics. DNA concentrations were quantified using a Nano Drop Lite and adjusted to reach approximately 20 ng/µL by diluting them in sterile biomolecular grade water.

#### 3.3.3.3.2 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) was first developed by Vaneechoutte *et al.* (1993) for identifying *Mycobacterium* species to the strain level, and has been used successfully to type *Acinetobacter* species (Vaneechoutte *et al*., 1995). Similar to RFLP (restriction fragment length polymorphism) the technique involves using primers directed at the conserved regions at the ends of the 16s gene, followed by digestion using tetra-cutter restriction endonuclease enzymes. The pattern obtained is representative of the species analysed.

Extraction of the DNA for ARDRA was carried out using MicroLysis™: which is a simple one-step method and DNeasy ™ Blood & Tissue kit as described in section 3.3.3.3.1-b.

### a. MicroLysis Method

A single colony from each bacterium was suspended into 20 µL of MicroLysis solution in a 0.2 mL PCR tube and placed into a thermal cycler (G-Storm model GS04822), following conditions as in table 3.5. At the end of this process, extracted DNA was ready to use for PCR process.

Table 3.5: Microlysis conditions for one cycle.

| Steps | Temperature (°C) | Time (minutes) |
|---|---|---|
| 1 | 65°C | 5 min |
| 2 | 96°C | 2min |
| 3 | 65°C | 4min |
| 4 | 96°C | 1min |
| 5 | 65°C | 1 min |
| 6 | 96°C | 0.5 min |
| 7 | 20°C | hold |

### b. Primers and PCR preparation

The universal primers 5'TGGCTCAGATTGAACGCTGGCGGC-3' (5' end of 16S rRNA gene) and 5' TACCTTGTTACGACTTCACCCCA-3' (3' end of 16S rRNA gene) (koeleman *et al.,* 1998) were used. The PCR process was set up as described in Vaneechoutte *et al.* (1993), except that the initial denaturation step was increased by one minute, table 3.6.

Table 3.6: Conditions for amplified ribosomal DNA restriction analysis (ARDRA)

| **Step** | **Temperature (°C)** | **Time (min)** | **Number of cycles** |
|---|---|---|---|
| Initial denaturation | 95.0 | 6 | 1 |
| Denaturation | 95.0 | 0.45 | 35 |
| Annealing | 50.0 | 0.45 | |
| Elongation | 72.0 | 1.0 | |
| Final extension | 72.0 | 7 | 1 |
| Store | 4.0 | Infinite | |

**c. Electrophoresis process and visualization**

Separation and visualisation of the amplified DNA fragments was performed as in Section 3.3.3.3.1-**d**. except that the gel was prepared using 3% agarose in 10X TBE buffer (Tris 89 mM-Boric acid 89 mM–EDTA 2 mM). TBE was also used as the gel buffer during electrophoresis.

**d. Preparation of restriction enzymes (RE)**

PCR products exhibiting a band at 1500bp characteristic of the 16S rDNA amplicon were restricted separately with the restriction enzymes *MspI, AluI, CfoI, RsaI, MboI* by incubating them with the sample DNA following the manufacturer's instructions. Each sample reaction is a mix of 1-3 µL of amplified DNA and 1 µL of restriction enzyme made up to a final volume of 20 µL with molecular grade water. The restriction mixtures were incubated at 37°C from 2 to 3 hours before gel electrophoresis visualisation process.

### 3.3.3.4 Determination of tolerance levels of phenol-degrading bacteria

Tolerance to phenol of the isolated phenol-degrading strains was estimated by incubating the bacterial cells in concentrations of phenol ranging from 100 to 400 mg/L. 1 mL of stored cells were inoculated into 9 mL of nutrient broth in a 15 mL volume of polyethylene tube and incubated at 37$^{o}$C for 24 hours to harvest a fresh culture. 1 mL of fresh culture was added to another 25 mL of nutrient broth for further incubation and cell growth was evaluated according to absorbance measurements at 600 nm.

Cells were harvested once the exponential phase was reached by centrifugation at 10000 rpm for 10 minutes, discarding the supernatant and retaining the pelleted cells. These were washed by re-suspension in 20 mL of sterile 0.9% sodium chloride solution, followed by centrifugation to obtain a fresh cell pellet. The cells were then re-suspended in 5 mL of sterilised MSM, and 1 mL of each suspension was transferred into 250 mL conical flasks made up to 100 mL with MSM supplemented with phenol at concentrations of 100, 200, 300, and 400 mg/L with 0.5% of glucose as a complementary source of carbon. All cultures were incubated in an orbital incubator at 120 rpm and 37$^{o}$C. Assessment of phenol tolerance was documented by determination of cell growth by measuring the absorbance at 600 nm every 24 hours with an assessment of phenol degradation using the 4-aminoantipurine method (EPA, 1978 ; Rice *et al.*, 2012) at different concentrations of phenol, see section (3.2.7.2).

## 3.4 Production of CCCs with the isolated bacteria

### 3.4.1 Preparation, production and characterisation

The protocol in section (3.2.2) was used to prepare the pelleted cells of the isolated strain showing robust tolerance to high concentrations of phenol. Two activated crosslinking polymers CCC3 and CCC4 were prepared as described in section 3.2.3 and used to produce CCC samples as in section 3.2.5. The

selection of crosslinking polymers was depended on the results obtained from crosslinking the commercial strains *P. mendocina*, *Rh. koreensis* and a mixture of both. Evaluation of CCC sample characteristics was performed using methods described in section 3.2.6.

### 3.4.2 Determination of the effect of incubation conditions on phenol degradation

CCC samples using the isolated bacteria were tested to assess the effect of incubation conditions on the cells' phenol degrading efficiency. CCC samples were incubated in sodium hydrogen carbonate buffer with 50 mg/L of phenol and tested under optimum conditions for eight cycles, as in section 3.2.8. To evaluate the effect of medium type on the metabolic activity of the cells CCC samples were incubated in mineral salt medium (MSM) supplemented with phenol instead of sodium hydrogen carbonate buffer and tested for ten cycles under the same conditions in a static incubator.

Evaluation of the effect of pH on CCC samples was done by incubating the samples in 40 mL of MSM supplemented with 50 mg/L of phenol and adjusting the pH to 6, 7, 8 and 9, incubated at 20$^{o}$C and 37$^{o}$C. An assessment of the effect of incubation temperature on CCC samples was done by incubating the samples in 40 mL of MSM supplemented with 50 mg/L of phenol adjusted to pH 7 and incubated at 20$^{o}$C, 30$^{o}$C and 37$^{o}$C for the crosslinking polymers CCC3 and CCC4, with orbital shaking at 100 rpm.

## 3.5 Storage of CCC samples

Two methods were used to store the crosslinked cell-cryogels samples after production: freezing them at -80$^{o}$C and the freeze-drying (lyophilisation) method. CCC samples of *Rh. koreensis* and mixture of *P. mendocina* and *Rh. koreensis* were placed into polyethylene tubes with 10 mL of sodium hydrogen carbonate buffer supplemented with 5% glycerol and frozen in liquid nitrogen before storing them in a freezer at -80$^{o}$C.

The freeze-drying method was applied only to the CCC samples of the isolated strain. The process started directly after finishing cryogelation process. The CCC samples were transported immediately into the freeze dryer for 3 or 4 days, then removed from the glass tubes containing the CCC and transported to sterilised polyethylene tubes of 50 mL volume and labelled as follows: polymer type, name of strain and store date; the samples were stored at room temperature for future use.

## 3.6 Assessment of incubation media after degradation of phenol

The final concentrations of phenol and its possible derivatives in water were estimated using HPLC analysis. Sample preparation included the dilution of the solution in pure methanol, freezing it at -20$^{o}$C for several hours, followed by centrifugation and filtering through a 0.22 µm filter to remove proteins and other unwanted compounds which might affect the column. The samples were also analysed by gas chromatography–mass spectrometry (GC-MS) to identify any products produced during the pathway of phenol degradation. 25 mL of each sample were filtered and 0.5 g of NaCl was added. Five drops of 37% HCl were added before extraction of the samples with 2 mL of ethyl acetate. The extracted layer was pipetted off and stored in 2 mL Eppendorf tubes and kept at -20$^{o}$C before sending them to Environmental Laboratory Ltd. to do the GC-MS analysis.

## 3.7 Production of CCCs within Kaldnes carriers (CCCs-KC)

### 3.7.1 Preparation and synthesis of CCCs-KC samples

The first step in this experiment was the selection of one type of crosslinking polymer solution. Due to insignificant differences between the activated polymers that were used to crosslink the bacterial cells, CCC3 (PVA-al 1% +

PEI-al 0.25%) was selected for this experiment. The preparation of bacterial cells to crosslink with polymers was performed by adding approximately 100 μL of *Acinetobacter radioresistens* stored at -80°C into 10 mL of TSB which was incubated at 30°C for 24 hours. Procedures in section (3.2.2) and (3.2.5) were followed to prepare and produce suspensions of CCC-KC.

Kaldnes carriers (7 mm long with inner diameter of 10 mm and an outer diameter of 12 mm) were arranged in a vertical position inside plastic tubes of 12.46 mm diameter or 5 mL volume syringes closed at one end with a rubber stopper in the bottom side. The crosslinked suspensions were transferred to completely fill the tubes especially the empty spaces of the Kaldnes carriers and quickly transported into cryostat bath to start the cryogelation process as shown in figure 3.4.

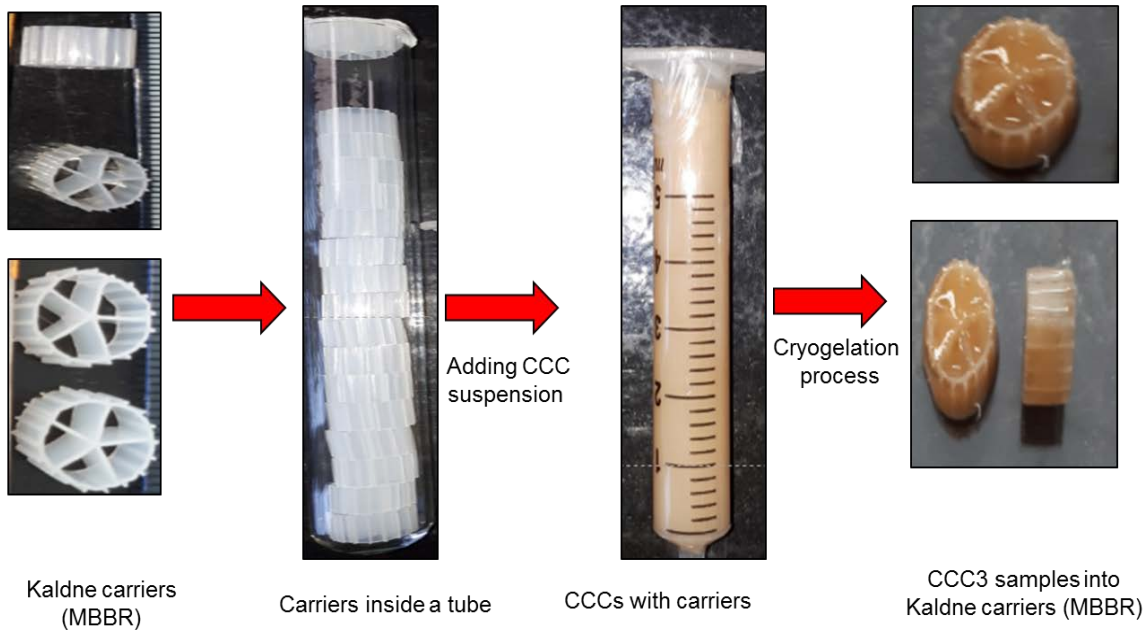



Figure 3.4: Production of CCC samples in Kaldnes carriers (MBBR)

## 3.7.2 Collections of water samples

Three types of water samples were collected from the environment during the end of October and the beginning of November 2017. Water samples were collected in accordance with standard protocols (Anon, 2012). Grab samples

(x3) of approximately 1 litre volume were collected in pre-sterilised plastic bottles in accordance with standard protocols and were transported to the laboratory in the dark at stored at 4$^{o}$C until further use.

- Ground water samples were collected in 1 litre plastic bottles by pumping water from a depth of 21 meters from a permanent borehole located at Preston Park, Brighton city, East Sussex, United Kingdom.

- Surface water samples for the phenol degradation studies were collected from two different rivers in East Sussex County (UK) – the Ouse and Cuckmere rivers. Samples were taken from the River Ouse in the village of Piddinghoe, 3 km from the estuary at Newhaven. The Ouse is the second largest river in the county of East Sussex in southern England, and drains into the English Channel an area of 396 km$^2$ to its tidal limit. This site is affected by inputs from biologically treated municipal wastewater (e.g. at Scaynes Hill, about 24-32 km upstream from the sampling site) and by diffuse pollution from animal rearing along the river's course (mainly cattle and sheep). The salinity ranges between 0.6 and 16 ppt dependent on the tide.

  Meanwhile, other surface water samples collected from Arlington Reservoir on the south of the Arlington village along the Cuckmere River and to the north of the Eastbourne to Lewes railway line. Arlington reservoir is filled with water pumped from Cuckmere River when the flows are high via straight channels to the east. One of two sites maintained by South East Water to supply water for people in south Sussex is designated a Site of Special Scientific Interest for its wild life.

- Tap water supplied by Southern Water Services Ltd. Registered in England No. 2366670. Samples were collected from taps located in EPHREG laboratory, Cockcroft Building, University of Brighton.

### 3.7.3 Initial preparation of the environmental water samples

Initially, all water samples were filtered using Whiteman filter paper (70 mm dimeter) to remove all suspended particles, except for tap water which was used directly. The pH of all samples was measured using a digital pH meter following calibration with calibration solutions of pH 4, 7 and 10. The water samples were evaluated for the presence of phenol or its derivatives using the 4-aminoantipyrine method and analysis using VIS-spectrophotometry at 510 nm as previously described in section 3.2.7.2.

Samples were also prepared for the estimation of nutrient concentrations. Triplicate water samples from each location were filtered using 0.22 µm Millex filters with a GP filter unit. 5 mL of each filtered water sample was transferred into 5 mL polyethylene vials and covered with a stopper prior to analyse by ion chromatography (IC). A standard curve was prepared at the following concentrations 5, 10, 20, 50,100 mg/L to assess seven ions: bromide, chloride, fluoride, nitrate, nitrite, phosphate and sulphate that could be detected by IC; distilled water was used as a blank.

The final assessment was the estimation of the concentration of carbon via the total organic carbon test. The environmental water samples were prepared by filtering 20 mL volume of water from each location, transferring them into 25 mL volume glass tubes which were sent to the Environmental Laboratory Ltd. for analysis. The principle of determination of TOC concentrations in water sample is by measuring the concentration of carbon dioxide released by chemical oxidation of the organic carbon in the sample. When the oxidation is complete the carbon dioxide is purged from the solution and routed to the NDIR (non-dispersive infrared detector) which is sensitive to the specific absorption of the wavelength for carbon dioxide using an Aurora 1030 wet oxidation analyser.

### 3.7.4 Determination of phenol concentrations by CCCs within Kaldnes carriers (CCCs-KC)

Following the preparation of CCCs-KC and the cryogelation process, the samples were washed with distilled water to remove un-crosslinked cells. 40 mL of the filtered environmental water from each location spiked with 50 mg/L of phenol were placed in 50 mL polyethylene tubes. The CCCs-KC samples were incubated with orbital stirring at 100 rpm and at 30ºC, without adjustment the initial pH values

Water samples of all the types of collected water without CCC or CCCs-KC were spiked with phenol, were also assessed for phenol concentrations. The reason behind this was to determine the ability of indigenous microbes in the water sample to degrade phenol and used as a control. The percentage of phenol consumption by CCCs-KC was measured three times a day as explained in section 3.2.7.2. CCCs-KC were examined for four cycles each cycle represents a day, which means fresh water samples spiked with phenol at 50 mg/L concentration were used and the same CCCs-KC samples without reactivation, just washing them with distilled water to remove the split cells and re-incubated.

Finally, estimation of the viability of CCCs-KC by the MTT assay (Patel *et al.*, 2013) was also performed following the same protocol as in section 3.2.6.1. Assessment of viable cells in the CCCs-KC was performed twice, before and after incubation with phenol, for all types of water samples and the difference between values was used to estimate the viable cells

## 3.8 Statistical analysis

Analysis of data in this research was performed using the statistical package IBM® SPSS version 24 with a significance level set at 5%. Normality and ANOVA tests were applied to analyse the data. One-way and two-way repeated analysis of ANOVA test were used, each analysis was chosen depending on the experiment design.

# Chapter 4 : Production of a 3D macroporous bioreactor using commercial bacterial species for phenol degradation

## 4.1 Introduction

The production and efficiency of 3D macroporous crosslinked cell-cryogels using two phenol-degrading bacterial strains is documented in this chapter. The bioremediation process is enhanced by immobilisation of the microbial biomass, physical isolation of the microbial cells from the external medium and a high ratio of biomass to polymer; these factors makes them easy to handle and minimises losses during the washing out stage (Sheehan, 1997; Wang, 2002). Such immobilisation systems have been widely investigated in medical, biological and biotechnological applications (Prieto*, et al.,* 2002; Zajkoska, *et al.,* 2013; Kotresha and Vidyasagar, 2014) and the immobilisation of bacterial cells within cryogel materials is a novel approach for biotechnological applications (Philp, *et al.*, 2003; Lozinsky, 2004).

Cryogels have desirable characteristics compared to other macroporous hydrogels, such as high mechanical strength during twisting, elongating and squeezing, which minimise damages to structure and characteristic properties of cryogels include simplicity of synthesis by solubility in water and their reaction in the unfrozen reaction to form a thin layer around the template of ice crystals (Okay and Lozinsky, 2014). These unique features of cryogels make them potential carriers and scaffolds to protect and enhance microbial activity and a promising tool to remove specific contaminants from industrial wastewater and mitigate environmental pollution (Savina *et al.*, 2016).

Cross-linking is one of many strategies employed to immobilise bacterial cells via a chemical reaction between the bacterial cell walls and cross-linking polymer to form a macroporous structure. The concept of crosslinking cells is based on the reaction between amino groups on the surface of bacterial cell

walls with aldehyde groups on the crosslinking polymer (Lozinsky, 2004; Kirsebom *et al.*, 2009).

Bioremediation methods have advanced in the last three decades as a result of reaching final point of mineralization process via microbial species as an efficient degrading agents comparative with previous methods used to clear the environment (O 'sullivan, 1998; Nair *et al.*, 2008). Bacterial genera including *Pseudomonas, Rhodococcus, Acinetobacter* and *Bacillus* have been shown to utilize phenol as a source of energy. However, high concentrations of phenols can inhibit or eliminate their metabolic ability and cause cell death (Boopathy, 2000; Krastanov *et al.*, 2013).

## 4.2 Aim and objectives

The aim of this chapter is to synthesis macroporous cryogels using two commercial bacterial strains known as degraders of aromatic hydrocarbons compounds. Different crosslinking systems will be employed and their ability to degrade phenol with fresh and frozen crosslinked samples determined.

To achieve the aim of this experiment the following objectives were applied:

- Crosslinking of cells and polymers using the freeze-thaw process at (-12$^{o}$C) was applied to immobilise the bacterial cells.
- Evaluation of four types of crosslinking systems at various concentrations for each bacterial strain.
- Estimation of cell viability before and after incubation with phenol using Laser Confocal Scan Microscopy (LCSM) and the MTT assay.
- Assessment of the mechanical properties of the CCC samples by rheometry before and after incubation with phenol.
- Evaluation of the morphological structure of CCC samples by Scanning Electron Microscopy (SEM).

- Determination of the efficiency of the CCC samples to consume phenol under optimum conditions of 30ºC and pH 7± 0.05 for cells suspensions (planktonic) and crosslinked cell-cryogels samples for freshly prepared and samples stored at -80ºC.

## 4.3 Results

### 4.3.1 Production and stability of crosslinked cells cryogels (CCC)

Thirty-six samples of 3D macroporous cryogels were produced using *Pseudomonas mendocina* and *Rhodococcus koreensis* which are known for their efficiency to degrade aromatic hydrocarbons. A 1:1 v/v mixture of the two species and four type of crosslinking solutions were prepared and evaluated, in total twelve samples for each bacterial strain. Cells were crosslinked through the chemical reaction between aldehyde groups from the crosslinking polymers with amino groups on the external surface of bacterial cells (Roberts and Caserio, 1977; Rogers, 1983; Aragão Börner *et al.*, 2014) via the cryogelation process, as presented in figure 4.1.

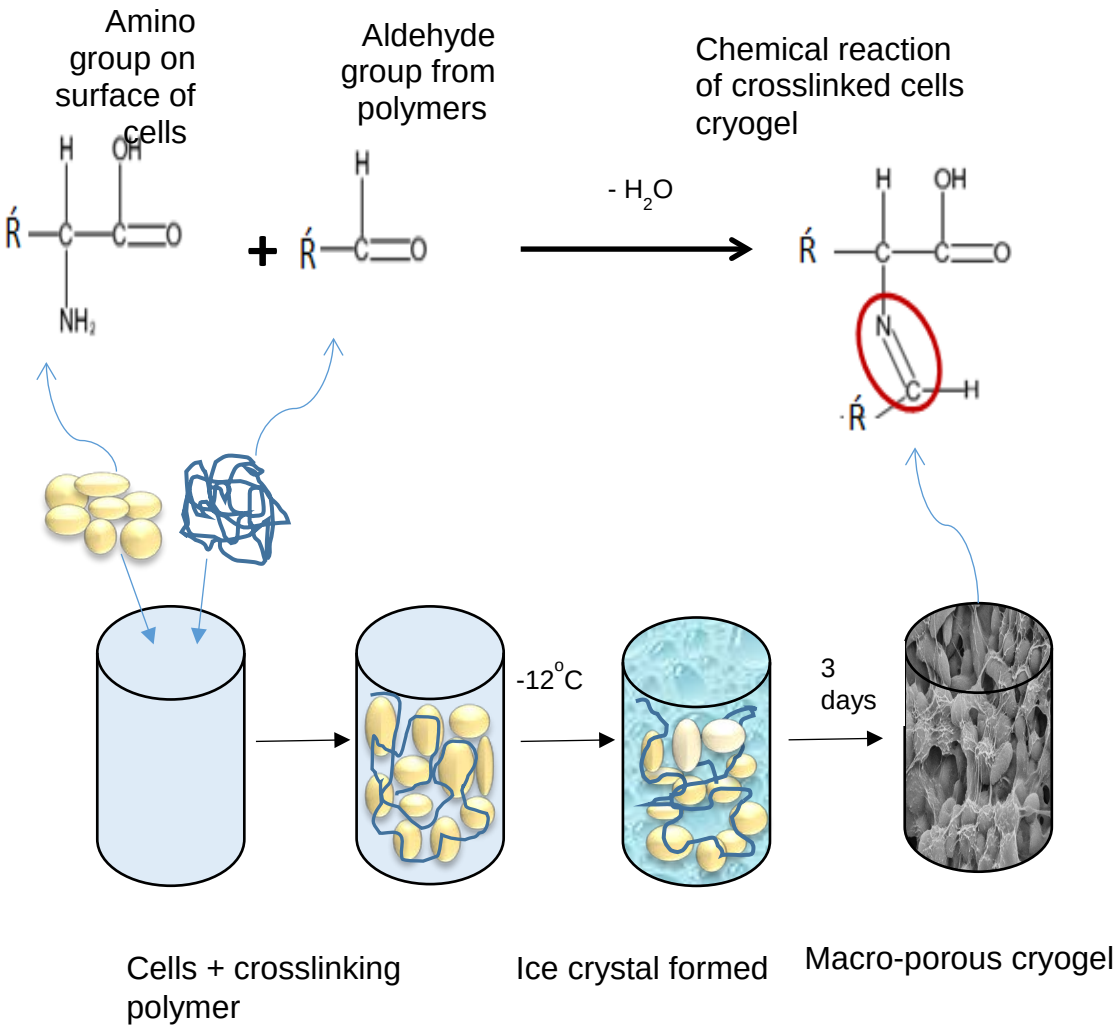



Figure 4.1: Illustration of crosslinked cells cryogels process with structure of chemical reaction.

Initially 0.5 to 1.5 % polymer solutions were used to immobilise the high-density bacterial cells and the optimum concentrations and ratios to form stable monolithic cryogel structures evaluated (results are not shown). CCC samples crosslinked with GA at a concentration of 0.5% and a combination of (PVA 1% + GA 0.5%) termed CCC1 and CCC2, respectively, formed weak structures that broke down after using them to degrade phenol and it was not possible to reuse them in the bioremediation process.

In contrast, activated crosslinking polymers solutions [polyvinyl alcohol (PVA-al) and polyethyleneimine (PEI-al)] were produced in the following concentrations

(PVA-al 1%+ PEI-al 0.25%) and (PVA-al 0.5% + PEI-al 0.6%) termed CCC3 and CCC4, respectively. These polymer mixtures resulted in the formation of monolithic structures which maintained their shape and scaffold structure over five cycles of incubation in phenol spiked buffer (figure 4.2).

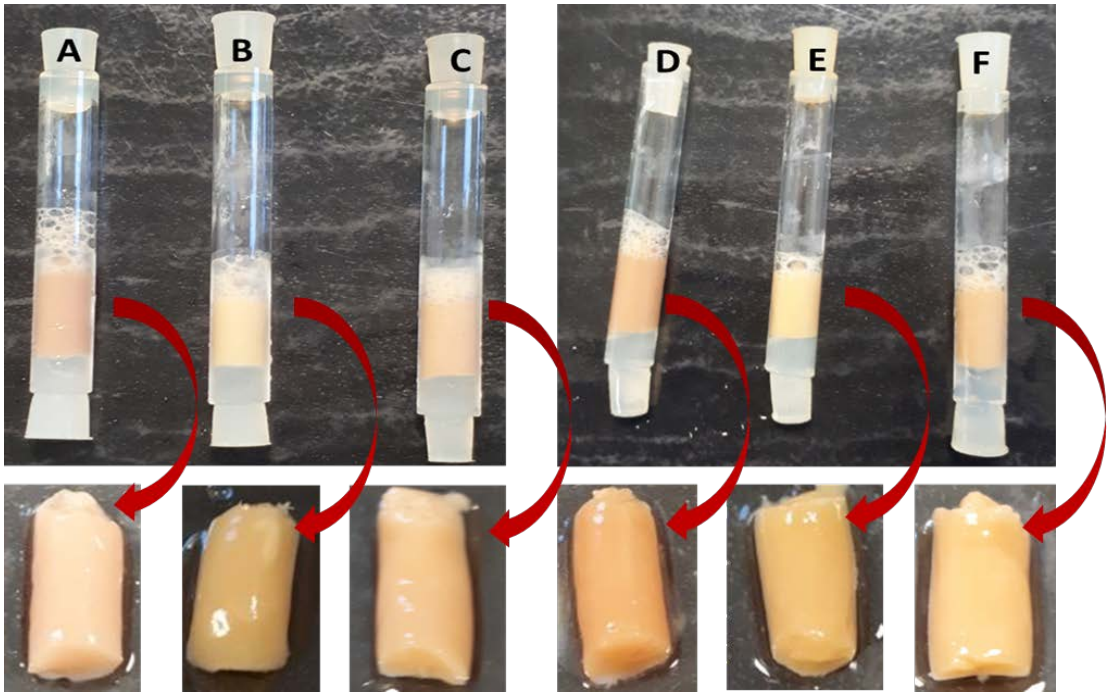



Figure 4.2: Production of crosslinked cells cryogels with two percentages of crosslinking polymers (CCC3): *P. mendocina* (A), *Rh. koreensis* (B), mixed cells (C) and CCC4: *P. mendocina* (D), *Rh. koreensis* (E), mixed cells (F).

### 4.3.2 Viability of CCCs samples

Assessment of the viability of the crosslinked cell-cryogels was carried out using the Live/Dead Baclight kit and confocal laser scanning microscopy. Figure 4.3 shows that most cells of *P. mendocina*, *Rh. koreensis* and a mixture of both were viable (stained green) after cross-linking and freeze-thawing. However, a few areas were stained red which indicates dead and injured cells (Xu *et al.,* 2011).

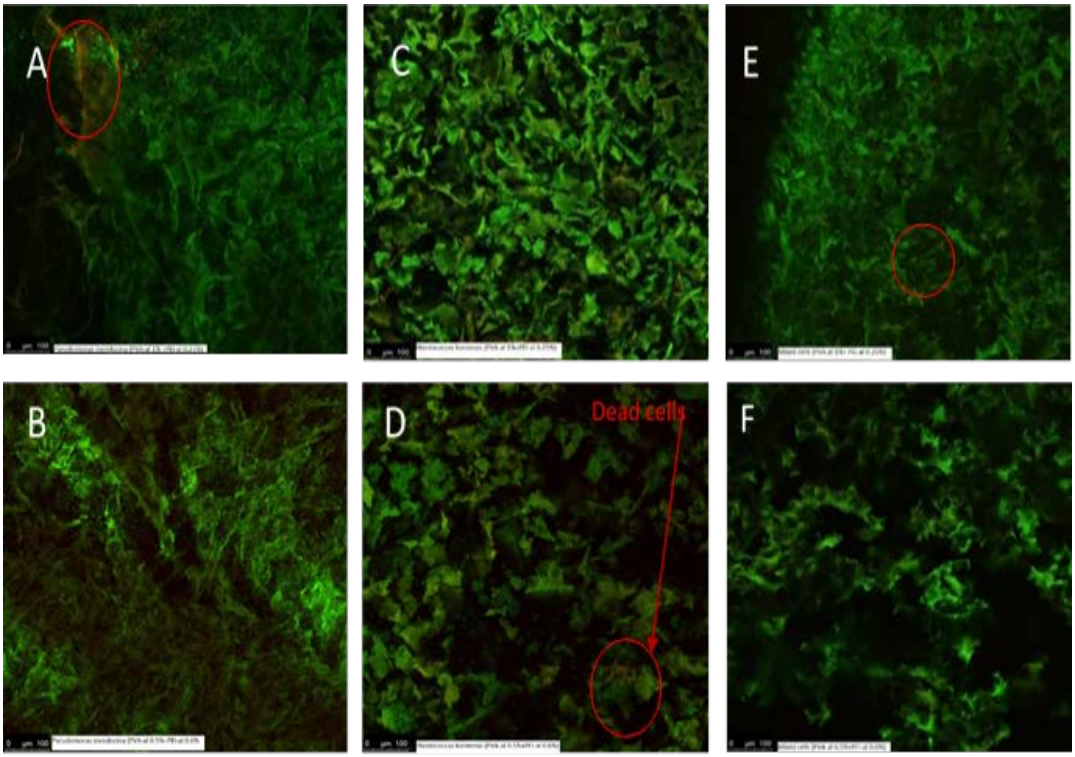



Figure 4.3: CLSM images of crosslinked cells cryogel stained by Live/Dead kit: P. mendocina (A-B), Rh. Koreensis (C-D) and mixed cell (E-F) crosslinked with (PVA-al 1% + PEI-al 1%) (A, C and E) and with (PVA-al 0.5% +PEI-al 0.6%) (B, D and F).

The impact of the freezing/thawing process and the crosslinking polymers used on the number of live cells was assessed using the MTT assay (figures 4.4 and 4.5).

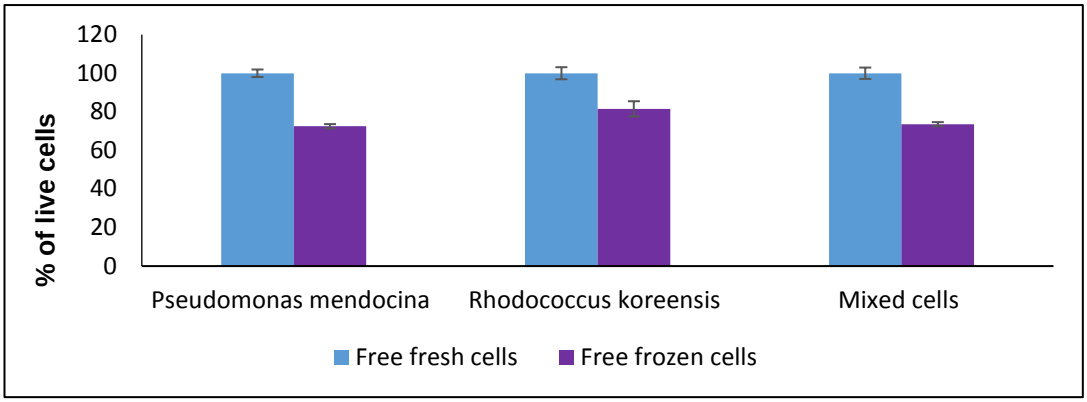



Figure 4.4: The percentage of live cells: free cells and frozen free cells estimated by using MTT assay.

Figure 4.4 shows the effect of freezing on the cells, the percentages of live cells in the same batch of *P. mendocina*, *Rh. koreensis* and their mixture (1:1 v/v). After freezing viability ranged from 70% to 80%, indicating a reduction in cell numbers and viability during the freezing process.

Figure 4.5 illustrates the effect of the cryogelation process on the live cells. Results of the MTT assay for samples CCC1, CCC2 and the combination of two activated polymers with various percentages of CCC3 and CCC4 using free frozen cells as a control at 100% showed that percentages of individual live cells of *P. mendocina* and *Rh. koreensis* ranged from 45% to 68%. In contrast, mixed samples of free frozen cells showed viability of 29% to 51% only.

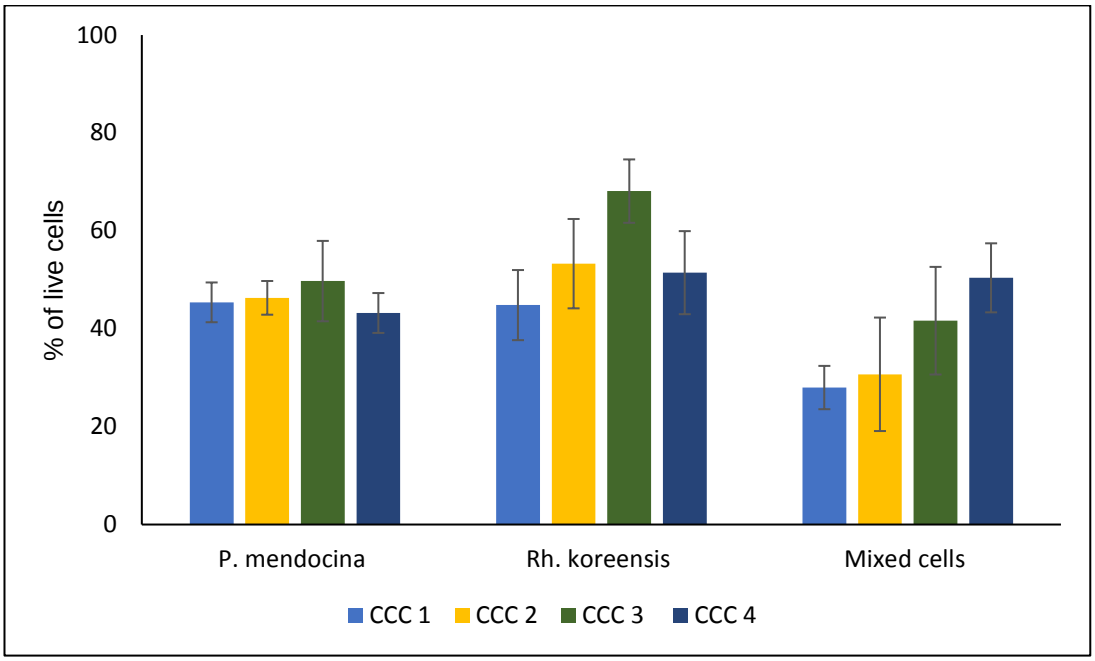



Figure 4.5: Percentages of live cells of *P. mendocina*, *Rh. koreensis* and their mixture estimated by using MTT assay for samples crosslinked with CCC1, CCC2, CCC3 and CCC4; frozen free cells were taken as (100%).

### 4.3.3 Mechanical properties of CCC samples

Evaluation of the mechanical behaviour of CCC samples are important in assessing their potential utility in industrial applications. Based on rheological analysis the results showed that storage or elastic modulus G' and the loss or

viscous modulus G'' appeared as a quasi-linear trend over frequencies between 0.1 and 1 Hz for all crosslinking polymer solutions for *P. mendocina*, *Rh. koreensis* and their mixture in a 1:1 volume. The dominance of G' over G'' for all bacterial strains confirmed that the samples formed gelled structures.

Crosslinking the cells with CCC1 showed higher values of G' and G'' with mixed cells compared to samples prepared with individual cells; similar behaviour was also noticed for CCC2 (figure 4.6 E and F). Activated polymer CCC3 did not show a considerable difference between bacterial types, whereas, crosslinking polymer CCC4 showed the highest values for samples formed from *Rh. koreensis* (figure 4.6, C and D). Samples produced from both CCC1 and CCC2 maintained their monolithic structure only for two days in the biodegradation cycle and then disintegrated gradually until they collapsed completely on the fifth day of incubation. Over the same time, samples of CCC3 and CCC4 showed better performance regarding their stability and reusability over the five weeks of incubation with phenol (50 mg/L) in carbonate buffer.

Another assessment of the mechanical proprieties of the crosslinked cell-cryogels was performed after incubation with phenol, using the oscillation frequency sweep test on CCC3 and CCC4 samples (figure 4.7). CCC samples of *P. mendocina* produced from crosslinking polymer systems CCC3 and CCC4 presented higher values for both moduli, G' and G'' compared to mixed samples (*P. mendocina* and *Rh. koreensis)* and individual samples of *Rh. koreensis*. The percentages of reduction in G' and G'' values for the *Rh. Koreensis* samples were determined as 11.75 % and 24.49 % for samples crosslinked with CCC3, 51.7% and 54.58% for samples crosslinked with CCC4, respectively. The calculations based on rheology assessment of initial fresh samples prepared after cryogelation process, figure 4.7 C and D.

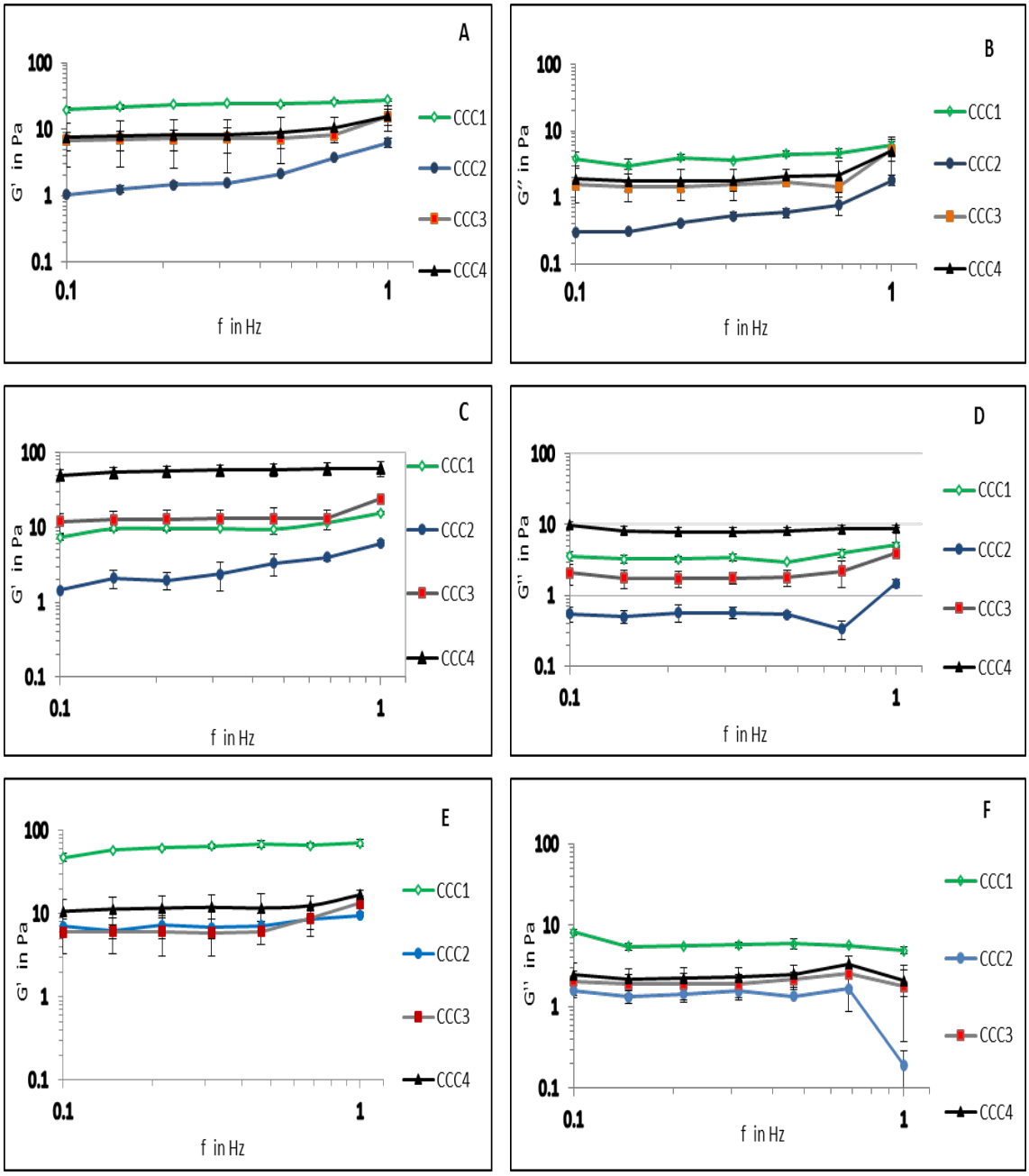



Figure 4.6: Rheology diagrams for *Pseudomonas mendocina* (A-B), *Rhodococcus koreensis* (C-D) and mixed cultures (E-F) crosslinked with CCC1, CCC2, CCC3 and CCC4, (n=3) before incubation with phenol.

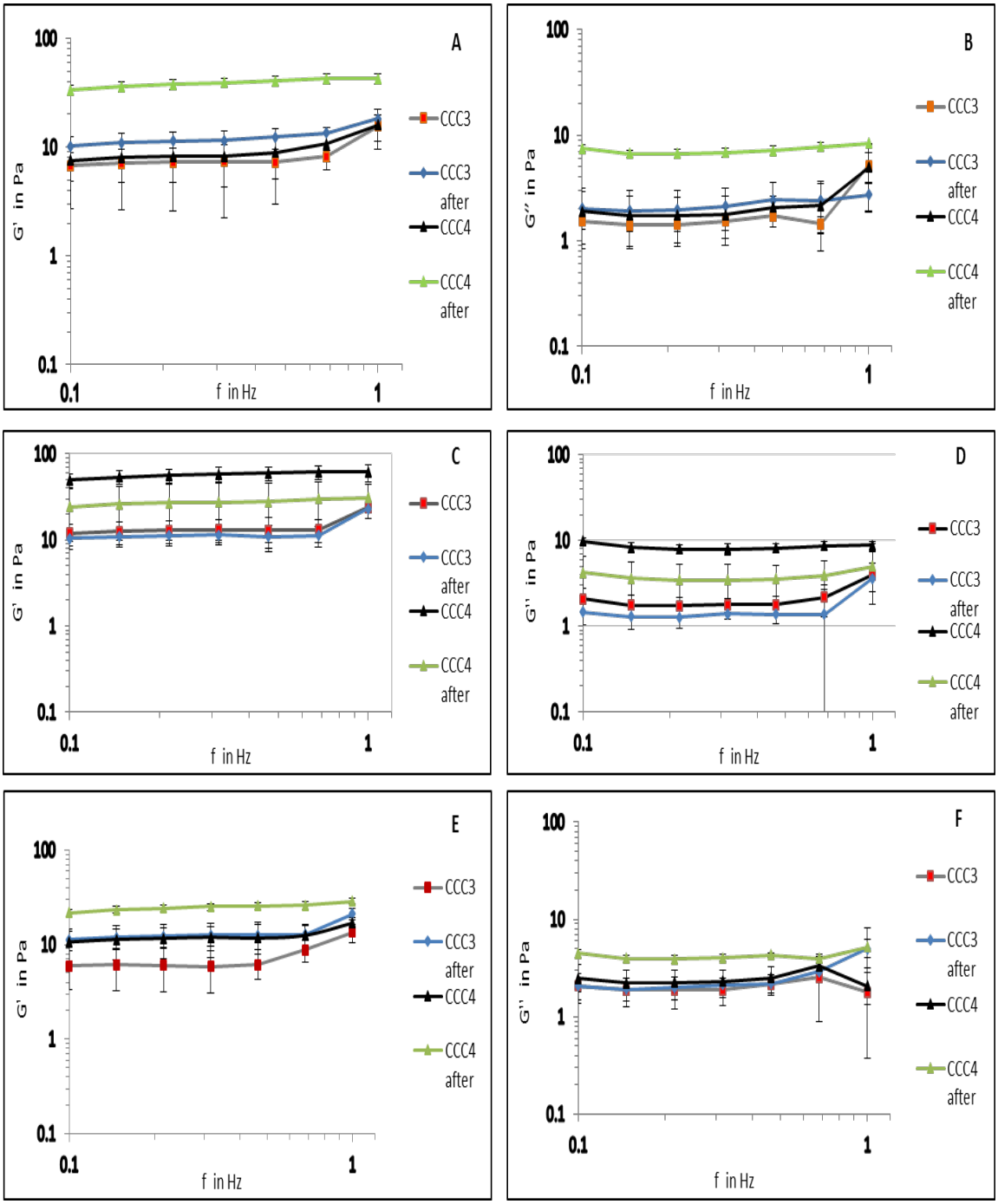



Figure 4.7: Rheology diagrams for *Pseudomonas mendocina* (A-B), *Rhodococcus koreensis* (C-D) and mixed cultures (E-F) crosslinked with CCC3 and CCC4, (n=3) before and after incubation with phenol.

### 4.3.4 Morphological structure

Scanning electron microscopy (SEM) images were taken only for CCC samples formed by activated crosslinking polymers CCC3 and CCC4 at two different concentrations. The SEM images of *P. mendocina* (figures 4.8, A and C), and *Rh. koreensis* (figure 4.9 E and G) and mixed samples (figure 4.10, I and K) revealed the macroporous structure of the cryogels. Cells were closely attached to each other forming a 3D structure with well-developed channels as a result of the cryo-structuration process. A visible thin layer of cross-linking polymer covering the cells was observed for all samples crosslinked with CCC3 polymers (figures: 4.8 B, 4.9 F and 4.10 J). Whereas, samples crosslinked with CCC4 showed a denser organization of cells with a decrease in the visible amount of crosslinking polymer on the cell surfaces (figures: 4.8 D, 4.9 H and 4.10 L).

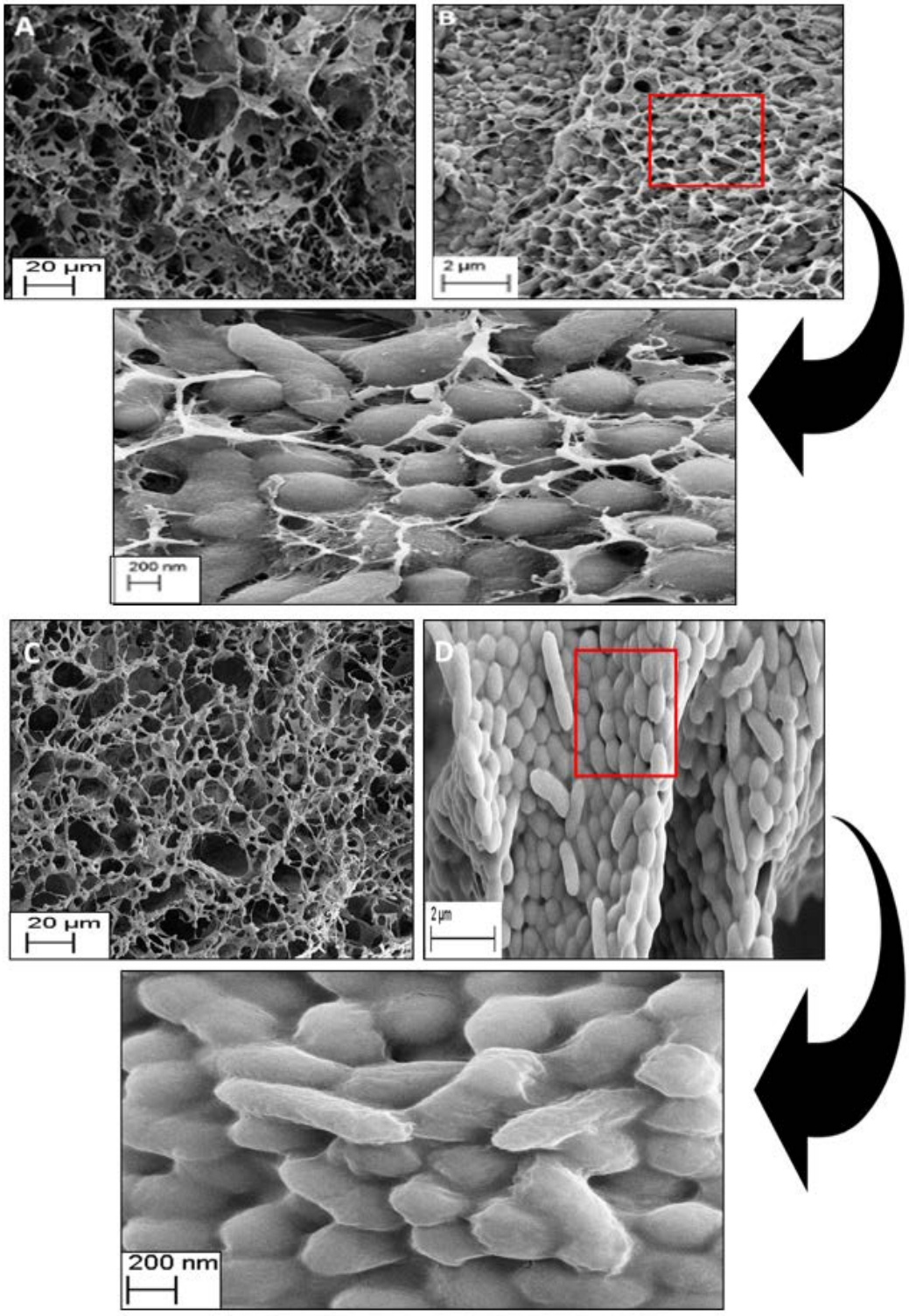



Figure 4.8: SEM images of *Pseudomonas mendocina*: (A, B) crosslinked with (CCC3) and (C, D) crosslinked with (CCC4).

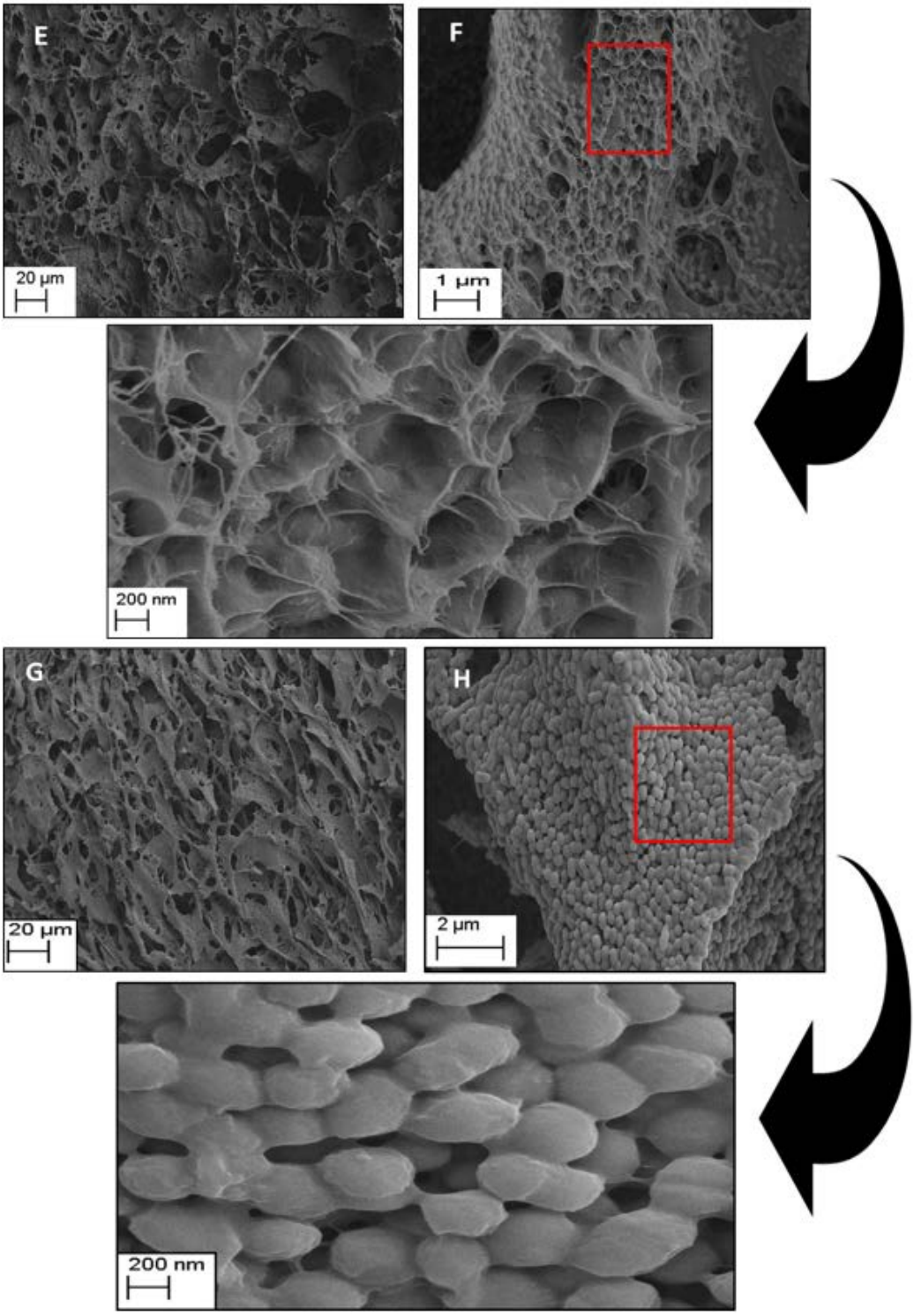



Figure 4.9: SEM images of *Rhodococcus koreensis*: (E-F) crosslinked with (CCC3) and (G-H) crosslinked with (CCC4).

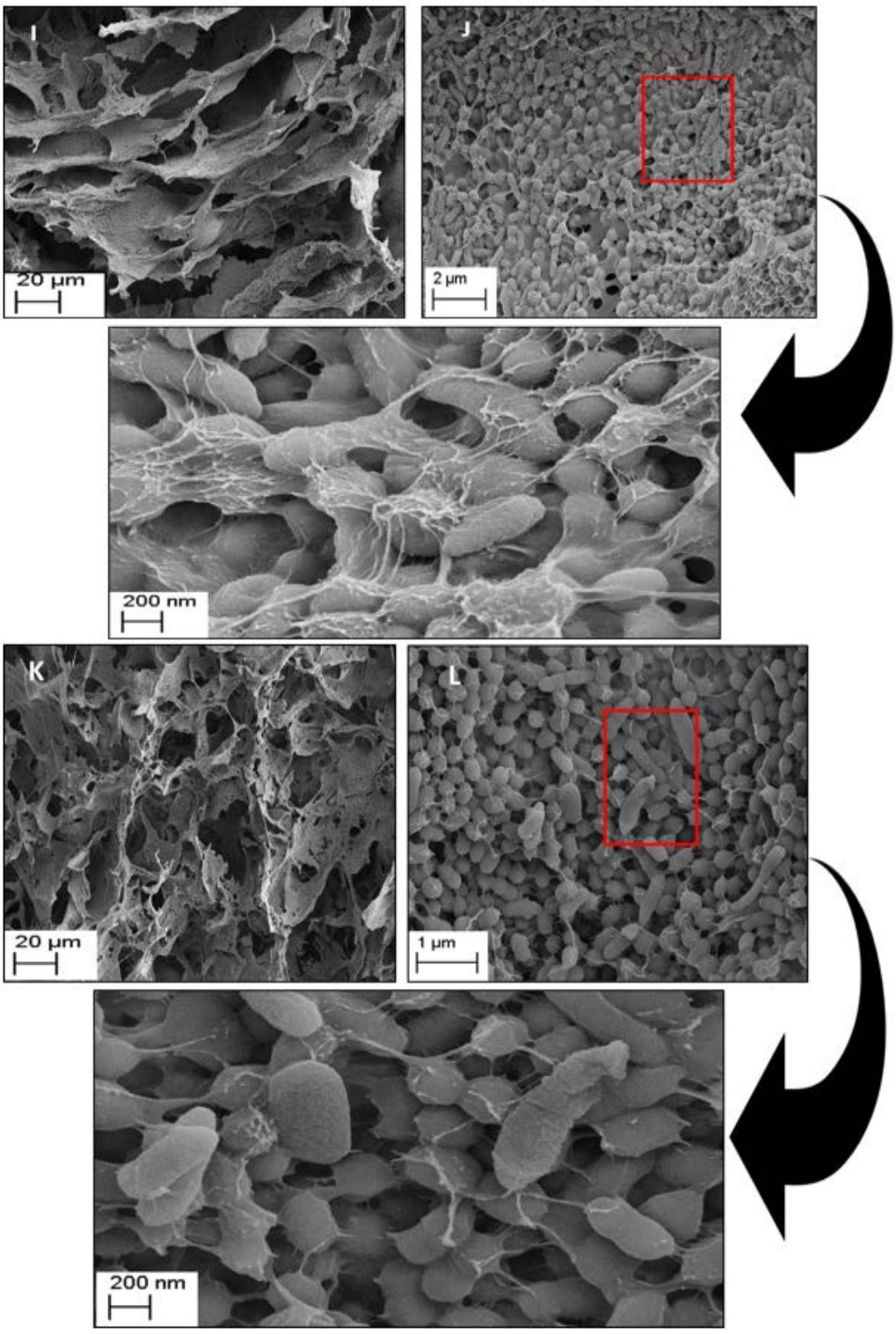



Figure 4.10: SEM images of mixed cells (*P. mendocina and Rh. koreensis*): (I-J) crosslinked with (CCC3) and (K-L) crosslinked with (CCC4).

## 4.3.5 Efficiency of bacterial cells to degrade phenol

### 4.3.5.1 Suspension of free cells

*P. mendocina* and *Rh. koreensis* have a documented ability to degrade aromatic compounds (Yoon *et al.*, 2000; Tian *et al.*, 2002). For this research, an estimation of their efficiency and of their mixture, to degrade phenol is plotted in figure 4.11.

In this experiment, suspension of pelleted cells of both individual strains and their mixture were incubated with sodium carbonate buffer supplemented with 50 mg/L of phenol under optimum conditions to check the cells' activity in the same conditions as crosslinked cells-cryogels. The results showed that free cells required more than a week to completely consume 50 mg/L phenol.

Approximately five days were required for both strains to adapt to the new environmental conditions before the exponential phase was observed. These results agreed with Ingraham *et al.* (1983) who stated that bacteria have a relatively long lag phase during adaptation.

In figure 4.11, *P. mendocina* cells showed slightly faster adaptation compared to *Rh. koreensis* alone and in with mixed cells. The latter, however, showed a steeper exponential phase compared with individual strains, probably because of competition between both species (Reardon *et al.*, 2002).

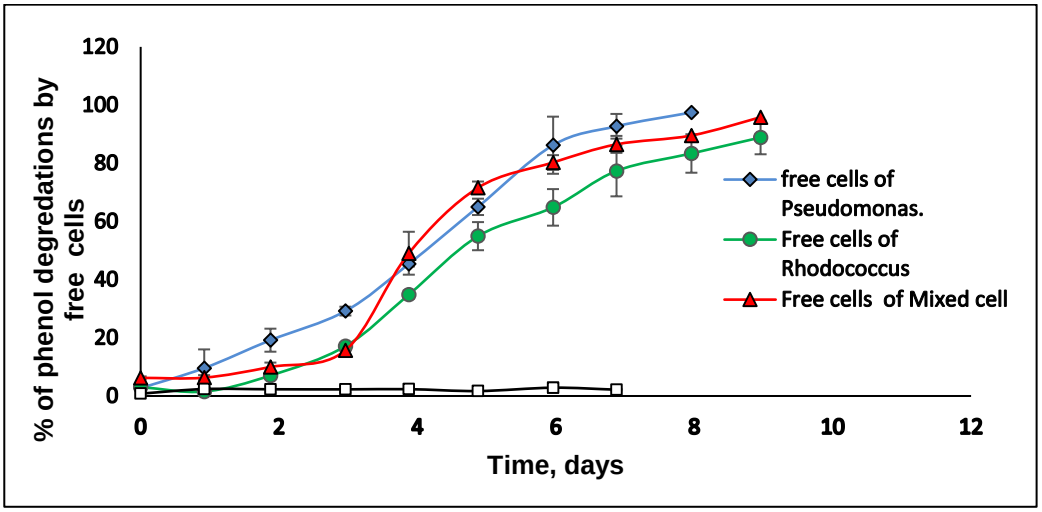



Figure 4.11: Degradation of phenol by the suspension of *P. mendocina*, *Rh. koreensis* and their mixture (1:1). (n=2).

#### 4.3.5.2 Fresh CCC samples

An initial assessment of phenol degradation by fresh CCC samples containing *P. mendocina* and *Rh. koreensis* crosslinked with the four crosslinking systems was made (results not shown). All CCC samples were used directly after preparation (freshly made) and there was no prolonged storage under the cryo-conditions. CCC samples crosslinked by GA (CCC1) and a combination of PVA+GA (CCC2) were destroyed at the end of first cycle for both strains and could not be reused for subsequent cycles.

Other samples prepared using *P. mendocina* using a combination of PVA-al + PEI-al (CCC3 and CCC4) showed better morphological stability and the remediation continued for seven days. Furthermore, they were reused for several cycles (figure 4.12). A slower rate of phenol degradation was observed during the first cycle which gradually increased over the subsequent slightly dropping again in the fifth bioremediation cycle. Phenol degradation rates for cells crosslinked with CCC3 for five cycles was as following: 0.005, 0.01, 0.011, 0.011, 0.012 mg/L. h with an average of 0.0098 mg/L/h. Fr CCC4 samples the rates were 0.0094 mg/h as an average of five cycles (0.0032, 0.0081, 0.01, 0.013 and 0.0122 mg/L/h. . Overall, the structural integrity and metabolic activity

of crosslinked cell-cryogels samples synthesised using (PVA-al 1% + PEI-al 0.25%) and (PVA-al 0.5 % + PEI-al 0.6 %) were stable over the five cycles of incubation (figure 4.12). Freshly prepared crosslinked cells showed faster adaptation to phenol spiked in bicarbonate buffer compared to free cells, although the samples were not activated after each cycle.

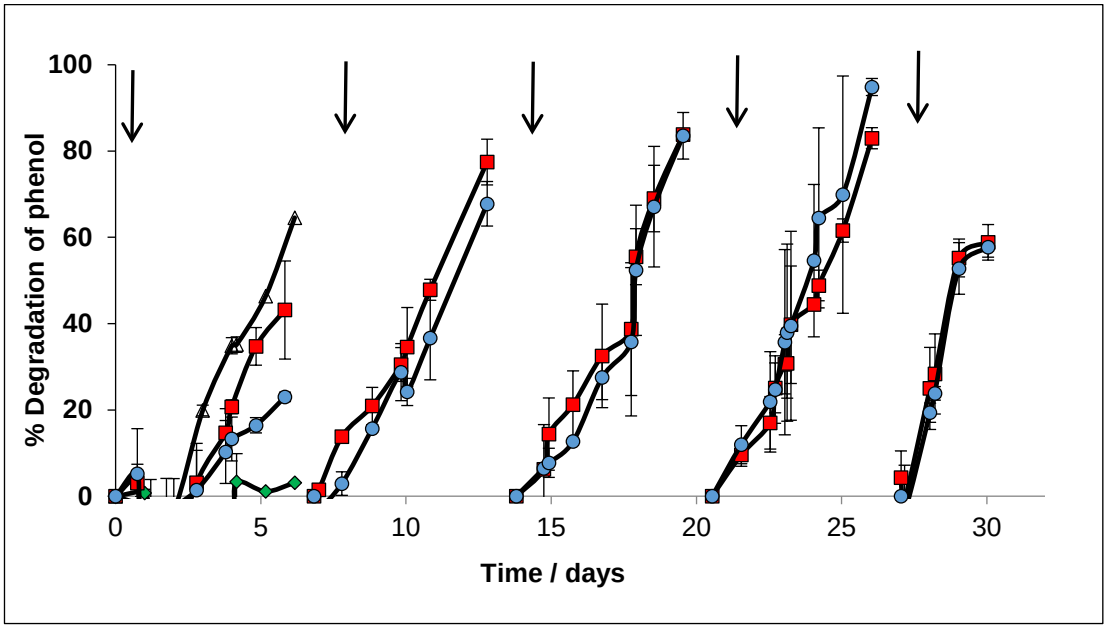



Figure 4.12: Percentage of phenol degradation in repeated treatment cycles by P. mendocina crosslinked with (CCC1 (∆), CCC2 (♦), CCC3 (■) and CCC4 (●) with a cross-linked number of cells equal to 5E+08 for all samples (n=2). * the arrow represents a new cycle.

### 4.3.5.3 The efficiency of CCC samples after storage in a frozen state.

The efficiency of CCC samples to degrade phenol after storage under frozen conditions was also evaluated. CCC samples of *Rh. koreensis* and a mixture of *P. mendocina* and *Rh. koreensis* (1:1) were thawed and tested for phenol degradation after storage at -80$^{o}$C for 4 weeks or more. Frozen samples of the cell mixture (*Rh. koreensis* and *P. mendocina* (1:1 volume) crosslinked with

polymers CCC1 and CCC2 were stored for four weeks at -80ºC. Following thawing at room temperature their structure was totally destroyed, whilst those crosslinked with CCC3 and CCC4 maintained their structures and shapes as one monolithic piece. Mixed cells crosslinked with CCC3 and CCC4 were stored for four weeks and tested for phenol degradation (figure 4.13). They showed a slower degradation rate of phenol during the first week of incubation, but over the following weeks showed a quicker degradation of phenol (0.002, 0.0097, 0.0107, 0.012 and 0.0132 mg/L/h) with an average for five cycles 0.0091 mg/L/h for samples crosslinked with CCC3 and 0.0002, 0.0045, 0.004, 0.0114, 0.0124 mg/L/h with as average of 0.0065 mg/h for samples crosslinked with CCC4.

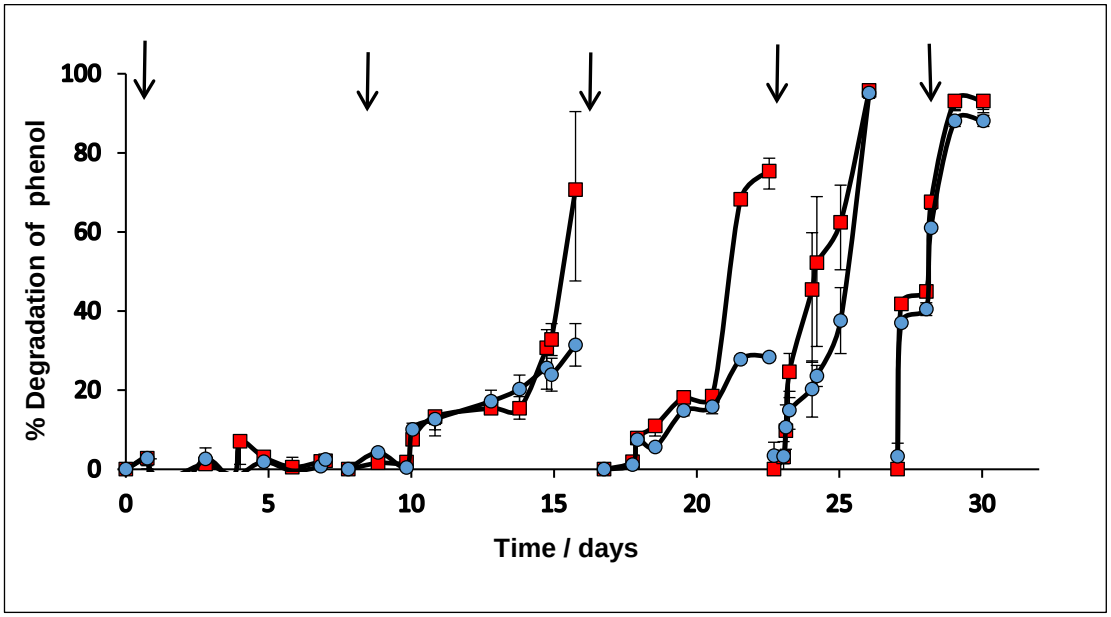



Figure 4.13: Degradation of phenol in repeat treatment cycles by mixed cells (*Rh. koreensis* and *P. mendocina*) crosslinked with CCC3 (■) stored in a frozen state at -80ºC (number of crosslinked cells = 2E+07); and CCC4 (●) stored in a frozen state at -80ºC.

Similar behaviour was also noted for *Rh. koreensis* that was stored for 6 weeks at -80ºC. It took over a week for them to recover and adapt to the incubation conditions with 50 mg/L of phenol in carbonate buffer (figure 4.14). Fresh

samples of CCC1 and CCC4 showed a faster degradation of phenol compared with frozen cryogels. Using the 4-amino antipyrine method the absorbance measurements for samples at the end of the bioremediation cycle were zero or slightly negative, showing that there was only a trace amount of phenol left after incubation over 6 days at 30$^{o}$C in the presence of carbonate buffer.

The amount of degradation rate for samples crosslinked with CCC3 was as following: 0.001, 0.00172, 0.0097, 0.01, 0.02 mg/L/h with an average for five cycles 0.007 mg/L/h, while the degradation rate for five cycles of CCC4 samples was as following: 0.0003, 0.003, 0.0088, 0.0102, 0.021mg/L/h with an average 0.0072mg/L/h. The near-complete degradation of phenol was confirmed using HPLC analysis (at a detection limit of 0.05 mg/L).

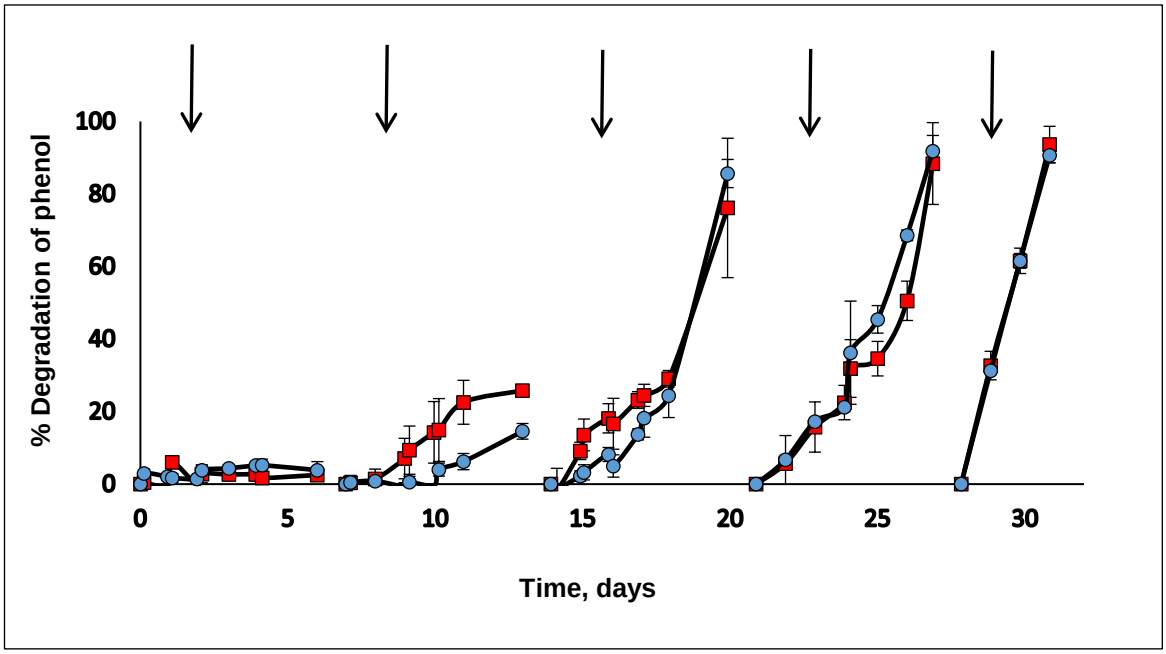



Figure 4.14: Degradation of phenol in repeat treatment cycles by *Rh. koreensis* crosslinked with CCC3 (■) and CCC4 (●) stored in a frozen state (-80$^{o}$C) with an initial number of cells equal to 5E+08.

#### 4.3.5.4 Estimation of viability of cells after phenol degradation

The viability of cross-linked cell-cryogels after exposure to 50 mg/L phenol solution was also assessed after a cycle of incubation using the MTT Assay (figure 4.15). The results show the percentage of live cells decreased for all crosslinked cell-cryogels containing *P. mendocina*, *Rh. koreensis* and their mixture (1:1) for both CCC3 and CCC4.

The percentage of viable cells of *P. mendocina, Rh. Koreensis* and mixed cells crosslinked with CCC3 decreased by more than 50% and less than 35% for CCC4 compared with initial percentages of fresh prepared samples

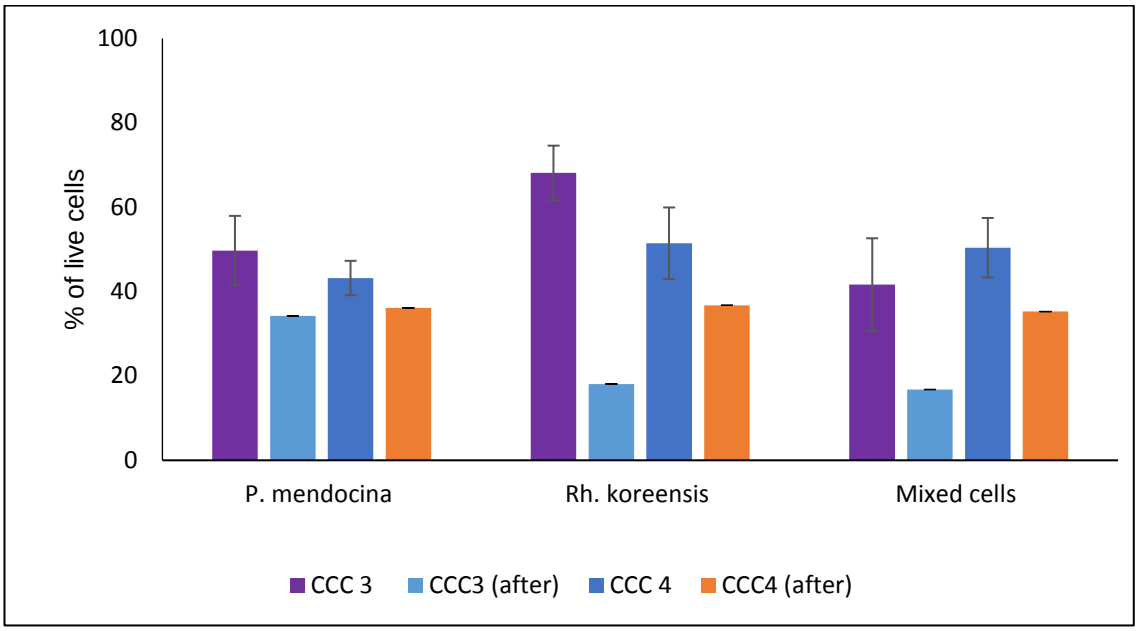



Figure 4.15: The viability of CCC samples with *P. mendocina, Rh. koreensis* and their mixture (1:1 volume) crosslinked with CCC3 and CCC4 before and after phenol degradation and relative to the number of cells used for cryogel preparation, taken as 100%.

## 4.4 Summary of results

The main findings in this chapter are summarised in the following points:

- Production of stable CCC samples of *P. mendocina, Rh. koreensis* and a mixture of both was achieved using the activated crosslinking polymers; CCC3 at concentration (PVA-al 1%+ PEI-al 0.25%) and CCC4 at concentration (PVA-al 0.5% + PEI-al 0.6%).
- Frozen suspension of cells showed about 30% less viability comparative with non-frozen cells. In addition, crosslinked cell-cryogels using both crosslinking polymers showed lower viable percentages for individual strains 45% to 68% and from 29% to 51% for mixed cells.
- Gel mechanical structures were confirmed, by demonstrating the elastic module G' over loose module G'' of CCC samples for all types of crosslinking polymers. However, CCC1 and CCC2 samples showed weaker stability following incubation with phenol in bicarbonate buffer and samples began to disintegrate after two days. On the other hand, activated polymers showed good ability to maintain their structure even after reuse for five cycles.
- Mechanical properties of CCC samples of *P. mendocina* was increased around two or three times of original percentage after incubation with phenol. Similar behaviour were also noticed for CCC samples of mixed cells but in less percentage. Meanwhile, *Rh. koreensis* samples that have decreased for G' about 11.75% and for G'' 24.49% for samples crosslinked with CCC3. Meanwhile, a decrease of 51.7% were noticed for G' and 54.58% for G'' for samples crosslinked with CCC4.
- Images of scanning electron microscopy SEM confirmed the CCC samples had a macroporous morphologies. CCC3 polymer were obviously appeared on the cell-cryogel surface, whereas, CCC4 were much tighter on the cells surfaces.
- Evaluation of the cells efficiency to degrade phenol showed that free cells required approximately ten days to degrade 50 mg/L phenol in sodium bicarbonate buffer under optimum conditions.

- Fresh CCC samples using *P. mendocina* produced by activated polymers CCC3 and CCC4 showed better performance to degrade phenol under the same conditions used for free cells. CCC3 & CCC4 were reused for five cycles, but CCC1 and CCC2 continued only for a week before being completely by the end of the.
- Frozen CCC samples using *Rh. koreensis* and mixed cells showed a slower rate of phenol degradation during the first cycle of incubation. Phenol degradation rates increased after the second cycle to achieve complete phenol degradation in the following cycles.
- The viability percentages of all CCC samples after incubation with 50 mg/L phenol were decreased comparative with percentages of CCC samples before incubation with phenol. The results showed that *P. mendocina* had lower percentages of dead cells with both polymers compared to *Rh. koreensis* and mixed cells which showed cell mortality of approximately 50% for CCC3 and 15% for CCC4 samples.

# Chapter 5 : Isolation, selection and identification of phenol degrading bacteria from oil-contaminated soil

## 5.1 Introduction

Following the successful production of crosslinked cell-cryogel samples (CCCs) using purchased commercial strains of *P. mendocina and Rh. koreensis* (chapter four), it was decided to test the efficacy of an indigenous bacterial strain isolated from oil contaminated soil. The premise being that indigenous and autochthonous bacteria from contaminated sites might be more effective in phenol degradation as they have adapted to those environmental conditions.

Therefore, in this chapter the results of the isolation and identification of phenol degrading bacteria from oil contaminated soil is presented. The utilisation of microorganisms as tools to remediate environmental contamination from industrial processes has attracted attention of many researchers (Vidali, 2001; Koutny *et al.*, 2003; Singleton, 2004; Arutchelvan *et al.*, 2005; Kotresha and Vidyasagar, 2008; Li Jing *et al.*, 2017a; Al-Hadithi *et al.*, 2017). Samples from contaminated soils (Koutny *et al.*, 2003), sediments and wastewaters (Arutchelvan *et al.*, 2005) and various sludges (Mao *et al.*, 2015) have been investigated as unique sources for the isolation of bacterial species with the ability to transfer complex xenobiotic materials into simple compounds (Koutny *et al.*, 2003).

Several bacterial species have been isolated and applied in various ways to treat phenol contamination in the environment, such as *Pseudomonas spp.* (Koutny *et al.*, 2003; Lakshmi *et al.*, 2009; Mohanty and Jena, 2017), *Rhodococcus spp*. (Yoon *et al.*, 2000a; Kuyukina *et al.*, 2009; Hristov *et al.*, 2016), *Bacillus spp*. (Banerjee and Ghoshal, 2011; Topalova *et al.*, 2014) and *Acinetobacter spp*. (Yamaga *et al.*, 2010; Jiang *et al.*, 2013; Fatajeva *et al.*, 2014; Al-Hadithi *et al.*, 2017). These bacteria possess proficient enzyme pathways with the ability to

transform complex aromatic compounds into simpler forms (Coates *et al.*, 1997; Fritsche and Hofrichter, 2008; Ahmad *et al.*, 2017). No specific individual or groups of bacteria have been found to be universally applicable for bioremediation, since the isolation site may have an impact on the organisms' properties (Nawawi, 2014).

Therefore, the isolation of microbes from contaminated sites might provide potentially more active tools for the bioremediation of specific contaminated environments.

## 5.2 Aim and objectives

The aim of this chapter is the isolation and selection of a robust phenol-degrading bacteria from oil contaminated soil samples. The bacteria isolated will be identified using standard biochemical and molecular methods.

The following objectives will be used to achieve this aim:

- Collection of oil contaminated soil samples from different locations in Basrah city/ southern Iraq, near the South Oil Company (SOC) (August 2015).
- Isolation of robust bacterial species with a high efficiency to degrade phenol. Screen and select the best performing and phenol-tolerant strain(s) to degrade phenol.
- Identify the bacterial strain(s) using morphological and biochemical tests including Gram stain, oxidase test, catalase test, motility test, phenylalanine deaminase test, API 20E and API 20NE kits.
- Apply genomic identification tests 16SrDNA polymerase chain reaction and amplified restriction DNA analysis (ARDRA) for identification to the strain level.

## 5.3 Results

### 5.3.1 Morphological and biochemical identification of the phenol-degrading bacteria

Following 24 hours incubation at 37ºC on TSA and MSM agar plates supplemented with 100 mg/L of phenol and 0.5% glucose, colonies of suspected phenol degrading bacteria were observed. Their morphology was yellowish-white to pale yellow colonies approximately 1 mm in diameter with a smooth surface and convex shape. Results of Gram staining showed small Gram-negative short rounded rods (coccobacilli). Their microscopic morphology was confirmed using laser scanning confocal microscopy (LSCM) at 20X magnification. The cells showed a diplococcus morphology, 1.19-1.30 µm long and from 876 nm-1.06 µm wide (figure 5.1, C and D).

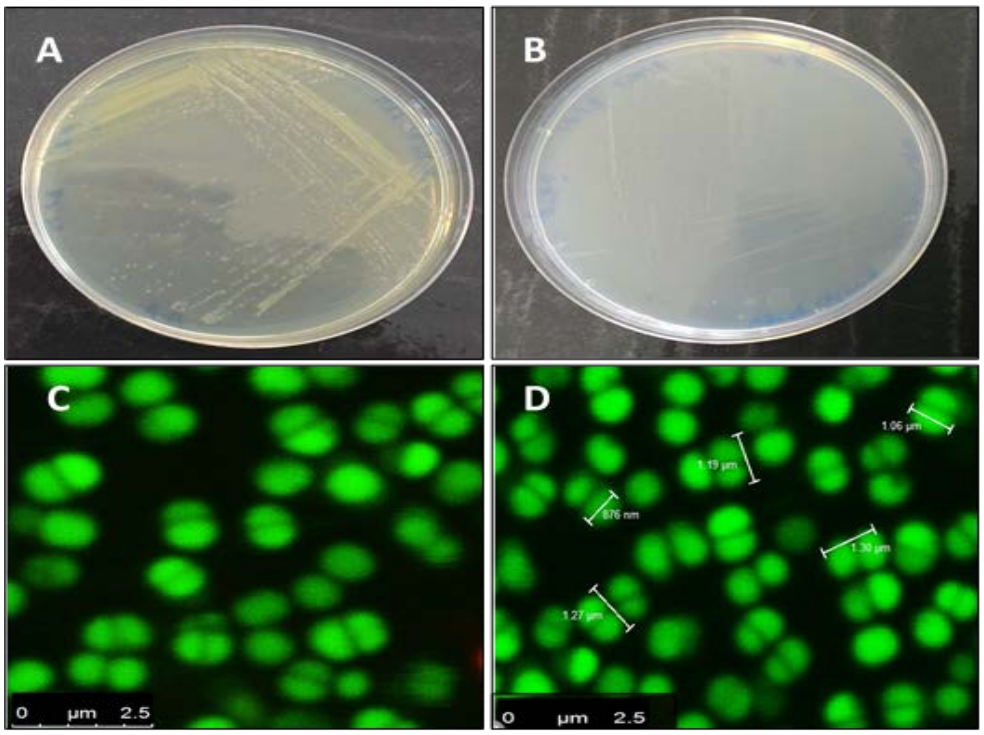



Figure 5.1: Morphological structure of isolated phenol degrading bacteria: A) shape and colour of colonies on TSA plate, B) colonies on MSM with phenol and

glucose, C) cells shape under LSCM, and D) width and length measurement of the cells.

Colonies on MacConkey agar plates were colourless, indicating to non-lactose fermenting cells. Moreover, colonies on 5% sheep blood agar plates were pale-yellow without any haemolysis indicating the bacterial cells were non-haemolytic (gamma haemolysis). (Figure 5.2 A, B).

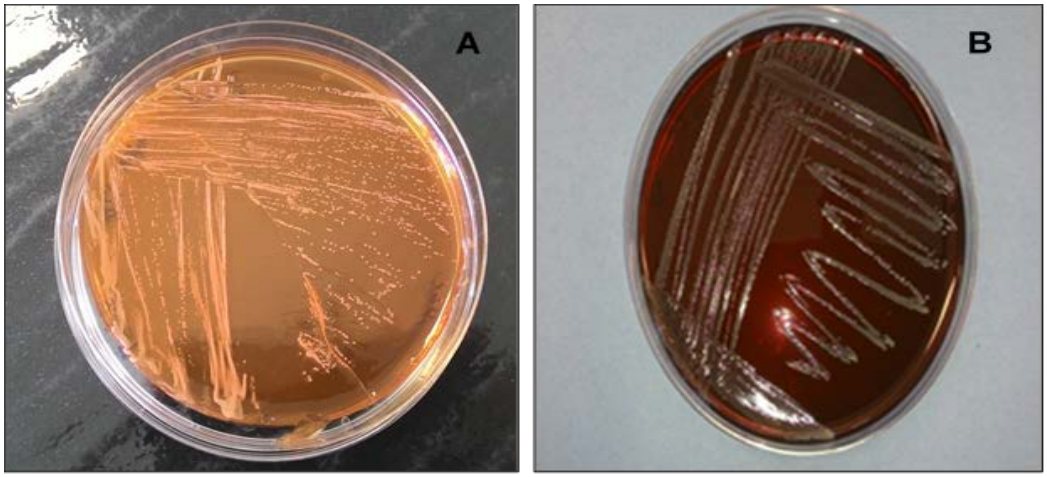



Figure 5.2: Growth of bacterial cells on differential-selective agar plates: A) MacConkey agar plate and B) Blood agar plate 5% sheep.

Other results were as follows: motility test in the agar-tube diffusion demonstrated the non-motile nature of the cells after 24 hours incubation at 37$^{o}$C; the catalase test was positive, and the oxidase and phenylalanine deaminase tests were negative (figure 5.3).

To further identify the genus and species of the isolated Gram-negative bacterium, the API 20E biochemical test strip for Gram-negative bacteria (*Enterobacteriaceae*) was applied. The results following incubation were converted to numbers (API-Analytical Profile Index) and used to identify the genus and species of the bacterium via the API online database (Biomeriexu, 2015). Unchanged colours due to non–fermentative reactions in many of the micro-tubes after 48 hours incubation figure 3D precluded a definitive identification for fermentative *Enterobacteriaceae.*

Consequently, the API 20NE test for Gram-negative non-fermentative bacteria was employed and the results of reaction and interrogation of the online database indicated that the unknown phenol-degrading bacterium had a 96.7% percentage of similarity to *Acinetobacter radioresistens*.

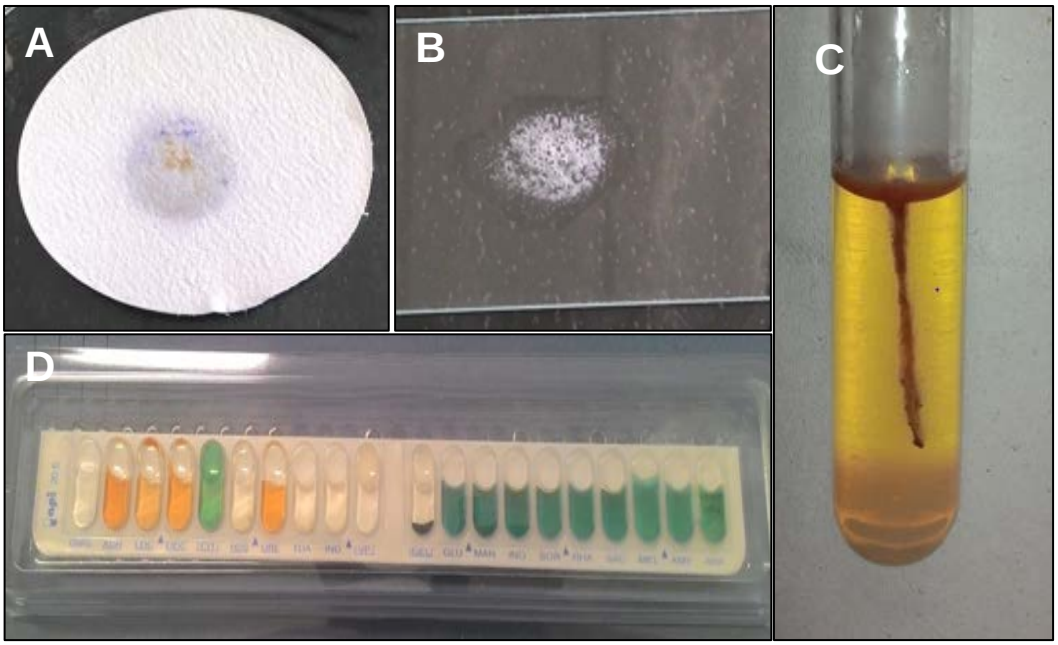



Figure 5.3: Biochemical tests: A) Oxidase test, B) Catalase test, C) Motility test and D) API 20E test strip after adding test reagents and incubating at 37ºC for 48 hours.

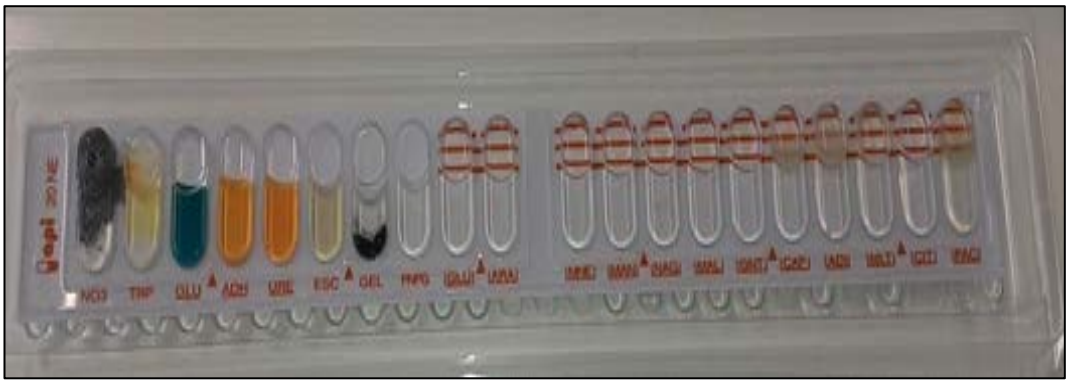


Figure 5.4: API20NE strip test after 48 hours incubation at 37ºC and adding test reagents.

### 5.3.2 Genetic identification

The 16S rDNA amplicon from the isolated presumptive strain of *Acinetobacter radioresistens* (A1, A2) was electrophoresed with two known strains of *Acinetobacter spp.* (W and R) obtained from the University of Brighton culture collection in replicate samples. Amplicons with a molecular size of 1500bp indicated the presence of 16srDNA for all *Acinetobacter* strains with absence of any bands in control samples (figure 5.5).

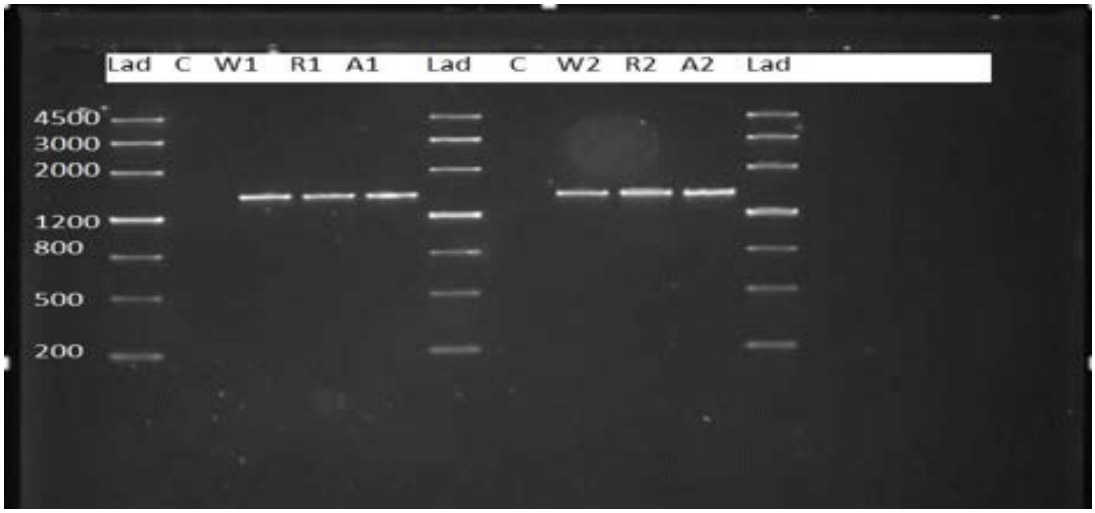



Figure 5.5: 16S rDNA amplicons of Acinetobacter spp. on 1.5 % agarose gel.

Strains W1 and 2 and R1 and 2 are *Acinetobacter* spp. provided from the culture collection; A1 and 2 are the presumptive strain of *Acinetobacter radioresistens* isolated from oil-contaminated soil. C is a DNA negative control.

Sequence analyses of the 16S rDNA amplicons were carried out by Eurofins Genomics, and the sequence data compared with data in Gene-bank database using the RDP web site:

http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp. The data for W1 showed a similarity range of 98-99.9% for *A. lowffii (T); DSM2403; X81665* and R 1 as 97.9-98.8% for *A. radioresistens (T); DSM6976; X81666* for the culture collection strains. Whereas, the isolated strain from oil-contaminated soil

showed 98-99% similarity to *A. radioresistens (T); DSM6976; X81666.* All sequence analysis data are presented in appendix 2.

Confirmation of these results was done by applying the ARDRA method. 16S rDNA PCR was applied as a first step in the ARDRA typing method to ascertain the presence of DNA amplicons at 1500bp. The results of 16S rDNA PCR electrophoresis for both methods Dneasy and Microlysis extraction kits were shown in figure 5.6.

Both methods produced amplicons at 1500bp, although the DNA extracted by MicroLysis was less well defined with an extra band for strain W. Amplicons from the Dneasy kit were clearer, stronger and lacked the anomalous band. For this reason, DNA extracted with the Dneasy Kit was used for subsequent DNA extractions for the ARDRA method. ARDRA was performed by mixing 2 or 3 µL of the amplified DNA with 1 µL of each restriction enzyme and following incubation for two hours, electrophoresed. A few bands appeared due to the cutting of the DNA at specific locations depending on the type of restriction enzyme. In this case, bands were seen only for *Acinetobacter spp.* W and R but not for the strain isolated from oil-contaminated soil (figure 5.7).

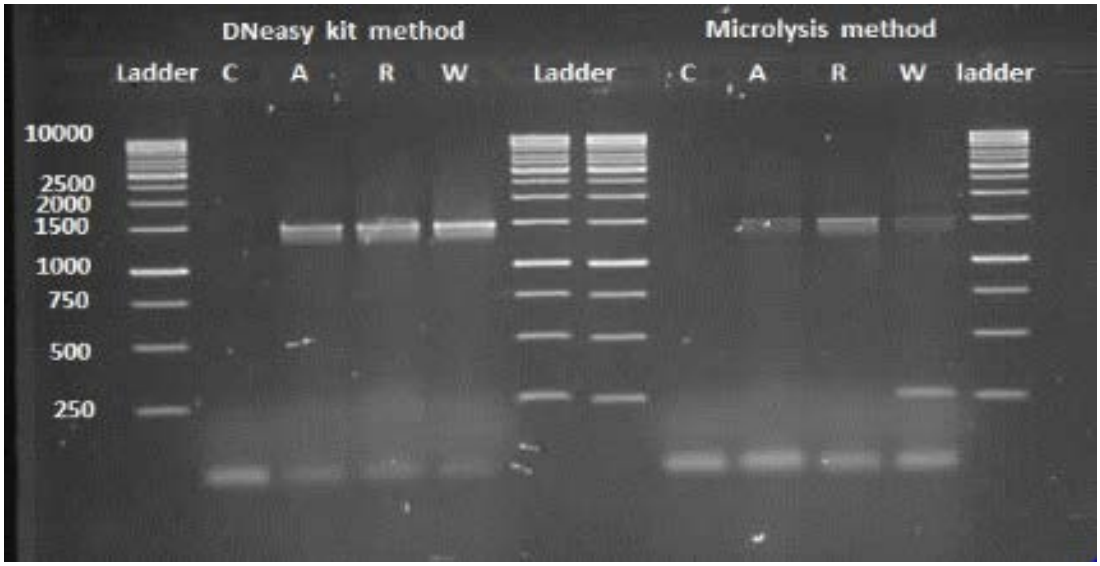



Figure 5.6: Amplification of 16S rDNA gene of presumptive *Acinetobacter* species: A, W and R on 3% agarose gel; C: DNA negative control.

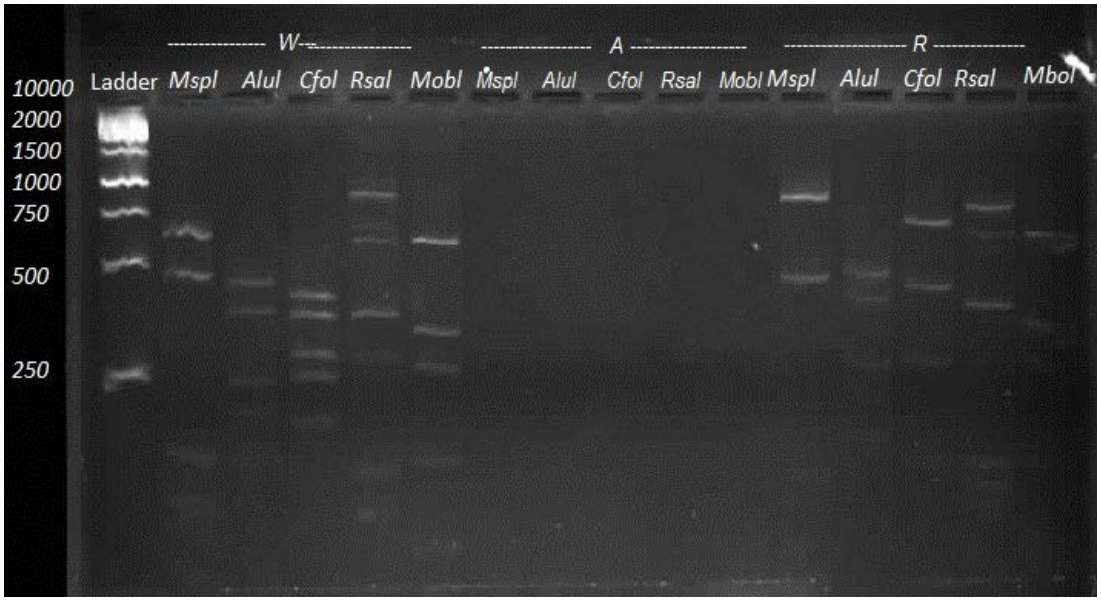



Figure 5.7: Amplification of DNA of *Acinetobacter spp*. W, A and R cut with restriction enzymes on 3% agarose gel.

Therefore, the experiment was repeated using the same incubation conditions with two concentrations of DNA and incubation time. Bands for A1 appeared as a result of adding 2 µL of DNA with the incubation time reduced to 1.5 hour, whilst for A2 the volume of the amplified DNA was increased to 3 µL and the incubation time extended to 3 hours.

The results of these condition are shown in figure 5.8. For A1 some bands appeared only for samples incubated with *MspI*, *CfoI* and *RsaI*, whilst conditions for A2 led to clear bands for all the restriction enzymes. The location of the DNA bands for each restriction enzyme was compared to the restriction patterns for *Acinetobacter spp*. assigned previously to genomic species according to Dijkshoorn *et al.* (1998). The band sequences were converted to numbers that represent patterns of bands obtained for each type of restriction enzyme, described in table 5.1.

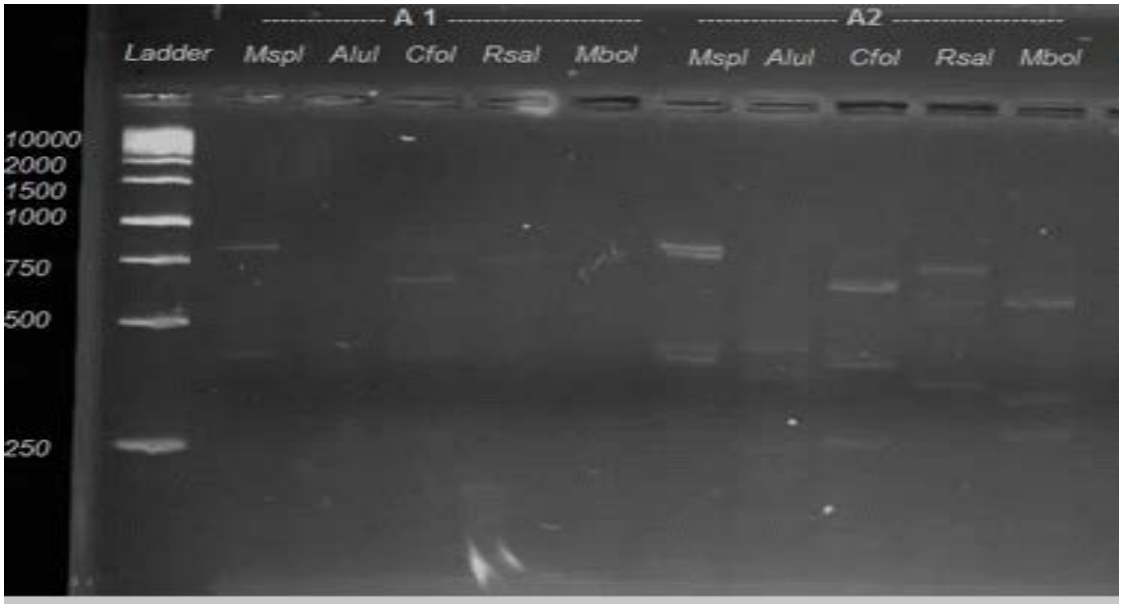



Figure 5.8: Amplification DNA of isolated strain of *Acinetobacter spp*. cut with restriction enzymes on 3% agarose gel under different conditions: A1) 2 µL of DNA and incubation for 1.5 hour, A2) 3 µL of DNA and incubation time for 3 hours.

Table 5.1: ARDRA profile for Acinetobacter spp. as previously identified to genomic species

| **Genomic species** | **Enzymes pattern** | | | | | **Ref.** | **Strain type** |
|---|---|---|---|---|---|---|---|
| | ***CfoI*** | ***AluI*** | ***MboI*** | ***RsaI*** | ***MspI*** | | |
| *A. lowffii* *(W)* | 3 | 3 | 2 | 1 | 2 | *NCTC *5866$^T$* | Culture collection |
| *A. radioresistens* (R) | 7 | 3 | 2 | 2 | 3 | **IAM131 86T | |
| ***A. radioresistens*** **(A)** | **7** | **3** | **2** | **2** | **1** | ***SEIP 12.81** | **Isolated** |

*NCTC: National Collection of Type Cultures, London, United Kingdom

****IAM**: Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan

***** SEIP, Service des Enterobacteries de I' Institut Pasteur, Paris, France (Dijkshoorn *et al.*, 1998).

### 5.3.3 Tolerance to elevated concentrations of phenol

A tolerance assessment of the isolated phenol degrading bacterium identified as *Acinetobacter radioresistens* was carried out to check the ability to degrade phenol at higher concentrations. Results of triplicate samples of pelleted cells after incubating in MSM broth supplemented with concentrations of phenol ranging between 100 to 400 mg/L for each experiment were plotted as the relationship between cell growth and phenol consumption as a negative association. Consumption of phenol was increased parallel with increasing the growth of cells, figure 5.9.

At 100 mg/L phenol the bacterial cells adapted quickly unlike samples incubated at high concentrations (400 mg/L) of phenol. An extension of the lag phase as concentrations of phenol were increased indicated the cells' requirement for adaptation to the new conditions.

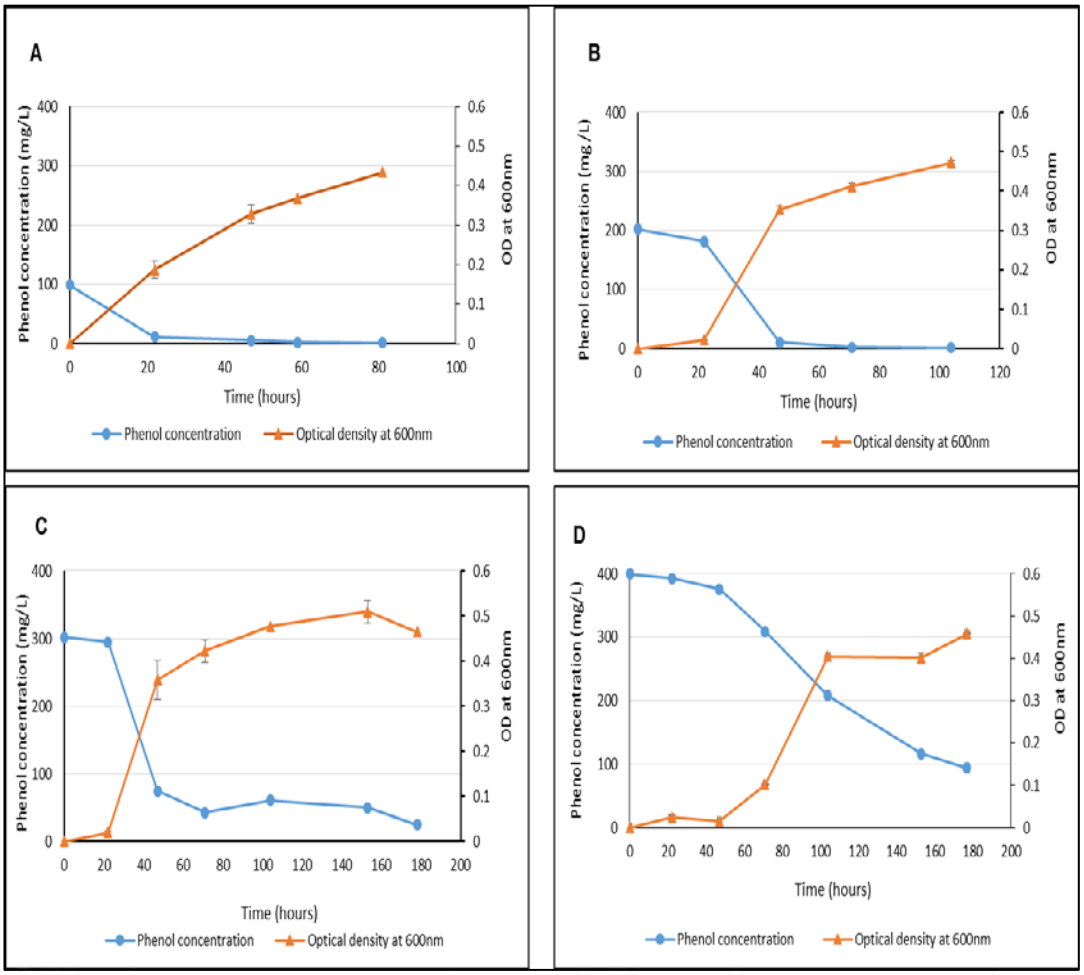



Figure 5.9: Phenol tolerance for the isolated bacterial strain at a range of different concentrations of phenol: A) 100mg/L, B) 200 mg/L, C) 300 mg/L and D) 400mg/L.

## 5.4 Summary of results

Results of this chapter can be summarised in the following points:

- The isolated bacterial strain grew on tryptone soya agar (TSA) and mineral salt medium (MSM) agar supplemented with 100 mg/L of phenol and 0.5% of glucose.
- Results of the Gram stain, and growth on selective and differential agars indicated that the isolated bacterium was a Gram negative short rod (coccobacillus) in the early stages of growth becoming more coccoid at the stationary phase. It was also a non-lactose fermenter and non–haemolytic.

- Other characteristics included a positive catalase test, negative oxidase and phenylalanine deaminase tests, and a lack of motility.
- A presumptive initial identification was not achieved using the API20E biochemical strip after 48 hours incubation because the unknown strain was non-fermentative, thus the API20NE test for non-fermentation bacteria was applied, resulting in a 96.7% similarity to Acinetobacter radioresistens.
- 16SrDNA amplicons from the isolated presumptive strain Acinetobacter radioresistens were electrophoresed with two known strains of Acinetobacter spp. and all produced a 1500bp amplicon indicative of 16S rDNA .
- Sequence analysis of the extracted and purified 16SrDNA amplicons was carried by Eurofins Genomics. The data were compared with the Gene-bank database on the RDP website, and the results of duplicate samples of the culture ‘W’ showed a similarity of 98 to 99.9% to Acinetobacter lowffii; the strain ‘R’ had a 97.9 to 98.8% similarity to *Acinetobacter radioresistens*, which was similar to the isolated strain ‘A’ in with 98 to 99% similarity to Acinetobacter radioresistens.
- Confirmation the identity of A. radioresistens was achieved by Amplified Ribosomal DNA Restriction Analysis (ARDRA) by applying five restriction enzymes Mspl, Alul, Cfol, Rasl, andMobl. Electrophoresis results were compared with band patterns for each restriction enzyme assigned previously for Acinetobacter spp. (Dijkshoorn et al., 1998).
- The isolated strain showed tolerance and the ability to degrade 100 mg/L to 400 mg/L phenol, and exhibited a longer lag phase with increasing concentrations of phenol.

# Chapter 6 : 3D macroporous cryogels of *Acinetobacter radioresistens* to degrade phenol

## 6.1 Introduction

This chapter presents details on the production of 3D macroporous cryogels using *Acinetobacter radioresistens* isolated from oil-contaminated soil and the assessment of their ability to degrade phenol under various conditions. *Acinetobacter* have been used in several biotechnical and environmental applications (Navon-Venezia *et al.*, 1995; Pessione *et al.*, 1999; Boswell *et al.*, 2001; Griva *et al.*, 2003; Divari *et al.* , 2003).

## 6.2 Aim and objectives

The aim of this chapter is to produce 3D macroporous crosslinked cell- cryogels using the indigenous, phenol-degrading strain of *Acinetobacter radioresistens* isolated and identified in chapter five.

To achieve this aim the following objectives will be met:

- Using the modified crosslinking polymers CCC3 and CCC4 (will be used to produce 3D monolithic CCC samples containing the indigenous, phenol-degrading strain of *Acinetobacter radioresistens.*
- The CCC sample swill be evaluated for cell viability using the MTT assay, their mechanical properties by rheometry, their morphological structure using scanning electron microscopy (SEM) and their efficiency to degrade phenol.
- An assessment of the effect of media composition, pH and temperature on their efficiency to degrade phenol and the viability of the bacterial cells.
- The viability of the CCC samples and their efficiency to degrade phenol under optimum conditions will be assessed following storage as freeze-dried samples.

## 6.3 Results

## 6.3.1 Characterization of crosslinked cells samples

### 6.3.1.1 Mechanical properties

The mechanical properties of crosslinked cell-cryogels (CCC) containing *A. radioresistens* were evaluated by subjecting four samples of each type of polymer to rheometry, and the average value was plotted and presented in figure 6.1. Generally, for all samples a liner shape of storage module (elastic) G' was commanded loose module (viscous) G'' for both type of polymers (CCC3 and CCC4) at frequencies ranged between 0.1 -1 Hz and confirmed their gelled structure.

Figure 6.1A and B, show the results of the evaluation of the G' and G'' moduli for samples of the crosslinking polymers CCC3 and CCC4. CCC3 samples showed better mechanical performance than CCC4 samples at an initial evaluation following the cryogelation process.

A second assessment of mechanical behaviour of the CCC samples was performed after incubation with phenol in MSM for one week under optimum conditions (figure 6. 1, C and D). CCC4 samples kept their structure and mechanically increased about 28.3% for G' and 23.5% for G'' comparative with initial values before incubation. Whilst, CCC3 samples showed an unconventional curve that decreased about 68% and 13.6% after incubation with 50 mg/L of phenol at MSM for both moduli G' and G'' moduli, respectively.

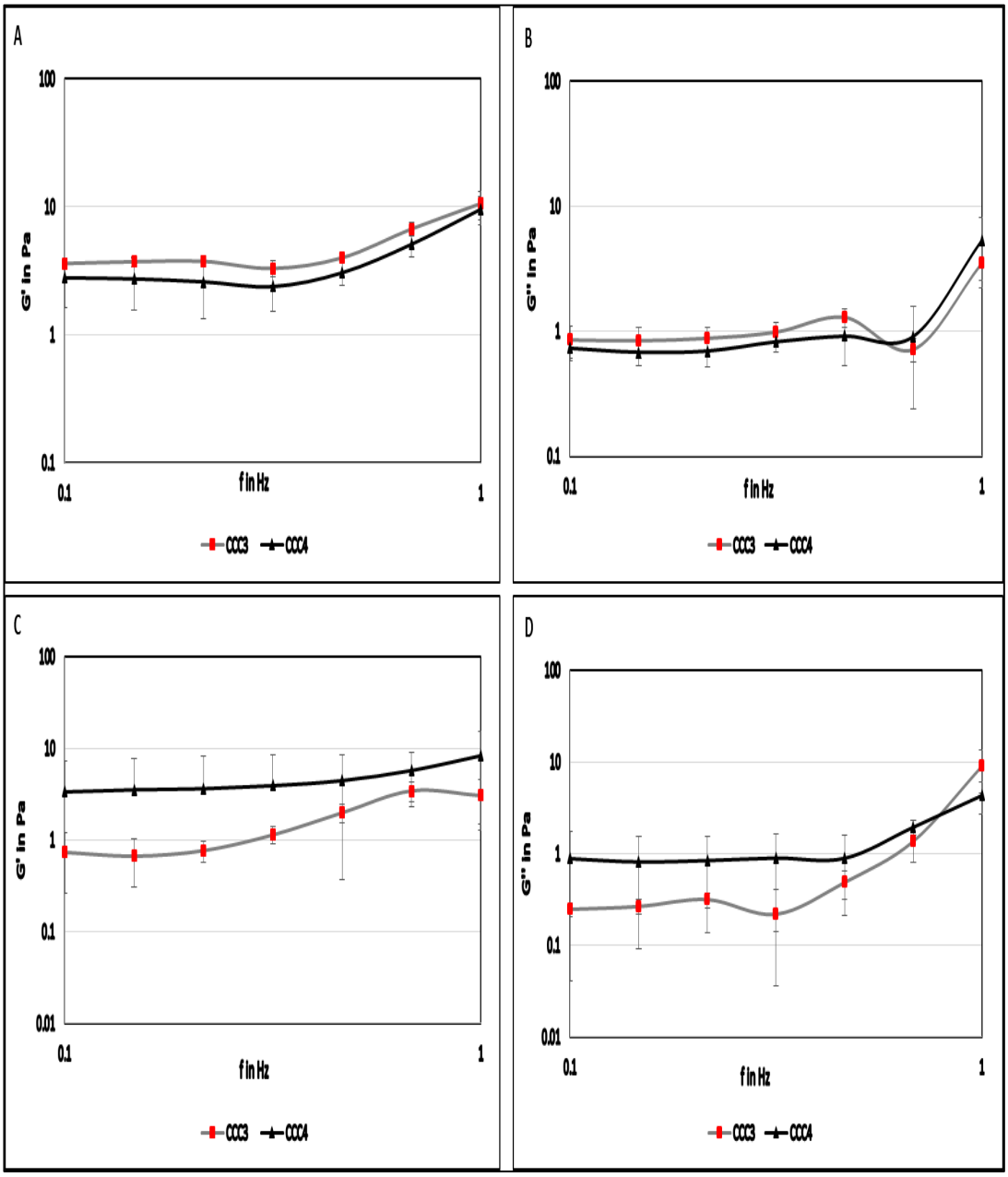



Figure 6.1: Mechanical properties of crosslinked cells samples for *A. radioresistens.* A and B) fresh samples (and C and D) after incubation with phenol.

### 6.3.1.2 Viability

Assessment of cell viability was recorded for samples made with crosslinking polymers CCC3 and CCC4, with frozen planktonic cells as a control using the MTT assay. Triplicate samples for each crosslinking polymer were used to assess the initial percentage of viable cells at the end of cryogelation process.

The viability results for CCC3 (PVA-al 1%+ PEI-al 0.25%) and CCC4 (PVA-al 0.5% + PEI-al 0.6%) samples are presented in figure 6.2. CCC3 samples showed 87% viability and CCC4 samples 81%, compared to free (planktonic) frozen samples at 100%.

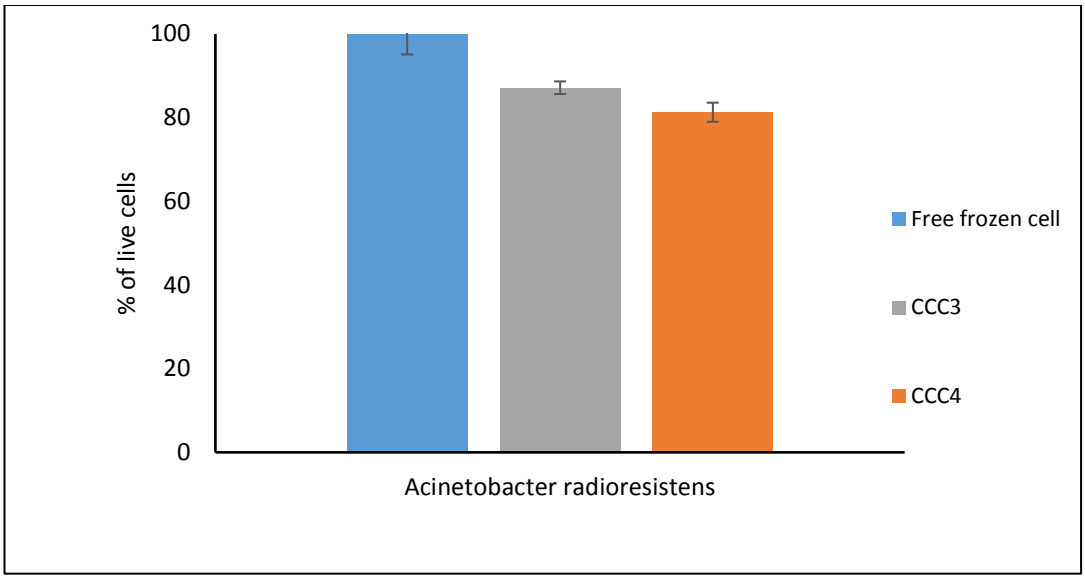



Figure 6.2: Viability of cells for *A. radioresistens* crosslinked with activated polymers

### 6.3.1.3 Morphological structure

The morphology of the crosslinked cell-cryogels with *Acinetobacter radioresistens* were evaluated through scanning electron microscopy (SEM). SEM images displayed the macropours structure of samples produced with the crosslinking polymers CCC3 and CCC4. The bacterial cells were organised as layers connected at multipoints around spaces formed as a result of melting ice crystals at room temperature (figure 6.3).

Macroporous structure and a tight arrangements of cells can be seen at higher magnifications for both CCC3 and CCC4. In CCC3 samples, all the cells were covered with visible polymers, whereas samples produced from CCC4 had less polymer on the surface and the bacterial cells were clearly organised.

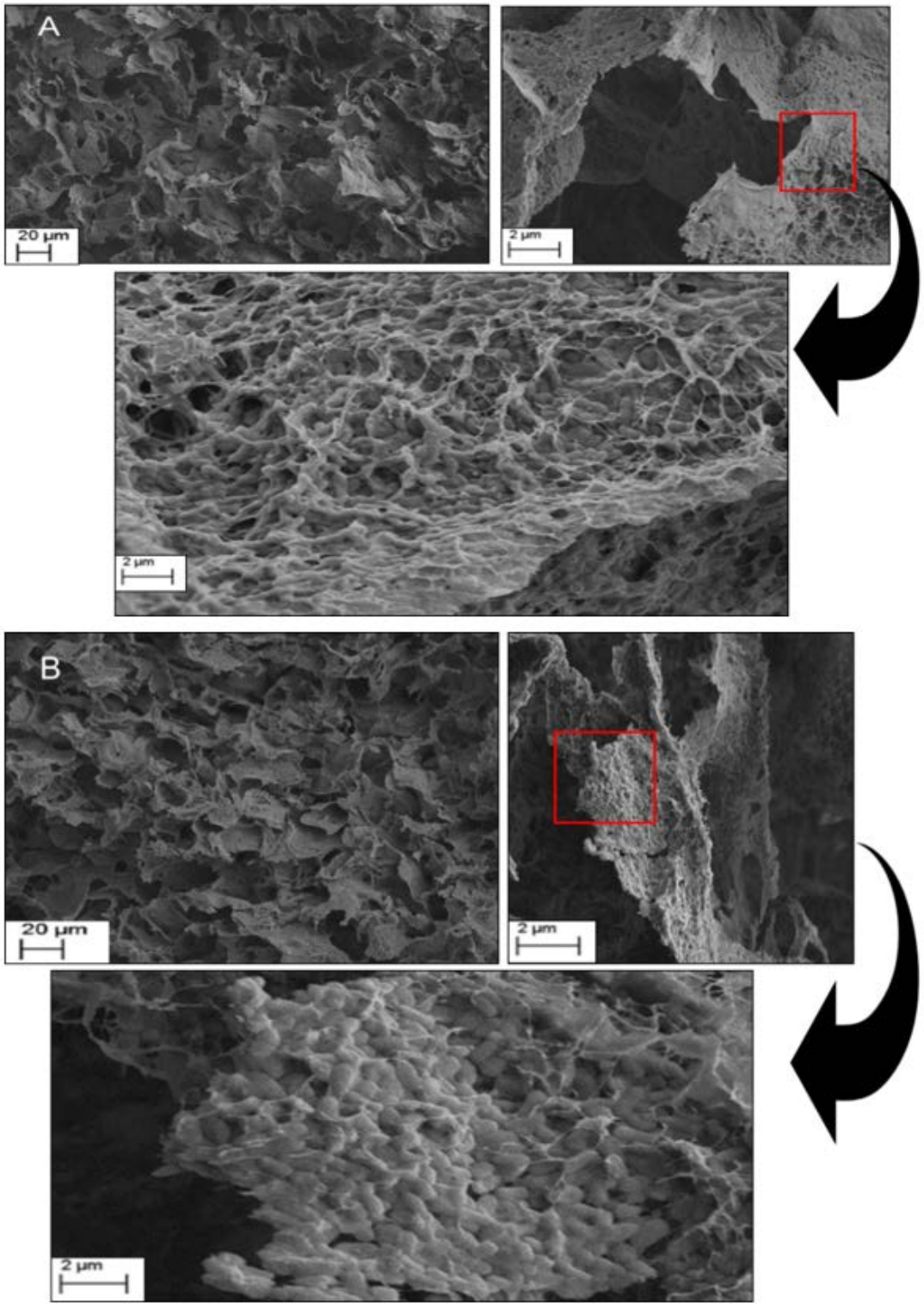



Figure 6.3: SEM images of *Acinetobacter radioresistens* crosslinked by modified crosslinking polymers: A) CCC3 and B) CCC4, various magnifications of images started with 500X, 5.00 KX and 10.00 KX, respectively

A second assessment of the morphology of the CCC samples after exposure to phenol was done by reimaging the samples after a week of incubation with 50mg/L phenol in MSM broth under optimum conditions. SEM images at low magnification showed that both CCC samples maintained their macroporous structure and shape as monolithic units. At higher magnifications cells appeared slightly thicker and were covered with transparent veil in addition to polymers compared with the non-phenol exposed samples in figure 6.3 A and B. It was difficult to distinguish individual cells among the cells with high adhesion (figure 6.4, C and D).

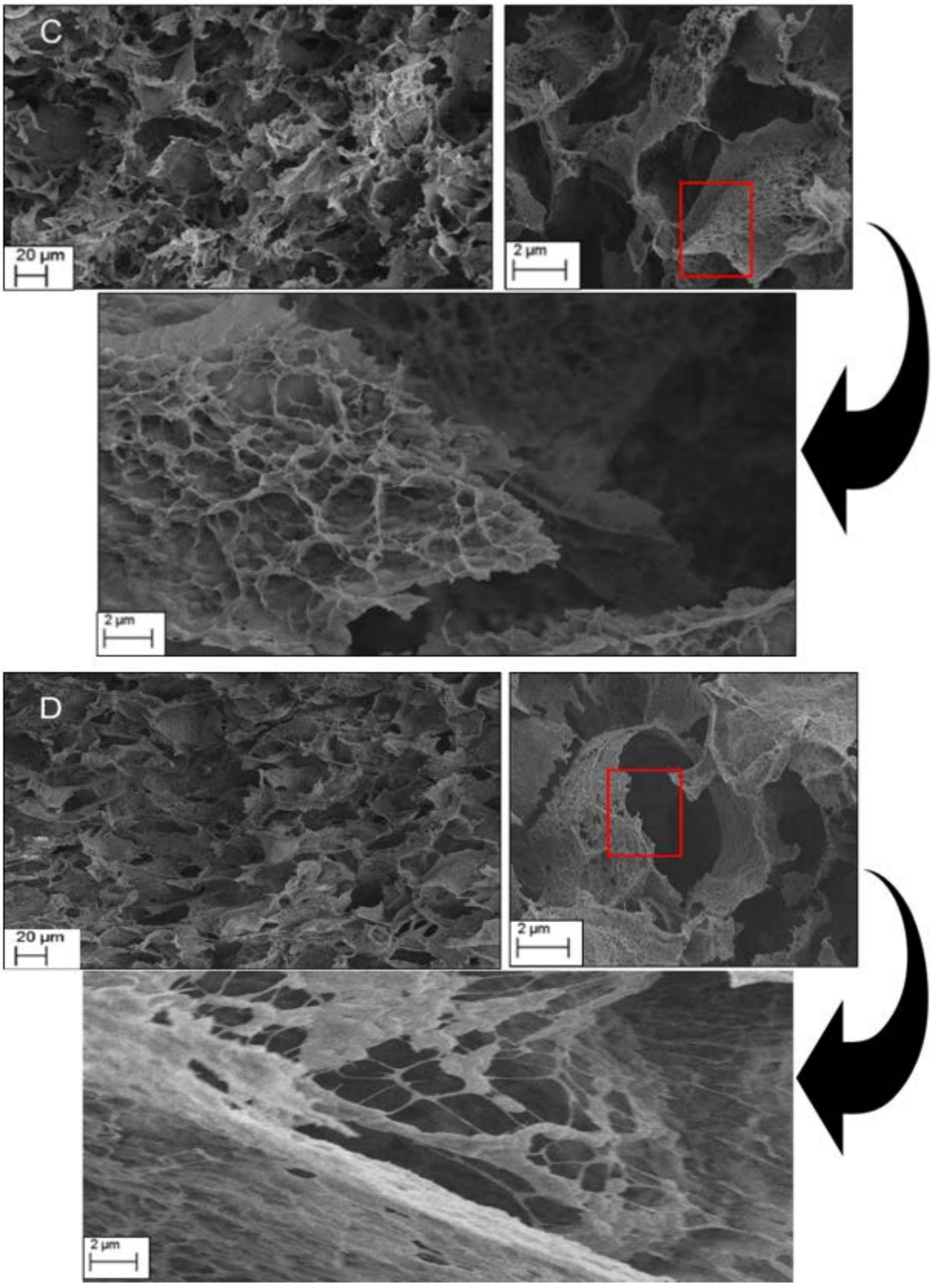



Figure 6.4: SEM images of *Acinetobacter radioresistens* after a week of incubation at 50 mg/L of phenol. The samples crosslinked by C) CCC3 and D) CCC4, at various magnifications 500X, 5.00 KX and 10.00 KX, respectively.

## 6.3.2 Effect of physiochemical factors on phenol degradation

### 6.3.2.1 Incubation media

Results of degradation of phenol by crosslinked cells cryogel samples produced by *A. radioresistens* and incubated into two different media are presented in figures 6.5 and 6.6.

Figure 6.5 shows the crosslinked samples CCC3 and CCC4 incubated in sodium carbonate buffer and tested for eight consecutive cycles without reactivation of samples between cycles.

The first cycle showed a slow rate of phenol degradation for both crosslinking polymers CCC3 and CCC4 (figure 6.5, A and B). Thereafter, the degradation performance improved in the following cycle to reach 96% degradation for CCC3 and 47% for CCC4 samples. The repetitive cycles had steeper degradation rates to reach approximately 95% for the remaining cycles except for last cycles for both crosslinking polymers. The final cycles for all samples showed slower rates of phenol degradation. Although they reached to 92% degradation for CCC3 and 90% CCC4 at the end of the incubation period, the rate was slower compared to the four previous cycles the average removal of phenol for eight cycles of CCC3 and CCC4 samples were 0.0086 mg/L/h and 0.0083 mg/L/h, respectively.

Statistical analysis by ANOVA showed a significant difference between the means of each cycle of the same crosslinking polymer $p<0.05$ level at 95% confidence level -for the degradation of phenol between repeated cycles. Furthermore, the results showed a non-significant difference between CCC3 and CCC4 polymers.

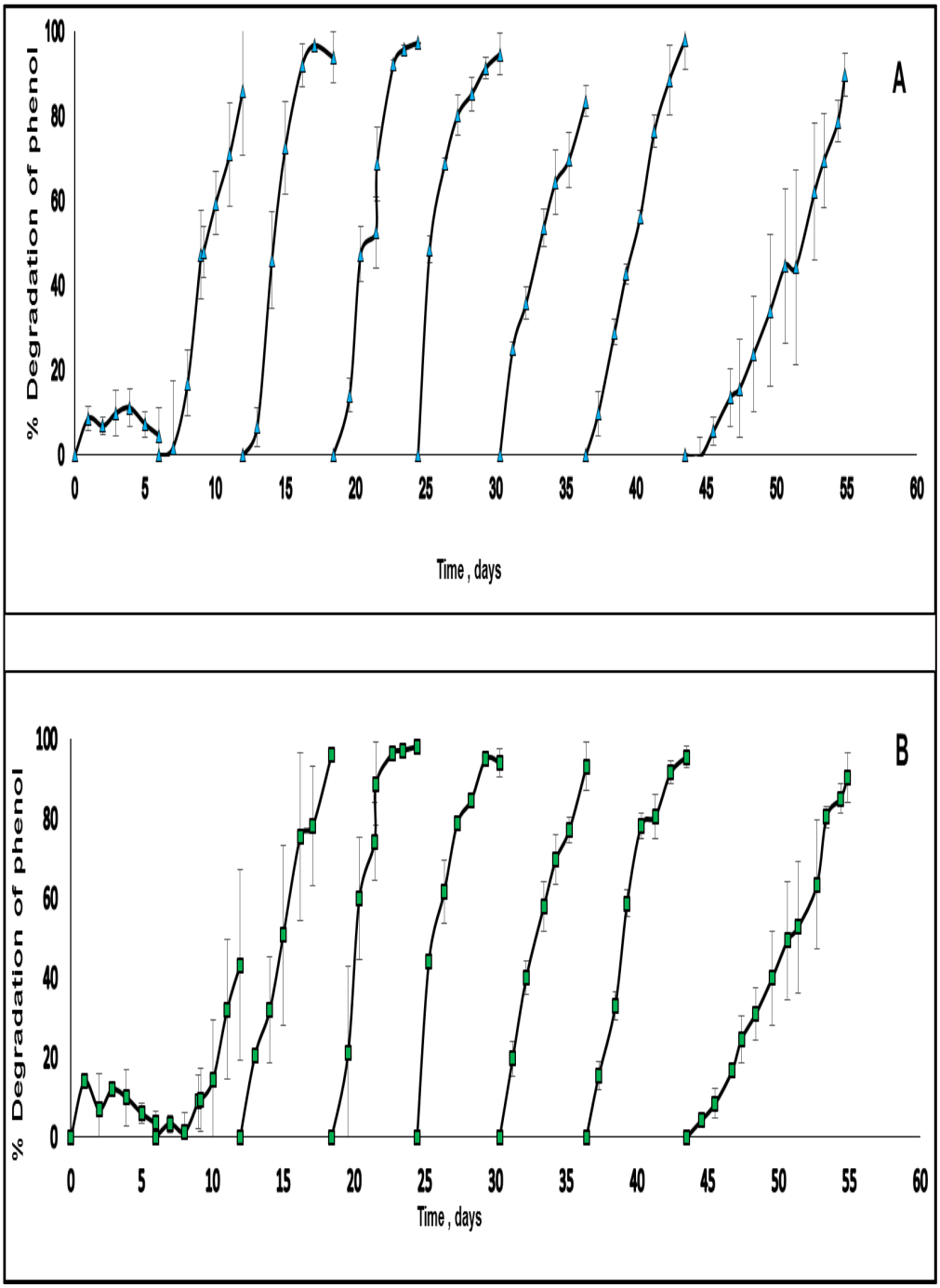



Figure 6.5: Cycles of crosslinked cells cryogel samples of *Acinetobacter radioresistens* incubated in sodium carbonate buffer with 50 mg/L of phenol, A) crosslinked by CCC3, B) crosslinked by CCC4.* each curve represent a new cycle of phenol degradation

CCC samples with *A. radioresistens* were also prepared and tested for phenol degradation efficiency in mineral salt medium (MSM) under the same optimum conditions for ten cycles and the results shown in figure 6.6, A and B. Faster phenol-degradation efficiency was recorded, with the first cycle using both types of polymers took three days. The following cycles showed faster ability to degrade phenol completely after 48 hours incubation under the same optimum conditions with a degradation rate to remove phenol by CCC3 samples is 0.038 mg/L/ h and 0.0353 mg/L/ h for CCC4 samples.

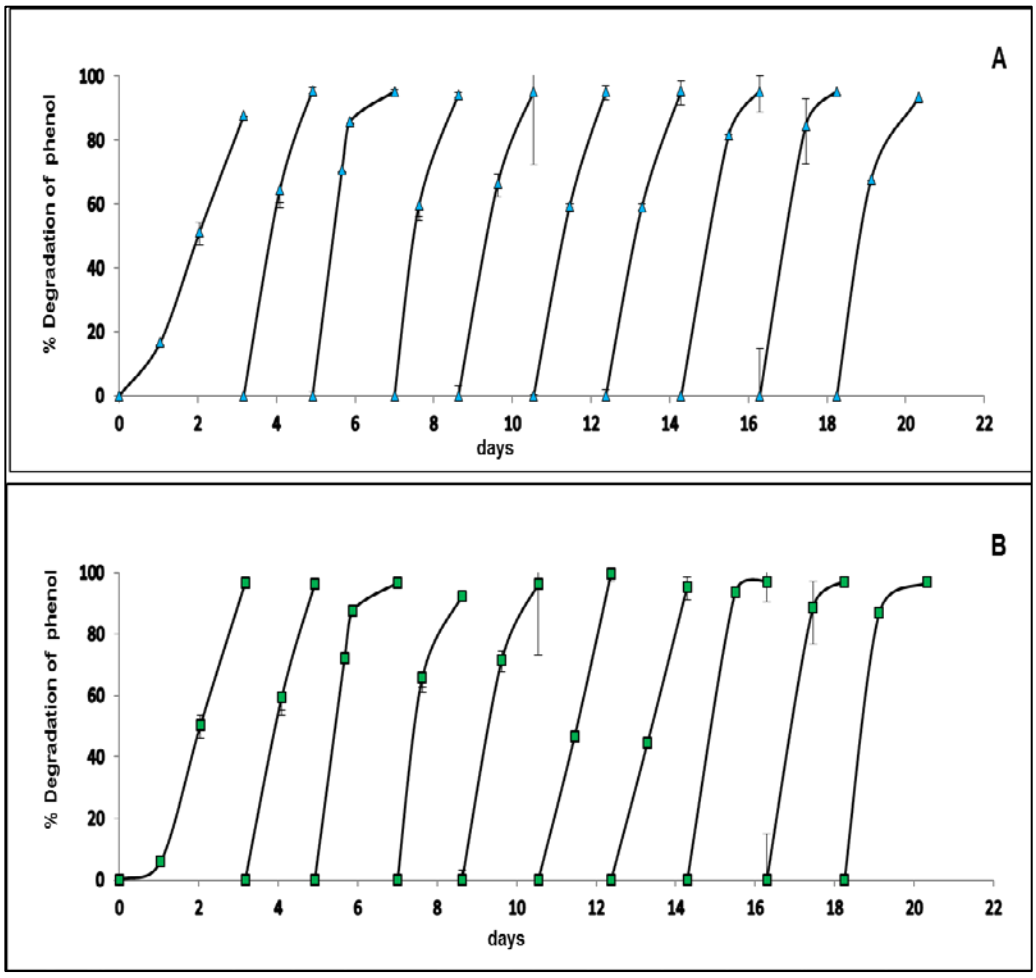



Figure 6.6: Reuse of crosslinked cell cryogel samples of Acinetobacter radioresistens for ten cycles. A) CCC3, B) CCC4 incubated with 50 mg/L phenol in MSM broth under optimum conditions.

These results confirmed that media composition has a marked effect on the cells' efficiency and degradation rate. Statistical analysis using two-way repeated ANOVA analysis at the 95% confidence interval showed a significant difference among tested cycles of MSM broth and sodium carbonate buffer for each type of CCC samples at $p<0.05$ level.

### 6.3.2.2 pH

As mentioned before, the evaluation of phenol degradation was set at optimum conditions of pH 7±0.2 and 30$^{o}$C as recommended in the literature (Wang *et al.*, 2007; Hoodaji *et al.*, 2013). Results of triplicate samples for both types of CCC samples under different pH conditions are presented in figure 6.7.

pH of 6, 7, 8 and 9 were used at 20$^{o}$C and 37$^{o}$C for 5 days and are presented in figure 6.7. At 20$^{o}$C temperature, the samples crosslinked with CCC3 showed degradation rates as following: 0.004, 0.018, 0.013, 0.010 md/L/h, respectively. Other CCC samples formed by CCC3 were tested again at 37$^{o}$C performed a degradation rates as following: 0.0035, 0.028, 0.0255, 0.012 mg/L/h and quicker phenol degradation was seen in samples incubated at pH 7 and 8, compared to other degradation rate of samples incubated at pH 9 and pH 6as shown in figure 6.7, B and D, which is higher comparative with samples incubated at 20 $^{o}$C.

Meanwhile, phenol consumption by samples crosslinked with CCC4 (figure 6.7, A and B) at 20$^{o}$C and 37$^{o}$C showed degradation rates at pH 6-9, as following; 0.0045, 0.0158, 0.0136, 0.0098 mg/L/h and 0.0056, 0.0268, 0.027, 0.011 mg/L/h, respectively. However, the percentage degradation reached only 80% for polymers incubated at pH 7 and 8. Approximately 60% degradation was observed for both samples incubated at pH 9 and 30% degradation for samples incubated at pH 6.

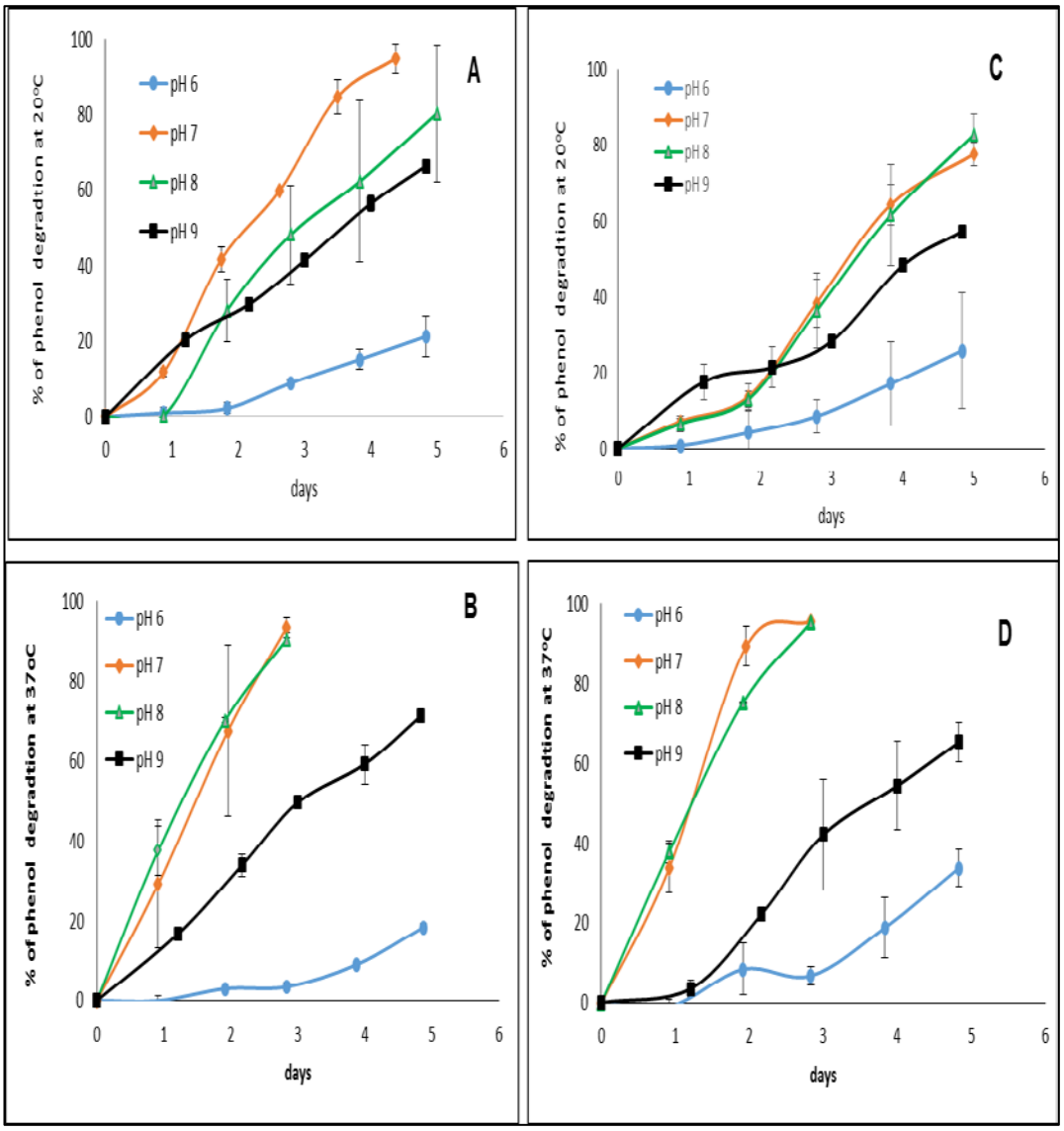



Figure 6.7: Efficiency CCC samples of *Acinetobacter radioresistens* at pH 6, 7, 8 and 9 at 20ºC and 37ºC, samples were crosslinked with CCC3 (A and B) and CCC4 (C-D).

Results of the effect of pH on the viability of cells was also assessed at the end of incubation time using the MTT assay after three consecutive cycles at 20ºC and 37ºC (figure 6.8). High percentages of viable cells were recorded for samples incubated at 20ºC for both crosslinking polymers; in contrast, low percentages were noticed for samples incubated at 37ºC (figure 6.8, A and B).

The percentages of viable cells in the CCC samples incubated at 20$^{o}$C, were 38.8% for CCC3 and 30.6% for CCC4 at pH 6, and 31.2% for CCC3, and 33.6% for CCC4 at pH 9. At pH 8 the percentages were 67.8% for CCC3, and 61.1% for CCC4, and at pH7 58.8% for CCC3, and 56% for CCC4. Comparable behaviour at 37$^{o}$C was also noticed for CCC samples incubated at various pH values. But the percentages of viable cells were reduced for all pH values when they were incubated at 37$^{o}$C. As shown in figure 6.8 B, low percentages such as 4.6% for CCC3, 4% for CCC4 at pH 6 and17% for CCC3 and 7% for CCC4 at pH 9 and 7.3% for CCC3 and 7.8% for CCC4 at pH 7. In contrast, samples at pH 8 shown high percentages 33.3% for CCC3 and 23.2% for CCC4 comparative with other samples at the same temperature.

According to statistical analysis, there was a significant difference in phenol degradation rates at different pH at both temperatures, moreover, the interaction between pH and temperature was also significant at the $p<0.05$ level.

It was also noticed that the pH of the media after one day of incubation with phenol for all samples decreased approximately from 0.2 to 0.5 compared with the initial pH values adjusted at the beginning of the experiment at 6, 7, 8 and 9. This confirms the effect of pH on the phenol degrading efficiency parallel with increasing temperature. However, increasing the temperature led to a reduction in the viability of cells (figure 6.8).

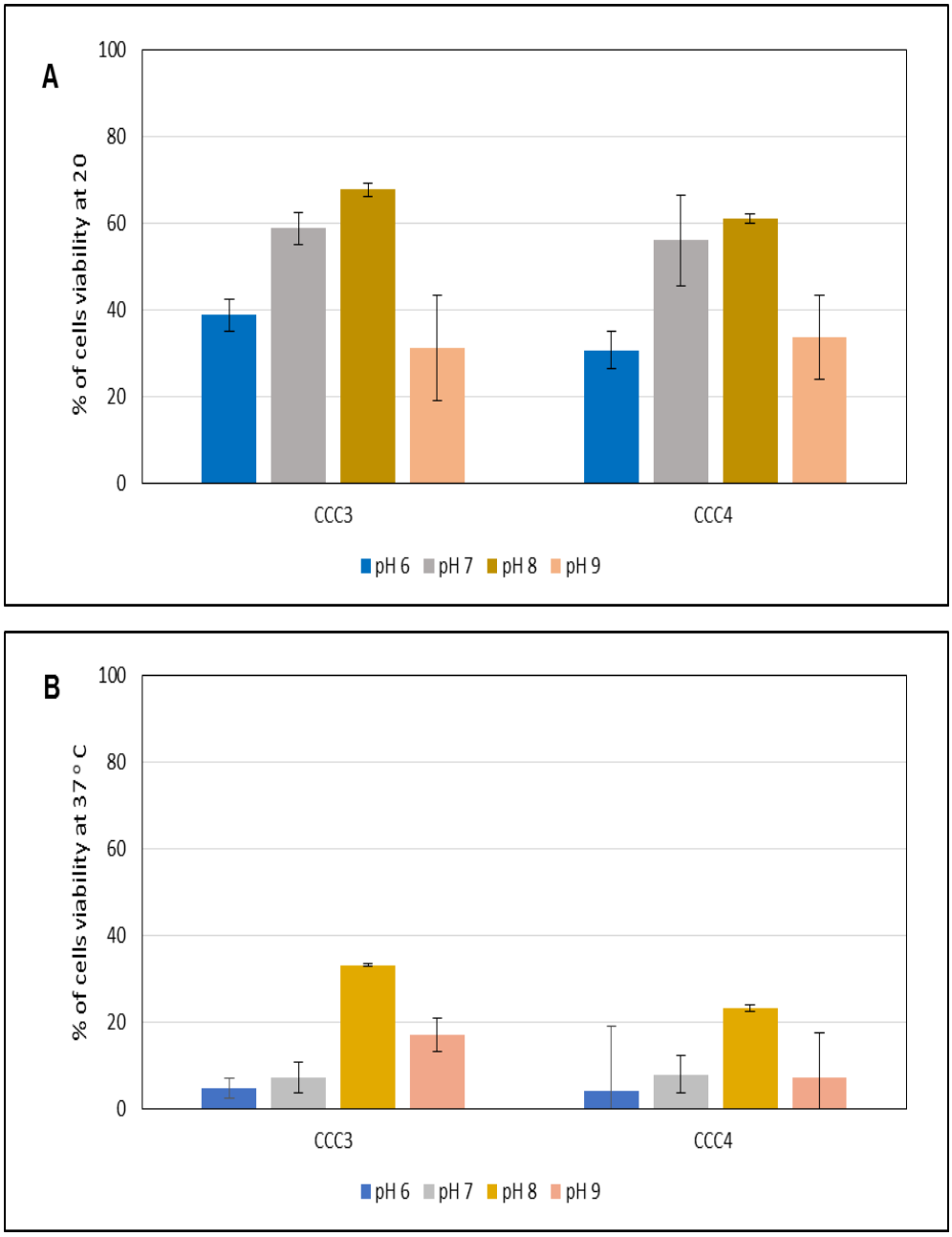



Figure 6.8: Percentages of viable cells of *A. radioresistens* incubated at different pH values to degrade phenol, at 20ºC and 37ºC. Initial viability percentages of crosslinked samples were used to determine the percentages after incubation.

### 6.3.2.3 Temperature

Temperature was considered as an important factor that could have an effect on the metabolic activity and viability of the bacterial cells. Therefore, an assessment of its impact on CCC samples was determined by evaluation at 20, 30, and 37$^{o}$C, with the pH at neutral; the results for CCC3 and CCC4 are shown in figure 6.9. Both types of sample required a longer time to complete phenol degradation at 20$^{o}$ C with better performance for CCC3 than CCC4. On the other hand, more rapid degradation for phenol was noticed for samples incubated at 37$^{o}$C comparative with samples incubated in 30$^{o}$C temperature.

In figure 6.9 A, it can be seen that CCC3 (PVA-al 1%+ PEI-al 0.25%) showed a linear and constant rate of degradation of 0.0182, 0.0236 and 0.0283 mg/L/h, meanwhile, less activity was noticed for samples formed by CCC4 (PVA-al 0.5% + PEI-al 0.6%), as shown in figure 6.9 B with degradation rates of 0.0158, 0.0261 and 0.0269 mg/L/h at 20$^{o}$C , 30$^{o}$C and 37$^{o}$C, respectively. Assessment of cell viability at the end of incubation was done to determine the optimum temperature to maintain large numbers of live cells. Results showed that samples incubated at 30$^{o}$C had higher percentage viability. Low temperatures had a negative impact on the live cells but not as much as higher temperatures significantly reduced their viability (figure 6.10). Although, low percentages of viable cells were recorded at 37$^{o}$C, the percentage of phenol degradation was higher due to continuous activity of remaining viable cells in crosslinked-cells cryogels, and adaptation of new cells to the environment. Additionally, dialysed dead cells in the batch culture could increase the carbon source in media, which may enhance the activity of remaining cells.

These results demonstrate the impact of temperature on the phenol degradation rate and cell viability. Statistically, temperature played a significant role on degradation rates of phenol.

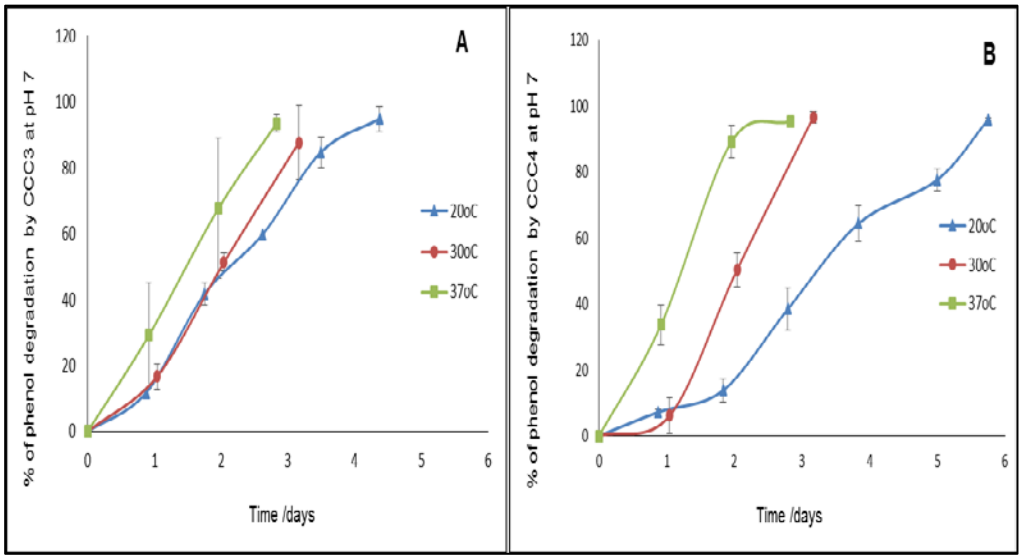



Figure 6.9: Effect of temperature on the degradation of phenol at pH 7 by CCC samples containing *A. radioresistens* at pH 7, A) CCC3, B) CCC4.

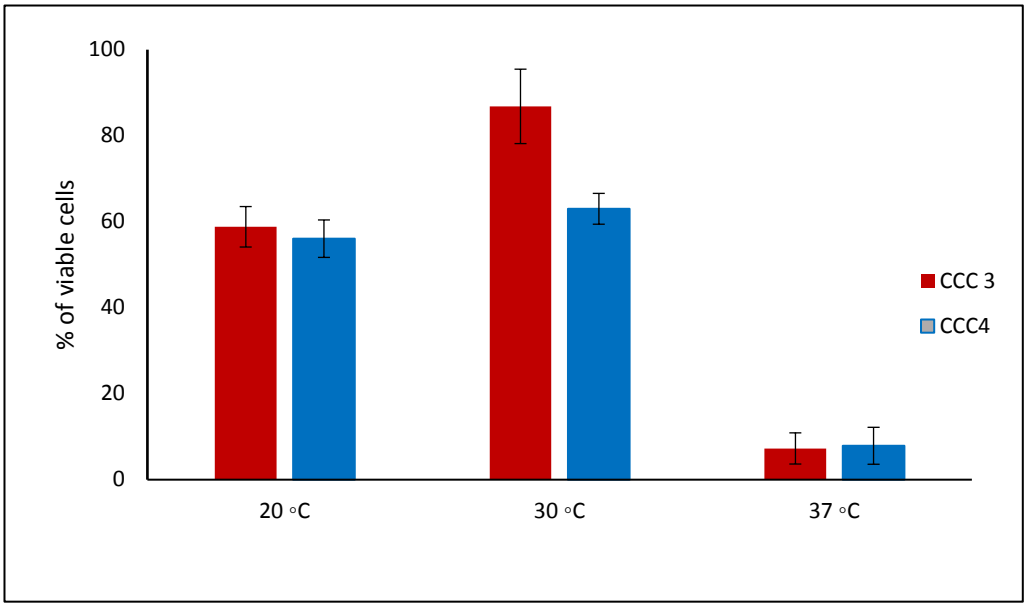



Figure 6.10: Percentage cell viability at 20, 30 & 37ºC and pH 7.

### 6.3.3 Efficiency of freeze-dried CCC samples

Assessment of phenol degradation by freeze-dried CCC samples with Acinetobacter radioresistens are presented in figure 6.11. The efficiency to degrade phenol was recorded after 24 hours incubation for four consecutive cycles (figure 6.11, A). The percentage degradation in the first cycle did not exceed 1.5% for either polymer, then percentages increased to 4.7%, 4% for CCC3 and 30%, 28.8% for CCC4 respectively. Over the following cycles efficiency of 77.5% for CCC3 and 71% for CCC4 was achieved by the fourth cycle. These results suggest that the bacterial cells required more time to adapt to the new environment.

Assessment of viability was also recorded for both samples in two states: i) initial freeze-dried samples, and ii) freeze-dried samples after phenol degradation as shown in figure 6.12. These both percentages were calculated depending on the initial percentages of fresh CCC samples. The percentages of viable cells after freeze-drying process were tolerable to continue the degradation process.

Following the four cycles of incubation with phenol CCC samples were also evaluated for viability of cells to estimate the effect of phenol concentration on CCC samples. The results showed that viability of freeze-dried CCC samples before incubation with phenol were 47.7% and 67.4% for both types of crosslinking polymers CCC3 and CCC4, respectively. The percentages were reduced to 26% and 28.4% for CCC3 and CCC4 after incubation with phenol, respectively.

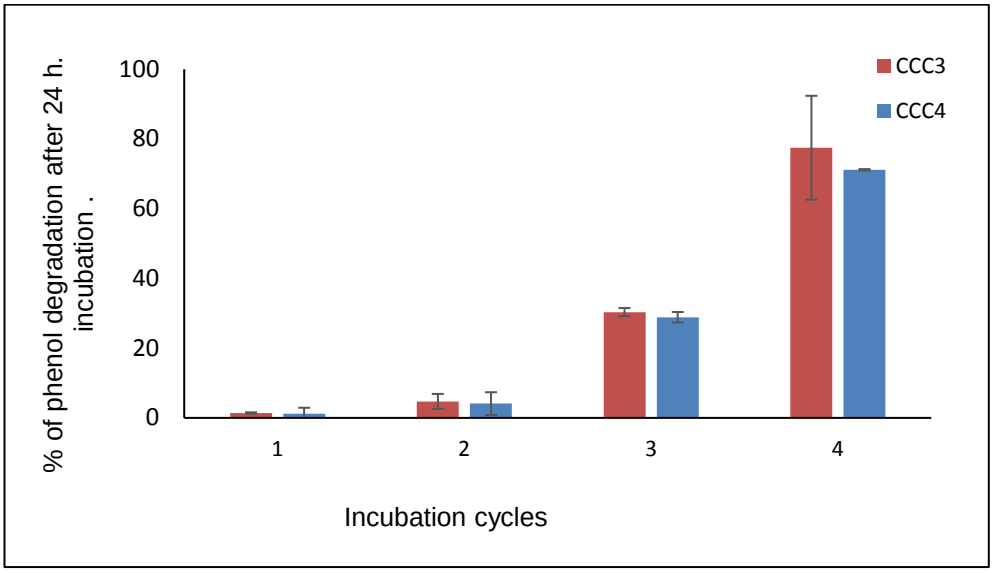



Figure 6.11: Percentage of phenol degradation by freeze-dried CCC samples of *Acinetobacter radioresistens* after 24 hours of incubation into MSM broth.

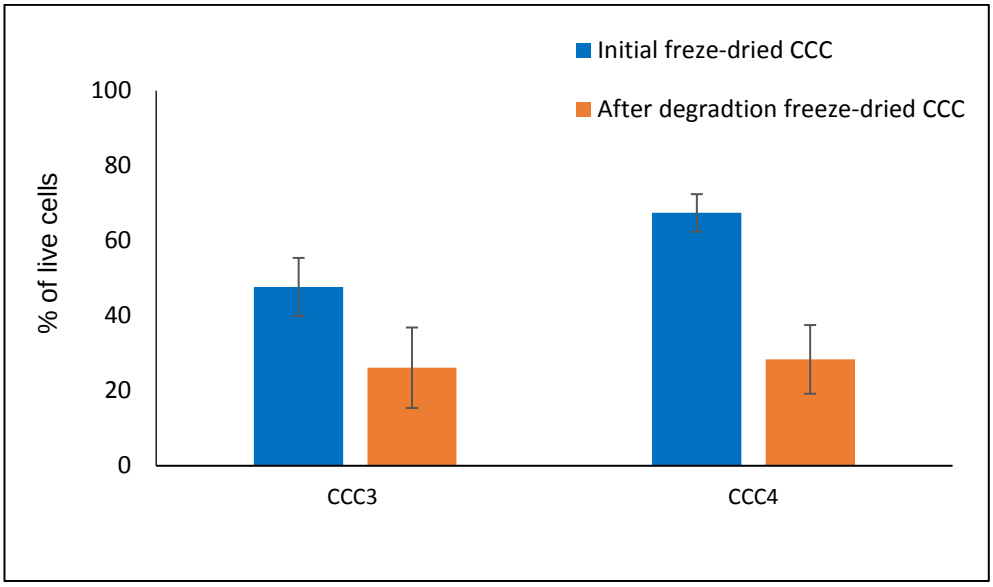



Figure 6.12: Comparative of freeze-dried samples viability before incubation (initial) and after four incubation cycles with phenol.

## 6.4 Summary of results

The main finding of this chapter are summarised in the following points:

- Modified polymers CCC3 (PVA-al 1% + PEI-al 0.25%) and CCC4 (PVA-al 0.5% + PEI-al 0.6%) were used to produce crosslinked cell cryogel samples containing *Acinetobacter radioresistens*.
- The mechanical properties, morphology, viability, and phenol degrading efficiency was determined for the CCC samples.
- Measurement of G' and G'' moduli demonstrated the gelled structure of CCC samples. Their mechanical properties changed after incubation with phenol, with CCC4 showing an enhancement in their mechanical features, whereas CCC3 samples exhibited a decrease of about 68% in elasticity and 13.6% in viscosity after incubation.
- The viability of initial CCC samples CCC3 and CCC4 reduced by approximately 20% compared to control samples, and CCC3 showed better viability after incubation with phenol compared to CCC4 samples.
- The morphology of the CCC samples as a 3D macroporous structure was confirmed by SEM images. The layers of crosslinked cells formed by the modified polymers after incubation with phenol became thicker and it was difficult to distinguish between cell borders as a result of the production of extracellular materials.
- The effect of media composition, pH, temperature and phenol degrading efficiency were evaluated. The type of incubation media had the greatest impact on the cells' capability to degrade phenol. For example, CCC samples in MSM required three days to remove 50mg/L phenol; in contrast, CCC samples in sodium carbonate buffer required seven days for the same outcome.
- The pH of the media impacted the cells' efficiency to degrade phenol, and also their viability. Results showed a significant difference between samples adjusted in different pH at the same temperature and media. The results showed that optimal pH for CCC sampled of *A. radioresistens* is ranged between 7 and 8. Lower efficiency to degrade phenol were recorded as long as the samples incubated in MSM adjusted at pH lower than 7 or higher than 8.

- Temperature played a significant role in the efficiency of the cells and their viability. At 20oC the CCC activity and cell viability decreased, whereas at 37$^{o}$C the degradation rate increased as did the percentages of dead cells. This suggests that the optimal temperature for maximum efficiency and viability was 30$^{o}$C.
- Using CCC samples in the freeze-dried state to degrade phenol were acceptable, although they required more time to reactivate. The percentage of phenol degradation achieved by the third cycle was similar to fresh samples, and the majority of the cells remained viable after incubation with phenol for four cycles.

# Chapter 7 : Application of CCCs within Kaldnes carriers (CCCs-KC) to degrade phenol spiked environmental water samples

## 7.1 Introduction

In this chapter, results are presented on the application of Kaldnes carriers as a protective shell or scaffold for the crosslinked cell-cryogels and the utility of these composite materials in degrading phenol spiked in real environmental water. Treatment of polluted water by bioremediation has been extensively developed and promoted over the last few decades to provide a more effective and lower cost method for providing safe drinking water and to reduce the impact of human activities on sources of water (Alexander, 1999; Vidali, 2001; Al-Hadithi *et al.,* 2017). One of the many strategies that has been applied is the moving bed bio-reactor (MBBR), which is considered a successful strategy to improve biological remediation. This is as a result of providing a protective environment for the microbes involved in the bioremediation process which helps establish the biomass and enables reuse without activation.

The carriers have a small foot-print, and reasonable cost efficiency and are flexible and easy to use and control (Nicholas, 2016). At the end of 1980s and in the early 1990s in Norway, MBBR was applied to improve the biological treatment process in 1988 as a result of co-operation work between the Norwegian University of Science and Technology and the Norwegian company AnoxKaldnes AS (European Patent no. 0,575,314, US Patent no. 5,458,779). Since then this product has gained several patents across the world following successful pilot treatment of municipal and industrial wastewaters. The MBBR technology is based on small cylindrical plastic carriers which are seeded with active sludge or active organisms, and which are inserted as freely moving substrates into wastewater treatment tanks (Rusten *et al.*, 2006; Ayati *et al.*, 2007; Borkar *et al.*, 2013; Francis and Sosamony, 2016).

These carriers were developed and designed with two different shapes with various properties, named Kaldens ® and Natrix ® (Maurer *et al.*, 2001) and described in detail in figure 7.1. They are shaped and designed as small plastic cylinders or tubes made of polyethylene with density less than water (approximately 1 g/cm$^3$) and a diameter or length ranging from 1 to 5 cm. The inner surfaces are divided into four or more sections to increase the inner surface area, which increases the quantity of biomass or biofilm attached or suspended in the reactors (Rusten *et al.,* 2006; Ayati *et al.,* 2007). A range of different shapes have been produced including cylinders for wastewater treatment (Dong *et al.,* 2015).

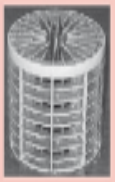
| Parameter | AnoxKaldnes type K1 | AnoxKaldnes type K3 | Natrix type O | Natrix type F3 |
|---|---|---|---|---|
| Shape |  |  |  |  |
| Length (mm) | 7 | 12 | 50 | 37 |
| Diameter (mm) | 10 | 25 | 60 | 46 |
| Maximum fillinf degree | 70% | 70% | 60% | 60% |
| Effective specific surface (m$^2$/m$^3$ carriers) | 500 | 500 | 300 | 200 |

Figure 7.1: Characterisation of Kaldens® and Natrix® carriers produced by the AnoxKaldnes Company (Falletti, 2013).

Researchers have investigated the use of these carriers to improve the removal rate of contaminants from wastewater. Of most relevance to the current thesis, Hosseini and Borghei (2005) investigated this technique to remove various concentrations of phenolic compounds with different hydraulic retention times. In addition to evaluating the inhibitory effect of phenol on the wastewater

treatment process, how the bacterial cells react toward these carriers and how they attach to them was also reported. Similarly, Li *et al.* (2011) set up a laboratory-scale pilot moving bed bio-reactor to remove phenols, thiocyanate and ammonium from coal-gasification wastewater. The performance of suspended and attached biomass in MBBR carriers were also studied and compared. Another paper by Accinelli *et al.* (2012) evaluated this technique using carriers prepared from bioplastic-based products to remove bisphenol A., oseltamivir and atrazine from wastewater compared to control samples. Results showed that the removal percentage of these compounds were significantly increased, therefore, authors recommend the use of biodegradable carriers inoculated with bioremediation microbes.

Koc-Jurczyk and Jurczyk (2017) studied the effect of packing volume of Kaldnes media on the efficiency of landfill leachate treatment. According to their results, increasing the volume of the Kaldnes media will result in a decrease in the rate of activated sludge reaction. Further research was also published on the removal of spiked phenol (ranging from 0-500 mg/L) from prepared saline wastewater using MBBR after inoculating the carriers with activated sludge from a wastewater treatment plant (Ahmadi *et al.*, 2017).

Most published research uses Kaldnes carriers to generate a biofilm contained within the carriers by inoculating them with activated sludge or active bioremediation microorganisms. These carriers however also offer an opportunity to provide a protective shell or scaffold for cryo- and hydrogels, to allow their use in more aggressive physical settings such as in moving, stirred tank or fluidised bed reactors. In this chapter a novel composite system will be created using Kaldnes carriers as scaffolds to surround the monolithic structure of crosslinked cell- cryogel samples for use in MBBR systems to remove phenol spiked into water samples collected from various environmental sources.

## 7.2 Aim and objectives

The aims of this chapter are to produce monolithic units of CCC samples of Acinetobacter radioresistens inside Kaldnes carriers (CCCs-KC) and their use in a moving bed bio-reactor (MBBR) to remediate phenols in polluted water.

The objectives to achieve this aim are as follows:

- Select an appropriate crosslinking polymer to crosslink the bacterial cells. Based on previous results (chapter 6), the CCC3 type (PVA-al 1%+ PEI-al 0.25%) was chosen.
- Inoculate purchased Kaldnes carriers with a suspension of CCC samples before operating the cryogelation process.
- Collect ground water, river water and tap water samples from the environment as manufactured water.
- Examine the key physicochemical parameters of the environmental water samples, including pH, initial concentration of phenols, concentration of total organic compounds (TOC) and anions by ionic chromatography.
- Assess the efficiency of CCCs incorporated into Kaldnes carriers (CCCs-KC) to degrade phenol in waters, and determine the viability of cells before and after the degradation process.

## 7.3 Results:

### 7.3.1 Initial evaluation of environmental water samples

The measured pH of environmental water samples is shown in table 7.1. The results showed that all samples were neutral to alkaline, ranging between 7.5 and 8.1 ±0.03. This pH range is suitable for Acinetobacter radioresistens to effectively degrade phenol as confirmed in chapter six.

Table 7.1: pH values for four sources of environmental water samples

| **Source of samples** | **Ground water** | **Ouse River** | **Cuckmere River** | **Tap water** |
|---|---|---|---|---|
| **pH values ±0.03** | 7.81 | 8.02 | 7.86 | 7.67 |

Results of the initial analysis for phenol and its derivatives in the water samples confirmed the absence of these compounds at concentrations of more than 1 mg/L by VIS-spectrophotometry. Results for water samples analysed by ion chromatography (IC) showed the presence of chloride, fluoride, nitrate and sulphate ions, although other anions such as bromide, nitrite and phosphate were not detected. However, some ions such as fluoride were only detected in certain sample types such as river water samples and not detected in ground water and tap water samples.

Similarly, nitrate was not detected in the River Ouse samples, whereas other samples showed concentrations of 4.19 mg/L for ground water, 6.34 mg/L for the River Cuckmere and 55.26 mg/L for tap water. The highest concentrations of chloride were recorded for the River Ouse samples at 92.1 mg/L, followed by 80.7 mg/L for the River Cuckmere, 52 mg/L for tap water and 39.5 mg/L for ground water. The highest concentration of sulphate (59 mg/L) was measured

in the River Cuckmere samples and the lowest concentration of 23.4 mg/L recorded for tap water samples. Over all most anions did not exceed the limits of world health organisation (WHO) except nitrate in tap water (WHO, 2003; WHO, 2004; WHO, 2011).

The results of TOC analysis are shown in figure 7.2. River Samples from the rivers Ouse and Cuckmere showed the highest concentrations of TOC with concentrations of 9.3 mg/L and 6.9 mg/L respectively. The concentration of TOC in groundwater was 3.5 mg/L and 2.9 mg/L recorded for tap water. TOC analysis results for initial water samples showed that the river samples contained higher concentrations of carbon sources.

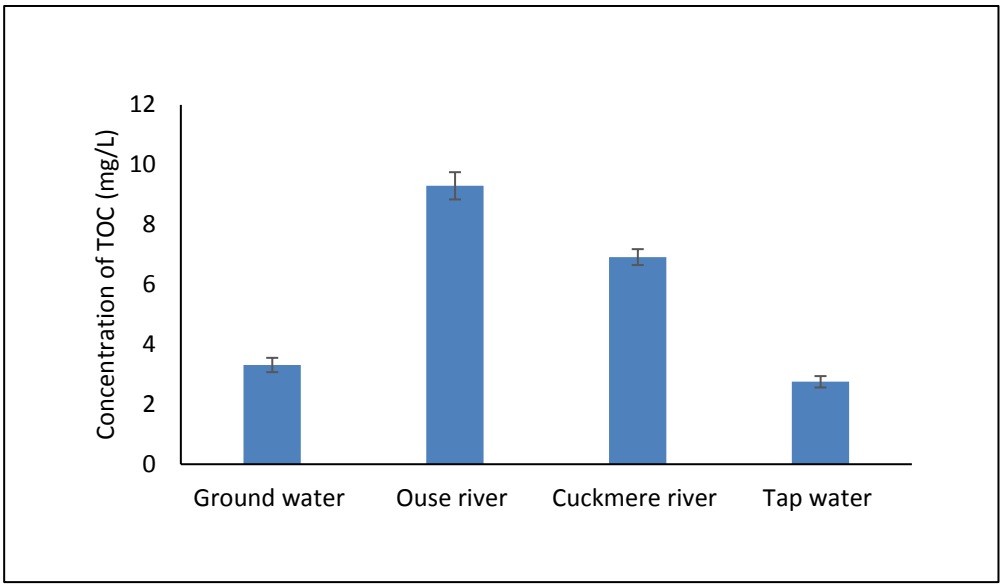



Figure 7.2: Concentrations of total organic carbon in environmental water samples at four different locations.

### 7.3.2 Efficiency of CCCs in Kaldnes carriers (CCCs-KC) to degrade phenol

Crosslinked cell-cryogel samples (CCCs) of *A. radioresistens* in Kaldnes carriers were evaluated for their efficiency to degrade phenol spiked into environmental water samples. The results are shown in figure 7.5. In summary, freshly prepared crosslinked cell cryogel samples inside MBBR carriers were active and started to degrade phenol after three hours of incubation for the environmental water samples (ground water from Preston Park and Rivers, Cuckmere and Ouse). Samples incubated with tap water showed slower rates of phenol consumption.

Measurement of absorbance of phenol for all samples after 24 hours of incubation with CCCs-KC confirmed the complete degradation of phenol (i.e. where the absorbance reading was zero or negative). Similar behaviour was also noticed when incubation with 50 mg/L of phenol was repeated for the second and third days. Whereas, on the fourth day after incubation with phenol the CCC samples showed slower activity in all samples. Degradation rate of phenol was calculated as average of four cycles for ground water, Cuckmere and Ouse Rivers and tap water as following: 0.072, 0.0763, 0.073 and 0.042 mg/L/h, respectively.

The percentage of phenol removal from the environmental water samples was reduced to 53.6% for samples incubated in tap water, and 48.9% for ground water on the fourth cycle of incubation (figure 7.3, A and B). In contrast, other samples from the Rivers Cuckmere and Ouse incubated showed complete degradation after 24 hours of incubation on the fourth cycle (figure 7.3, C and D), although with a slower rate of degradation compared to the other cycles.

The viability of the CCC samples incorporated into MBBR was assessed after four cycles, whereby (as noted above) each cycle consisted of 24 hours of incubation with phenol, and each day new water samples were spiked with 50 mg/L concentration of phenol. The final percentages of viable cells were

compared with initial percentages of cells before incubation, which were used as a control to estimate the percentage of remaining live cells after the degradation process (figure 7.4).

As can be seen in figure 7-4, approximately 50% viability was observed for CCCs-KC incubated in both river water samples, followed by 38% viability for samples incubated in ground water, and a relatively low cell viability of 24% for samples incubated in tap water.

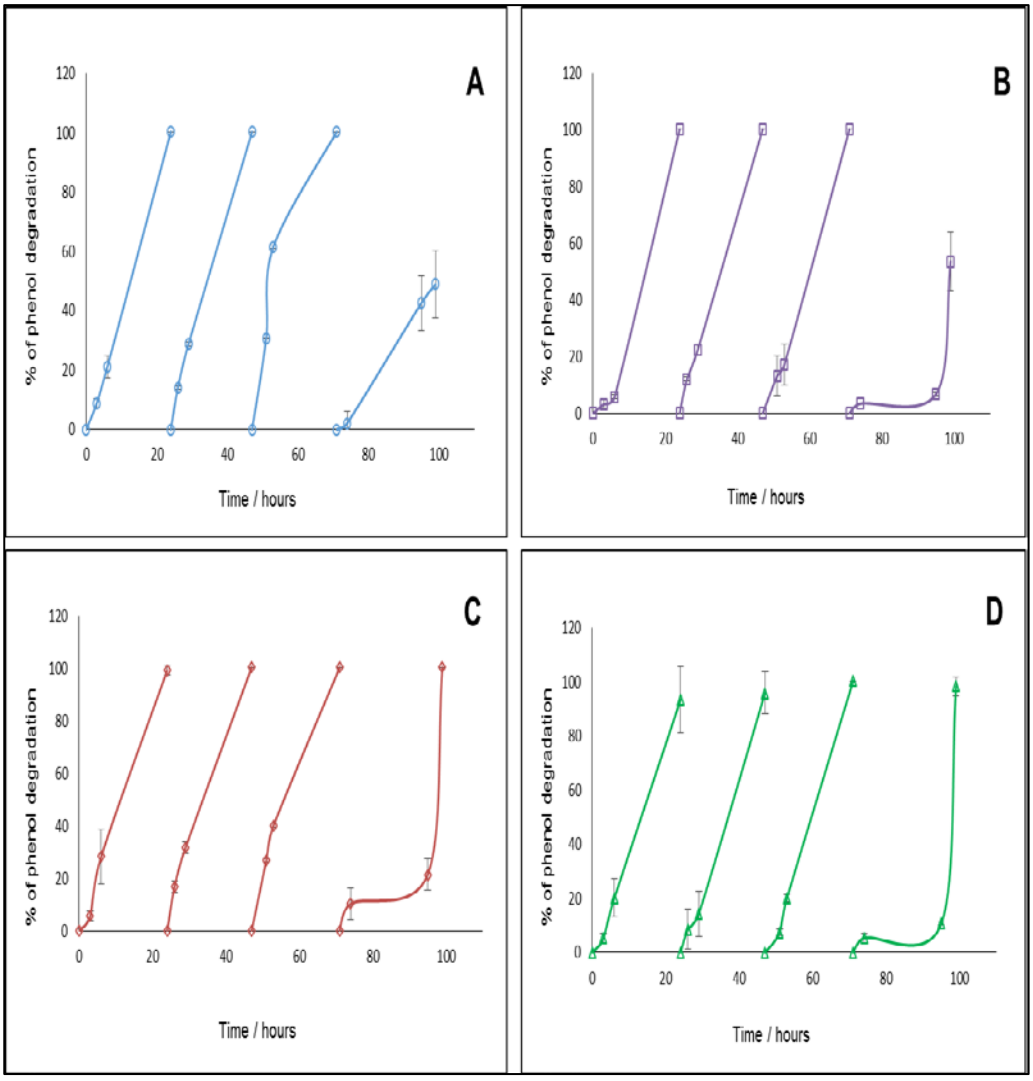



Figure 7.3: Percentage of phenol degradation in environmental water samples by CCC samples incorporated into MBBR carriers; A) ground water, B) tap water, C) River Cuckmere and D) River Ouse.

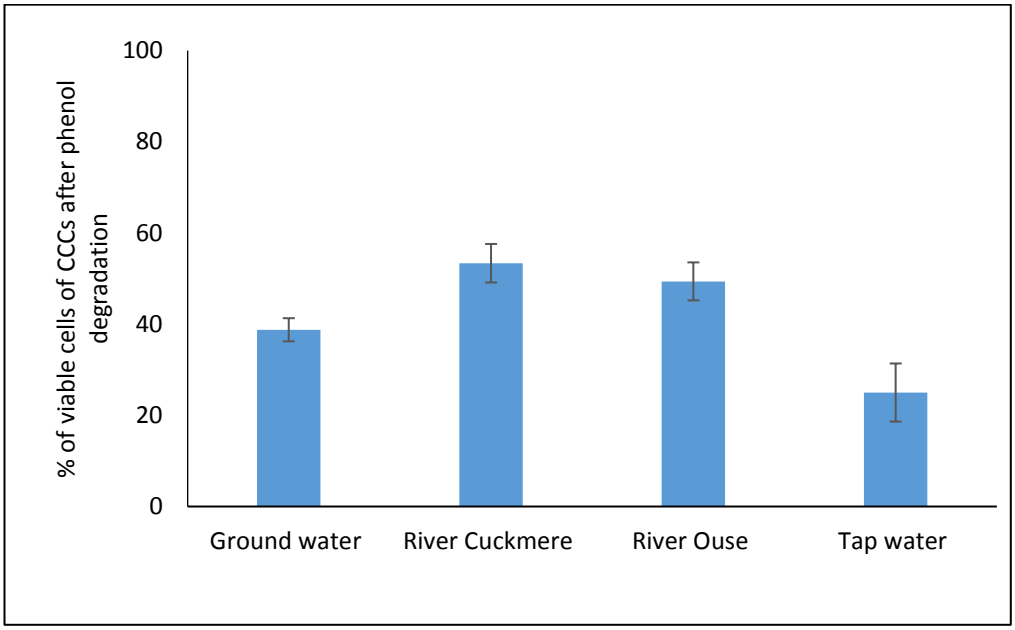



Figure 7.4: Viability of crosslinked cells cryogel of *Acinetobacter radioresistens* crosslinked with CCC3 (PVA-al 1% + PEI-al 0.25%) after 4 cycles: each cycle is one day's incubation with phenol spiked water samples.

## 7.4 Summary of results

The main finding of this chapter was summarised in the following points:

- The pH values of water samples collected from the environment ranged between 7.6 and 8, which is considered appropriate for the metabolism of *Acinetobacter radioresistens* according to the data presented in section 6.3.2.
- VIS-spectrophotometric analysis of the collected water samples showed the absence of phenol and its derivatives at concentrations above 1 mg/L.
- Ion chromatographic analysis of the water samples showed the presence of the ions chloride, fluoride, nitrate and sulphate, none of which exceeding the standard limits of the EPA, whilst bromide, nitrite and phosphate were not detected in all the samples.
- Assessment of total organic carbon (TOC) in the environmental water samples showed concentrations of 9.3 mg/L for samples collected from the

River Ouse compared to 6.9 mg/L for the River Cuckmere and 3.5 mg/L in ground water samples; and the lowest concentrations recorded were 2.9 mg/L for tap water.

- The efficiency of crosslinked cell-cryogel samples incorporated into Kaldnes carriers (CCCs-KC) for phenol degradation in a moving bed bioreactor (MBBR) was high. The samples showed a more rapid consumption of phenol than the CCC alone (without Kaldnes carriers) for all types of water samples. A percentage degradation of up 10% was achieved after three hours of incubation under orbital conditions.
- The final percentages of phenol consumption were approximately similar in all three cycles for all types of water. However, during the fourth cycle IN tap water and ground water the percentage of degradation dropped compared to that with river water.
- The percentage viability of crosslinked cell-cryogel samples incorporated with Kaldnes carriers (CCCs-KC) incubated in river water samples were recorded at 49.4% for the River Ouse and 53.4% for the River Cuckmere. Meanwhile, 38.8% viability was recorded for samples incubated in ground water and the lowest viability of 24.9% registered for samples incubated in tap water. These percentages were based on comparison with the initial percentage of cells before incubation.

# Chapter 8 : Discussion, conclusions and recommendations for future work

## 8.1 Discussion

The investigation of using a new bioremediation strategy supported with low concentrations of polymers to remedy pollutants, in precise phenol, was presented in this thesis. In this chapter, all methods and protocols in earlier chapters are critically evaluated and recommendations for further investigations based on the outcome of this research are also presented.

This chapter also discusses how the results were reported in the previous chapters. Started with choosing the suitable polymers and right concentrations to form 3D crosslinked cell-cryogels samples for both commercial *Pseudomonas mendocina*; *Rhodococcus koreensis* and their mixture and autochthonous indigenous strain *Acinetobacter radioresistens.* And finished with testing there efficiency to degrade 50 mg/L of phenol spiked at real environmental water samples collected from different sources.

This research has provided a novel bioremediation method by forming scaffolds of crosslinked cell-cryogels, which mainly comprise live cells crosslinked with low concentrations (less than 2%) of the modified polymers PVA-al and PEI-al. With respect to post-use disposal, the cryogels developed in this research degraded quicker than cryogels which used larger amounts of polymer and crosslinking agents. Furthermore, the percentage of polymers used enable natural biological breakdown. Finally the research provides information about the morphological and mechanical features of macroporous crosslinked cryogels and for the first time, the viability of the cells within the CCC samples.

### 8.1.1 Crosslinking polymers for 3D CCC samples

The basic concept of crosslinking strategy applied in this research was based on the chemical reaction between aldehyde groups as a part of the crosslinking agent linking the polymer structure and amino groups located on the surface of bacterial cells. In contrast to other approaches, the research presented here uses low concentrations of polymers and a high density of cells. The percentage of polymer recommended for the production of 3D synthetic cryogels is up to 1% (Lozinsky, 2014). For glutaraldehyde (GA), which is a common and efficient crosslinking agent used to link cells, proteins, enzymes and polymers was 0.5% (Hughes and Thurman, 1970; Damink *et al.*, 1995; Migneault *et al.*, 2004; Matty *et al.*, 2017). This figure is suggested because of its characteristic as a small molecule that can easily penetrate the cell membrane and disrupt metabolic pathways even at low concentrations, resulting in cell death (ATSDR, 2017). Therefore, low concentrations of GA have been used in previous research to eliminate its toxic effects (Hughes and Thurman, 1970; Damink *et al*., 1995; Migneault *et al.,* 2004; Kirsebom *et al.,* 2009; Aragão Börner *et al.,* 2014; Zaushitsyna *et al.*, 2017).

The use of a non-toxic and efficient crosslinking system is an important step for environmental applications. Therefore, GA and two other polymers, polyvinyl alcohol (PVA) and linear polyethyleneimine (PEI) previously recommended as non-toxic, biocompatible polymers, were investigated. (PVA) have been shown to an efficient agent to crosslink biomaterials (Kuyukina *et al.*, 2006; Gutiérrez *et al.,* 2007; Plieva *et al*., 2008a; Abdeen *et al.*, 2014) because of its stability and biocompatibility and nontoxic properties (Lozinsky and Plieva, 1998; DeMerlis and Schoneker, 2003). PEI was also used in this study because of its solubility in water, possession of a large number of amine groups (-NH2) and prior use in biological applications (Bečka *et al.*, 2003; Lei and Segura, 2009; Ling *et al.*, 2015).

Due to the large number of hydroxyl and amine groups in PVA and PEI, they are amenable to modification and use in the crosslinking process. Residual amounts

of unreacted groups or crosslinking agent remaining after crosslinking may have negative impact on live cells in biological applications (Hassan and Peppas, 2000). Thus, when GA is used as a crosslinking agent, during the cryogelation process pre-concentration of all components occur in the frozen sample in the so called ‘non-frozen liquid micro-phase’ and the concentration in the sample will increase during the freezing process, with potential impacts on cell metabolism and viability (ATSDR, 2017). To avoid this situations modified polymers of PVA and PEI with aldehyde functionality (PVA-al and PEI-al) were used.

Figure 8-1 A and B describes the chemical reaction of PVA and PEI activated with GA to produce PVA-al and PEI-al. Both polymers were purified after the modification by dialysis to remove any unreacted molecules of GA (Rao *et al*., 2006; Figueiredo *et al.,* 2009).

Two combinations of modified polymers, CCC3 (PVA-al 1% + PEI-al 0.25%) and CCC4 (PVA-al 0.5% + PEI-al 0.6%) at two different concentrations were used to increase the mechanical stability of cryogel due to an increase in the number of interactions points between the aldehyde groups on the polymer and the amino groups on the surface of the cells under sub-zero temperature via cryogelation process.

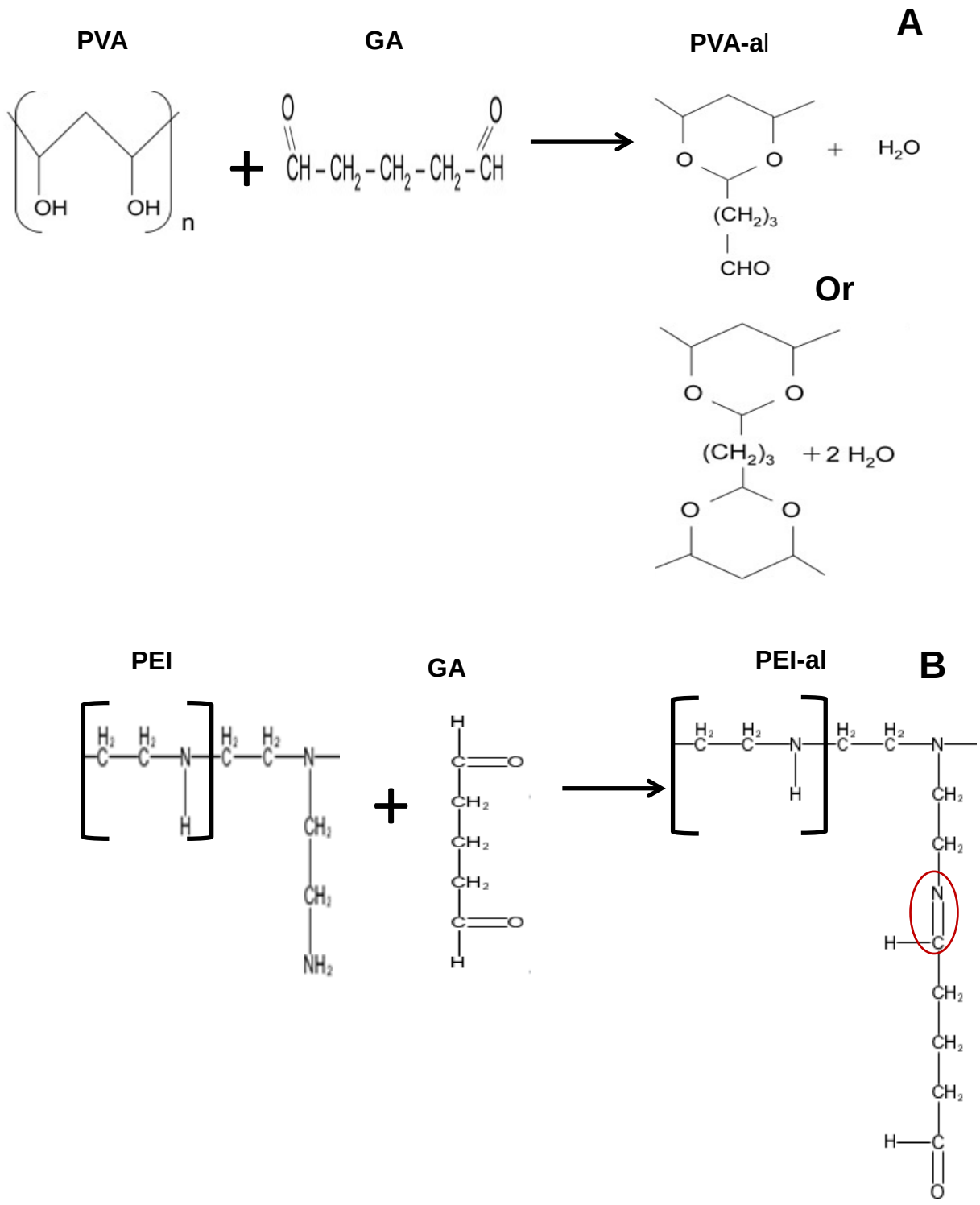



Figure 8.1: Mechanism of the reaction between: A) polyvinyl alcohol (PVA) and glutaraldehyde (GA) (Figueiredo *et al.,* 2009), B) polyethyleneimine (PEI) and glutaraldehyde (GA) (Srinivasa Rao *et al.,* 2006).

The final product is the formation of a stable cryogel comprising crosslinked polymer and cells. Specific properties of natural and synthesised cryogels include variable pore size, and good mechanical properties. Furthermore, their utilisationin different technological application such as bioreactors, for cell separation and as tissue engineering scaffolds (Plieva *et al.,* 2008; Hixon *et al.,* 2017) influenced this research to use the same process to produce macroporous cryogel structures made of live bacterial cells and small percentages of activated crosslinking polymers.

### 8.1.2 Characterizations of CCC samples using commercial bacterial strains

A general assessment of cell viability was performed by staining the cryogel samples with the Live/Dead Bac Light kit and CLSM imaging. The green colour in crosslinked cell cryogels (CCC) stained with the Live/Dead Bac Light kit confirmed the presence of live cells, whilst red spots indicated dead cells between the layers of the cryogels (Berney *et al.*, 2007). This method allows the visualisation of both live and dead cells in the material, but it is not quantitative. In contrast, the MTT assay used in this study provided quantitative data for cell viability.

The stability and mechanical properties of crosslinked cell cryogels can be influenced by different factors and conditions such as the chemical properties of polymers, concentrations used, and the type and shape of bacterial cells. Crosslinking polymer CCC1 (GA 0.5%) showed higher values of G’ and G’’ with mixed species (*Rh. koreensis, P. mendocina*) compared to single species alone, probably due to the different cell morphologies, which made it easier for CCC1 to spread via the spaces between the cells during the cryogelation process (Lazaridou and Biliaderis, 2004); similar behaviour also was noted for CCC2 (PVA 1% + GA0.5%). Meanwhile, the modified polymers CCC3 (PVA-al 1% + PEI-al 0.25%) and CCC4 (PVA-al 0.5% + PEI-al 0.6%) showed approximated value of G’ and G’’ for *P. mendocina* and mixed samples, whereas, *Rh. koreensis* samples presented a higher value.

Furthermore, the concentration and the molecular weight of the polymer solution might also effect the G' and G'' values. Converting the water into ice crystals with higher concentrations of polymer promoted greater intermolecular connections which helped maintain structural integrity on thawing (Lozinsky, 2004). Therefore, CCC1 and CCC2 showed less ability to maintain their structures, similarly to results published by Zaushitsyna *et al.* (2017) that confirmed the fragile nature of crosslinked cell cryogels produced using CCC1. Despite the lower molecular weight of crosslinking agents, the concentrations used are still the main factors influencing the stability of cryogel samples. This is in contrast to Giannouli and Morris (2003) who stated that further incremental concentrations of crosslinking polymers may reduce the G' and G'' values. The modified polymers CCC3 and CCC4 theoretically contains more active aldehyde groups, especially CCC4 compared to CCC1 and CCC2, which showed effective interactions between the active groups during freezing conditions at -12$^{o}$C) leading to stabile structures and reusability to degrade phenol over five cycles.

The storage (elastic) modulus G' for CCC3 and CCC4 were comparable to other cryogels such as Fmoc-Phe-Phe (Berillo *et al.*, 2012b) prepared via self-assembly of a 1% wt/v kefiran solution, and up to 0.5 % of Xanthan at -20$^{o}$C (Piermaria *et al.*, 2008). Also, the type of bacterial genus (Gram positive or Gram negative) may have an influence on chemical reactions during the crosslinking process due to the different nature of compounds located on the cells' surface. Bio-surfactant materials produced by cells extracellularly or as a part of cell membranes are could be anionic, neutral or cationic (of which the latter contains the amino groups) (Abdeen *et al.*, 2014). *Rhodococcus sp*. is one of the cationic bacterial groups, therefore the reaction between both groups (aldehydes and amines) increased and explains the higher viscoelasticity (G') of samples prepared from *Rh. koreensis* and modified polymers CCC4 and CCC3 compared with samples produced from CCC1 and CCC2.

After incubation with phenol in buffer, samples of *P. mendocina* and mixed cells showed a higher G' value, and slightly lower G'' value, compared with initial values (figure 4.7, A, B, E and F). The much larger lipopolysaccharide (LPS)

layer located in the outer membrane of Gram negative cells is considered a first barrier of defence for the cells when environmental conditions are changed as a part of their metabolic activities to adapt to a new habitat (Rosenfeld and Shai, 2006; Sperandeo *et al.*, 2009; Silhavy *et al.*, 2010). The much smaller LPS layer in Gram positive cells results in reduced values of G' and G'' compared with the initial samples (Singleton, 2004). However, the percentage reduction did not have an influence on their 3D structure and they maintained their stability which was confirmed by reusing them for up to five cycles.

It was clear in the SEM images that cells crosslinked with CCC4 were much more closely packed probably because the number of active groups in the PEI-al structure increased the number of reaction points between cells and crosslinking polymer. Meanwhile, the samples crosslinked with CCC3 (PVA-al 1% + PEI-al 0.25%) appeared in SEM images as if they were covered with a layer of polymer. It is thought that this is a result of the reaction between the activated PVA-al polymer and other polymer molecules instead of cells (figure 4.8, A and B); only a limited number of reaction points on the outer surface of cells could react with the activated polymers even though low concentrations of PEI-al were used to increase the active groups.

### 8.1.3 Phenol degradation by CCC samples using commercial strains of phenol-degrading bacteria

An assessment of the bacterial cells' efficiency to degrade phenol in both free and crosslinked cells cryogels, was performed as presented in section 4.3.5. Free (planktonic) cells survived and adapted, although it took more than nine days to complete the degradation of 50 mg/L phenol in carbonate buffer solution. *P. mendocina* cells showed more rapid adaptation probably due to the nature of the outer membrane structure (Rogers, 1983). Compared to *Rh. koreensis* and mixed cells (Sperandeo *et al.*, 2009; Silhavy *et al.*, 2010). Mixed cells showed a steeper exponential phase as a result of competition between the two bacterial strains (O'Sullivan, 1998). Fresh crosslinked cell-cryogels of *P. mendocina* required approximately seven days to complete one cycle and could be reused

for up to five cycles of phenol degradation although with a slow degradation rate during the first cycle.

Several reasons can be attributed to explain the slower degradation rate during the first remediation cycle, such as the freezing conditions, the properties of the crosslinking polymer and the incubation media and conditions. The formation of ice crystals and a lack of oxygen during the cryogelation process might damage the cell membrane and reduce the percentage of live cells. This was confirmed by analysis of the cells' viability for both free and frozen cells (figure 4.4). The percentage viabilities decreased to 43-49% for *P. mendocina*, 44%-68% for *Rh. koreensis* and 28%-50% for mixed cells when cells were crosslinked compared to free frozen cells (figure 4.5). The results in figure 4.15 showed a further reduction when the samples were refrozen and stored at -80$^{o}$C. The decrease in the number of viable cells explains the slower degradation of phenol using mixed cells and *Rh. koreensis* alone which had been refrozen for 4 and 6 weeks.

The characteristics of the crosslinking agent or polymer also may affect cell viability and the stability of CCCs samples. For instance, glutaraldehyde has been used as a main crosslinking agent in CCC1 or combined with another crosslinking polymer such as PVA in CCC2, and part of the activated polymer components in CCC3 and CCC4. Despite using low concentrations of GA, it reduced cell viability because of its ability to inhibit the metabolic activity of cells following penetration through the cell walls causing their death (ATSDR, 2017). An additional factor to consider is the surrounding environment; incubation the CCCs samples of all strains with 50 mg/L of phenol, negatively impacted cell viability due to its toxicity to live organisms even at low concentrations of 1 mg/L (Luttrell, 2003; Santos *et al.*, 2004; Kulkarni and Kaware, 2013).

Previous research (Hughes and Thurman, 1970; Bečka *et al.*, 2003; Gutiérrez *et al.*, 2007; Figueiredo *et al.*, 2009) demonstrated that using glutaraldehyde or combining it with other polymers could result in stable macroporous units of crosslinked cells, whereas our results showed that such units were unstable, fragile and disintegrated during the first cycle of incubation. CCC samples

produced using a combination of activated polymers were more stable, sustainable, and reusable and had good cell viability. In this experiment the final percentages of live cells after phenol degradation for samples crosslinked with CCC3 were 34.3%, 18.2% and 16.78% compared to initial percentage cells viability before phenol degradation of 49.7%, 68.1% and 41.6%. Meanwhile, the final percentages for samples crosslinked with CCC4 were 36.1%, 36.7% and 35.2% compared to initial percentages of 43.2%, 51.5% and 50.4% for *P. mendocina*, *Rh. koreensis* and mixed cells, respectively.

The number of viable cells was sufficient to enable reuse for up to five sequential cycles with similar efficiencies for degrading phenol for both crosslinking polymers. The difference observed before and after degradation indicates the effect of the environment on the cells' viability. CCC samples incubated in sodium bicarbonate buffer only may delay their activity and resistance to environment factors. However, the remaining number of the cells in CCC samples adapted to that environment and were able to degrade phenol for the rest of the cycles. Another benefit of these CCC is the ability to be stored after production, transported and then reactivated for use at the site of application (e.g. industrial facility, wastewater treatment works etc.).

### 8.1.4 Isolation and identification of phenol-degrading bacteria from oil-contaminated sites

The isolation of an indigenous and autochthonous bacterial strain from oil-contaminated soil with the ability to degrade phenol was successfully achieved via using a mineral salt medium (MSM) as an enrichment medium to grow cells after adding phenol as a main source of carbon (Ahmad *et al.*, 2011; Jiang *et al.*, 2013). MSM broth was used at low concentrations only to maintain the continuity of metabolic activity of the cells, and to encourage only those cells to grow that have the efficiency to degrade phenol in that medium. As mentioned in the initial results of isolating phenol degrading bacteria (section 5.3.1), there little or no growth on MSM agar plates supplemented with phenol only. For that reason, glucose at 0.5 g/L was added to the media at the beginning of the

isolation process, to help the bacterial cells to survive until they adapted to the new environment and become able to metabolize phenol. Basic identification tests for the phenol-degrading bacteria isolated from oil-contaminated soil were applied. The presumptive identification through microscopic and macroscopic morphology, Gram stain, and basic biochemical tests suggested that the strain was an unspecified member of the genus *Acinetobacter*.

Discrimination of the isolated *Acinetobacter species* to strain level was performed using genomic identifications tests. Confidence in the PCR results is assured via choosing the conditions suitable for each bacterial strain. Therefore, different protocols with various conditions have been applied for the identification of *Acinetobacter sp.* (Joshi and Deshpande, 2011; Lorenz, 2012; Khosravi *et al.*, 2015). Furthermore, the choice of primers, DNA extraction method and the conditions for the PCR can affect the results. For these reasons, the use of appropriate universal primers for *Acinetobacter sp*. and high-quality kit to extract DNA and appropriate conditions for PCR led to a robust identification of the isolated *Acinetobacter* strain. In these experiments, comparing the DNA sequences obtained with all *Acinetobacter* strains on the database at the RDP website indicated a high percentage similarity with known bacterial strains.

Consequently, restriction enzyme analysis using the ARDRA method was applied to provide a more robust identification of the isolated strain (Vaneechoutte *et al.*, 1993; Telenti *et al.*, 1993; Vaneechoutte *et al.*, 1995; Dijkshoorn *et al.*, 1998). However, the outcome of ARDRA can be affected by factors like those for PCR. In addition, the volume of amplified DNA mixed with the restriction enzymes and incubation times employed play an important role in this process. If the volume of DNA or restriction enzymes and the duration of the restriction reaction is incorrect, it can lead to failure, for example as occurred with strain A (figure 5.8). However, other researchers have suggested the use of other methods instead of ARDRA. Koeleman *et al.* (1998) recommended the use of amplified fragment length polymorphism analysis (AFLP) as a quick, simple and accurate method for typing *Acinetobacter* species, meanwhile,

Khosravi *et al.* (2015) stated that partial sequencing of the *rpoB* and *16S rRNA* genes differentiated *Acinetobacter* species to the strain level comparative with ARDRA which is less able to assign *Acinetobacter sp.* isolates to the strain level. Nevertheless, the profile indicated by ARDRA was adequate for identification of the autochthonous strain isolated from oil-contaminated soil in Basra, Iraq.

Assessment of the bacterium's tolerance to high concentrations of phenol indicated a reverse relationship between the growth of cells and phenol concentrations. Probably the main reason for the low growth of cells is the toxicity effect of phenol on the metabolic activity of living cells (Luttrell , 2003; Lu *et al.*, 2008). The low growth of cells is associated with high concentrations of phenol, which is shown clearly by the extension of the lag phase observed in the growth curve. This phenomenon is in agreement with Singleton (2004), and Mara and Horan (2003), whom both stated that bacterial species need extra time to adapt to the variable conditions of their new environment.

### 8.1.5 Characterisation of CCC samples from the isolated, indigenous phenol-degrading strain

As previously mentioned, the selection of crosslinking polymers to produce stable macroporous cryogels was the first step in this experiment. Subsequently from the results obtained from applying the commercial strains, the two modified polymers CCC3 (PVA-al 1%+ PEI-al 0.25%) and CCC4 (PVA-al 0.5% + PEI-al 0.6 %) were used to form CCC samples with *A. radioresistens.* Characterisation of CCC samples began with an assessment of mechanical properties that showed samples of CCC4 and CCC3 were mechanically stable and have a gelled structure as indicated by the elasticity module G' and viscous module G'' (Franck, 2004). However, these mechanical properties changed after incubation with phenol in MSM. For CCC4 samples, both moduli increased, whereas both moduli decreased for CCC3 probably because less connected points between the cells and reacted polymers which could effect on the mechanical behaver after incubation. CCC4 polymers as previously proposed have more active aldehyde groups in its structure because of the PEI-al structure which can react

with the amine groups on the surface of cells at different connected points. Moreover, *A. radioresistens* has the ability to produce extracellular materials, mainly polysaccharide associated with protein; this tend to act as a stabiliser when exposed to different pH and temperature (Navon-Venezia *et al.*, 1995; Margesin *et al.*, 2013; Fatajeva *et al.*, 2014). These extracellular materials combined with a good crosslinking polymer could increase the mechanical features of the CCC samples. CCC3 samples, which have fewer aldehyde groups due to the structure of PVA-al which may lead to fewer connected points thus reducing the elasticity and viscosity characteristics of the CCC3 samples following incubation with phenol.

These explanations were partially confirmed by SEM images which illustrated the organisation of the cells and their association with polymers before and after incubation with phenol. The changes in the thickness of the cell surface layer following incubation with phenol make them difficult to recognise as individual cells within the CCC samples (figure 6.4, C and D).

### 8.1.6 Effect of incubation conditions on CCC samples of *A. radioresistens*

The efficiency and rate of phenol degradation depends on factors including the availability of nutrients, ambient temperature and pH. In this experiment, it was noted that phenol degradation by CCC samples with *A. radioresistens* in mineral salt medium (MSM) supplemented with phenol was all that was necessary for the bacterial cells to remain viable (Wang *et al.*, 2007; Mukred *et al.*, 2008; Al-Hadithi *et al.*, 2017). Whereas, CCC samples in sodium carbonate buffer required more time to begin and maintain the degradation process, most likely due to the lack of necessary growth elements. All the samples in MSM under the same optimum conditions (pH 7 and 30$^{o}$C took less than the half period to activate than that for sodium carbonate buffer. Mara and Horan (2003) and Singleton (2004) both highlighted that the absence or insufficient amounts of essential salts or mineral elements in the media could reduce their efficiency

and cell activity. Adams *et al.* (2015) reported that these elements could function as electron donors during the bioremediation process.

The pH of the medium is another factor that has an influence on cell growth and metabolic activity. Most living cells grow best at or nearly a neutral value (Mara and Horan, 2003). Results in this experiment using crosslinked cell-cryogels were comparable to other studies (Arutchelvan *et al.*, 2005; Mukred *et al.*, 2008; El-Naas *et al.*, 2009; Ahmad *et al.*, 2011; Hoodaji *et al.*, 2013; Al-Hadithi *et al.*, 2017) and exhibited their best performance at a pH from 7 and 8.The phenol hydroxylase enzyme responsible for the cleavage of phenol is effected by pH, and previous research shows that the optimum pH for the phenol hydroxylase enzyme from *Pseudomonas sp.CF600* and *Acinetobacter radioresistens S13* is 7.4 and 7.5, respectively (Divari *et al.*, 2003; Kagle and Hay, 2006). Furthermore, Ahmad *et al.* (2017) stated that the pH for phenol hydroxylase from *Acinetobacter sp.* strain AQ5NOL1 changed depending on the buffer used. For example, the optimum enzyme activity ranged from 6.5 to 7.5 for phosphate buffer, and from 7 to 7.5 for Tris-HCL buffer. Overall, effect of pH on the enzymes plays a major role in the bioremediation process.

Lower efficiencies of phenol degradation were recorded at pH 6 and 9, subsequently the effect of pH on the ionization process that may impact on the interaction and binding of various molecular processes and in turn affect the metabolic pathway in addition to denaturing vital enzymes (Hoodaji *et al.* , 2013; Shah, 2014). Furthermore, most acids below pH 4 and bases higher than pH 9 can penetrate easily into cells (Kotresha and Vidyasagar, 2008). Finally, changes in pH could lead to denaturing of proteins that may impact negatively on microorganism's cell walls and metabolic processes (Mohanty and Jena, 2017).

Temperature is also a factor that has a great impact on cell viability and is associated with pH. Normally, the optimum temperature for growing cells ranges from 25$^{o}$C to 35$^{o}$C (Mara and Horan, 2003) whereas to achieve effective bioremediation the ideal temperature ranges from 30$^{o}$C to 37$^{o}$C (Mukred *et al.*,

2008; Lakshmi *et al.*, 2009). In this research, CCC samples of *A. radioresistens* were tested at the optimal temperature of 30ºC. Further investigations were carried out to elucidate the effect of temperature on the degradation process by running the bioreactors at 20ºC and 37ºC.

The best degradation performance was recorded for samples incubated at 37ºC for both type of polymers, followed by 30ºC and 20ºC. Similar results were published by Mukred *et al.* (2008) where higher percentages of crude oil degradation in wastewater were observed when the strains were incubated at 37ºC (which is considered the 'normal' temperature used in industrial processes to degrade crude oil in wastewater): 72% with *Acinetobacter faecalis* (WD2), 65% *Staphylococcus sp.* (DD3), 60% *Neisseria 141 longate* (TDA.4.2) and 52% *Pseudomonas putida* (TAM 4.4). Whereas, Hoodaji *et al.* (2013) stated that most degradation of phenol by *Alcaligenes sp.* ATHE8 isolated from coke processing wastewater occurred at 35ºC and pH 7; samples from the same batch incubated at 20ºC and 45ºC required longer time to degrade the same concentrations of phenol.

The rate of reactions within the degradation process are controlled by enzymes produced by the bacterial cells. Scopes (2013) highlighted that the phenol hydroxylase enzyme was affected by temperatures above 23ºC by disturbing their stability, and further increases led to a loss of activity. In addition, Ahmad *et al.* (2011) declared that at temperatures below 20ºC or higher than 37ºC, *Acinetobacter sp*. strain AQ5NOL 1 cells had a reduced efficiency to degrade phenol.

These results generally explain the outcomes of this experiment, although the CCC samples using either of the selected crosslinking polymers were more efficient to degrade phenol at 37ºC. Nevertheless, there was a reduction in cell viability compared to CCC samples incubated at 20ºC and 30ºC. Moreover, cell viability for samples incubated at 20ºC was decreased compared to samples incubated at 30ºC. This confirms that determining the optimum incubation

temperature for each bacterial strain used is important to enable the required enzymes to continue their activity without a loss of efficiency.

In general, the results obtained agreed with the conclusion of Mohanty and Jena (2017) that pH, temperature and initial substrate concentration play a key role in the degradation rate of phenol.

### 8.1.7 Freeze-dried samples

Assessment of CCC samples to degrade phenol in the freeze-dried state was important to verify as lyophilisation is an easy technique to enable storage of the samples at room temperature when produced in large quantities. Although low degradation rates were recorded for the first cycle of remediation, an increase in these rates occurred over the following cycles as a result of adaptation of the lyophilised cells to the new environment, especially when optimal conditions are available (Hottes *et al.*, 2013; Mao *et al.*, 2015).

However, any live cells remaining after the preparation and freeze-drying conditions were able to degrade phenol for four cycles in a gradual enhancement of activity. Decreasing the quantity of viable cells to approximately to half of the initial cell count could be due to reasons such as phenol toxicity (Luttrell, 2003; Duan *et al.*, 2017) or adverse incubation conditions (Hoodaji *et al.*, 2013) .

### 8.1.8 Evaluation of initial properties of environmental water samples

Measuring the pH of the initial water samples collected from the environment was a necessary step to assess its effect on cell activity. The impact of pH on the organisms living in aquatic environments is due to its effect on solubility and biological availability of the nutrients (phosphorus, nitrogen and carbon) and heavy metals that are used by living organisms (Taylor, 1962; Mara and Horan, 2003; Singleton, 2004; Kurniawan *et al.*, 2018). As shown in (table 7.1), the pH

of the environmental water samples ranged between 7.5 and 8.1, which is normally acceptable for most of living organisms (Edstrom Industries, 2003). Moreover, this range of pH is suitable for the growth and activity of *Acinetobacter radioresistens* growth as presented in chapter six. The highest phenol-degrading ability of CCC samples with *A. radioresistens* at pH ranges from 7- 8 was similarly to the ranges that reported by Fatajeva *et al.* (2014) , who used two strains of *Acinetobacter sp*. NJ5 and N3 to degrade oil-derived hydrocarbons that reached higher degradation percentages 15.9% at pH 8 and 13.7% at pH 7. Similarly, Li *et al.* (2017) stated that the high percentage to degrade phenol by the isolated strain FB-2 was 78% at pH 7 then 71.4% at pH 8. Meanwhile, Shah (2014) reported that pH 7.5 is the optimum pH for *Pseudomonad spp*. to obtain higher degradation rate of phenol at percentage 8.2.63%.

Water is crucial for all living organisms and it is rarely pure and non-contaminated. Therefore, identification of the ions and elements found in these water samples was necessary to assess their impact on the CCC's efficiency to degrade phenol. Regulatory or guideline limits for these ions vary according to region or regulatory body. For instance, chloride and sulphate concentrations should be less than 250 mg/L in drinking water or potable tap water, while fluoride should show concentrations less than 2-4 mg/L; similarly, nitrate limits are 10 mg/L for drinking water (United States Environmental Protection Agency, 2003) , and the limit for nitrate in surface water is from 0-18 mg/L (WHO, 2011). However, limits for nitrate were raised to 50 mg/L or below 100 mg/L in Europe Union (EU) member states according to World Health Organisation's guideline value for drinking water (Drinking Water Inspectorate, 2017) . The limit values for sulphate range from 0-630 mg/L according to GEMS/ water (a global network of water monitoring stations) (WHO, 2004) and less than this for ground water. In this research the concentrations of ions such as chloride, fluoride, sulphate and nitrate (section 7.1.2) were less than the limit values determined by these organisations.

Determination of total organic carbon (TOC) in the environmental water and tap water samples was also an essential requirement to assess the total level of organic contamination in the water samples. The results of the TOC assays for these samples showed that concentrations exceeded the limits legislated by US Purified Water that recommended that a concentration of 0.05 mg/L as the standard limit for TOC in drinking water (Edstrom Industries, 2003). In contrast, the European Union published new guidelines for the concentrations of TOC in water which ranged between 0.1-10 mg/L for drinking water, river water, and ground water, and for purified water the concentration range was reduced to 1-500 µg/L (Visco *et al.*, 2005; Whitehead, 2018). The results for TOC in this experiment for environmental and tap water samples showed that the concentrations did not exceed the standard limits.

In general, values of the concentrations of anions, TOC and pH values will fluctuate depending on prevailing biotic and abiotic factors. For instance, an increase in rainfall may increase the level of contamination as a result of runoff from agriculture and urban runoff, or may result in an increase in faecal pollution as a result of the input of human and animal wastes (Drinking Water Inspectorate, 2017).

### 8.1.9 Phenol degradation by CCCs within Kaldnes carriers (CCCs-KC)

Phenol degradation rates in spiked environmental water samples were successfully increased by using monolithic units of CCC samples embedded within Kaldnes carriers in a moving bed bioreactor (MBBR) system. They required less than 24 hours to achieve 100% degradation. CCC samples of *A. radioresistens* without Kaldnes carriers (described in section 6.3.1) required at least 48 hours to complete the degradation of phenol, even though they were incubated with minimal salt medium (MSM) under optimum conditions.

In this study, number of reasons can explain this positive impact of using Kaldnes carriers as supports for the CCCs. For example, Kaldnes carriers

showed good potential to enhance the phenol degradation rates and reduce the incubation times by acting as barriers or protective shells. The main function of Kaldnes carriers utilised in this study is to maintain the structural integrity of the crosslinked cell-cryogels and to prevent them from collapsing during incubation under orbital conditions. The carriers also provided a scaffold for cell growth for cells that could attach to it with the shell providing protection against mechanical disruption in the MBBR. Other research has used Kaldnes carriers in MBBR systems as a basic support to build biofilm layers inside the carriers and to reduce reloading active sludge overtime to treat industrial effluents (Hosseini and Borghei, 2005; Ayati *et al.*, 2007; Francis and Sosamony, 2016; Koc-Jurczyk and Jurczyk, 2017). Another beneficial impact of using Kaldnes carriers is that orbital incubating in MBBR enhances the cells efficiency by providing the cells with oxygen by keeping the media aerated (Brock *et al.*, 1988; Singleton, 2004). This ensures that most cells will be provided with nutrients and oxygen during incubation (Bates *et al.*, 2016). Furthermore, this shaking movements prevents free cells of other microbes that exist in the environmental water to form biofilms on the Kaldnes carriers, which may negatively affect the efficacy of the CCCs-KC.

Environmental factors such as temperature, pH and the nutrients available in the environment also play an important role cell viability during exposure to toxic compounds such as phenol. In this work, incubation temperatures were setup depending on the results in presented in section 6.3.3. The pH values of the environmental water samples were in the same range as the optimum pH values determined as suitable for *Acinetobacter radioresistens* (section 6.3.2). These factors and the presence of total organic carbon (a minimum of 2.75 mg/L in tap water and a maximum of 9.3 mg/L in Ouse River water) plus other elements indicated by ion chromatography, could enhance and boost the metabolic activity of cells and enhance the rate of breakdown of pollutants (Vidali, 2001). Similarly, other research confirmed that adding 0.5 mg/L glucose to the media as a source of organic carbon (Lakshmi *et al.*, 2009; Chakraborty *et al.*, 2010) or bicarbonate as an inorganic carbon (Li *et al.*, 201 1) increased the rate of

phenol degradation. This effect was also noticed in this study at the beginning of the isolation process. Thus, the more rapid and efficient degradation of spiked phenol at 50 mg/L by CCCs-KC was a consequence of the combination of all these factors.

The presence of other microorganisms in the environmental water samples could have an influence on the CCCs-KC ability to initiate degradation activity in a short time, because of competition between the microbial species. This observation is supported by data from samples incubated in tap water, which showed the lowest percentage of phenol degradation after six hours incubation. The percentage was 5.8% compared to other water samples which showed a 20-28% efficacy, especially in the initial cycle. The efficiency increased over the next two cycles as a result of adaptation to the new environment (Hottes *et al.*, 2013). Hoodaji *et al.* (2013) also found that isolating a native bacterium from contaminated areas can increase the degradation efficiency for phenol. The strain of *Acinetobacter radioresistens* used in this experiment was isolated from oil contaminated soil from Iraq, and this ability to adapt and live under such conditions could be an additional factor to explain the acceleration in the degradation of phenol observed with the autochthonous isolate compared to the purchased phenol-degrading bacteria.

During the protocol of adding 50 mg/L phenol in a new cycle every day, by the fourth cycle of degradation the CCCs-KC samples required more time to complete degradation than in the previous cycles. The reason behind this is believed to be that the number of viable cells decreased during the incubation period, which was confirmed by comparing the final percentage viabilities with initial viabilities, which was set as 100% as shown in figure 7.4. The toxicity of phenol is perhaps the main reason for the reduction in cell viability (Ren, 2003; Atsdr, 2008; Duan *et al.*, 2017). However, the numbers of remaining viable cells were enough to complete the degradation of phenol.

In summary, the degradation rates of phenol increased during this research programme. Several factors could impact the cells' activity. For instance,

degradation rates of phenol were 0.0098, 0.0093 mg/L/h for *P. mendocina*, 0.0091, 0.0065 mg/L/h for mixed cells and 0.007, 0.0072 mg/L/h for *Rh. koreensis* for both CCC3 and CCC4 when they were incubated in carbonate buffer only. This was also observed for *A. radioresistens,* where the degradation rates reached 0.038 and 0.035 mg/L/h for CCC3 and CCC4 when they were incubated in MSM buffer. The rates were variable during incubation at different pH and temperatures. When tested with water from the environment (rather than spiked water samples) the degradation rate increased to approximately 0.07 mg/L/h for ground water and water from the rivers Cuckmere and Ouse. Meanwhile, degradation in tap water was 0.04 mg/L/h, because chlorine residues in water reduced the number of cells and limited their activity (Virto *et al.,* 2005). However, because of the existence of other carbon sources cell activity could be increased.

During this research the concentration of oxygen was not controlled and may have limited the cells' activity. As long as the strains tested were aerobic, oxygen is necessary to initiate the degradation pathway for phenol to achieve phenol mineralisation (Fritsche and Hofrichter, 2000; Nair *et al*., 2008; Seo *et al.,* 2009). Also, it can be determined how many concentrations of oxygen are required for phenol mineralisation to $CO_2$ by calculating the number of moles. For instance, according to this equation:

**$C_6H_5OH + 7O_2 = 6CO2 + 3H_2O + \Delta$**

Seven moles of oxygen require to breakdown one mole of phenol, so to calculate the concentration of oxygen in (mg/L) following formula should be applied:

*COD or OD (mg/L) = (C/FW)*(RMO)*(32) =

C= concentration of oxidised compounds.

FW= Formula weight of oxidised compounds

RMO= Ratio of the moles of oxygen to moles of oxidised compounds in reaction to $O_2$.

Therefore, to oxidise 50 mg/L of phenol:[50 (mg/L) / 94 (mg/mmole of phenol)] * [7* 32(mg of O2/mmole of phenol)] = **119 (mg/L) of $O_2$** is needed (CE251 Environmental Engineering Fundamentals, n.d.).

## 8.2 Conclusions

The production of monolithic units of crosslinked cell-cryogels with a 3D macroporous structure using commercial bacterial strains (*P. mendocina* and *Rh. koreensis*) or from indigenous bacterial strain of *Acinetobacter radioresistens* isolated from oil contaminated soil, supported by low concentrations of activated polymers was a successful strategy for the bioremediation of phenol in water. Characterisation of the CCC samples and evaluation of their efficiency for phenol degradation under different conditions such as incubation medium, temperature and pH were achieved. An examination of these units in real environmental water samples showed great promise and potential for use in wastewater treatment. The main finding can be summarised as follows:

- This research showed that low concentrations of crosslinking agents and polymers, i.e. less than 2%, can produce monolithic macroporous cryogels predominantly comprising of live bacterial cells. The nature of the polymers and their properties play an important role in the immobilisation of the bacterial cells as a result of the crosslinking strategy. Ordinary crosslinking systems such as glutaraldehyde (GA 0.5%) or combination of polymer and glutaraldehyde (PVA 1% + GA 0.5%), named in this research as CCC1 and CCC2, respectively, were not practical to produce robust, maintainable and reusable samples of crosslinked cells cryogels. In contrast, reverse modified polymers (PVA-al 1% + PEI-al 0.25%) and (PVA-al 0.5% + PEI-al 0.6%) termed CCC3 and CCC4 showed better performance and sustainable characteristics such as good mechanical properties, macroporous structure and good cell viability during testing and evaluation.
- Fresh CCC samples produced with *P. mendocina* showed a rapid rate of phenol degradation at concentrations of 50 mg/L, whereas CCC samples with

*Rh. koreensis* and mixed cells of *P. mendocina* and *Rh. koreensis* frozen at -80$^{o}$ C of showed a delayed response and required at least a week to reactivate and start normal metabolic activity. Therefore, a longer time is required to degrade phenol if the CCC are frozen rather than prepared freshly.

- The isolation of indigenous and autochthonous bacterial strains from contaminated sites is a good method for obtaining microbes suitable for the bioremediation process since the organisms have adapted to such environments. Initially, MSM media supplemented with phenol and 0.5% of glucose for the initial isolation of the microbes enhanced the growth of bacterial strains able to tolerate high concentrations of phenol.
- The application of physiochemical tests is not adequate enough to give a robust identification of the isolated phenol degrading bacteria. Therefore, standard genomic identification methods were used such as 16S rDNA PCR and sequencing and Amplified rDNA (Ribosomal DNA) Restriction Analysis (ARDRA) to give a strain level of identification of *Acinetobacter radioresistens.*
- CCC samples of *A. radioresistens* showed good morphological and mechanical properties in parallel with samples produced with *P. mendocina* and *Rh. koreensis* and their mixture, by using modified crosslinking polymers CCC3 (PVA-al 1% + PEI-al 0.25%) and CCC4 (PVA-al 0.5% + PEI-al 0.6%). Their mechanical properties increased after incubation with phenol as the microbial cells proliferated in the new environment under optimum conditions.
- The optimum pH for *A. radioresistens* to obtain the highest phenol-degrading efficiency is between pH 7 and pH 8. Any value between that ranges would be suitable for their metabolic activity at the optimum temperature of 30$^{o}$ C. However, temperatures above or below or above may affect negatively the viability of cells.
- Employing Kaldnes carriers as a scaffold and physical protector for *A. radioresistens* in crosslinked cell cryogels samples (CCCs-KC) was a successful strategy. Evaluation of their ability to degrade phenol spiked in real water samples collected from rivers, ground water and tap water, showed quicker degradation rates compared to the CCC samples without carriers.

The conclusion from the points above is that crosslinked cell-cryogels developed utilising indigenous bacteria isolated from the contaminated area, combined with low ratios of crosslinking polymer is a promising tool for the bioremediation of phenol and its derivative in water and for wastewater treatment. The overall efficiency of the process is enhanced and more rapid when the CCCs are combined with Kaldnes carriers.

## 8.3 Recommendations for future work

Recommendations for further research are determined according to the results presented in this thesis. A few questions are raised and to extend the novelty and efficacy of crosslinked cryogel in Kaldnes carriers (CCC-KC) for water or wastewater treatments, further research is required on the following aspects:

- Methods to extend the expiry date /'shelf-life' of the synthesised activated crosslinking polymers.
- Does increasing the concentration of aldehyde groups provide more crosslinking reaction points? Theoretically this should help build stronger macroporous structures.
- How can the mechanical properties, porosity, flow rate and viability of cells in the CCCs be improved to degrade higher concentrations of phenol?
- Further investigations are required on the metabolic pathway(s) of *Acinetobacter radioresistens* for phenol degradation to gain a clearer view about the phenol degradation outcomes and by- products.
- Further research is needed to assess the effect of different shapes of Kaldnes carriers on the efficiency of the CCC samples. What morphological changes occur inside the structure of the CCCs-KC during and after degradation of phenol in environmental water?
- Further research is required to select the best process to storage the crosslinked cells cryogel samples for future use with high concentrations of viable cells

# References


ABDEEN, Z., EL-SHESHTAWY, H.K. & MOUSTAFA, Y., (2014). Enhancement of Crude Oil Biodegradation by Immobilizing of different Bacterial Strains on Porous PVA Hydrogels or Combining of them with their produced Biosurfactants. *Petroleum & Environmental Biotechnology,* 05**,** 192.

ABDELWAHAB, O., AMIN, N. & EL-ASHTOUKHY, E.Z., (2009). Electrochemical removal of phenol from oil refinery wastewater. *Journal of Hazardous Materials,* 163**,** 711.

ABUBAKAR, A. & SHUKOR, M.Y., (2017). Phenol removal via cellular immobilization: A review. *Bioremediation Science and Technology Research,* 5**,** 1.

ACCINELLI, C., SACCÀ, M.L., MENCARELLI, M. & VICARI, A., (2012). Application of bioplastic moving bed biofilm carriers for the removal of synthetic pollutants from wastewater. *Bioresource Technology,* 120**,** 180.

ADAMS, G.O., FUFEYIN, P.T., OKORO, S.E. & EHINOMEN, I., (2015). Bioremediation, biostimulation and bioaugmention: a review. *International Journal of Environmental Bioremediation & Biodegradation,* 3**,** 28.

AHMAD, S., SYED, M., ARIF, N., SHUKOR, M. & SHAMAAN, N., (2011). Isolation, identification and characterization of elevated phenol degrading Acinetobacter sp. Strain AQ5NOL 1. *Australian Journal of Basic and Applied Sciences,* 5**,** 1035.

AHMAD, S.A., SHAMAAN, N.A., SYED, M.A., KHALID, A., AB RAHMAN, N.A., KHALIL, K.A., DAHALAN, F.A. & SHUKOR, M.Y., (2017). Meta-cleavage pathway of phenol degradation by Acinetobacter sp. strain AQ5NOL 1. *Rendiconti Lincei,* 28**,** 1.

AL-HADITHI, H.T., AL-RAZZAQ, E.A. & FADHIL, G.F., (2017). Bioremediation of polycyclic aromatic hydrocarbons by Acinetobacter species isolated from ecological sources. *Journal of Environmental Biology* 38**,** 785.

ALEXANDER, M., (1999). *Biodegradation and bioremediation,* London, Acadmic press

ARAGÃO BÖRNER, R., ZAUSHITSYNA, O., BERILLO, D., SCACCIA, N., MATTIASSON, B. & KIRSEBOM, H., (2014). Immobilization of Clostridium acetobutylicum DSM 792 as macroporous aggregates

through cryogelation for butanol production. *Process Biochemistry,* 49**,** 10.

ARUNKUMAR, D. & ANITHA, A., (2014). Biodegradation of phenol and emulsification properties of native microorganisms from coal carbonization plant. *International Journal of Applied Biology and Pharmaceutical Technology,* 5**,** 249.

ARUTCHELVAN, V., KANAKASABAI, V., NAGARAJAN, S. & MURALIKRISHNAN, V., (2005). Isolation and identification of novel high strength phenol degrading bacterial strains from phenol-formaldehyde resin manufacturing industrial wastewater. *Journal of Hazardous Materials,* 127**,** 238.

ATLAS, R.M. & PHILP, J., (2005). *Bioremediation. Applied microbial solutions for real-world environmental cleanup,* Washington, ASM Press.

ATSDR (2008). Toxicological profile for phenol. Georgia.: Division of toxicology and environmental medicine/applied toxicology branch, US Department of health and human services.

ATSDR (2011). Phenol. Georgia Agency for toxicsubstances & disease registry

AYATI, B., GANJIDOUST, H. & MIRFATAH, M., (2007). Degradation of aromatic compounds using moving bed biofilm reactors. *Iranian Journal of Environmental Health Science & Engineering,* 4**,** 107.

BANERJEE, A. & GHOSHAL, A.K., (2010). Phenol degradation by Bacillus cereus: pathway and kinetic modeling. *Bioresource Technology,* 101**,** 5501.

BARLOW, J., JOHNSON, J.A.P., BELCHER, S. & FENTON, S. (2007). Early life exposure to phenols and breast cancer risk in later years: Fact sheet on phenols. p.1-20.

BASHA, K.M., RAJENDRAN, A. & THANGAVELU, V., (2010). Recent advances in the biodegradation of phenol: a review. *Asian Journal of Experimental Biological Sciences,* 1**,** 219.

BAYRAM, E., UZUN, L., TÜRKMEN, D., YıLMAZ, E., GALAEV, I.Y., DENIZLI, A. & BEKTAŞ, S., (2007). Poly (acrylamide-allyl glycidyl ether) Cryogel as a Novel Stationary Phase for Chlorophenol Adsorption. *Hacettepe Journal of Biology and Chemistry,* 35**,** 219.

BELLAR, T.A., LICHTENBERG, J.J. & KRONER, R.C., (1974). The occurrence of organohalides in chlorinated drinking waters. *American Water Works Association,* 66**,** 703.

BERILLO, D., ELOWSSON, L. & KIRSEBOM, H., (2012). Oxidized dextran as crosslinker for chitosan cryogel scaffolds and formation of polyelectrolyte complexes between chitosan and gelatin. *Macromolecular Bioscience,* 12**,** 1090.

BERRIDGE, M.V., HERST, P.M. & TAN, A.S., (2005). Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnology Annual Review.* Elsevier.

BERRY, H., COOK, A. & WILLS, B., (1956). Bactericidal activities of soap-phenol mixtures. *Journal of Pharmacy and Pharmacology,* 8**,** 425.

BIOMERIEXU. (2015). *API & ID 32 identification databases* [Online]. Available: **http://www.biomerieux-diagnostics.com/sites/clinic/files/9308960-002-gb-b-apiweb-booklet.pdf** [Accessed 07/02/2018.

BOHDZIEWICZ, J., KAMIŃSKA, G. & TYTŁA, M., (2012). The removal of phenols from wastewater through sorption on activated carbon. *Architecture Civil Engineering Environment,* 2**,** 89.

BOOPATHY, R., (2000). Factors limiting bioremediation technologies. *Bioresource Technology,* 74**,** 63.

BORDEN, R.C., (2017). Natural bioremediation of hydrocarbon-contaminated ground water. *Handbook of Bioremediation (1993).* CRC Press.

BORKAR, R., GULHANE, M. & KOTANGALE, A., (2013). Moving bed biofilm reactor–a new perspective in wastewater treatment. *IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)* 6**,** 15.

BROCK, T., MADIGAN, M., MARTINKO, J. & PARKER, J., (1988). *Growth and its control,* Prentice-Hall,, Englewood Cliffs, NJ.

CE251 ENVIRONMENTAL ENGINEERING FUNDAMENTALS. (n.d.). *CE3501 Environmental Engineering* [Online]. Available: **http://www.cee.mtu.edu/~mtauer/classes/ce3501/hw/HW2solutions.htm** [Accessed 06th of October 2018 ].

CHANDRA, R., YADAV, S., BHARAGAVA, R.N. & RAI, V., (2011). Phenol degradation by Paenibacillus thiaminolyticus and Bacillus cereus in axenic and mixed conditions. *World Journal of Microbiology and Biotechnology,* 27**,** 2939.

COATES, J.D., WOODWARD, J., ALLEN, J., PHILP, P. & LOVLEY, D.R., (1997). Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Applied and Environmental Microbiology,* 63**,** 3589.

COICO, R., (2005). Gram staining. *Current Protocols in Microbiology* Appendix 3**,** Appendix 3C.

DAKHIL, I.H., (2013). Removal of phenol from industrial wastewater using sawdust. *International Journal of Engineering and Science,* 3**,** 25.

DIJKSHOORN, L., VAN HARSSELAAR, B., TJERNBERG, I., BOUVET, P.J.M. & VANEECHOUTTE, M., (1998). Evaluation of Amplified Ribosomal DNA Restriction Analysis for Identification of Acinetobacter Genomic Species. *Systematic and Applied Microbiology,* 21**,** 33.

DIMITRIOS, B., (2006). Sources of natural phenolic antioxidants. *Trends in Food Science & Technology,* 17**,** 505.

DIVARI, S., VALETTI, F., CAPOSIO, P., PESSIONE, E., CAVALETTO, M., GRIVA, E., GRIBAUDO, G., GILARDI, G. & GIUNTA, C., (2003). The oxygenase component of phenol hydroxylase from Acinetobacter radioresistens S13. *European Journal of Biochemistry,* 270**,** 2244.

DIX, P., (1981). Cross-resistance in cell lines of Nicotiana sylvestris selected for resistance to individual antibiotics. *Annals of Botany,* 48**,** 321.

DRINKING WATER INSPECTORATE, D. (2017). *Nitrate and private water supplies* [Online]. Available: **http://www.dwi.gov.uk/stakeholders/guidance-and-codes-of-practice/pws-nitrates.pdf** [Accessed 19th of June 2018].

DUAN, W., FANPING, M., LIN, Y. & WANG, G., (2017). Toxicological effects of phenol on four marine microalgae. *Environmental Toxicology and Pharmacology,* 52**,** 170.

DUBEY, S.K. & HUSSAIN, A. (2013). Phenol Biodegradation: A review. *Proc. of the International conference on Advances in Civil, Structural and Environmental Engineering--ACSEE 2013.* China

EL-NAAS, M.H., AL-MUHTASEB, S.A. & MAKHLOUF, S., (2009). Biodegradation of phenol by Pseudomonas putida immobilized in polyvinyl alcohol (PVA) gel. *Journal of Hazardous Materials,* 164**,** 720.

EL-NAAS, M.H., AL-ZUHAIR, S. & ALHAIJA, M.A., (2010). Removal of phenol from petroleum refinery wastewater through adsorption on date-pit activated carbon. *Chemical Engineering Journal,* 162**,** 997.

EPA. (1978 ). *Method 420.1: Phenolics (Spectrophotometric, Manual 4 AAP With Distillation)* [Online]. USA Environmental Protection Agency (EPA). Available: https://**www.epa.gov/sites/production/files/2015-08/documents/method_420-1_1978.pdf**. [Accessed 5th of May 2015].

ERTURK, G. & MATTIASSON, B., (2014). Cryogels-versatile tools in bioseparation. *Journal Chromatogr A,* 1357**,** 24.

FATAJEVA, E., GAILIŪTĖ, I., PALIULIS, D. & GRIGIŠKIS, S., (2014). The use of Acinetobacter sp. for oil hydrocarbon degradation in saline Waters. *Biologija,* 60**,** 3.

FERREIRA, V.B., DA SILVA, T.T.C., COUTO, S.R.M. & SRUR, A.U.O.S., (2015). Total phenolic compounds and antioxidant activity of organic vegetables consumed in Brazil. *Food and Nutrition Sciences,* 6**,** 798.

FRANCIS, A. & SOSAMONY, K., (2016). Treatment of pre-treated textile wastewater using Moving bed bio-film reactor. *Procedia Technology,* 24**,** 248.

FRANCK, A. (2004). Understanding rheology of structured fluids. Book of TA instruments, pp.1-7.

FRITSCHE, W. & HOFRICHTER, M., (2000). Aerobic degradation by microorganisms. *Biotechnology* 11**,** 144.

FULEKAR, M., (2012). *Bioremediation technology: recent advances,* New Delhi, India, Springer Science & Business Media.

GAD, N.S. & SAAD, A.S., (2008). Effect of environmental pollution by phenol on some physiological parameters of Oreochromis niloticus. *Global Veterinaria,* 2**,** 312.

GAMI, A.A., (2014). Phenol and its toxicity. *Journal of Environmental Microbiology and Toxicology,* 2**,** 11.

GIANNOULI, P. & MORRIS, E.R., (2003). Cryogelation of xanthan. *Food Hydrocolloids,* 17**,** 495-501.

GODDARD, P.A. & MCCUE, K.A., (2001). Phenolic compounds. *Disinfection, sterilization, and preservation. .* Philadelphia: Lippincott Williams & Wilkins.

GUN'KO, V.M., SAVINA, I.N. & MIKHALOVSKY, S.V., (2013). Cryogels: morphological, structural and adsorption characterisation. *Advances in Colloid and Interface Science,* 187**,** 1.

HASSAN, C.M. & PEPPAS, N.A., (2000). Structure and applications of poly (vinyl alcohol) hydrogels produced by conventional crosslinking or by freezing/thawing methods. *In:* J.Y. CHANG, D. Y. G., M.J. HAN, C.M. HASSAN, J. KIM, B. LEE, Y. LEE, N.A. PEPPAS, R.P. QUIRK, T. YOO (ed.) *Biopolymers· PVA Hydrogels, Anionic Polymerisation Nanocomposites.* New York, Berline, : Springer Science & Business Media.

HASSAN, H., JIN, B., DONNER, E., VASILEIADIS, S., SAINT, C. & DAI, S., (2018). Microbial community and bioelectrochemical activities in MFC for degrading phenol and producing electricity: Microbial consortia could make differences. *Chemical Engineering Journal,* 332**,** 647.

HENDERSON, T.M., LADEWIG, K., HAYLOCK, D.N., MCLEAN, K.M. & O'CONNOR, A.J., (2013). Cryogels for biomedical applications. *Journal of Materials Chemistry B,* 1**,** 2682-2695.

HIXON, K.R., LU, T. & SELL, S.A., (2017). A comprehensive review of cryogels and their roles in tissue engineering applications. *Acta Biomaterialia,* 62**,** 29.

HOODAJI, M., TAHMOURESPOUR, A. & ESKANDARY, S., (2013). Effect of environmental conditions on phenol biodegradation by newly isolated bacterium (Alcaligenes Sp. ATHE8) from coke processing wastewater. *Journal of Residuals Science & Technology,* 10.

HOSSAIN, M.M., ISLAM, K.N. & RAHMAN, I.M., (2012). An overview of the persistent organic pollutants in the freshwater system. *In:* DR. VOUDOURIS (ED.) (ed.) *Ecological Water Quality-Water Treatment and Reuse.* InTech.

HOSSEINI, S.H. & BORGHEI, S.M., (2005). The treatment of phenolic wastewater using a moving bed bio-reactor. *Process Biochemistry,* 40**,** 1027.

HRISTOV, A.E., CHRISTOVA, N.E., KABAIVANOVA, L.V., NACHEVA, L.V., STOINEVA, I.B. & PETROV, P.D., (2016). Simultaneous Biodegradation of Phenol and n-Hexadecane by Cryogel Immobilized Biosurfactant Producing Strain Rhodococcus wratislawiensis BN38. *Polish Journal of Microbiology,* 65**,** 287.

HUANG, W.-Y., CAI, Y.-Z. & ZHANG, Y., (2009). Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. *Nutrition and Cancer,* 62**,** 1.

HYOUNG-JOON, J., YOUNG SOO, Y. & HYEONSEONG, B., (2010). Monolithic Macroporous Carbon Cryogel Prepared from Natural Polymers. *Journal of the Korean Physical Society,* 57**,** 1950.

INGAVLE, G.C., BAILLIE, L.W.J., ZHENG, Y., LIS, E.K., SAVINA, I.N., HOWELL, C.A., MIKHALOVSKY, S.V. & SANDEMAN, S.R., (2015). Affinity binding of antibodies to supermacroporous cryogel adsorbents with immobilized protein A for removal of anthrax toxin protective antigen. *Biomaterials,* 50**,** 140.

INGRAHAM, J.L., MAALØE, O. & NEIDHARDT, F., (1983 ). *Growth of the Bacterial Cell* Sinauer Associates

JAHANGIRI, E., REICHELT, S., THOMAS, I., HAUSMANN, K., SCHLOSSER, D. & SCHULZE, A., (2014). Electron beam-induced immobilization of laccase on porous supports for waste water treatment applications. *Molecules,* 19**,** 11860.

JIANG, L.C., RUAN, Q.P., LI, R.L. & LI, T.D., (2013). Biodegradation of phenol by using free and immobilized cells of Acinetobacter sp BS8Y. *Journal of Basic Microbiology,* 53**,** 224.

JIANG, Y., DENG, T., SHANG, Y., YANG, K. & WANG, H., (2017). Biodegradation of phenol by entrapped cell of Debaryomyces sp. with nano-Fe3O4 under hypersaline conditions. *International Biodeterioration & Biodegradation,* 123**,** 37.

JOSHI, M. & DESHPANDE, J., (2011). Polymerase chain reaction: methods, principles and application. *International Journal of Biomedical Research,* 2**,** 81.

KAGLE, J. & HAY, A.G., (2006). Phenylacetylene reversibly inhibits the phenol hydroxylase of Pseudomonas sp. CF600 at high concentrations but is oxidized at lower concentrations. *Applied Microbiology and Biotechnology,* 72**,** 306.

KARAKAYA, S., (2004). Bioavailability of phenolic compounds. *Critical Reviews in Food Science and Nutrition,* 44**,** 453.

KARIMI, M. & HASSANSHAHIAN, M., (2016). Isolation and characterization of phenol degrading yeasts from wastewater in the coking plant of Zarand, Kerman. *Brazilian Journal of Microbiology,* 47**,** 18.

KAVITA, R. & GEETA, D., (2014). Bioremediation and Biodegradation of Pesticide from Contaminated Soil and Water - A Noval Approach. *International Journal of Current Microbiology and Applied Science,* 3**,** 23.

KHOSRAVI, A.D., SADEGHI, P., SHAHRAKI, A.H., HEIDARIEH, P. & SHEIKHI, N., (2015). Molecular methods for identification of acinetobacter species by partial sequencing of the rpoB and 16S rRNA genes. *Journal of Clinical and Diagnostic Research: JCDR,* 9**,** DC09.

KIM, K.H., JAHAN, S.A., KABIR, E. & BROWN, R.J., (2013). A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. *Environment International* 60**,** 71.

KOBAYASHI, H. & RITTMANN, B.E., (1982). Microbial removal of hazardous organic compounds. *Environmental Science & Technology,* 16**,** 170A.

KOC-JURCZYK, J. & JURCZYK, L., (2017). The effect of Kaldnes bio filter media packing volume on the effeciency of maunicipal landfill leachate treatment. *Carpathian Journal of Earth and Environmental Sciences* 12**,** 77.

KOELEMAN, J.G., STOOF, J., BIESMANS, D.J., SAVELKOUL, P.H. & VANDENBROUCKE-GRAULS, C.M., (1998). Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification ofAcinetobacter genomic species and typing ofAcinetobacter baumannii. *Journal of Clinical Microbiology,* 36**,** 2522.

KOTRESHA, D. & VIDYASAGAR, G., (2008). Isolation and characterisation of phenol-degrading Pseudomonas aeruginosa MTCC 4996. *World Journal of Microbiology and Biotechnology,* 24**,** 541.

KOTRESHA, D. & VIDYASAGAR, G., (2014). Isolation and characterisation of phenol-degrading Pseudomonas aeruginosa MTCC 4996. *World Journal of Microbiology and Biotechnology,* 24**,** 541.

KOTRESHA, D. & VIDYASAGAR, G.M., (2014). Phenol degradation by immobilised cells of Pseudomonas areuginosa MTCC 4996. . *International Journal of Development Research,* 4**,** 1439.

KOUTNY, M., RUZICKA, J. & CHLACHULA, J., (2003). Screening for phenol-degrading bacteria in the pristine soils of south Siberia. *Applied Soil Ecology,* 23**,** 79.

KOVACIC, P. & WESTON, W., (2017). Phenolic antioxidants as drugs for Alzheimer's disease: oxidative stress and selectivity. *Novel Approaches in Drug Designing and Development,* 2**,** 555598.

KRASTANOV, A., ALEXIEVA, Z. & YEMENDZHIEV, H., (2013). Microbial degradation of phenol and phenolic derivatives. *Engineering in Life Sciences,* 13**,** 76.

KRISHNAN, S., MANAVATHU, E.K. & CHANDRASEKAR, P.H., (2005). A comparative study of fungicidal activities of voriconazole and amphotericin B against hyphae of Aspergillus fumigatus. *Journal of Antimicrobial Chemotherapy,* 55**,** 914.

KUJAWSKI, W., WARSZAWSKI, A., RATAJCZAK, W., POREBSKI, T., CAPAŁA, W. & OSTROWSKA, I., (2004). Removal of phenol from wastewater by different separation techniques. *Desalination,* 163**,** 287.

KULKARNI, C., (2017). Phytochemical analysis and total phenol content in Daucus carota Linn. *Phytochemical Analysis,* 2**,** 74.

KULKARNI, S.J. & KAWARE, J.P., (2013). Review on research for removal of phenol from wastewater. *International journal of scientific and research publications,* 3**,** 654.

KUMAR, A., BISHT, B., JOSHI, V. & DHEWA, T., (2011). Review on bioremediation of polluted environment:A management tool. *International Journal of Environmental Sciences,* 1**,** 1079.

KUMARI, J. & KUMAR, A., (2017). Development of polymer based cryogel matrix for transportation and storage of mammalian cells. *Scientific Reports,* 7**,** 41551.

KURNIAWAN, S.B., PURWANTI, I.F. & TITAH, H.S., (2018). The effect of pH and aluminium to bacteria isolated from aluminium recycling industry. *Journal of Ecological Engineering,* 19**,** 154.

KUYUKINA, M.S., RUBTSOVA, E.V., IVSHINA, I.B., IVANOV, R.V. & LOZINSKY , V.I., (2009). Selective adsorption of hydrocarbon-oxidizing Rhodococcus cells in a column with hydrophobized poly(acrylamide) cryogel. *Journal of Microbiological Methods,* 79**,** 76.

LA SPINA, R., TRIPISCIANO, C., MECCA, T., CUNSOLO, F., WEBER, V. & MATTIASSON, B., (2014). Chemically modified poly(2-hydroxyethyl methacrylate) cryogel for the adsorption of heparin. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 102**,** 1207.

LAKSHMI, M.C., SRIDEVI, V., NEHARIKA, E., BEENA, C., RAO, M.N. & SWAMY, A., (2009). Effect of temperature and carbon source on phenol degradation by Pseudomonas aeruginosa (NCIM 2074) and Pseudomonas desmolyticum (NCIM 2028) and their comparison. *International Journal of Chemical Sciences,* 7**,** 2591.

LAZARIDOU, A. & BILIADERIS, C.G., (2004). Cryogelation of cereal β-glucans: structure and molecular size effects. *Food Hydrocolloids,* 18**,** 933.

LEE, S. & SPEIGHT, J.G., (2000). *Environmental technology handbook,* New York, Taylor and Francis

LI, J., ZHOU, Q.-B., WANG, X. & ZHANG, Y.-H. (2017). Isolation of Highly Efficient Phenol Degradation Strain and Characterization of Degradation of Phenol. *IOP Conference Series: Earth and Environmental Science.* IOP Publishing.

LI, S., CHENG, C. & THOMAS, A., (2017). Carbon-Based Microbial-Fuel-Cell Electrodes: From Conductive Supports to Active Catalysts. *Advanced Materials,* 29**,** 1602547.

LIU, D.-M., CHEN, J. & SHI, Y.-P., (2018). Advances on methods and easy separated support materials for enzymes immobilization. *Trends in Analytical Chemistry* 102**,** 332.

LIU, Y., ZHANG, A. & WANG, X., (2009). Biodegradation of phenol by using free and immobilized cells of Acinetobacter sp. XA05 and Sphingomonas sp. FG03. *Biochemical Engineering Journal,* 44**,** 187.

LOZINSKY, V.I., (2002). Cryogels on the basis of natural and synthetic polymers: preparation, properties and application. *Russian Chemical Reviews,* 71**,** 489.

LOZINSKY, V.I., (2004). What new opportunities the use of diverse polymeric cryogels opens for the immobilization of molecules and cells. *Hemijska Industrija,* 58**,** 111.

LOZINSKY, V.I., (2014). A brief history of polymeric cryogels. *In:* OKAY, O. (ed.) *Polymeric Cryogels. Advances in polymer science* New York: Springer Cham.

LOZINSKY, V.I., GALAEV, I.Y., PLIEVA, F.M., SAVINA, I.N., JUNGVID, H. & MATTIASSON, B., (2003). Polymeric cryogels as promising materials of biotechnological interest. *Trends in Biotechnology,* 21**,** 445.

LU, D., ZHANG, Y., NIU, S., WANG, L., LIN, S., WANG, C., YE, W. & YAN, C., (2012). Study of phenol biodegradation using Bacillus amyloliquefaciens strain WJDB-1 immobilized in alginate–chitosan–alginate (ACA) microcapsules by electrochemical method. *Biodegradation,* 23**,** 209-219.

LU, G.-H., WANG, C. & GUO, X.-L., (2008). Prediction of Toxicity of Phenols and Anilines to Algae by Quantitative Structure-activity Relationship. *Biomedical and Environmental Sciences,* 21**,** 193.

LUKA, Y., HIGHINA, B. & ZUBAIRU, A., (2018). Bioremediation: A Solution to Environmental Pollution-A Review. *American Journal of Engineering Research (AJER),* 7**,** 101.

LUTTRELL, W.E., (2003). Toxic tips: phenol. *Chemical Health and Safety,* 5**,** 20.

MACFADDIN, J.F., (2000). *Biochemical tests for identification of medical bacteria,* London, Philadelphia, Lippincott Williams & Wilkins.

MAHVI, A.H. & MALEKI, A., (2010). Photosonochemical degradation of phenol in water. *Desalination and Water Treatment,* 20**,** 197.

MAO, Z., YU, C.Y. & XIN, L.L., (2015). Enhancement of Phenol Biodegradation by Pseudochrobactrum sp through Ultraviolet-Induced Mutation. *International Journal of Molecular Sciences* 16**,** 7320.

MARA, D. & HORAN, N.J. (eds.) (2003). *Handbook of water and wastewater microbiology,* London: Academic Press, An Imprint of Elsevier.

MARGESIN, R., MOERTELMAIER, C. & MAIR, J., (2013). Low-temperature biodegradation of petroleum hydrocarbons (n-alkanes, phenol, anthracene, pyrene) by four actinobacterial strains. *International Biodeterioration & Biodegradation,* 84**,** 185.

MARTINS, S.C.S., MARTINS, C.M., FIÚZA, L.M.C.G. & SANTAELLA, S.T., (2013). Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology,* 12**,** 4412.

MCQUARRIE, D.A., ROCK, P.A. & GALLOGLY, E.B. (2011). Aromatic Hydrocarbons ,Interchapter H. *General Chemistry,Interchapters Online.* Fourth Edition ed.: University Science books.

MEIKAP, B. & ROY, G., (1997). Removal of Phenolic Compounds From Industrial Waste Water by SemifluidizedBed Bio-Reactor. *Journal of the Institution of Public Health Engineers (IPHE),* 1997**,** 54.

MENDONÇA, E., MARTINS, A. & ANSELMO, A.M., (2004). Biodegradation of natural phenolic compounds as single and mixed substrates by Fusarium flocciferum. *Electronic Journal of Biotechnology,* 7**,** 38.

MENDOZA, L., MAMO, G., FLORES, N., GIMENEZ, A. & HATTI-KAUL, R., (2011). Laccase mediator system for activation of agarose gel: Application for immobilization of proteins. *Journal of Molecular Catalysis B: enzymatic,* 68**,** 270.

MICHAŁOWICZ, J. & DUDA, W., (2007). Phenols-Sources and Toxicity. *Polish Journal of Environmental Studies,* 16**,** 347.

MOHANTY, S.S. & JENA, H.M., (2017). Biodegradation of phenol by free and immobilized cells of noval Pseudomonas sp. NBM11. *Brazilian Journal of Chemical Engineering,* 34**,** 75.

MOHITE, B.V., JALGAONWALA, R.E., PAWAR, S. & MORANKAR, A., (2010). Isolation and characterization of phenol degrading bacteria from oil contaminated soil. *Innovative Romanian Food Biotechnology,* 7**,** 61.

MOLLAEI, M., ABDOLLAHPOUR, S., ATASHGAHI, S., ABBASI, H., MASOOMI, F., RAD, I., LOTFI, A.S., ZAHIRI, H.S., VALI, H. & NOGHABI, K.A., (2010). Enhanced phenol degradation by Pseudomonas sp. SA01: gaining insight into the novel single and hybrid immobilizations. *Journal of Hazardous Materials,* 175**,** 284.

MOWNICA, B. & MANIMEKALAI, B., (2017). Performance Evaluation of Fluidised Bed Biofilm Reactor using Kaldnes K1 for Domestic Wastewater. *IJSRD - International Journal for Scientific Research & Development,* 5

MROZIK, A., MIGA, S. & PIOTROWSKA-SEGET, Z., (2011). Enhancement of phenol degradation by soil bioaugmentation with Pseudomonas sp. JS150. *Journal of Applied Microbiology,* 111**,** 1357.

NAIR, C.I., JAYACHANDRAN, K. & SHASHIDHAR, S., (2008). Biodegradation of phenol. *African Journal of Biotechnology,* 7**,** 4951.

NASSEF, E.M.R., (2014). Removal of polyaromatic hydrocarbons from waste water by electrocoagulation. *Journal of Petroleum and Gas Engineering,* 5**,** 32.

NAWAWI, N.M., (2014). Isolation and characterization of phenol-degrading microorganism: Recent Advances. *Journal of Environmental Bioremediation and Toxicology,* 2.

NICHOLAS, N. (2016). *MBBR Wastewater Treatment - 5 Top Advantages of advanced moving bed bioreactor systems* [Online]. Available: https://**www.linkedin.com/pulse/mbbr-wastewater-treatment-5-top-advantages-nick-nicholas** [Accessed 4th July 2018].

O'SULLIVAN, M. 1998. *The Degradation of Phenol and Mono-Chlorophenols by a Mixed Microbial Population.* degree of Doctor of Philosophy, Dublin City University.

OKAY, O. & LOZINSKY, V.I., (2014). Synthesis and structure–property relationships of cryogels. *In:* OKAY, O. (ed.) *Polymeric cryogels, macroporous gels with remarkable properties.* Switzerland, : Springer International publishing

OLANIRAN, A.O. & IGBINOSA, E.O., (2011). Chlorophenols and other related derivatives of environmental concern: properties, distribution and microbial degradation processes. *Chemosphere,* 83**,** 1297.

ÖNNBY, L., (2016). Applications of Cryogels in Water and Wastewater Treatment. *Supermacroporous Cryogels.* pp.331.

OZTOPRAK, Z., HEKIMOGLU, T., KARAKUTUK, I., TUNCABOYLU, D.C. & OKAY, O., (2014). Porous rubber cryogels: effect of the gel preparation temperature. *Polymer Bulletin,* 71**,** 1983.

PANAGOS, P., VAN LIEDEKERKE, M., YIGINI, Y. & MONTANARELLA, L., (2013). Contaminated sites in Europe: review of the current situation based on data collected through a European network. *Journal of Environmental and Public Health,* 2013**,** 11.

PATEL, N., OUDEMANS, P.V., HILLMAN, B.I. & KOBAYASHI, D.Y., (2013). Use of the tetrazolium salt MTT to measure cell viability effects of the bacterial antagonist Lysobacter enzymogenes on the filamentous fungus Cryphonectria parasitica. *Antonie Van Leeuwenhoek,* 103**,** 1271.

PEREZ-JIMENEZ, J., NEVEU, V., VOS, F. & SCALBERT, A., (2010). Identification of the 100 richest dietary sources of polyphenols: an application of the Phenol-Explorer database. *European Journal of Clinical Nutrition,* 64 Suppl 3**,** S112.

PHILP, J.C., BALMAND, S., HAJTO, E., BAILEY, M.J., WILES, S., WHITELEY, A.S., LILLEY, A.K., HAJTO, J. & DUNBAR, S.A., (2003). Whole cell immobilised biosensors for toxicity assessment of a wastewater treatment plant treating phenolics-containing waste. *Analytica Chimica Acta,* 487**,** 61.

PLIEVA, F.M., GALAEV, I.Y. & MATTIASSON, B., (2007). Macroporous gels prepared at subzero temperatures as novel materials for chromatography of particulate-containing fluids and cell culture applications. *Journal of Separation Science,* 30**,** 1657.

PLIEVA, F.M., GALAEV, I.Y., NOPPE, W. & MATTIASSON, B., (2008). Cryogel applications in microbiology. *Trends in Microbiology,* 16**,** 543.

PLIEVA, F.M., KIRSEBOM, H. & MATTIASSON, B., (2011). Preparation of macroporous cryostructurated gel monoliths, their characterization and main applications. *Journal of Separation Science,* 34**,** 2164.

PLIEVA, F.M., OKNIANSKA, A., DEGERMAN, E. & MATTIASSON, B., (2008). Macroporous gel particles as robust macroporous matrices for cell immobilization. *Biotechnology Journal* 3**,** 410.

PLOTKIN, J.S. (2016). *What's New in Phenol Production?* [Online]. American Chemical Society. Available: https://**www.acs.org/content/acs/en/pressroom/cutting-edge-chemistry/what-s-new-in-phenol-production-.html** [Accessed 17th July 2018].

POTTERS, G. (2013). Marine Pollution. 1st edition ed ed.: eBooks at bookboon.com.

PRADEEP, N., ANUPAMA, S., NAVYA, K., SHALINI, H., IDRIS, M. & HAMPANNAVAR, U., (2015). Biological removal of phenol from wastewaters: a mini review. *Applied Water Science,* 5**,** 105.

PRIETO, M.B., HIDALGO, A., RODRIGUEZ-FERNANDEZ, C., SERRA, J.L. & LLAMA, M.J., (2002). Biodegradation of phenol in synthetic and industrial wastewater by Rhodococcus erythropolis UPV-1 immobilized in an air-stirred reactor with clarifier. *Applied Microbiology and Biotechnology,* 58**,** 853.

RATHORE, P. & SINHA, K., (2014). Screening and Characterization of Xenobiotic (Phenol) Degrading Bacteria with Reference to Bioremediation. *Indian Journal of Applied Research* 4**,** 36.

REARDON, K.F., MOSTELLER, D.C., ROGERS, J.B., DUTEAU, N.M. & KIM, K.H., (2002). Biodegradation kinetics of aromatic hydrocarbon mixtures by pure and mixed bacterial cultures. *Environmental Health Perspectives,* 110**,** 1005.

REN, S., (2003). Phenol mechanism of toxic action classification and prediction: a decision tree approach. *Toxicology Letters,* 144**,** 313.

RENGARAJ, S., MOON, S.-H., SIVABALAN, R., ARABINDOO, B. & MURUGESAN, V., (2002). Agricultural solid waste for the removal of organics: adsorption of phenol from water and wastewater by palm seed coat activated carbon. *Waste Management,* 22**,** 543.

RENUKAPRASAD, M.T., ISMAILSAB, M., REDDY, P.V., BABURAO, K., NAYAK, A.S. & KAREGOUDAR, T., (2017). A comparative study of utilization of single and mixed phenolic compounds by individual and mixed culture. *3 Biotech,* 7**,** 215.

RESHMA, J., THANGA, V. & MATHEW, A., (2014). Phenol degradation by bacteria isolated from coir retting beds of south kerala coast. *Journal of Global Biosciences,* 3**,** 935.

RICE, E.W., BAIRD, R.B., DATON, A.D. & CLECERI, L.S. (eds.) (2012). *Standard methods for the examination of water and waste water* Washington: American public health association, American water works association and Water environment federation

RISS, T., MORAVEC, R., NILES, A., DUELLMAN, S., BENINK, H., WORZELLA, T. & MINOR, L. (2016). Cell viability assays, 2004.

ROBERTS, J.D. & CASERIO, M.C. (1977). Basic principles of organic chemistry WA Benjamin, Inc. **http://resolver.caltech.edu/CaltechBOOK:1977.001**.

ROCHA-GUZMÁN, N.E., HERZOG, A., GONZÁLEZ-LAREDO, R.F., IBARRA-PÉREZ, F.J., ZAMBRANO-GALVÁN, G. & GALLEGOS-INFANTE, J.A., (2007). Antioxidant and antimutagenic activity of phenolic compounds in three different colour groups of common bean cultivars (Phaseolus vulgaris). *Food chemistry,* 103**,** 521.

ROGERS, H. (1983). Bacterial cell structure. Washington: Amarican socity for microbiology

RUAN, B., WU, P., CHEN, M., LAI, X., CHEN, L., YU, L., GONG, B., KANG, C., DANG, Z., SHI, Z. & LIU, Z., (2018). Immobilization of Sphingomonas sp. GY2B in polyvinyl alcohol–alginate–kaolin beads for efficient degradation of phenol against unfavorable environmental factors. *Ecotoxicology and Environmental Safety,* 162**,** 103.

RUIZ, Y., MEDINA, L., BORUSIAK, M., RAMOS, N., PINTO, G. & VALBUENA, O., (2013). Biodegradation of polyethoxylated nonylphenols. *ISRN Microbiology,* 2013**,** 9.

RUSTEN, B., EIKEBROKK, B., ULGENES, Y. & LYGREN, E., (2006). Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural Engineering,* 34**,** 322.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T., (1989). *Molecular cloning: a laboratory manual,* Cold spring harbor Laboratory press.

SARAVANAN, P., PAKSHIRAJAN, K. & SAHA, P., (2008). Kinetics of phenol and m-cresol biodegradation by an indigenous mixed microbial culture isolated from a sewage treatment plant. *Environmental Sciences,* 20**,** 1508.

SATCHANSKA, G., TOPALOVA, Y., DIMKOV, R., GROUDEVA, V., PETROV, P., TSVETANOV, C., SELENSKA-POBELL, S. & GOLOVINSKY, E., (2015). Phenol degradation by environmental bacteria entrapped in cryogels. *Biotechnology & Biotechnological Equipment,* 29**,** 514.

SAVINA, I.N., INGAVLE, G.C., CUNDY, A.B. & MIKHALOVSKY, S.V., (2016). A simple method for the production of large volume 3D macroporous hydrogels for advanced biotechnological, medical and environmental applications. *Scientific Report* 6**,** 21154.

SCOPES, R.K., (2013). *Protein purification: principles and practice,* New York, Springer Science & Business Media.

SELVI, A., BANERJEE, M. & DAS, N., (2015). Degradation of cefdinir from pharmaceutical waste water using immobilized Candida sp. SMN04 and biofilm formed on gravels. *Journal of Applied Pharmaceutical Science* 5**,** 073.

SEMPLE, K.T., CAIN, R.B. & SCHMIDT, S., (1999). Biodegradation of aromatic compounds by microalgae. *FEMS Microbiology Letters,* 170**,** 291.

SEO, J.-S., KEUM, Y.-S. & LI, Q.X., (2009). Bacterial degradation of aromatic compounds. *International Journal of Environmental Research and Public Health,* 6**,** 278.

SHAH, M.P., (2014). Microbiological Removal of Phenol by an Application of Pseudomonas spp. ETL. An Innovative Biotechnological Approach Providing Answers to the problems of FETP. *Journal of Applied & Environmental Microbiology,* 1**,** 6.

SHAHAMAT, Y.D., FARZADKIA, M., NASSERI, S., MAHVI, A.H., GHOLAMI, M. & ESRAFILI, A., (2014). Magnetic heterogeneous catalytic ozonation: a new removal method for phenol in industrial wastewater. *Journal of Environmental Health Science and Engineering,* 12**,** 50.

SHEEHAN, D., (1997). *Bioremediation protocols,* Totowa, New Jersey, Humana Press.

SHOURIAN, M., NOGHABI, K.A., ZAHIRI, H.S., BAGHERI, T., KARBALLAEI, G., MOLLAEI, M., RAD, I., AHADI, S., RAHEB, J. & ABBASI, H., (2009). Efficient phenol degradation by a newly characterized Pseudomonas sp. SA01 isolated from pharmaceutical wastewaters. *Desalination,* 246**,** 577.

SILHAVY, T.J., KAHNE, D. & WALKER, S., (2010). The bacterial cell envelope. *Cold Spring Harbor perspectives in biology,* 2**,** a000414.

SIMONEIT, B.R., KOBAYASHI, M., MOCHIDA, M., KAWAMURA, K., LEE, M., LIM, H.J., TURPIN, B.J. & KOMAZAKI, Y., (2004). Composition and major sources of organic compounds of aerosol particulate matter sampled during the ACE-Asia campaign. *Journal of Geophysical Research: Atmospheres,* 109.

SINGH, H., (2006). *Mycoremediation: fungal bioremediation,* USA, John Wiley & Sons, INC., Publication

SINGH, S., SINGH, B.B. & CHANDRA, R., (2009). Biodegradation of phenol in batch culture by pure and mixed strains of Paenibacillus sp. and Bacillus cereus. *Polish Journal of Microbiology* 58**,** 319.

SINGLETON, P., (2004). *Bacteria in biology, biotechnology and medicine,* Chichester, John Wiley & Sons, Ltd.

SMITH, C.V., WILSON, J.D., LLADOS, F.T. & PAIKOFF, S.J. (2009). Substance-Specific Applied Research Program Priority Data Needs for:Phenol. Agency for Toxic Substances and Disease Registry/Division of Toxicology and Environmental Medicine (ATSDR/DTEM).

STEPANOV, N. & EFREMENKO, E., (2018). "Deceived" Concentrated Immobilized Cells as Biocatalyst for Intensive Bacterial Cellulose Production from Various Sources. *Catalysts,* 8**,** 33.

STRAIF, K., BAAN, R., GROSSE, Y., SECRETAN, B., EL GHISSASSI, F. & COGLIANO, V., (2005). Carcinogenicity of polycyclic aromatic hydrocarbons. *The Lancet Oncology,* 6**,** 931.

SUHAILA, Y.N., ROSFARIZAN, M., AHMAD, S.A., LATIF, I.A. & ARIFF, A.B., (2013). Nutrients and culture conditions requirements for the degradation of phenol by Rhodococcus UKMP-5M. *Journal of Environmental Biology,* 34**,** 635.

TAYLOR, A.C., (1962). Responses of cells to pH changes in the medium. *The Journal of Cell Biology,* 15**,** 201.

TIAN, L., MA, P. & ZHONG, J.-J., (2002). Kinetics and key enzyme activities of phenanthrene degradation by Pseudomonas mendocina. *Process Biochemistry,* 37**,** 1431.

TOPALOVA, Y., DIMKOV, R., TODOROVA, Y., DASKALOVA, E. & PETROV, P., (2014 ). Biodegradation of Phenol by Immobilized in Peo-CryogelBacillus Laterosporus BT-271in Sequencing Batch Biofilter. *Biotechnology & Biotechnological Equipment,* 25**,** 2613.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY, U. (2003). National Secondary Drinking Water Regulation.

VANEECHOUTTE, M., DE BEENHOUWER, H., CLAEYS, G., VERSCHRAEGEN, G., DE ROUCK, A., PAEPE, N., ELAICHOUNI, A. & PORTAELS, F., (1993). Identification of Mycobacterium species by using amplified ribosomal DNA restriction analysis. *Journal of Clinical Microbiology,* 31**,** 2061.

VANEECHOUTTE, M., DIJKSHOORN, L., TJERNBERG, I., ELAICHOUNI, A., DE VOS, P., CLAEYS, G. & VERSCHRAEGEN, G., (1995). Identification

of Acinetobacter genomic species by amplified ribosomal DNA restriction analysis. *Journal of Clinical Microbiology,* 33**,** 11.

VASUDEVAN, S., (2014). An efficient removal of phenol from water by peroxi-electrocoagulation processes. *Journal of Water Process Engineering,* 2**,** 53.

VIDALI, M., (2001). Bioremediation. An overview *Pure and Applied Chemistry,* 73**,** 1163.

WANG, H., CHENG, H., WANG, F., WEI, D. & WANG, X., (2010). An improved 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of Escherichia coli cells. *Journal of Microbiological Methods,* 82**,** 330.

WANG, J.L. (2002). Microbial immobilization techniques and water pollution technology. *3rd International Conference on Microwave and Millimeter Wave Technology.* Beijing: Science press.

WASI, S., TABREZ, S. & AHMAD, M., (2013). Toxicological effects of major environmental pollutants: an overview. *Environmental Monitoring and Assessment,* 185**,** 2585.

WEBER, M., WEBER, M. & KLEINE-BOYMANN, M. (2000). Phenol. *Ullmann's Encyclopedia of Industrial Chemistry.*

WEISBURG, W.G., BARNS, S.M., PELLETIER, D.A. & LANE, D.J., (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173**,** 697.

WEITZ, D., WYSS, H. & LARSEN, R., (2007). Oscillatory rheology: Measuring the viscoelastic behaviour of soft materials. *GIT Laboratory Journal Europe,* 11**,** 68.

WHITEHEAD, P. (2018). *Total orgainc carbon (TOC) and its measurment* [Online]. Available: https://**www.elgalabwater.com/blog/total-organic-toc** [Accessed 8th of June 2018 ].

WHO (1995). Global strategy on occupational health for all: the way to health at work, r. *ecommendation of the Second Meeting of the WHO Collaborating Centres in Occupational Health, 11-14 October 1994.* Beijing, China: World Health Organisation.

WHO (2004). Sulfate in Drinking-water, . *Background document for development of WHO Guidelines for Drinking-water Quality.* Geneva World Health Organization.

WHO (2011). Nitrate and Nitrite in Drinking-water, Background document for development of WHO Guidelines for Drinking-water Quality. Geneva: World Health Organisation.

WU, C., LIU, X., WEI, D., FAN, J. & WANG, L., (2001). Photosonochemical degradation of Phenol in water. *Water Research,* 35**,** 3927.

XU, F., SRIDHARAN, B., DURMUS, N.G., WANG, S., YAVUZ, A.S., GURKAN, U.A. & DEMIRCI, U., (2011). Living bacterial sacrificial porogens to engineer decellularized porous scaffolds. *PLoS One,* 6**,** e19344.

YAMAGA, F., WASHIO, K. & MORIKAWA, M., (2010). Sustainable biodegradation of phenol by Acinetobacter calcoaceticus P23 isolated from the rhizosphere of duckweed Lemna aoukikusa. *Environmental Science & Technology,* 44**,** 6470.

YANG, L.-H., ZHU, T.-T., CAI, W.-W., HAIDER, M.R., WANG, H.-C., CHENG, H.-Y. & WANG, A.-J., (2018). Micro-oxygen bioanode: an efficient strategy for enhancement of phenol degradation and current generation in mix-cultured MFCs. *Bioresource Technology,* BITE 20155, https://doi.org/10.1016/j.biortech.2018.07.025.

YAVUZ, Y., KOPARAL, A.S. & ÖĞÜTVEREN, Ü.B., (2010). Treatment of petroleum refinery wastewater by electrochemical methods. *Desalination,* 258**,** 201.

YOON, J.-H., KIM, S.B., CHO, Y.-G., LEE, S.T., KANG, S.-S. & PARK, Y.-H., (2000). Rhodococcus koreensis sp. nov., a 2, 4-dinitrophenol-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology,* 50**,** 1193.

YORDANOVA, G., IVANOVA, D., GODJEVARGOVA, T. & KRASTANOV, A., (2009). Biodegradation of phenol by immobilized Aspergillus awamori NRRL 3112 on modified polyacrylonitrile membrane. *Biodegradation,* 20**,** 717.

ZAJKOSKA, P., REBROS, M. & ROSENBERG, M., (2013). Biocatalysis with immobilized Escherichia coli. *Applied Microbiology and Biotechnology,* 97**,** 1441.

ZAUSHITSYNA, O., BERILLO, D., KIRSE0BOM, H. & MATTIASON, B., (2014). Cryostructured and Crosslinked Viable Cells Forming Monoliths Suitable for Bioreactor Applications. *Topics in Catalysis,* 57**,** 339.

ZAUSHITSYNA, O., DISHISHA, T., HATTI-KAUL, R. & MATTIASSON, B., (2017). Crosslinked, cryostructured Lactobacillus reuteri monoliths for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1, 3-propanediol from glycerol. *Journal of Biotechnology,* 241**,** 22.

# Appendices

## Appendix 1

### Determination of the growth curve of *Pseudomonas mendocina* (NCIMB 13264) and *Rhodococcus koreensis* (NCIMB 13709)

A growth curve for *P. mendocina* and *Rh. koreensis* was determined by picking a single colony of each strain and inoculation into individual sterile tubes containing 10 mL of tryptone soya broth (TSB), and incubated at 37$^{o}$ C for 24 hours. One ml from each tube was transferred to a conical flask containing 100 ml of TSB and incubated in an orbital incubator at 30$^{o}$C and 150 rpm. Cell growth was monitored by measuring the absorbance at a wavelength of 600 nm in an UV-spectrophotometer. Growth curves for both strains were prepared to estimate when the cells had reached the stationary phase.

At this phase, cells numbers have reached the maximum level to enable harvesting of the bacterial cells for the immobilisation process. Estimation of cell numbers was performed by counting the colonies (expressed as CFU/ml) using a colony counter for each dilution of the broth cultures spread and incubated on TSA. In parallel, the optical density was measured using a spectrophotometer at a wavelength of 600 nm (figure A1-1).

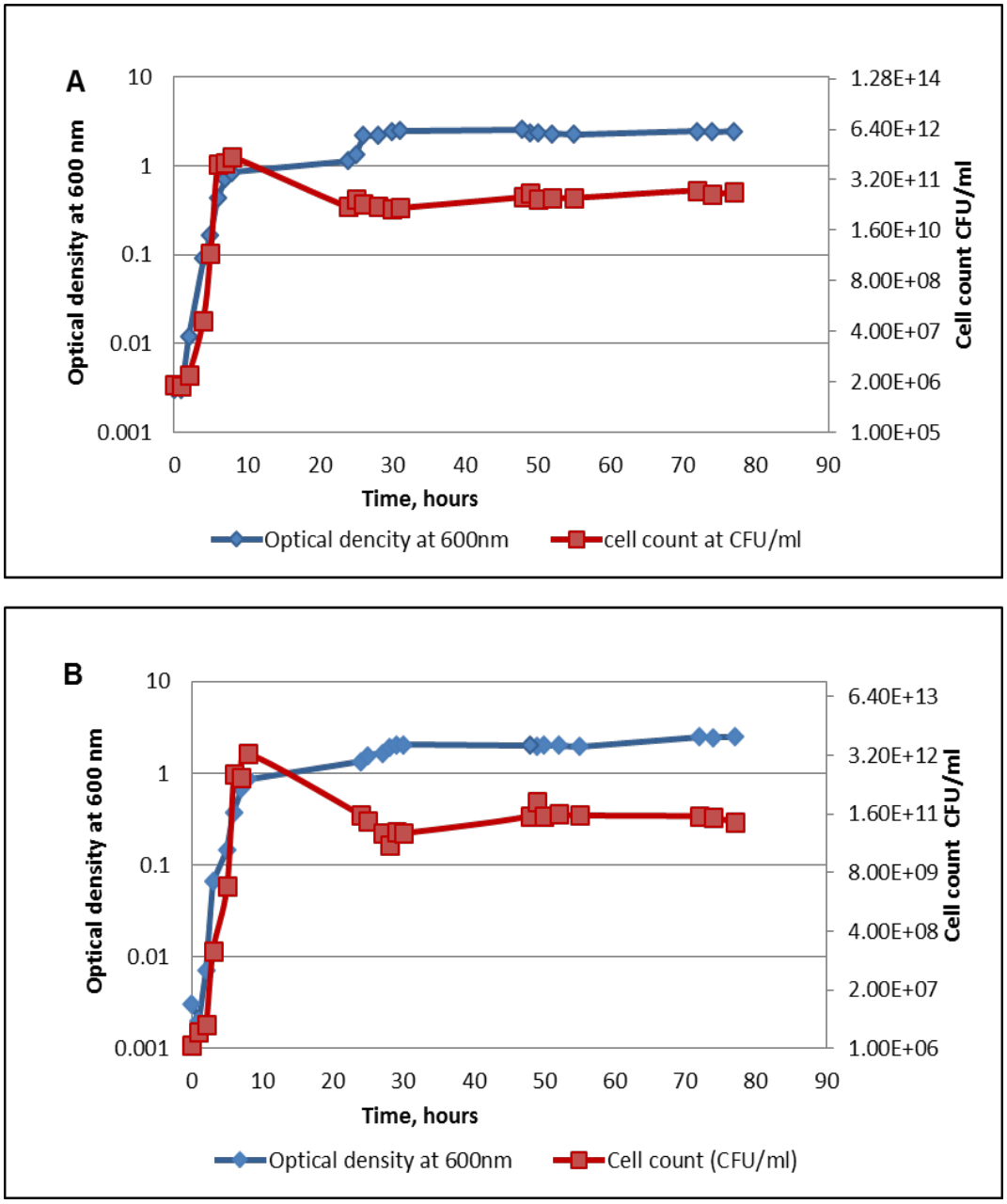



Figure A1.1: Growth curve for: A) *Pseudomonas mendocina* (13264) and B) *Rhodococcus koreensis* (13709)

**Phenol standard curve**

Standard curves for phenol in sodium hydrogen carbonate buffer solution and phenol in mineral salt medium MSM were plotted by adding 1 mL of phenol stock solution to a 250 mL conical flask and diluting the phenol solution to 100 mL, to give a concentration of 50 mg/L of phenol. Serial dilutions were prepared to produce phenol concentrations of 5, 10, 20, 30, 40, 50 mg/L and their absorbance was measured at 510 nm (figure A-2).

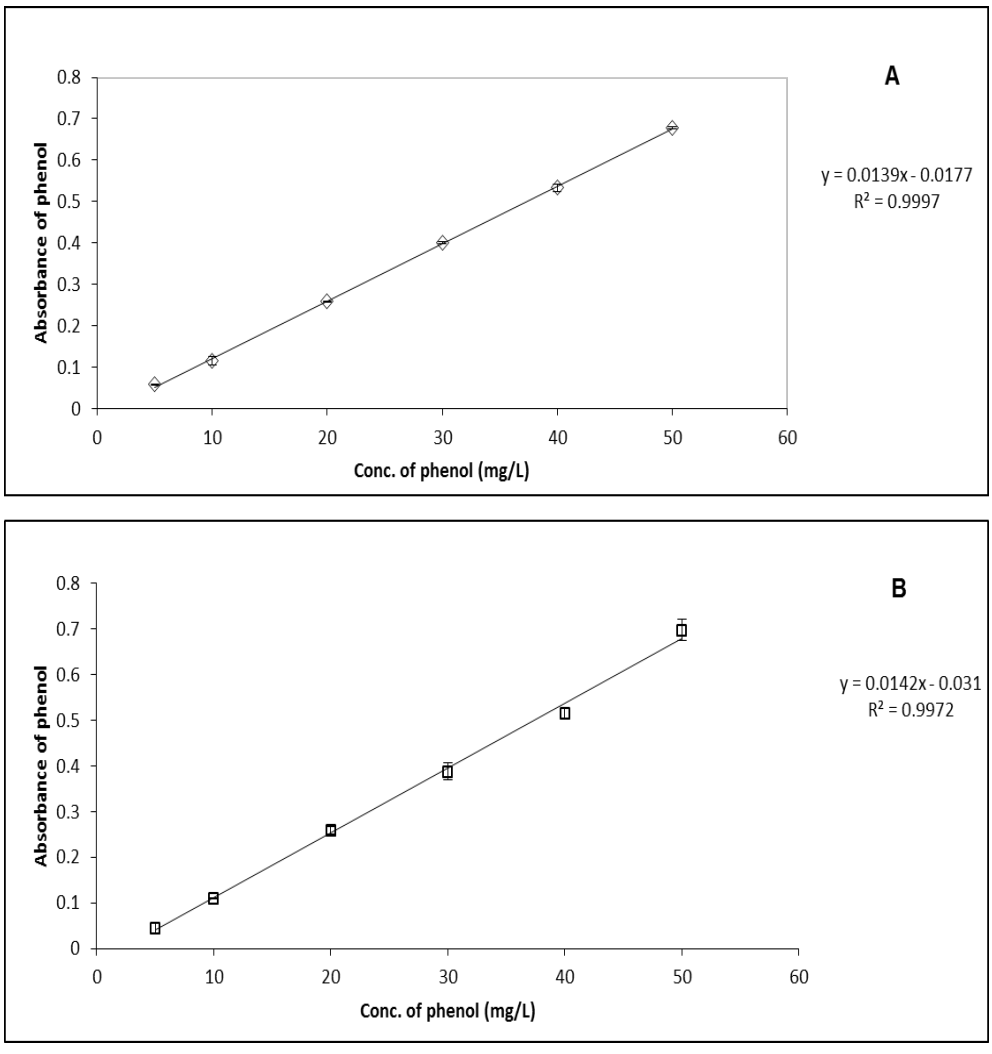



Figure A1.2: standard curve of phenol: A) in sodium carbonate buffer, B) in MSM.

## Appendix 2:

## Preparation of media

- **Nutrient broth medium (NB)**

Nutrient broth medium was prepared by mixing 0.3 g/L of peptone and 5 g/L of beef extract and sterilized by autoclave for 15 minutes at 121$^{o}$C (Ahmad, *et al.,* 2011).

- **Minimal salt medium (MSM)**

Minimal salt medium was prepared according to the method published by . The media comprised (g/L): $K_2HPO_4$ (0.4g), $KH_2PO_4$ (0.2g), NaCl (0.1g), $MgSO_4$ (0.1g), $FeSO_4.7H_2O$ (0.02g), $MnSO_4.H_2O$ (0.02g), $NaMoO_2.2H_2O$ (0.02g) and $(NH_4)2SO_4$ (0.4g). After the medium was autoclaved, 25mg/L of sterilized phenol (by filtration) was added to the media as a source of energy (Ahmad, *et al.,* 2011).

## . Morphological and biochemical tests

- **Gram staining**

Gram stain is a fundamental test to characterize the morphological structure of bacteria according to their cell-wall structure. Gram-positive cells are stained as purple to blue because of a thick peptidoglycan layer; Gram-negative cells are stain as red to pink as a result of a minimal peptidoglycan content layer (Coico, 2005).

Staining of cells was done by picking a single colony from plates and touched with a sterilized loop, emulsified in a drop of distilled water on glass slide to form a thin film and left to air dry. The slide was passed through a Bunsen burner flame for a few seconds to fix the cells onto the slide. The slide was covered with methyl violet solution for 1 minute and washed with water, followed with iodine solution for 1minute and washed off with 95% alcohol left on for a few

seconds only before rinsing the slide with distilled water. Finally, the safranin counter stain was applied for 1 minutes and washed with distilled water and left to dry. The stained dried slides were examined microscopically at 40x and 100x using oil immersion. This test was repeated twice using to bacterial strain *E.coli* and *staphylococcus aureus* as a control.

- **Differential and enriched media**

Differential and enriched media are used for identifying some microbial groups, due to special nutrients, dyes or chemicals that may enhance or inhibit the growth of some organisms and to change the colour of the colonies or the media. MacConkey's agar is both a selective and differential media, and is used for Gram negative bacteria to distinguish those bacteria that have the ability to ferment lactose (Collins and Lyne's, 2004). Blood agar is considered as an enriched growth media that can adi identification because of ability of some strains to lyse the red cells (Singleton, 2004). Both media were incubated at 37$^{o}$C for 18hr.

- **Oxidase test**

The oxidase test is used to determine if the bacteria can produce cytochrome c oxidase, which is an enzyme in the bacterial electron transport chain. Usually, bacteria that are oxidase positive are aerobic and can use oxygen as a terminal electron acceptor in respiration (Macfaddin, 2000).

A fresh solution of 1% tetramethyle p-phenylenediamine (TMPD) was prepared by dissolving 0.1 g of TMPD in 10 ml of distilled water. Subsequently, a small amount of freshly cultured bacteria was picked up with a sterile plastic loop and smeared onto a 5 cm diameter filter paper in a Petri dish, and impregnated with one drop of TMPD solution. The formation of a purple colour within a few seconds indicates a positive reaction.

- **Catalase test**

Bacteria living in oxygenated environments contain the catalase enzyme, which breaks down hydrogen peroxide. The catalase test alone cannot identify a particular organism, however, combining this test with others can aid the identification of certain genera and species (Macfaddin, 2000). Catalase production was examined by adding two drops of 3% hydrogen peroxide ($H_2O_2$) onto a microscope slide and mixed with a loop of purified colonies. The immediate production of oxygen gas bubbles is considered a positive reaction.

- **Motility test**

The motility test can also assist in identification. The test was performed in order to ascertain if the isolated Gram-negative bacteria was a member of the *Enterobacteriaceae* (positive for motility). The motility test medium was prepared from the following materials:

beef extract (3 g), pancreatic digest of casein (10 g), sodium chloride (5 g) and agar (4 g) dissolved in 1 L of boiling water to melt all the agar which was then supplemented with 5ml of 1% tri-phenyl tetrazolium chloride (TTC) (Macfaddin, 2000).

The prepared solution was added to 5 ml test tubes and autoclaved for 15 min at 121$^{o}$C at 15 psi pressure, and then left on racks to cool down. One colony was picked with a needle to inoculate the test tube deeply in a straight line to 1 cm above the tube base. Then, inoculated tubes were incubated at 37$^{o}$C for 24 hours or until the growth of cells was evident. Motile bacteria migrate from the inoculation line whereas non-motile bacteria are confined to the line.

- **Phenylalanine Deaminase Test**

The purpose of this test to identify and differentiate microorganisms that have the ability to reduce an amino group ($NH_2$) from the amino acid phenylalanine enzymatically by phenylalanine deaminase; these bacteria include the genera

*Proteus*, *Providencia*, and *Morganella* of the family *Enterobacteriaceae*, and some species of *Moraxella* (Macfaddin, 2000). A positive result in this test is a green colour that should appear after the addition of the $FeCl_3$ reagent produce phenyl pyruvic acid (a keto acid) and ammonia ($NH_3$)figure A2-1):

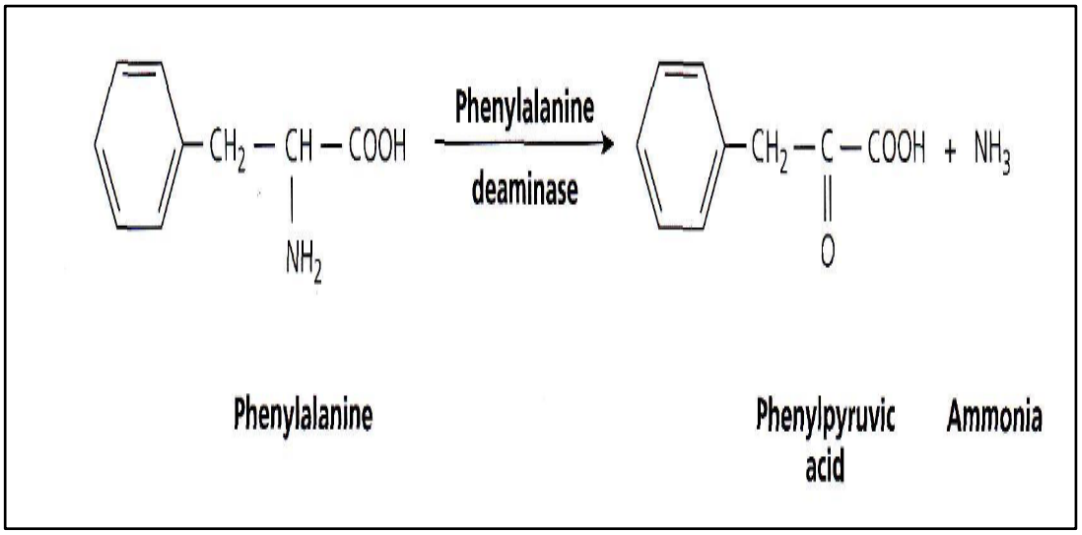



Figure A2.1: Reaction of phenylalanine deaminase test (Macfaddin, 2000).

Phenylalanine Deaminase agar was prepared by dissolving 2.3 g of agar in 100 mL of warm distilled water to melt the agar. 20 mL of the agar solution were transferred into a 50 ml test tube for sterilisation at 121°C and 15 psi pressure for 15 minutes. The tubes were sloped until they cooled and solidified. The tubes were inoculated with a fresh sample and incubated at 37ºC for 24 hours.

When growth appeared, five drops of ferric chloride 10% $FeCl_3$ was added to the slant tubes. A colour change from yellow to green within five minutes was considered positive; if the colour of colonies remained unchanged a negative test was recorded.

- **API 20E test**

The API 20E (bioMérieux) is a simple battery of biochemical tests to identify Gram negative bacterial strains belonging to the family *Enterobacteriaceae* and non-fastidious strains (Murray *et al.*, 1999). A well isolated pure colony was picked with a sterile toothpick and emulsified in 5 mL of 0.85% NaCl which was dispensed into the micro-tubes in the test strips according to the manufacturer's instructions.

The inoculated strips were incubated for 24 hours at 37$^{o}$C, but due to negative reactions after 24 hours, the strip was re-incubated for another 24 hours. At the end of incubation period reagents were added to specific micro tubes to produce enzymatic colour reactions as a results of fermentation of carbohydrate in micro tubes because of alteration of pH in the media. Stable and non-changing colours presented as a negative test.

- **API 20NE test**

The API 20NE test (bioMérieux) is another battery of biochemical tests used to identify non–fastidious and non-fermentation Gram negative bacteria or Gram negative bacterial species that do not belong to the family of *Enterobacteriaceae*.

The principle of this test is similar to API 20E and according to manufacture protocol, the results of this test are depending on the activity of enzymes and potential to ferment carbohydrates. After incubation the stripe at 37$^{o}$C for 48 hours, the test reagents were added to the micro-tubes and colour reactions were noted and recorded by following the protocol description, converted into 7-digit as it shown in the following image.

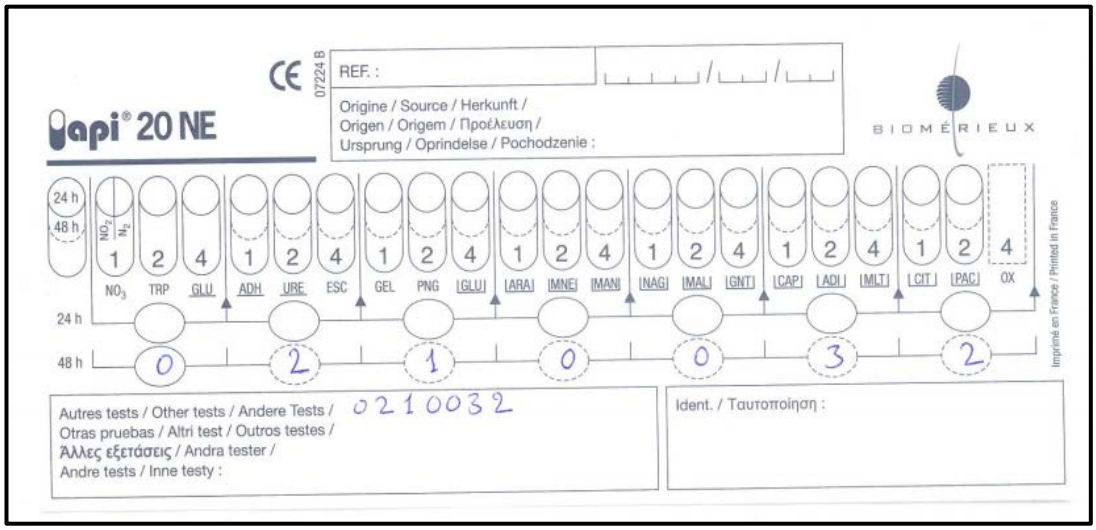



Figure A2.2: The 7-digit Analytical Profile Index number was compared with API data online to determine the identity of the bacterial stains.

## Genetic Identification

### DNA sequences

The sequence analyses lengths of the amplified DNA for the three samples are summarized in table (1).

**Table 1:** Length of sequence analyses for all *Acinetobacter* species by using the primers 27F and 1492R.

| Template | Primer | Status | LeftClip - RightClip | Length |
|---|---|---|---|---|
| A1 | 27F Lane | final result | 17 - 999 | 982 |
| A2 | 1492R(s) | final result | 13 - 970 | 957 |
| R1 | 27F Lane | final result | 17 - 1004 | 987 |
| R2 | 1492R(s) | final result | 14 - 968 | 954 |
| W1 | 27F Lane | final result | 19 - 915 | 896 |
| W2 | 1492R(s) | final result | 27 - 985 | 958 |

**A1:**

5’-ACCATGCAAGTCGAGCGGATGAAGGTAGCTTGCTACCGGATTCAGCGGCGGACGGGTGAG

TAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACGTTCCGAAAGGGGCGCTAATACCG

CATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTAATAGATGAGCCTAA

GTCGGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTG

AGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG

TGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG

CCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACCTAGATTAATACTTTAGGAT

AGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC

AGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCCAATTAA

GTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTGGCTAGAG

TATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAA

TACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGG

AGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGG

GGCCCTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGT

CGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT

TAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATACAGAGAACTTTCCAGAGA

TGGATTGGTGCCTTCGGGAACTC-3’

**A2:**

5’-GTAGCGTCCTCCTTGCGGTTAGACTACCTACTTCTGGTGCAACAAACTCCCATGGTGTG

ACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATTCTGATCCGCGATTAC

TAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTT

TTGAGATTAGCATCACATCGCTGTGTAGCAACCCTTTGTACCGACCATTGTAGCACGTGT

GTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCGTCCCCGCCTTCCTCCAGTTTGT

CACTGGCAGTATCCTTAAAGTTCCCACCCGAAGTGCTGGCAAATAAGGAAAAGGGTTGCG

CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACC

TGTATCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGTATGTCAAGGCC

AGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC

CCGTCAATTCATTTGAGTTTTAGTCTTGCGACCGTACTCCCCAGGCGGTCTACTTATCGC

GTTAGCTGCGCCACTAAAGCCTCAAGGGCCCCAACGGCTAGTAGACATCGTTTACGGCAT

GGACTACCAGGGTATCTAATCCTGTTTGCTCCCCATGCTTTCGTACCTCAGCGTCAGTAT

TAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTACGCATTTCACCGCT

ACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCCAACCAGTATCGAATGCAATTCC

CAAGTTAAGCTCGGGGATTTCACATTTGACTTAATTGGCCGCCTACGCACGCTTTACGCC

CAGTAAATCCGATTAACGCTCGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTT-3’

**R1:**

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GAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT

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GCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT

AATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATACAGAGAACTTTCCAGAGAT

GGATTGGTGCCTTCGGGAACTCTGATAC-3’

**R2:**

5’-GGTAGCGTCCTCCTTGCGGTTAGACTACCTACTTCTGGTGCAACAAACTCCCATGGTGTG

ACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATTCTGATCCGCGATTAC

TAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTT

TTGAGATTAGCATCACATCGCTGTGTAGCAACCCTTTGTACCGACCATTGTAGCACGTGT

GTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCGTCCCCGCCTTCCTCCAGTTTGT

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CCGTCAATTCATTTGAGTTTTAGTCTTGCGACCGTACTCCCCAGGCGGTCTACTTATCGC

GTTAGCTGCGCCACTAAAGCCTCAAGGGCCCCAACGGCTAGTAGACATCGTTTACGGCAT

GGACTACCAGGGTATCTAATCCTGTTTGCTCCCCATGCTTTCGTACCTCAGCGTCAGTAT

TAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTACGCATTTCACCGCT

ACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCCAACCAGTATCGAATGCAATTCC

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**W1:**

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GAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT

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TCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTGGCTAGAGT

ATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT

ACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACACTGAGGTGCGAAAGCATGGGGA

GCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGG

GCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTC

GCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG-3’

**W2:**

5’-ACGGTTAGACTACCTACTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTAC

AAGGCCCGGGAACGTATTCACCGCGGCATTCTGATCCGCGATTACTAGCGATTCCGACTT

CATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGCATCC

TCTCGCGAGGTAGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGTCGTA

AGGGCCATGATGACTTGACGTCGTCCCCGCCTTCCTCCAGTTTGTCACTGGCAGTATCCT

TAAAGTTCCCGGCTTAACCCGCTGGCAAATAAGGAAAAGGGTTGCGCTCGTTGCGGGACT

TAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTATGTAAGTTCC

CGAAGGCACCAATCCATCTCTGGAAAGTTCTTACTATGTCAAGACCAGGTAAGGTTCTTC

GCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTT

GAGTTTTAGTCTTGCGACCGTACTCCCCAGGCGGTCTACTTATCGCGTTAGCTGCGCCAC

TAAAGCCTCAAAGGCCCCAACGGCTAGTAGACATCGTTTACGGCATGGACTACCAGGGTA

TCTAATCCTGTTTGCTCCCCATGCTTTCGCACCTCAGTGTCAGTATTAGGCCAGATGGCT

GCCTTCGCCATCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCT

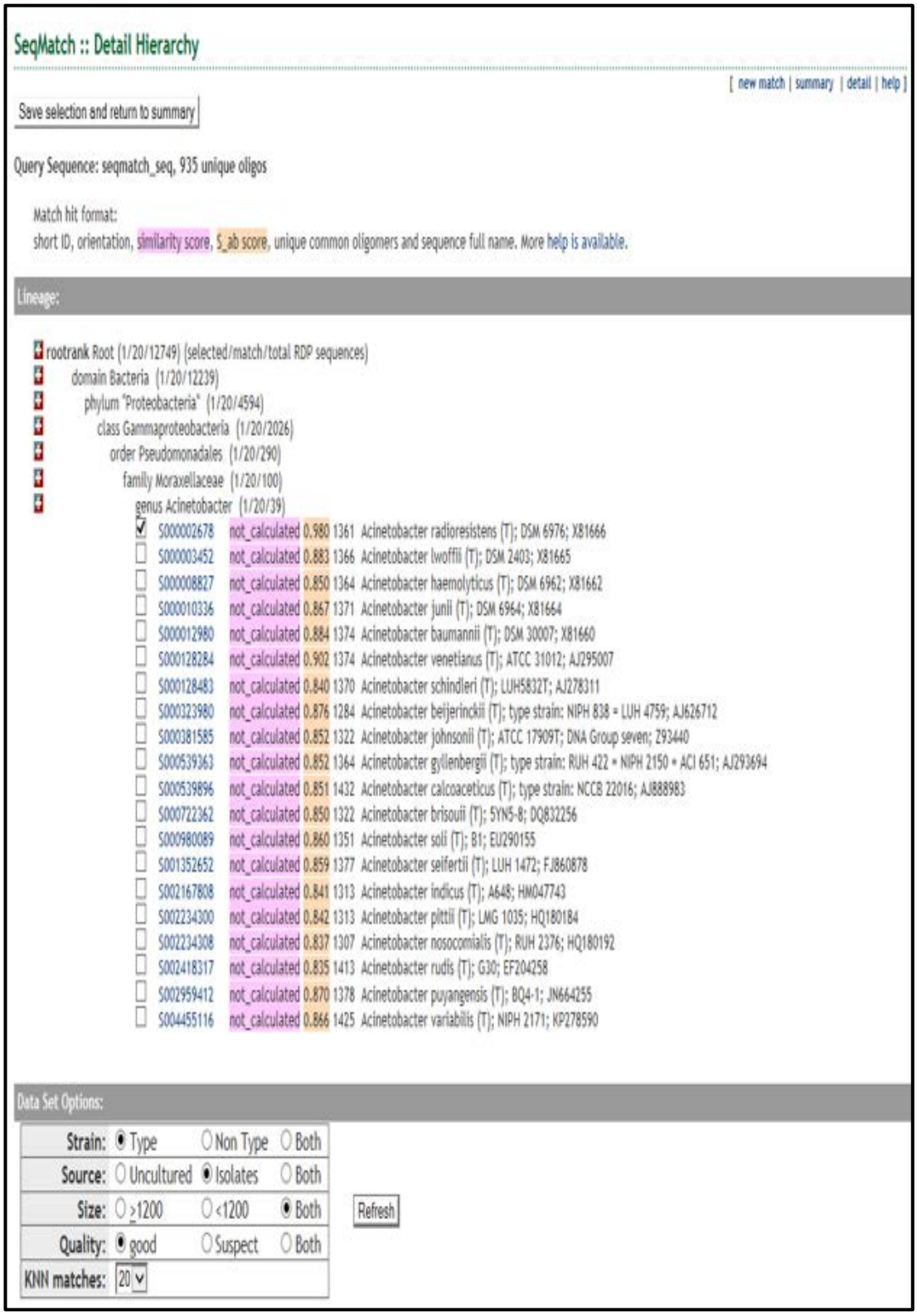
ACCATCCTCTCCCATACTCTAGCCAACCAGTATCGAATGCAATTCCCAAGTTAAGCTCGG

GGATTTCACATTTGACTTAATTGGCCACCTACGCGCGCTTTACGCCCAGTAAATCCGATT

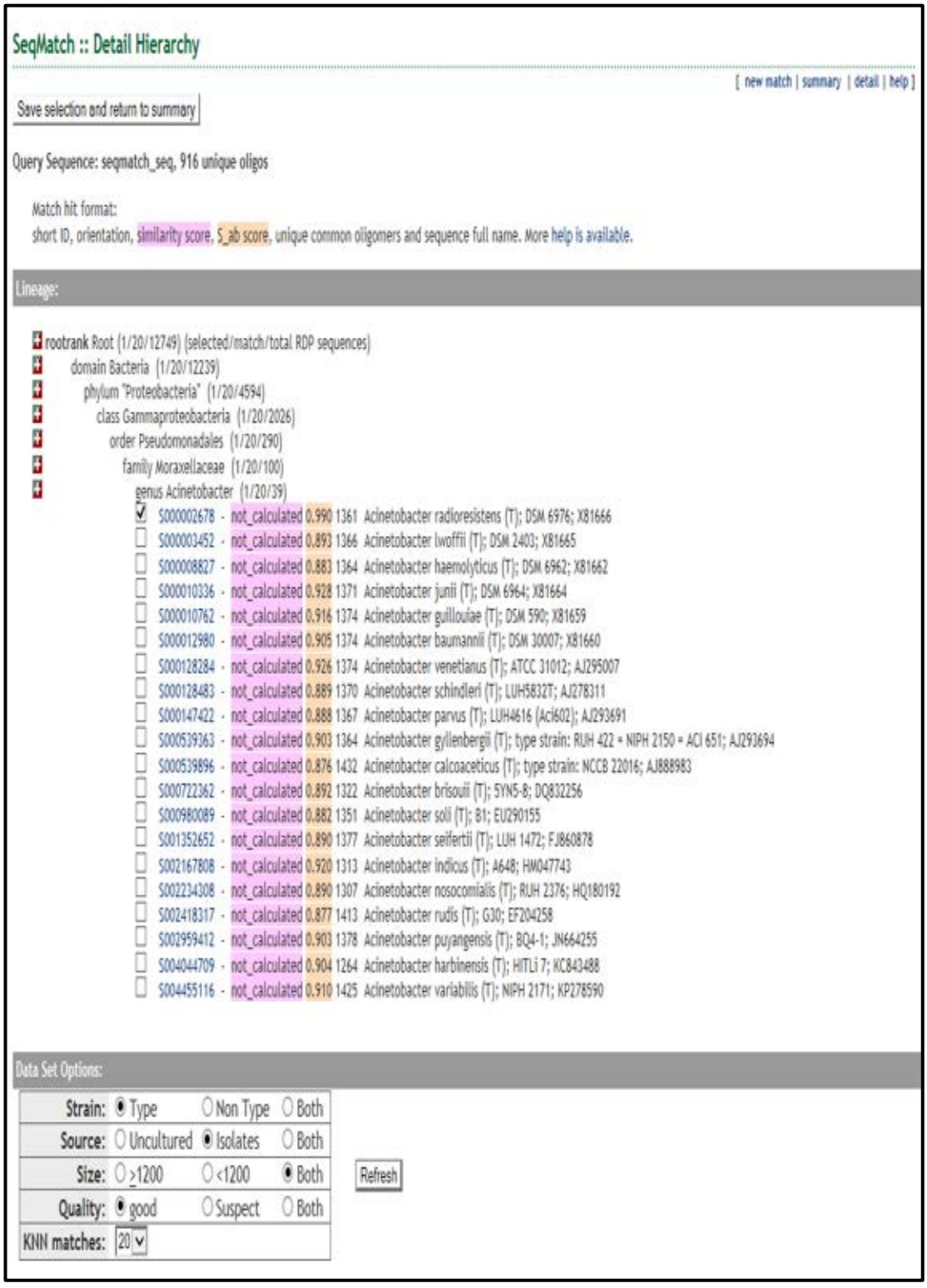
AACGCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTC-3’

The full sequences were uploaded online into Genebank database by using RDP website **http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp** to match the sequences.

The results were inserted in the following figures:




FigureA2.3: Results of matching the DNA sequences of sample **A1**



SeqMatch :: Detail Hierarchy

[ new match | summary | detail | help ]

Save selection and return to summary

Query Sequence: seqmatch_seq, 916 unique oligos

Match hit format:
short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:

rootrank Root (1/20/12749) [selected/match/total RDP sequences]
domain Bacteria (1/20/12239)
phylum "Proteobacteria" (1/20/4594)
class Gammaproteobacteria (1/20/2026)
order Pseudomonadales (1/20/290)
family Moraxellaceae (1/20/100)
genus Acinetobacter (1/20/39)

- S000002678 - not_calculated 0.990 1361 Acinetobacter radioresistens (T); DSM 6976; X81666
- S000003452 - not_calculated 0.893 1366 Acinetobacter lwoffii (T); DSM 2403; X81665
- S000008827 - not_calculated 0.883 1364 Acinetobacter haemolyticus (T); DSM 6962; X81662
- S000010336 - not_calculated 0.928 1371 Acinetobacter junii (T); DSM 6964; X81664
- S000010762 - not_calculated 0.916 1374 Acinetobacter guillouiae (T); DSM 590; X81659
- S000012980 - not_calculated 0.905 1374 Acinetobacter baumannii (T); DSM 30007; X81660
- S000128284 - not_calculated 0.926 1374 Acinetobacter venetianus (T); ATCC 31012; AJ295007
- S000128483 - not_calculated 0.889 1370 Acinetobacter schindleri (T); LUH5832T; AJ278311
- S000147422 - not_calculated 0.888 1367 Acinetobacter parvus (T); LUH4616 (Aci602); AJ293691
- S000539363 - not_calculated 0.903 1364 Acinetobacter gyllenbergii (T); type strain: RUH 422 = NIPH 2150 = ACI 651; AJ293694
- S000539896 - not_calculated 0.876 1432 Acinetobacter calcoaceticus (T); type strain: NCCB 22016; AJ888983
- S000722362 - not_calculated 0.892 1322 Acinetobacter brisouii (T); SYN5-8; DQ832256
- S000980089 - not_calculated 0.882 1351 Acinetobacter soli (T); B1; EU290155
- S001352652 - not_calculated 0.890 1377 Acinetobacter seifertii (T); LUH 1472; FJ860878
- S002167808 - not_calculated 0.920 1313 Acinetobacter indicus (T); A648; HM047743
- S002234308 - not_calculated 0.890 1307 Acinetobacter nosocomialis (T); RUH 2376; HQ180192
- S002418317 - not_calculated 0.877 1413 Acinetobacter rudis (T); G30; EF204258
- S002959412 - not_calculated 0.903 1378 Acinetobacter puyangensis (T); BQ4-1; JN664255
- S004044709 - not_calculated 0.904 1264 Acinetobacter harbinensis (T); HITLi 7; KC843488
- S004455116 - not_calculated 0.910 1425 Acinetobacter variabilis (T); NIPH 2171; KP278590

Data Set Options:

| | | | | |
|---|---|---|---|---|
| Strain: | Type | Non Type | Both | |
| Source: | Uncultured | Isolates | Both | |
| Size: | >1200 | <1200 | Both | Refresh |
| Quality: | good | Suspect | Both | |
| KNN matches: | 20 | | | |

Figure A2.4: Results of matching the DNA sequences of sample **A2**

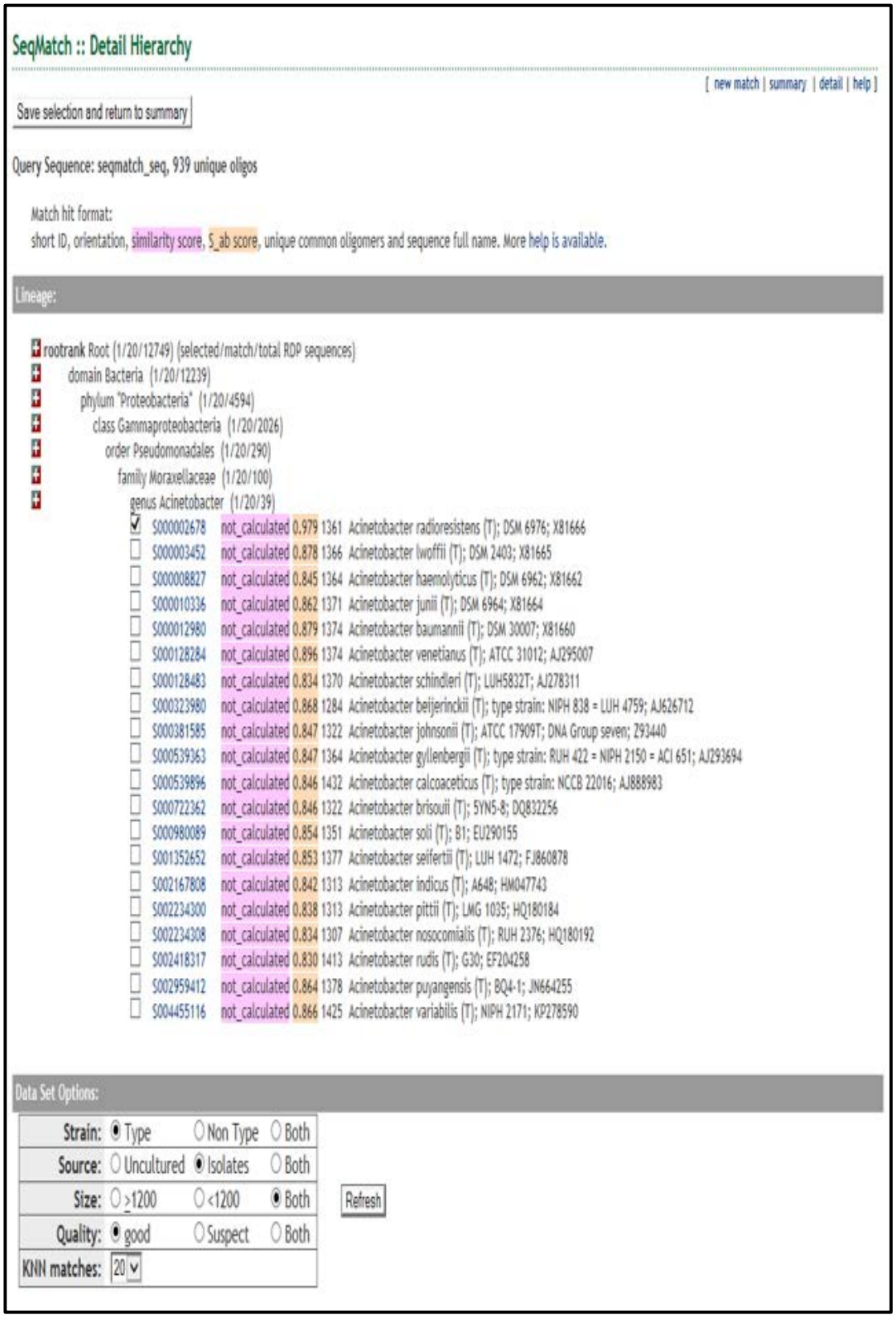
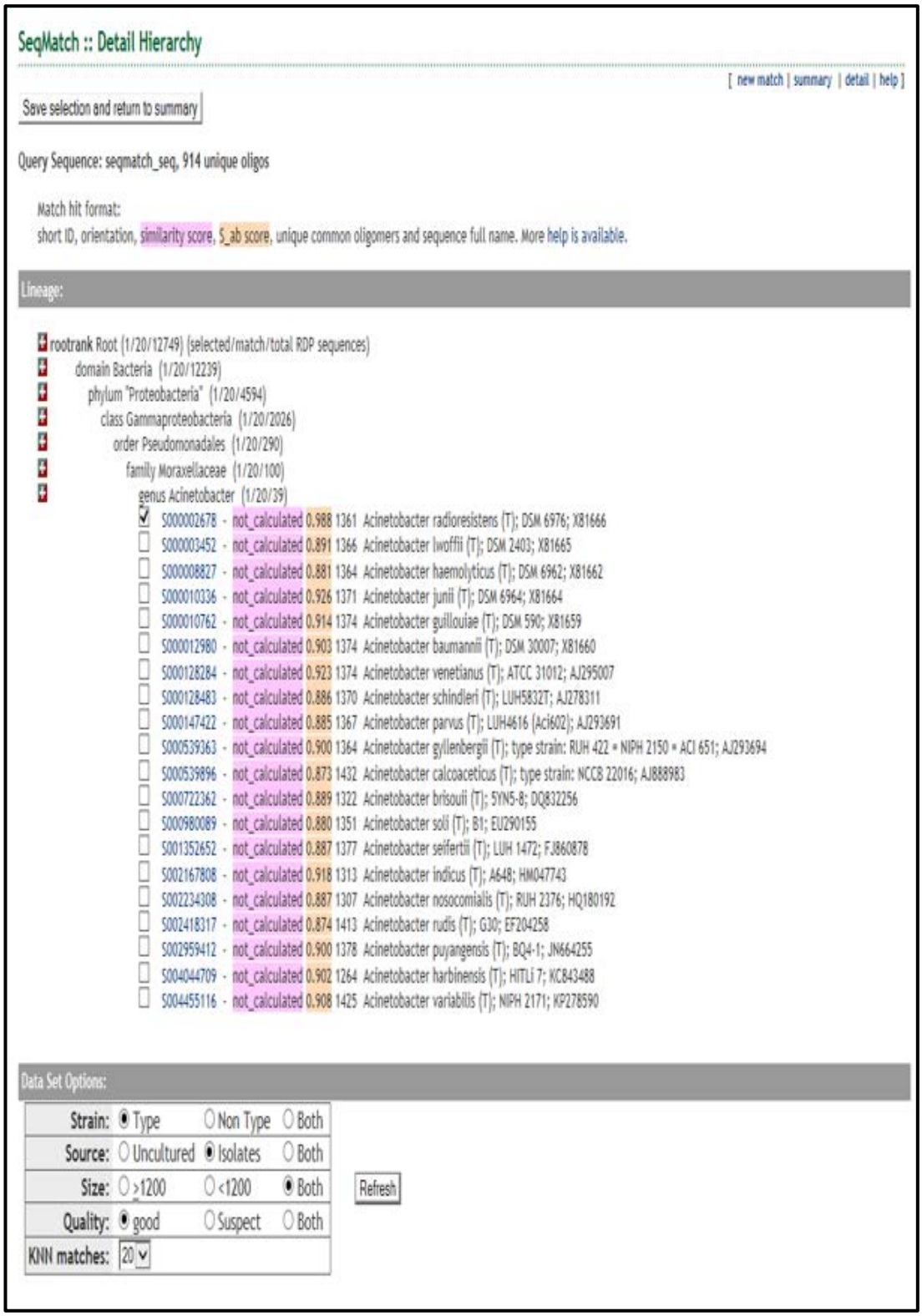



Figure A2.5: Results of matching the DNA sequences of sample **R1**




FigureA2.6: Results of matching the DNA sequences of sample **R2**

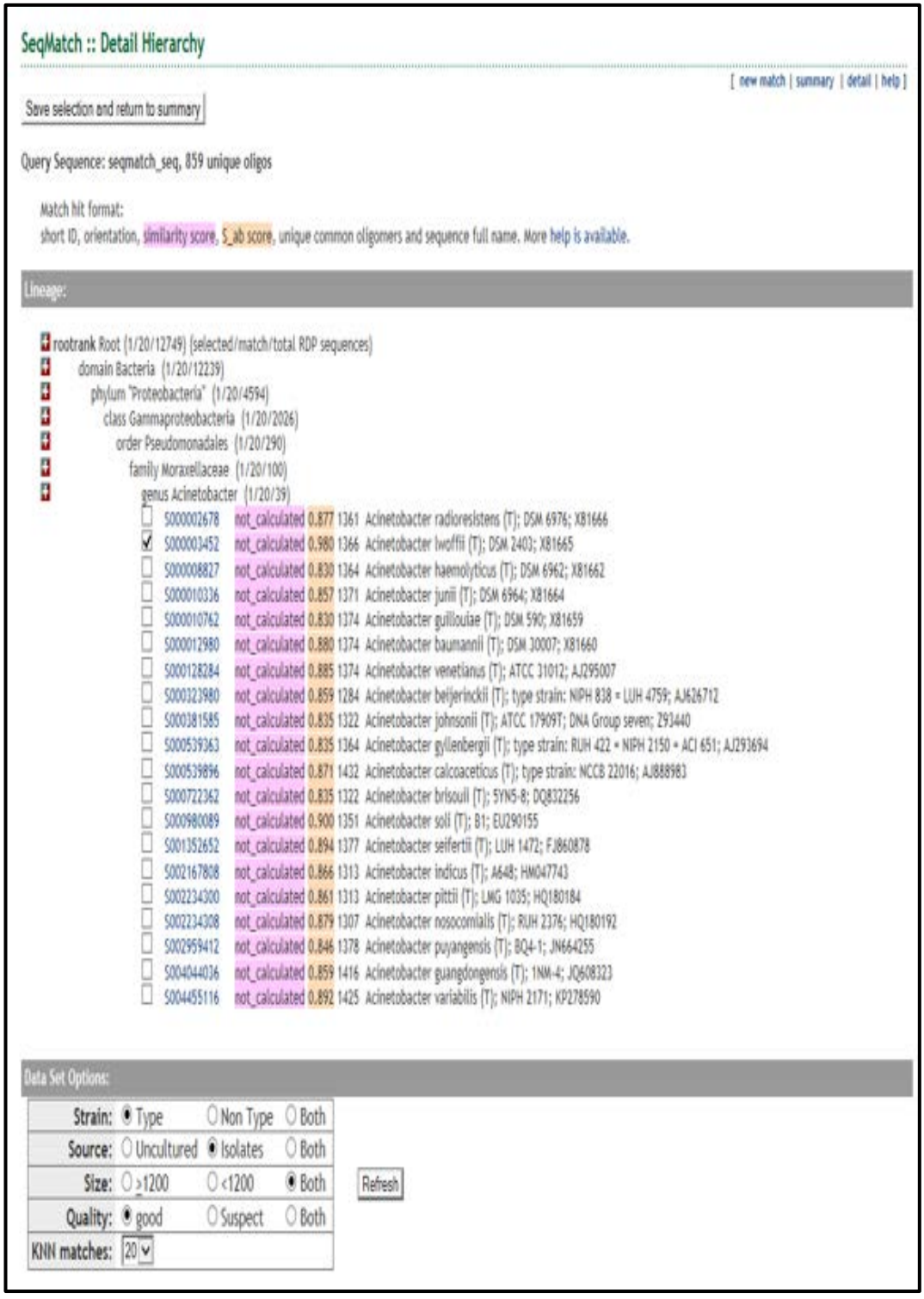



Figure A2.7: Results of matching the DNA sequences of sample **W1**

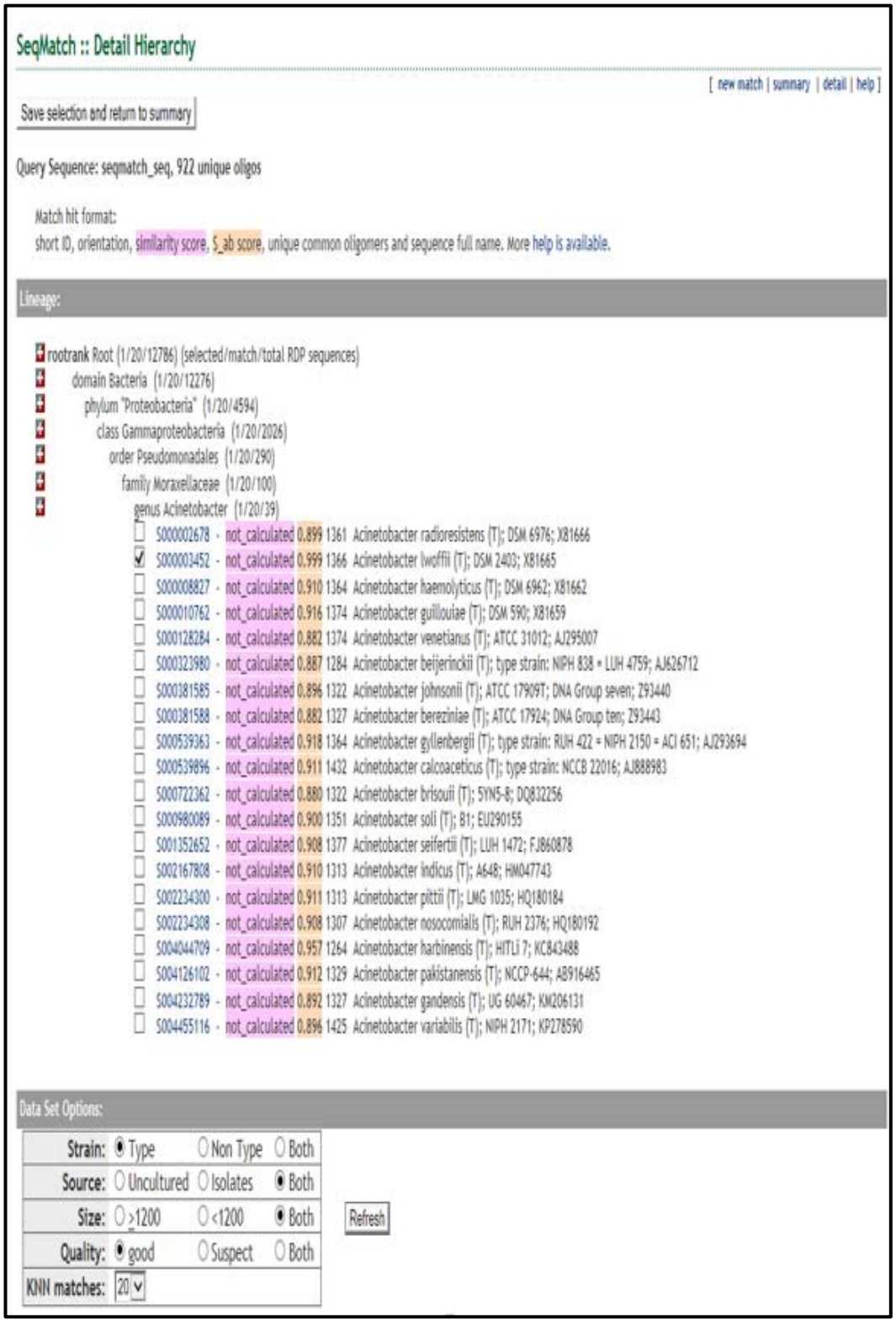



Figure A2.8: Results of matching the DNA sequences of sample **W2**

## Appendix 3:

### HPLC setting and analysis

The method details: column temperature 45$^{o}$C, sample temperature 25$^{o}$C, C) Gradient Start at 75.0, End at 44.0%, D) Gradient Start at 25.0, End at 56.0% for 9.5 minutes, and at composition 44%-56% C-D for 5 min. C) Gradient Start at 44.0, End at 0.0% with duration of 1.0 min and D) Gradient Start at 56.0%, End at 100.0%, for 1.0min. 5 minutes at 100% of CH3OH&1% HAc. C) Gradient Start at 0.0, End at 75.0% for 1.0min; D) Gradient Start at 100.0, End at 25.0% with duration of 1.0min.

The final concentration of phenol and its possible derivatives in water were also estimated using HPLC. A reverse phase C18 column was used with washes of 1% methanol & acetic acid and 1% aqueous acetic acid,75 /25 at a flow rate of 1 mL/min with a UV detector (275 nm). The injection volume of the sample was 40 µL in 50% methanol. Sample preparation included the dilution of the solution in pure methanol, freezing it at -20$^{o}$C for several hours, its centrifugation to remove proteins and filtering through a 0.22 µm filter to remove mechanical impurities and other unwanted compounds affecting the column. The retention time for the phenol was 5.5 -5.6 minutes.

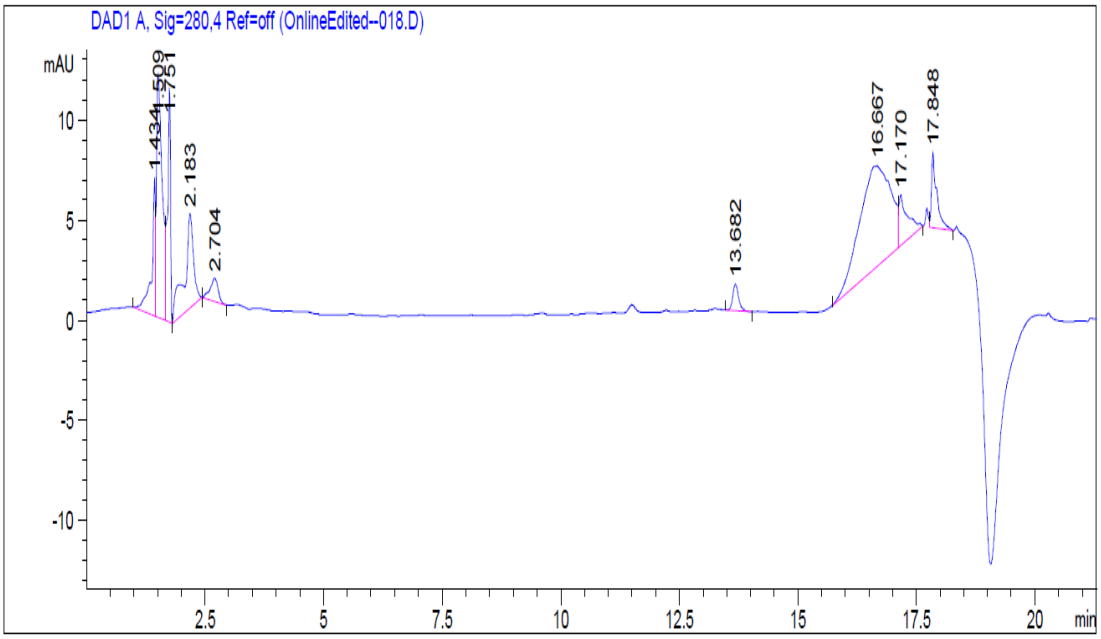



Figure A3.1: HPLC chromatogram of the solution after phenol degradation by the *Rhodococcus koreensis*

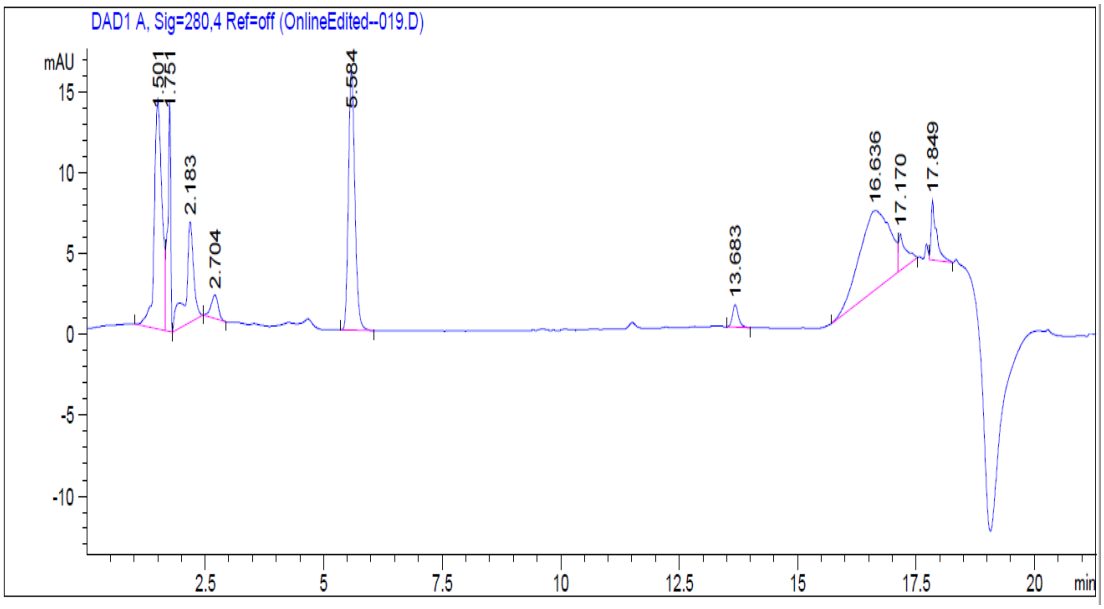



Figure A3. 2: HPLC chromatogram of the solution after phenol degradation *Pseudomonas mendocina*

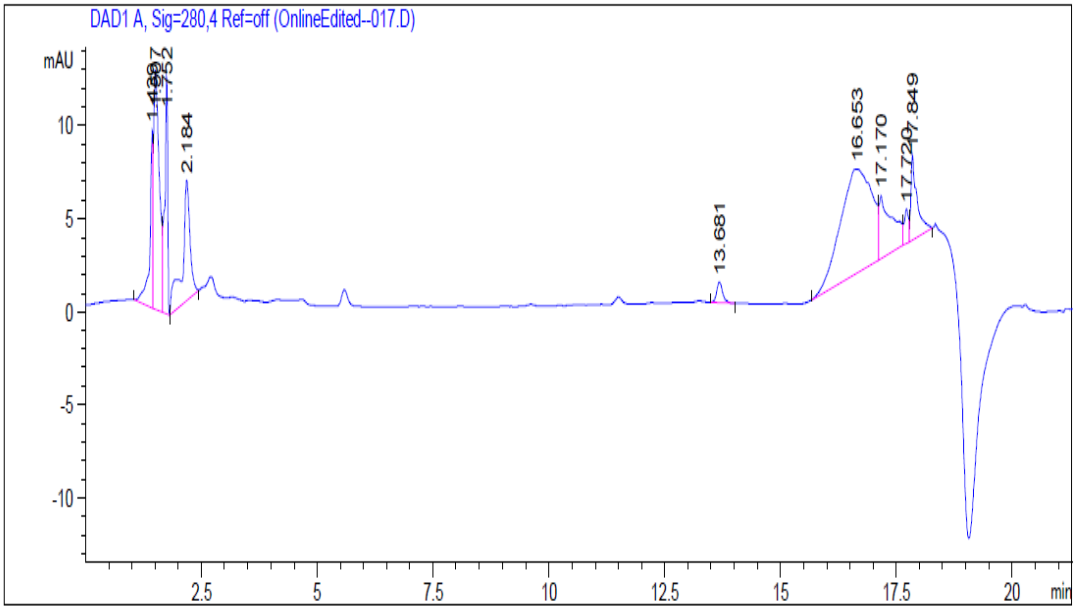



Figure A3. 3: HPLC chromatogram of the solution after phenol degradation by mixed cells (*Pseudomonas mendocina* and *Rhodococcus koreensis*)

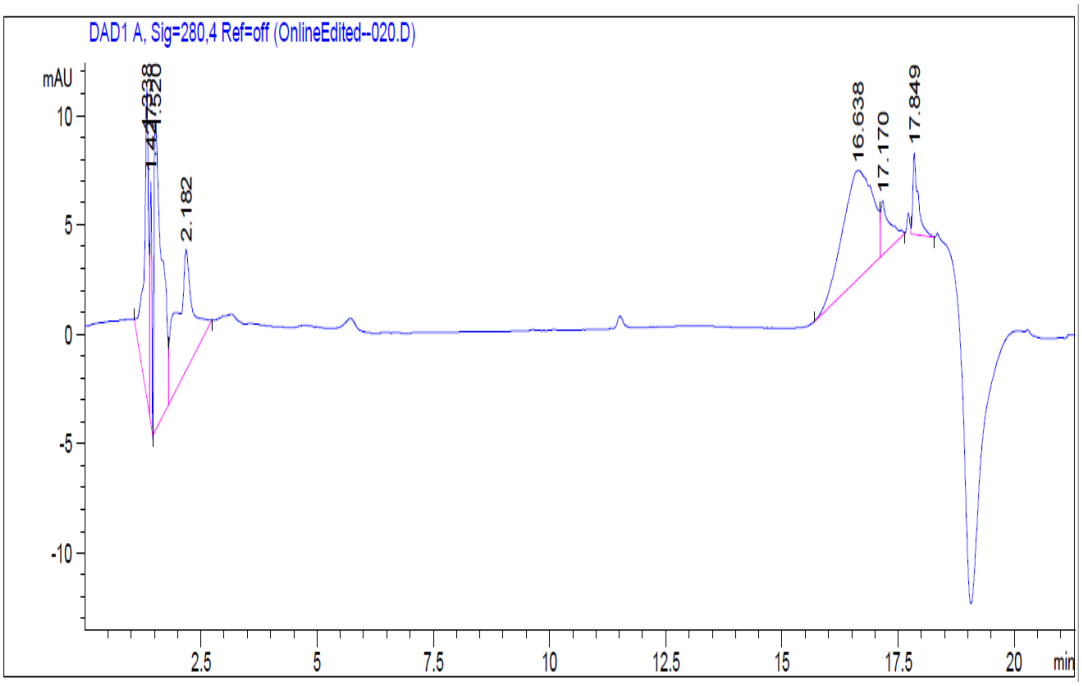



Figure A3. 4: HPLC chromatogram of the solution after phenol degradation by *Acinetobacter radioresistens*.