GROWTH OPTIMIZATION OF EXTREMOPHILIC *PRACOCCUS BARUCHII* LBOG37 AND DETECTION OF ACETYL COA ACTYLTRANSFERASE GENE

CHRISTOPHER OPUKO WESONGA

MASTER OF SCIENCE

(Biotechnology)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2016

Growth Optimization of Extremophilic *Pracoccus baruchii* Lbog37 and Detection of Acetyl Coa Actyltransferase Gene

Christopher Opuko Wesonga

A Thesis Submitted In Partial Fulfillment for the Degree of Master of Science in Biotechnology in the Jomo Kenyatta University of Agriculture and Technology

2016

Declaration

This thesis is my original work and has not been presented for a degree in any other University.

Sign …….. Date …….

Wesonga Opuko Christopher

This thesis has been submitted for examination with our approval as supervisors.

Sign …….. Date …….

Dr. Romano Mwirichia

Embu University, Kenya

Sign …….. Date …….

Dr. Justus Onguso

JKUAT, Kenya

Sign …….. Date …….

Dr. Ronald G. Maathai

MKU, Kenya

DEDICATION

To my beloved wife Mary Miriti Kendi who has instilled in me the vital lessons of life including the virtues of patience, honesty and hard work and my children Lewis Samuel, Jessica Nafuna and Christine Karimi who have been the source of my happiness and encouragement.

ACKNOWLEDGEMENT

I would like to thank my supervisors Dr. Mwirichia Romano, Dr. Justus Onguso, and Dr. Ronald Maathai who despite their busy schedule spared time to guide and show me the way forward when writing this thesis. My deep appreciation goes to my family for financial and moral support. I am grateful to my course mate Jacob Kamau for his continued encouragement, support and guidance. My sincere appreciation also goes to the staff of department of Molecular Genetics, National Museums of Kenya especially Dr. Githui Kem and Mrs. Ann Mwaura for their material and technical support during data collection. Not forgetting the staff of IBR laboratory especially Mr. Richard Rotich for his unending support during bench-work. God bless them all.

CONTENTS

LIST OF PLATES

LIST OF FIGURE

ACRONYMS

BLAST- Basic Local Alignment Search Tool

CAP - catabolite gene activator protein

CcpA - catabolite control protein

DNA – deoxyribonucleic acid

dNTP - deoxy-nucleotide phosphates

EPS - exopolysaccharide

HMG-CoA - 3-hydroxy-3-methylglutaryl-CoA

IPP - Δ^{3-}isopentyl pyrophosphate

MEGA – Molecular Evolutionary Genetics Analysis

MH – Modified Horikoshi

MVA - mevalonic acid

NCBI – National Center for Biotechnology Information

OD – optical density

ORF – Open Reading Frame

PCR - polymerase chain reaction

PHAs - Polyhydroxyalkanoates

PHB - Poly-(R)-3-hydroxybutyrate

PHBV - poly-ß-hydroxybutyrateco-3-hydroxyvalerate

RNA – ribonucleic acid

rRNA – ribosomal Ribonucleic acid

UV – ultra violet light

ABSTRACT

The genus *Paracoccus* is a group of *proteobacteria* which is distantly related to *Escherichia coli* based on the analysis of their 16s rDNA sequence. Microbial biosynthesis of natural products is an emerging area of metabolic engineering and industrial biotechnology that offers significant advantages over conventional chemical methods or extraction from biomass. However, metabolic engineering and industrial biotechnology is highly dependent on the existence of well characterized genes whose functions and characteristics are known. Thus, the potential of any gene to be utilized in the metabolic engineering requires its detection, isolation and eventual characterization. Although acetyl coA acetyltransferase (phaA) gene has been detected and isolated from other organisms, its detection from an extermophile such as Paraccocus baruchii LBOG37 is yet to be carried out. This study sought to optimize the growth conditions and of *Paraccocus baruchii* LBOG37anddetect *phaA* gene. The results indicated that indicated that *Paracoccus baruchii* LBOG37 can grow at 37^{o}C to 54^{o}C; at pH ranges from 9-11; and can utilize carboxymethyl cellulose, starch and glucose. The results also established that the 16S rDNA sequences obtained were related to other *Paracoccus* species. Moreover, phylogenetic analysis indicated that the gene sequences clustered closely to those in GeneBank. It was also established that the gene amplified was related to *phaA* gene from other organisms. It was concluded that the organism used in this study was indeed *Paracoccus sp* LBOG37. It was also concluded that *Paracoccus baruchii* LBOG37 is a versatile bacteria capable of utilizing carboxymethyl cellulose, starch and glucose as carbon sources and it grows optimally at 40 ^{o}C and pH 9.0 and 9.5. It was also concluded that *Paracoccus baruchii* LBOG37 has *phaA* gene.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The genus *Paracoccus* is a group of *proteobacteria* which is distantly related to *Escherichia coli* based on the analysis of their 16s rDNA sequence (Baker, Ferguson, Ludwig, Page, Richter & van Spanning, 1998). The genus *Paracoccus* currently comprises of over 40 recognized and validly named species, isolated from different environments in various geographical locations (Dziewit, Czarnecki, Wibberg, Radlinska, Mrozek, Schlüter, Szymczak, Pühler & Bartosik, 2014). Members of this genus exhibit a broad range of metabolic flexibility, especially in respiratory processes. For instannce, they can utilize nitrate, nitrite, nitrous oxide and nitric oxide as alternative electron acceptors in denitrification and they have the ability to use one-carbon (C1) compounds such as methanol or methylamine as electron donors to respiratory chains (Baker *et al*., 1998). Moreover, *Paracoccus* spp. as facultative chemolithoautotrophs may utilize reduced sulfur compounds such as thiocyanate, thiosulfate or elemental sulfur, molecular hydrogen and Fe(II) as energy sources (Dziewit *et al.*, 2014).

Paracoccus spp. are also able to use a broad range of organic compounds as their sole source of carbon and energy, including pollutants such as acetone, dichloromethane, formamide, N,N-dimethylformamide (DMF) and methylamine (Kelly, Euzeby, Goodhew & Wood, 2006). Another common feature of *Paracoccus* spp. is methylotrophy, defined as the ability to utilize reduced C1 carbon substrates containing no carbon-carbon bonds (including methane, methanol, methylated amines, halogenated methanes and methylated sulfur species) as their sole source of carbon and energy for growth.

Morphologically, all the species are coccoid, between 0.4–0.9 μm in diameter, or coccobacilli, up to 2 μm in length, and occur as single cells, pairs or clusters (Kelly, *et al*., 2006b). All are Gram-negative and most species are non-motile. All species grow aerobically on a wide range of organic substrates and some are capable of anaerobic growth with nitrate or nitrous oxide as the terminal oxidant, producing dinitrogen as the final product. None is known to be able to grow fermentatively. The optimum temperature for growth of all the species is in the range 25–37°C, and the pH for good growth ranges between pH 6.5–8.5 for different species, except for *P. alcaliphilus* (which is moderately alkaliphilic with an optimum of pH 8–9 and grows at pH 9.5, but only weakly at pH 7..

All organisms in the genus *Paracoccus* are characterized by a high guanine plus cytosine (G+C) content, are Gram negative, catalase positive, oxidase-positive bacteria that contain C18: 1ω7c as a major component of the cellular fatty acids and are metabolically versatile (Kelly *et al*., 2006b). Phylogenetic analyses of the16S rRNA gene sequences for the genus *Paracoccus* have demonstrated that all the described species of the genus *Paracoccus* form a coherent cluster within the α-3 subclass of the Proteobacteria with the closest relatives being members of the genus *Rhodobacter* (Kelly *et al*., 2006a).

Having a versatile metabolism, *Paracoccus* spp. play an important role in biogeochemical cycles and they have also been successfully employed in the biotreatment of contaminated environments, such as bioremediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs) using *Paracoccus* sp. HPD-2 (Sun, Luo, Teng, Christie, Jia & Li, 2013). Although *Paracoccus* spp. constitute an interesting and metabolically versatile group of bacteria with substantial biotechnological potential, little is known about the content and organization of their genomes. Only one complete genome of *Paracoccusdenitrificans* PD1222 has been deposited in the NCBI database ([GenBank:CP000489], [GenBank: CP000490] and [GenBank:CP000491]). This genome is composed of two chromosomes (ChI – 2.9 Mb & ChII – 1.7 Mb) and a single megaplasmid (plasmid 1) of 653 kb.

Much more is known about mobile genetic elements (MGEs) of *Paracoccus* spp. Baj, Piechucka, Bartosik and Wlodarczyk (2000) demonstrated that bacteria belonging to this genus usually harbor at least one plasmid (in most cases a megasized replicon, exceeding 100 kb). Several multireplicon strains carrying 4 or more plasmids were identified, including *P. aminophilus* JCM 7686. Dziewit *et al* (2014) reported that they had already obtained the sequences of 18 plasmids, ranging in size from 2.7 to 40 kb, that originate from different *Paracoccus* spp. Moreover, complex analyses of 25 *Paracoccus* spp. strains using trap plasmid systems have led to the identification and characterization of (i) 48 insertion sequences (ISs), (ii) a composite transposon Tn6097 carrying genetic modules involved in the arginine deiminase pathway and daunorubicin/doxorubicin resistance, (iii) 3 non-composite transposons of the Tn3 family, (iv) a transposable genomic island TnPpa1 (45 kb) and (v) several transposable modules (TMos) generated by a single copy of the IS1380 family insertion sequence. The findings outlined above suggest that horizontal gene transfer (HGT) events occur frequently in *Paracoccus* spp. genomes, which may explain their metabolic flexibility.

It is known that organisms isolated from extreme environment produce compounds such as carotenoids to help them survive the extreme conditions (Asker *et al*., 2012). Thus, having been isolated from extreme alkaline and thermophilic environment, it was anticipated that *Paraccocus* sp. LBOG37 would be capable of synthesising such compounds. To do the organism need acetyl coA acetyltransferase that is involved in initial stages of the synthesis of such compounds. Therefore, this study aimed at optimizing the growth conditions of *Paraccocus baruchii* LBOG37isolated from Lake Bogoria and thereafterdetect acetyl coA acetyltransferase gene responsible for the initiation of production of protective compounds such as carotenoids. Detection of this gene was vital because this was expected to set the stage for characterization of the gene and subsequent utilization in pathway engineering to increase production of various natural products such as acyclic carotenoids, bioplastics and butanol.

1.2 Problem Statement

Microbial biosynthesis of natural products is an emerging area of metabolic engineering and industrial biotechnology that offers significant advantages over conventional chemical methods or extraction from biomass (Joseph *et al.* 2006). However, metabolic engineering and industrial biotechnology is highly dependent on the existence of well characterized genes whose functions and characteristics are known. Thus, the potential of any gene to be utilized in the metabolic engineering requires its detection, isolation and eventual characterization. Although acetyl coA acetyltransferase (phaA) gene has been detected and isolated from other organisms, its detection from an extermophile such as Paraccocus baruchii LBOG37 is yet to be carried out. Thus, detection of this gene from Paraccocus baruchii LBOG37 was to act as a step for future combinatory biosynthesis. This would help in setting the stage for future pathway engineering to increase production of various natural products such as acyclic carotenoids, bioplastics and butanol whose natural production involves acetyl coA acetyltransferase enzyme at its initial stages.

1.3 Hypothesis

Paracoccus baruchiiLBOG37 has acetyl coA acetyltransferase (*phaA*) gene.

1.4 Objectives

To optimize the growth conditions and of *Paraccocus baruchii LBOG37* anddetect *phaA* gene

Specific Objectives

1. To confirm the identity of isolate *Paracoccus baruchii* LBOG37 obtained from the Genebank using partial sequencing of the 16S gene
2. To optimize for the growth conditions of *Paracoccus baruchii* LBOG37, that is pH, temperature and carbon source.

3. To detect the *phaA*gene in *Paracoccus baruchii* LBOG37

1.5 Justification

The increasing pollution of the environment by chemically produced plastics calls for production of biodegradable plastics. However, the production of such biodegradable plastics is hindered by their low molecular weight and the fragile nature of bioplastics produced from micro-organisms. Moreover, micro-organisms are not able to accumulate substantial amounts of bioplastics. Detection of phaA from *Paracoccus baruchii* which is thermophilic and extremophilic could set the stage for natural production of high molecular weight bioplastics. Bioengineering of genes involved in the production of bioplastics have indicated the possibility of increasing productivity of bioplastics. thus, detection and subsequent utilization of phaA from *Paracoccus baruchii* could be revolutionize the production of bioplastics and other natural products such as biofuel since this gene is at the center stage of all these.

CHAPTER TWO

LITERATURE REVIEW

Confirmation of microbial isolate using 16S rRNA gene

Micro-organisms have been classified and identified on the basis of a variety of phenotypic characteristics including morphology, gram-staining, growth, tolerance, metabolic and biochemical reactions. However, these methods of bacterial identification have major drawbacks. First, they cannot be used for non-cultivable organisms. Second, some micro-organisms exhibit biochemical characteristics that do not fit into patterns of any known genus and species. Third, identification of slow growing organisms would be extremely slow and difficult. Recently there has been a tendency to determine definitive classification and taxonomic assignment by nucleic acid hybridization, 16S rRNA sequence analysis, and other molecular genetic techniques. Comparative sequence analysis of ribosomal RNAs or the corresponding genes currently is the most widely used approach for the reconstruction of microbial phylogeny. Since the discovery of PCR and DNA sequencing, comparison of the gene sequences of bacterial species showed that the 16S rRNA gene is highly conserved within a species and among species of the same genus, and hence can be used as the new 'gold standard' for identification of bacteria to the species level. The properties of 16S rRNA properties predestine it as a universal phylogenetic marker. There are regions on 16S rRNA gene that are quite conserved and other which are variable. Comparing the base sequence of 16S rRNA of *Paracoccus baruchii* LBOG37 to those in the gene bank was expected to help in confirmation of its identity.

Mesbah, Abou-El-Ela and Wiegel (2006) investigated the phylogenetic diversity of the bacterial and archaeal community in the water and sediments of three large lakes of the Wadi An Natrun was using 16S rRNA clone libraries. Mesbah *et al* reported that the bacterial community was diverse: 769 clones formed 345 operational taxonomic units

(OTUs) defined at 99% 16S rRNA sequence identity. The 16S rRNA enabled the authors to identify operational taxonomic units related to the g-proteobacteria that were more abundant in the sediment of Lake Fazda and the d-proteobacteria that dominated the sediment of Lake UmRisha. Thus, 16S rRNA gene analysis can be used to confirm the identity of *Paracoccus baruchii* LBOG37.

Although 16S rRNA gene has been used a lot in micro-organisms as phylogenetic marker, a study by Osanjo, Muthike, Tsuma, Okoth, Bulimo, Lünsdorf, Abraham, Dion, Timmis, Golyshin and Mulaa (2009) reported two species, *Paraccocus bogoriensis* and *Paracoccus eastuari,* that had sequence similarity of 99% but genotypic and phenotypic differences. For instance, Osanjo *et al* indicated that the two species were different in terms of motility, C18: 1w7c fatty acid content, G + C content and temperature of growth. The authors argued that the metabolic variability between *P. bogoriensis and P. eastuari* were also striking since *P. aestuarii* could not assimilate any of the L-saccharides such as L-fucose, L-rhamnose, Larabinose, L- xylose or L-sorbose while *P. bogoriensis* utilized all of them. In *P. bogoriensis* did not utilize the structurally peculiar sugar *myo*-inositol and citrate which are carbon sources for *P. aestuarii*. This study demonistrated instances where 16S rRNA gene could not succinctly be used as a stand-alone phylogenetic marker. This implies that in some instances the use of 16S rRNA gene analysis need to be complemented with other tests such as biochemical tests. However, in this study it was expected that clustering of the 16S rRNA gene of *P. baruchii* with sequences in the GeneBank would act as a definitive confirmation of the organism. Nonetheless, phenotypic characteristic such as the color and growth temperatures of the organism were also expected to complement confirmatory tests based on phylogenetic analysis of 16S rRNA gene.

At the molecular level, comparison of 16S rRNA gene sequences of the *Paracoccus* have shown that clustering depicts ancestral relationships with the subclass of the proteobacteria with the closest relatives being the genus *Rhodobacter* (Tsubokura *et al.*, 1999). According to Kelly *et al* (2006) the 16S rRNA gene sequence similarity within

the genus *Paracoccus* is in the range of 93.5 - 99.8% .Osanjo *et al* (2009) argued that a high sequence similarity was not unusual among members of the *Paracoccus* genus. For example *P. carotinifaciens* and *P. marcusii* share 99.8% sequence similarity (Kelly *et al*., 2006). High sequence similarity does not therefore indicate species identity among the *Paracoccus.* Osanjo *et al* (2009) however, argues that the phylogenetic distinction among the *Paracoccus* species is supported by the long branches in the dendrogram , bootstrap values from maximum likelihood trees and posterior values from the Bayesian analysis. Thus, the length of branches in the dendrogram, bootstrap values from maximam likelihood trees were also used in this study to confirm the identity of *P. baruchii.*

Growth optimization of *Paraccocus*

Paracoccus species grow aerobically on a wide range of organic substrates and some are capable of anaerobic growth with nitrate or nitrous oxide as the terminal oxidant, producing dinitrogen as the final product (Kelly, *et al*., 2006). However, the organisms do not utilize similar carbon sources. The optimum temperature for growth of all the species is in the range 25–37°C, and the pH for good growth ranges between pH 6.5–8.5 for different species, except for *P. alcaliphilus* (which is moderately alkaliphilic with an optimum of pH 8–9 and grows at pH 9.5, but only weakly at pH 7.0. however, the optimum growth conditions vary from one species to another.

According to Urakami, Tamaoka, Suzuki and Komagata (1989) *Paracoccus alcaliphilus* has affinity for alkalinity. The growth of *P. alcaliphilus* in nutrient broth is very poor, but growth in nutrient broth adjusted to pH 9.0 is abundant. Growth of *P. alcaliphilus* in peptone water is also poor or nonexistent, but growth in peptone water adjusted to pH 9.0 is abundant. Urakami *et al* (1989) also reported that *P. alcaliphilus* reducesnitrate to nitrite. The organism does not hydrolyse gelatin and starch. *P. alcaliphilus* also utilizes L-Arabinose, D-xylose, D-glucose, D-mannose, D-fructose, D-galactose, D-sorbitol, D-mannitol, inositol, glycerol, succinic acid, citric acid, acetic acid, ethanol, and methanol

as carbon sources, but maltose, sucrose, lactose, trehalose, soluble starch, formic acid, dimethylamine, trimethylamine, methane, and hydrogen are not utilized.

Harker, Hirschberg and Oren (1998) reported that *Paracoccus marcusii* was an orange Gram negative coccus that appeared as a contaminant on a nutrient agar plate. The bacterium grows optimally at temperatures of between 25 – 30 ^{o}C. the bacterium utilizes D-glucose, D-fructose, D-galactose, D-mannose, L-arabinose, maltose, cellobiose, D-lactose, melibiose, sucrose, turanose, D-trehalose, gentiobiose, lactulose, D-gluconic acid, D-glucuronic acid, D-galacturonic acid, glycerol, erythritol, D-mannitol, D-sorbitol, xylitol, myo-inositol, adonitol, D-arabitol, propionic acid, cis-aconitic acid, citric acid, m-lactic acid, malonic acid, quinic acid, succinic acid, malic acid, formic acid, L-alanine and alaninamide as carbon sources.

Tsubokura, Yoneda and Mizuta (1999) described *Paracoccus carotinifaciens* as a Gram negative, aerobic, orange-pigmented, rod-shaped, motile and astaxanthin producing micro-organism. The optimal growth temperature for *P. carotinifaciens* is between 25 and 30 "C. No growth occurs at 37 "C. The organism is oxidase and catalase positive and urease negative. *P. carotinifaciens* utilizes D-glucose, D-mannose, D-mannitol, D-maltose, potassium gluconate and DL-malic acid.

Berry, Janssens, Humbelin, Jore, Hoste, Cleenwerck, Vancanneyt, Bretzel, Mayer, Lopez-Ulibarri, Shanmugam, Swings and Pasamontes (2003) reported that *Paracoccus zeaxanthinifaciens* grow weakly or not at all at 10°C, but grow well from 25 to 40°C. *P. zeaxanthinifaciens* also grow on Marine Agar supplemented with 3, 6 or 8%NaCl, and in Marine Broth having a final pH ranging from 6.1 to 9.1. Berry *et al* (2003) argued that this was in contrast to *Paracoccus marcusii* DSM 11574T and *Paracoccus carotinifaciens* E-396T which grow very poorly in the presence of 8% NaCl, and that P. marcusii DSM 11574T was not able to grow at pH 9.1. Growth of *Paracoccus zeaxanthinifaciens* is strictly aerobic. *Paracoccus zeaxanthinifaciens* does not also hydrolyse starch and gelatin but it reduces nitrate to nitrite. The organism also utilizes D-arabitol, D-galactose, a-D-glucose, myo-inositol, a-lactose, D-mannitol, D-melibiose,

D-trehalose, L-asparagine, L-aspartic acid, L-glutamic acid and L-pyroglutamic acid for growth..

Lee, Kim, Choi, Lee and Kim (2004) isolated *Paracoccus haeundaensis* from the Haeundae Coast, Korea. They described it as an aerobic, non-motile, Gram-negative, orange-pigmented, rod-shaped, astaxanthin-producing marine bacterium. The optimal growth temperature is 25 ^{o}C and no growth occurs below 10 ^{o}C or above 40 ^{o}C. The optimum NaCl concentration for growth is 1–6% (w/v). Optimal pH for growth is 8. The bacterium utilizes D-arabinose and galactose as carbon and energy sources. No growth occurs on D-glucose, lactose, maltose, sucrose, trehalose, D-mannitol, D-sorbitol, inositol, D-raffinose, D-fructose, D-mannose, dimethylformamide, L-glutamic acid, acetone, L-leucine, L-asparagine, L-rhamnose, salicin, D-cellobiose, adonitol, dulcitol, D-xylose or glycerol. Unlike *P. alcaliphilus* and *P. zeaxanthinifaciens*, *P. haeundaensis* utilizes starch as a carbon source and energy source.

Paracoccus marinus was described by Khan, Takaichi and Harayama (2008) as a Gram-negative, non-motile, catalase- and oxidase-positive, aerobic bacterium. The bacterium is circular, convex, smooth and dull orange in color. The organism is catalase and oxidase positive but negative for b-glucosidase and b-galactosidase. Growth occurs between 10 and 35 ^{o}C (optimum 25–35 ^{o}C) and at pH 6–9 (optimum pH 7.0–8.0). NaCl (1–4%, w/v) is required for growth (optimum 2–3%). The bacterium utilizes peptone, but not ammonium sulfate, sodium glutamate, sodium nitrate or Casamino acids as nitrogen sources. D-Fructose, D-galactose, D-glucose, lactate, pyruvate and asparagine are utilized as carbon sources, but D-arabinose, a-D-lactose, maltose, D-mannose, sucrose, trehalose, acetate, formate, propionate, succinate, arginine, ethanol, myo-inositol, Dmannitol and D-sorbitol are not utilized. Starch, gelatin, casein, chitin, DNA, aesculin, urea, arginine and Tweens 40 and 80 are not hydrolysed, but Tween 20 is hydrolysed. The bacterium does not reduce nitrate to nitrite.

Zheng, Wang, Chen, Wang, Xia, Fu, Zhang and Jiao (2011) described *Paracoccus beibuensis* as Gram-negative, non-motile, short rod-shaped or spherical bacterial strain

that accumulates poly-bhydroxybutyrate (PHB) granules. *P.beibuensis* grows at 10–30^{o}C, pH 6.0–8.0 and 0–15% NaCl; the optimal growth temperature, pH and salinity are 25 ^{o}C, pH 7.0 and 2–5% NaCl. The organism is positive for oxidase, catalase, gelatin hydrolysis and PHB production. This implies that unlike *P. alcaliphilus* and *P. zeaxanthinifaciens* which do not hydrolyse gelatin, the *P.beibuensis* does. Like *P. alcaliphilus* and *P. zeaxanthinifaciens*, *P.beibuensis* does not hydrolyse starch. *P.beibuensis* does not also hydrolyse Tween 80 and casein. Moreover, unlike *P. alcaliphilus* and *P. zeaxanthinifaciens*, *P.beibuensis* does not reduce nitrate to nitrite.

Acetyl-CoA acetyltransferase

Acetyl-CoA acetyltransferase is also known as Acetyl-CoA C-acetyltransferase, 3-ketothiolase or thiolase I (Hiroe, Tsuge, Nomura, Itaya, & Tsuge, 2012) Acetyl-CoA-acetyltransferase is encoded by *phaA* which catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. Acetyl-CoA acetyltransferase is the first enzyme involved in the biosynthesis pathway of poly-(R)-3-hydroxybutyrate (PHB) (Kocharin, Chen, Siewers & Nielsen, 2012). The second is the NADPH-dependent acetoacetyl-CoA reductase encoded by *phaB* which catalyzes the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA. Finally, PHA synthase encoded by *phaC*, catalyzes the polymerization of (R)-3-hydroxybutyryl-CoA monomers to PHB. PHB is the most common type of polyhydroxyalkanoates (PHAs) synthesized and accumulated by microorganisms like *Ralstonia eutropha*, *Bacillus megaterium* or *Pseudomonas sp*. as carbon and energy storage material in response to conditions of physiological stress (Steinbuchel *et al*. 1993). PHA are polyesters synthesized by more than 200 species of bacteria and are accumulated as intracellular carbon and energy storage materials (Hiroe, Tsuge, Nomura, Itaya, & Tsuge, 2012). Biodegradable PHB is linear polyester consisting solely of the stereospecific monomer, (R)-3-hydroxybutyric acid. It belongs to the group of short chain length PHAs consisting of C3-C5 hydroxyacid monomers. (Hankermeyer & Tjeerdema 1999; Melchiors *et al*. 1994).

The natural PHB producers like *R. eutropha, Bacillus megaterium* or *Pseudomonas sp*. are known to produce and accumulate PHB as a storage compound in response to nutrient imbalance caused by growth under conditions of carbon source excess but limitation in other essential nutrients (Steinbüchel & Hein 2001; Trotsenko & Belova 2000). Instead of employing the natural PHB producers, which can depolymerize PHB and use it as a secondary energy source, metabolic engineering can be used to transfer the PHB biosynthetic pathway to alternative hosts that may have advantage over the natural PHB producers, in particular the lack of enzymes for PHB depolymerization (Uchino & Saito 2006). Furthermore, by transferring to alternative hosts one may take advantage of a range of technologies developed for general platform cell factories like *Escherichia coli* and *Saccharomyces cerevisiae*. Thus, there have been a range of studies where PHB production has been evaluated in *E. coli* and further metabolic engineering has been carried out with the objective to improve the productivity. In a metabolic and kinetic study, a recombinant *E. coli* strain producing PHB was examined and compared to the native PHB producer, *R. eutropha*, and this study revealed that the PHB flux was highly sensitive to the acetyl-CoA/CoA ratio, the total acetyl-CoA plus CoA concentration and pH (van Wegen *et al*. 2001).

In recombinant *E. coli,* a mutation in arcA encoding a protein that regulates aerobic respiration under microaerobic conditions resulted in higher amounts of PHB accumulated in the cell (Nikel *et al*. 2006). Low agitation conditions had a positive effect on PHB synthesis from glycerol in recombinant *E. coli* carrying the *phaCAB* operon and *phaP* encoding a granule-associated protein (phasin) (de Almeida *et al*. 2010). Several studies also attempted to synthesize PHB in plants according to the aim to produce high amounts of PHB at lower costs compared to microbial fermentation, particularly in plant plastids where the biosynthesis of fatty acids from acetyl-CoA occurs (Bohmert *et al*. 2000; Nawrath *et al*. 1994; Petrasovits *et al*. 2012).

Many approaches have been followed to improve the production of PHB in yeast. Srienc and co-workers performed elementary mode analysis of a *S. cerevisiae* containing the

PHB synthesis pathway in order to identify new metabolic engineering targets (Carlson *et al*. 2002). The analysis suggested that the introduction of the ATP citrate-lyase reaction and the transhydrogenase reaction can improve the theoretical PHB carbon yield (Carlson *et al*. 2002). Acetyl-CoA serves as the precursor for the PHB biosynthesis pathway and increasing the availability of acetyl-CoA was proposed to improve PHB production (Carlson & Srienc, 2006; Suzuki *et al*. 2002). However, using enzyme inhibitors to reduce its consumption by other pathways or feeding of the substrate during cultivation would result in increasing production costs and may not be feasible for industrial applications. Kocharin, Chen, Siewers and Nielsen (2012) demonstrated metabolic pathway engineering by co-transformation of a plasmid containing the PHB biosynthesis pathway and an acetylcoenzyme A (acetyl-CoA) boost plasmid designated to improve the availability of cytoplasmic acetyl-CoA and hereby improve the productivity of PHB in S. cerevisiae. Introduction of the acetyl-CoA plasmid together with the PHB plasmid, improved the productivity of PHB more than 16 times compared to the reference strain used in this study, as well as it reduced the specific product formation of side products.

A homopolymer of (*R*)-3-hydroxybutyrate [P(3HB)] is the most common type of PHA that bacteria accumulate in nature, and its production has been extensively studied (Hiroe, Tsuge, Nomura, Itaya, & Tsuge, 2012). *Ralstonia eutropha* is the most widely studied native producer of P(3HB), and in this bacterium the polymer is synthesized from acetyl coenzyme A (acetyl-CoA). P(3HB) produced by native PHA-producing bacteria is a brittle and rigid material with low flexibility because of its high crystallinity. On the other hand, recombinant *Escherichia coli* harboring the *R. eutropha pha* operon (*phaC-phaA-phaB*) is capable of synthesizing ultrahigh-molecular-weight P(3HB). The ultrahigh-molecular-weight P(3HB) higher mechanical strength, and unlike lower-molecular-weight P(3HB) polymers, ultrahigh-molecular-weight P(3HB) can be processed into strong films or fibers by hot or cold drawing. Detection of *phaA* in *Paracoccus baruchii* which is extremophilic could help in production of natural ultra-high molecular-weight P(3HB) that has a higher mechanical strength.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Bacterial isolate revival

Paracoccus baruchii LBOG37 was originally isolated from Lake Bogoria. However, the bacterial isolate, *Paracoccus baruchii* LBOG37 glycerol stock was obtained from culture stocks at the Biochemistry department of University of Nairobi. The culture was then transported in a cool box to Jomo Kenyatta University of Agriculture and Technology (JKUAT), Institute for Biotechnology Research (IBR) laboratory. The bacterium was revived by inoculating in liquid modified Horikoshi medium and then incubating for 24 hours at 40oC. Each treatment in this study was replicated three times.The bacterium was then streaked on modified Horikoshi agar comprising of 10 g/l starch, 10 g/l NaCl, 5 g/l bacteriological peptone, 5 g/l yeast extract and 15 g/l bacto-agar (the liquid medium had the same ingredients except bacto-agar). The pH of the medium was adjusted to 9.0 using 2 M NaOH. This was followed by sterilization by autoclaving using Astel autoclave at 1bar for 15 minutes. The pH was measured using inoLab® pH 7110 benchtop meter after autoclaving. The sterile medium was poured on plates and allowed to solidify. The *Paracoccus baruchii* LBOG37isolate was streaked onto the solidified agar and incubated at 40^{o}C overnight in an incubator (applied biosystems, La Jolla CA USA). A single colony of *Paracoccus baruchii* LBOG37 from agar plate was used to inoculate LB broth pH 9.0 and incubated overnight with continuous shaking at 130 rpm in a Gallenkamp orbital incubator shaker (Sanyo Gallenkamp). Glycerol stocks of *Paracoccus baruchii* LBOG37 were prepared from the revived culture by mixing 500 µL of the overnight culture to 500µL of 50% glycerol in a 2 mL sterile microfuge tubes using sterile pipet tips and gently mix. (The 50% glycerol solution was made by diluting 100% glycerol in double distilled water and autoclaving). The mixture was then quickly placed into the -80

3.2 Biochemical characterization

Paracoccus baruchii LBOG37was grown on modified Horikoshi medium at 40^{o}C. The pH was adjusted using 2M NaOH. The growth of the *Paracoccus baruchii* LBOG37isolate was measured as optical density at a wavelength of 600 nm and measured for 48 hours at an interval of 0 h, 2 h, 4 h, 8 h, 24 h and 48 h. All experiments were carried out in triplicate and inoculations were done in laminar flow clean bench.The objective for biochemical characterization was to optimize growth conditions for *Paracoccus baruchii* LBOG37isolate. The factors optimized here included pH, temperature and carbon source.

3.2.1 Growth under different pH conditions

To test for the optimal pH for growth of the *Paracoccus baruchii* LBOG37, a 10µl aliquot of the revived culture was transferred into tubes containing 20ml MH liquid medium with final pH adjusted to 9.0, 9.5, 10.0, 10.5, and 11.0 respectively. The adjustment of pH had been done after autoclaving by addition of a Na_2CO_3/$NaHCO_3$ buffer. The cultures were then incubated with shaking (130rpm) at 40oC for 24 hrs. The bacterial growth at different pH was measured at OD 600nm after 24 hours using spectrophotometer.

3.2.2 Effect of temperature on growth

Effect of temperature on the *Paracoccus baruchii* LBOG37 isolate was monitored by inoculation of 10µl of revived isolate in tubes containing 20ml MH liquid medium and incubation at 37^{o}C,40^{o}C, 45^{o}C, 50^{o}C and 54^{o}C respectively with shaking at 130rpm. The optical density (OD) was then measured for 48 hours at intervals of 0h, 2h, 4h, 8h, 24h and 48h and at a wavelength of 600nm.

3.2.3 Effect of Carbon Source on Growth

Effect of sugar source on *Paracoccus baruchii* LBOG37isolate growth was monitored by inoculation of 10µl of revived isolate in tubes containing 20ml MH liquid medium with different carbon sources (10g/l carboxymethyl cellulose (Sigma), 10g/l starch

(RDH33615) and 10g/l glucose (sigma) which replaced the 10g/l of starch and incubation at 37^{o}C, 40^{o}C, 45^{o}C, 50^{o}C and 54^{o}C with shaking at 130rpm. Optical density (OD) was measured for 48 hours at an interval of 0h, 2h, 4h, 8h, 24h and 48h at a wavelength of 600nm to help in determination of optimum temperature. It should be noted that the decision to determine the sugar use at different temperatures rather than pH was informed by the fact that L. Bogoria where the isolate was isolated from has alkaline pH. The study used the optimum pH obtained at 40^{o}C. Thus, it was assumed that the optimum pH obtained would apply to all the three sugars used in this study.

3.3 Molecular Characterization

3.3.1 DNA Extraction Buffer

Genomic DNA extraction was carried as described previously by Rainey *et al* (1996). Prior to DNA extraction, the extraction buffer was prepared (see appendix 1). Solution A (500 µl) was placed in 1.5 ml Eppendorf tube. A loopful of the isolate from the plates was added to the solution and centrifuged at 13,000 rpm for a minute. The supernatant was discarded. The sample was re-suspended in 200 µl of solution A. This was followed by addition of 5 µl of lysozyme (20 mg/ml) and the mixture was mixed gently. A five (5) µl of RNAseA (20 mg/ml) was then added and mixed gently. The mixture was incubated at 37^{o}C for 1 hour. Solution B (600 µl) was added and mixed by inverting several times. A 10 µl of proteinase K (20 mg/ml) was added mixed gently and incubation was done at 55^{o}C for 30 minutes. The mixture was divided into two equal volumes for convenience and DNA was extracted by adding equal volumes of Phenol:Chloroform. The mixture was then centrifuged at 13,000 rpm for 15 minutes and the aqueous phase which contained crude DNA was carefully pipetted out. The extraction was repeated with Phenol:Chloroform and the DNA was then extracted with an equal volume of Chloroform:Isoamylalcohol (24:1). This was then centrifuged at 13,000 rpm for 15 minutes and the aqueous phase was pipetted out to a clean Eppendorf tube. The extraction was repeated with Chloroform:Isoamylalcohol (24:1) to remove all

the phenol from the DNA. An equal volume of Isopropanol and 0.1 volumes of 3M NaCl was added and left at -20^{o}C overnight. NaCl was added in order to neutralize the negative charges of the DNA molecule, making the separate DNA molecules more likely to collect together and become visible. The DNA samples were defrosted and centrifuged for 30 minutes at 13,000 rpm to pellet the DNA. The pellet was washed with 70% ethanol and centrifuged at 13,000 rpm for 5 minutes. The ethanol was pipetted out taking care that the DNA pellet was not dislodged. The washing was repeated and all the ethanol was pipetted. The pellet was air dried on the bench at room temperature for 20 minutes and was then dissolved in 50 µl of Tris EDTA (TE) buffer pre-warmed at 55^{o}C. The DNA obtained was stored at -20^{o}C for further molecular analysis. To check the quality of the extracted DNA, a five (5) µl of extracted DNA was mixed with 1 µl of 6X gel loading buffer. The mixture was then loaded into the wells in the gel (see appendix II for preparation of the agarose gel). A 1Kb ladder was loaded in one well and the gel was run at 100V for approximately 30 minutes. The gel was then observed under ultraviolet light using benchtop UV trans-illuminator and photographed.

3.3.2 The 16S rRNA *PCR*

The PCR was undertaken to amplify the 16S rRNA gene. The 16S rRNA gene was amplified in order to help in establishing whether the isolate was similar the same organism whose sequences were deposited at the Genebank through phylogenetic analysis of the sequences obtained. The amplification of 16S rRNA gene involved preparation of the master mix using 4.0 µl of 1X of green *PCR* buffer (Promega), 2.0 µl of 1.25 mM of deoxy-nucleoside triphosphates (dNTPs), 1.0 µl of 8F primer (AGAGTTTGATCCTGGCTCAG), 1.0 µl of 1492R primer (5′-TACCTTGTTACGACTT) 0.5 µl of Taq polymerase, 1.0 µl template and 10.5 µl of double distilled water. The total reaction volume was 20.0 µl. Amplification was done on a *GeneAmp*® 9700 Thermocycler as follows: initial denaturation at 96^{o}C for 5 minutes, denaturation at 96^{o}C for 30 seconds, annealing at 55^{o}C for 1 minute, extension at 72^{o}C for 1 minute and final extension at 72^{o}C for 5 minutes. The *PCR* was run for 30

cycles. The *PCR* amplicon was checked by loading 5 µl of *PCR* product into the wells of 1% Agarose gel and gel electrophoresis was run at 100V for approximately 30 minutes. The gel was then observed under ultraviolet light using benchtop UV trans-illuminator and photographed.

3.3.3 Gel Extraction and Sequencing

Gel electrophoresis of 16 sRNA *PCR* product was carried out using 1% Agarose gel. Each well in the gel was loaded with 15µl of *PCR* product. The gel was observed under UV light after running for approximately 45 minutes at 100V. DNA bands were exercised and placed in pre-weighed Eppendorf® tubes. Extraction of DNA from the agarose was done using the Qiaex gel extraction kit following the manufacturer's instructions (see appendix III). Purified 16S rRNA*PCR* products were sequenced at Macrogen laboratories, Korea using reverse primer.

3.4 The *crt*I Gene Amplification

OLIGO Primer Analysis software was used to design the following degenerate primers using *phaA* gene sequences from *Paracoccus marcusii:* 5' - GAT CCG GCG ACC TTG CGG - 3' (16F) and 5' - GGT GTT TCC GGC CAA GGA TG - 3' (19R). The master mix was made using 4.0 µl of 1X of green *PCR* buffer, 2.0 µl of 1.25 mM of deoxy-nucleotide phosphates (dNTPs), 1.0 µl of 16F primer, 1.0 µl of 19R, 0.5 µl of Taq polymerase, 1.0 µl template and 10.5 µl of double distilled water. The total reaction volume was 20.0 µl. The mix was placed in a *GeneAmp*®9700 thermocycler and run under the following conditions: initial denaturation at 96^{o}C for 5 minutes, denaturation at 96^{o}C for 1 minute, annealing at 55^{o}C for 1 minute, extension at 72^{o}C for 1 minute and final extension at 72^{o}C for 5 minutes. The *PCR* was run for 30 cycles. The DNA was checked by loading 5 µl on a 1% agarose gel.

3.5 *Crt*I*PCR* Product Purification

Gel electrophoresis of the *crt*I*PCR* product was carried out using 1% Agarose gel. Each well in the gel was loaded with 15 µl of *PCR* product. The gel was observed under UV light after running for approximately 45 minutes at 100V. DNA bands were exercised and placed in pre-weight eppendorf tubes. Extraction of DNA from the agarose was done using the Micro spin gel extraction kit (peqLab) following the manufacturer's instructions (see appendix IV). Purified *crt*I*PCR* products were sequenced at Macrogen laboratories, Korea.

3.6 Phylogenetic Analysis *of* 16S rRNA Sequences

The sequences of 16S rRNA gene were edited using Chromas lite software (http://www.technelysium.com.au). The edited sequences were subjected to BLASTn at NCBI website (http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi). The parameters for BLASTn analysis included other database and optimized for highly similar (megablast). The resulting sequences were aligned using Clustal tool in MEGA 5.2 software (Tamura, *et al.*, 2011). The resulting alignments were used to construct a phylogenetic tree using neighbor joining algorithm.

3.7 Phylogenetic Analysisof*crt*I Sequences

The sequences of *phaA* gene were edited using Chromas lite software (http://www.technelysium.com.au). The edited sequences were translated using the translate tool at the Expasy software (http://web.expasy.org/translate/). The open reading frames (ORFs) were subjected to BLASTp analysis at NCBI website (http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi) (Altschul, *et al.*, 1990**)**. The resulting sequences were aligned using Clustal W tool in MEGA 5.2 software (Tamura, *et al.*, 2011). The alignment were then used to construct phylogenetic tree.

3.8 Data Analysis

The data was analysed using Microsoft windows Excel. Data was presented in form of graphs.

CHAPTER FOUR

RESULTS

4.1 Bacterial Isolate Revival

The isolate was successfully revived in liquid medium. The isolate showed a characteristic orange colour when it was streaked and incubated on MH agar plates as shown in Figure 2.

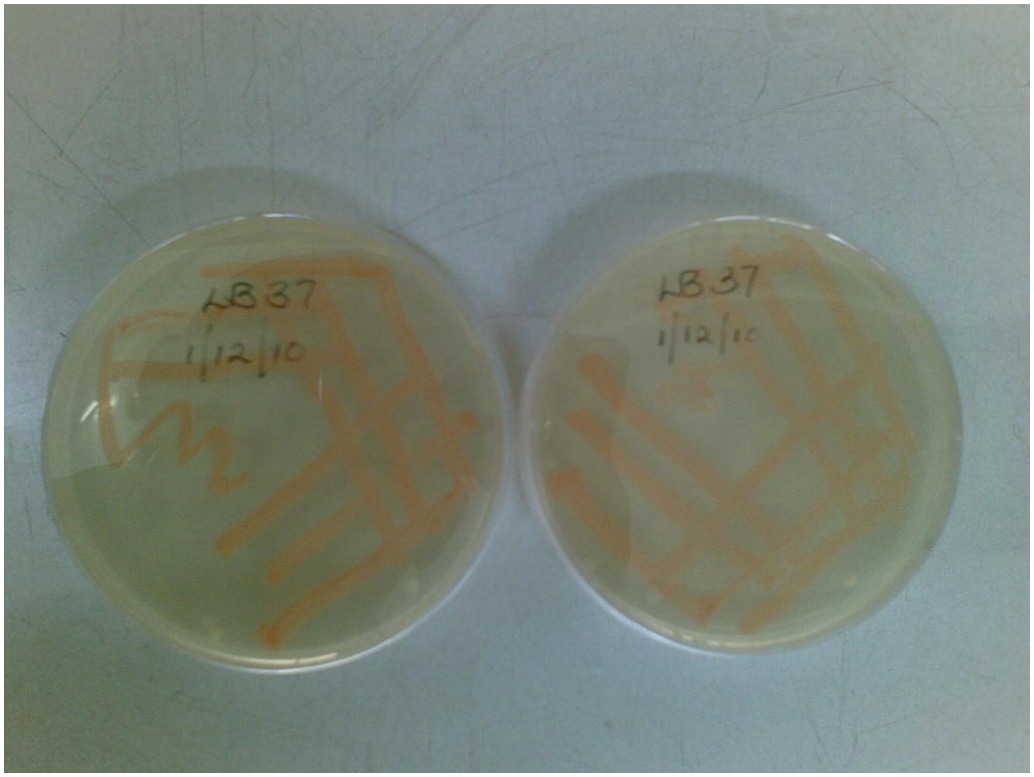

Plate 4.1: *Paracoccus baruchii*LBOG37growing on modified Horikoshi (MH) agar medium

Coloration in bacteria is usually an indicative of presence of caretonoids (Armstrong, 1997). Thus, it is possible that the bacteria produce carotenoids due to its characteristic orange color observed.

4.2 Temperature Optimization Using Different Sugars

Carboxymethyl-cellulose, glucose and starch were used as carbon sources in this study. Figure 4.2, 4.2, 4.3, 4.4, and 4.5 shows a summary of the results obtained at various temperatures. Figure 3 shows the growth of *Paracoccus baruchii* LBOG37 at 37^{o} C in different carbon source.

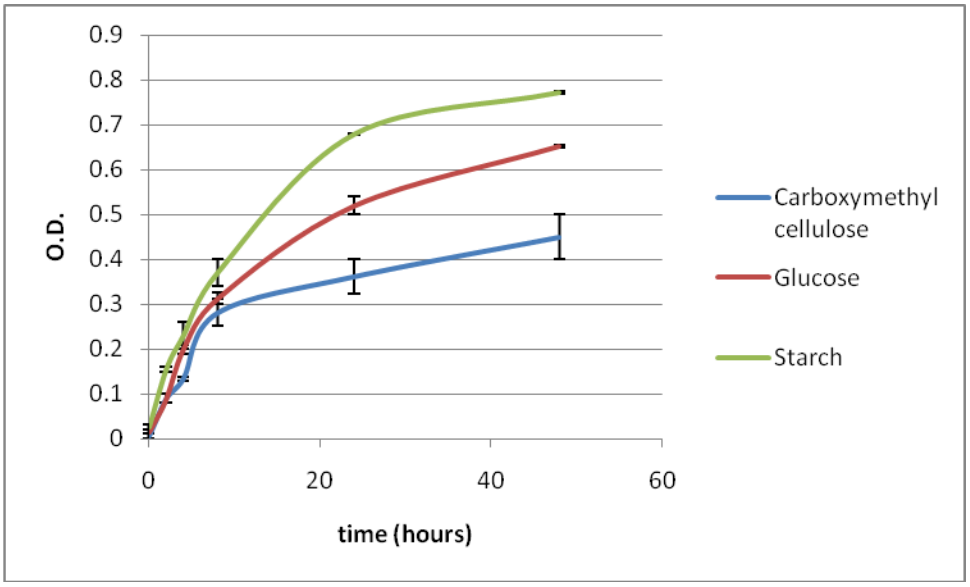

Figure 4.1: Optical density (absorbance at 600 nm) at different time intervals after incubation at 37 ^{o}C in different carbon sources

The results show that the growth of the bacteria had no observable lag phase. There was however, a progressive growth of the organism in the exponential phase after two hours incubation period in all carbon sources (Figure 4.1). Growth in carboxymethyl cellulose had the a shorter exponential phase as compared to growth in other carbon sources. The highest growth was recorded in carboxymethyly cellulose, followed by glucose. However, the recorded optical density in all carbon sources was less than 0.8 OD. The

growth reached optimum growth approximately after 24 hours incubation (Figure 4.1). The *Paracoccus baruchii* LBOG37was also grown at 40^{o}C. The results are illustrated in Figure 4.2.

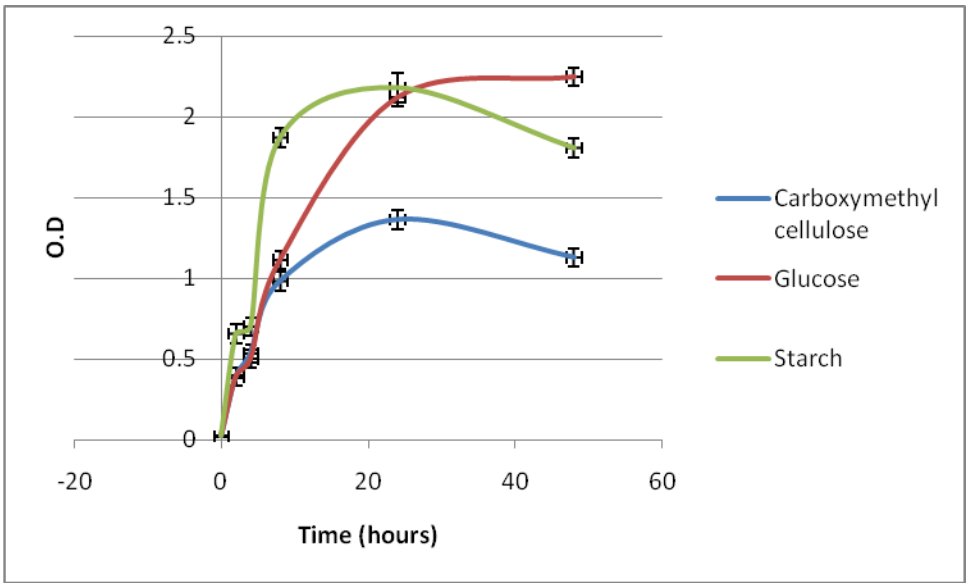

Figure 4.2: Optical density (absorbance at 600 nm) at different time interval after incubation at 40^{o} C in different sugar sources.

The results show that growth in all the three carbon sources had a shortened lag phase. This differs from growth at 37 oC where there was almost no observable lag phase. Again growth in starch had a shortened exponential phase as opposed to growth in glucose and carboxymethyl cellulose with growth in carboxymethyl cellulose recording a prolonged exponential phase. It should however be noted that, growth at this temperature (40o C) was higher than 1 optical density in all the three sugars with growth in both glucose and starch recording higher than 2.0 OD after 24 hours of incubation. Growth in starch and carboxymethyl cellulose began declining after incubating for 24 hours. On the other hand there was no observable decline in growth of the bacteria in glucose after incubating for 24 hours.

The *Paracoccus baruchii* LBOG37was further grown at 45^{o}C and the results were presented graphically in Figure 4.3.

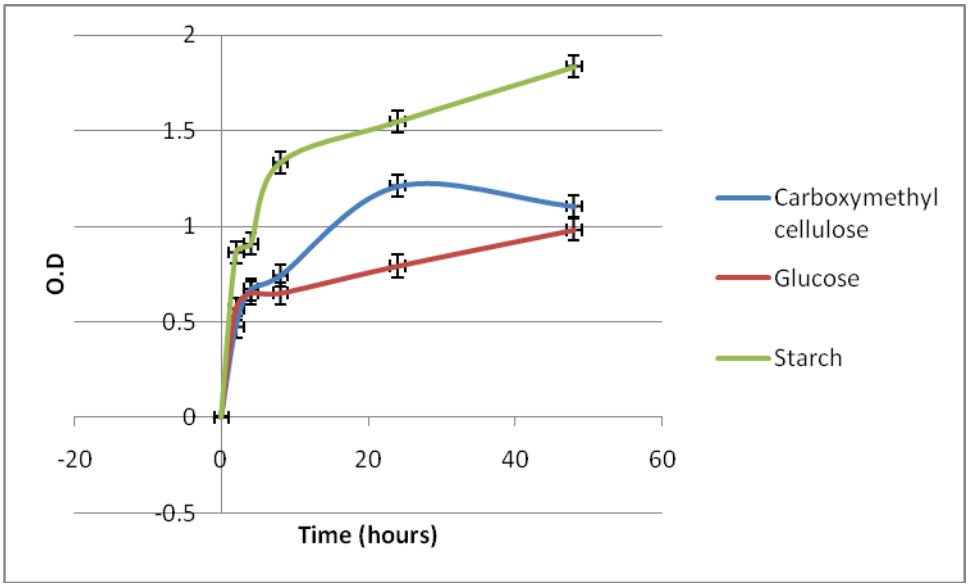

Figure 4.3: Optical density (absorbance at 600 nm) at different time interval after incubation at 45^{o}C in different sugar sources.

At 45oC growth in all the three carbon sources was below 2.0 OD after incubating for 48 hours. However, growth in carboxymethyl cellulose and in starch recorded growth of over 1.0 OD after incubating for 24 hours. The highest growth was recorded in starch which recorded OD of over 1.5. Again at this temperature, lag phase was not observable in all the three carbon sources. A decline in growth was only observed in carboxymethyl cellulose after incubating for 48 hours.

The *Paracoccus baruchii* LBOG37was also grown at 50^{o}C using the three sugars. The results are illustrated in Figure 4.4.

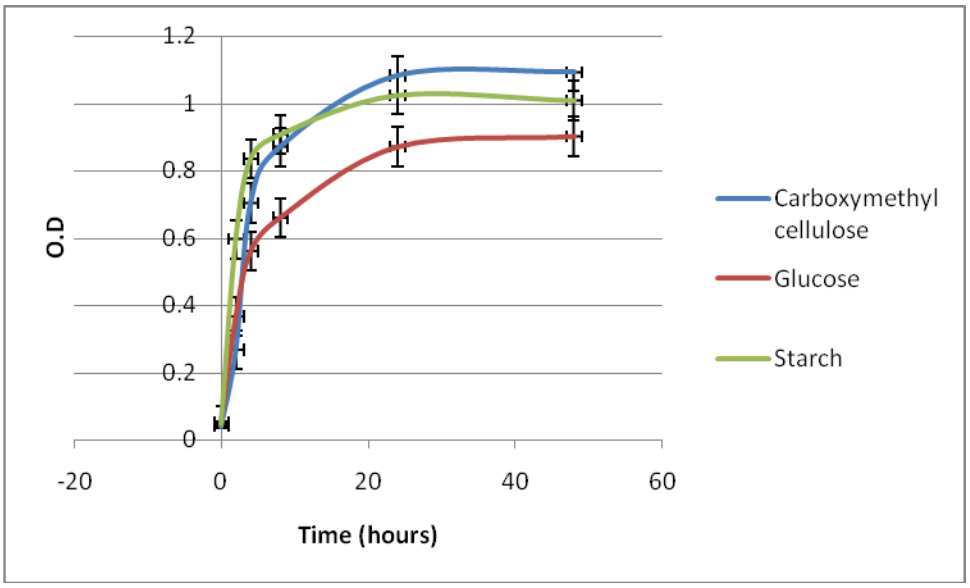

Figure 4.4: Optical density (absorbance at 600 nm) at different time interval after incubation at 50^{o} C in different sugar sources.

There was no observable lag phase growth on growth of the bacteria at 50o C. The exponential growth was also shortened with growth of the bacteria reaching optimal growth after about 8 hours of incubation. However, growth was greatly reduced in all the three carbon sources used. The highest growth was recorded in carboxymethyl cellulose which recorder slightly higher than 1 optical density at otmal growth. This was followed by growth in starch which also recorded slightly above 1.0 OD. However, growth in glucose was slightly below 1.0 OD. Very little decline in growth was observed in both carboxylmethyl cellulose and starch with no observable decline in growth of the bacteria in glucose after incubating for 48 hours.

Finally, the *Paracoccus baruchii* LBOG37was grown at 54^{o}C. The results were presented graphically in Figure 4.5.

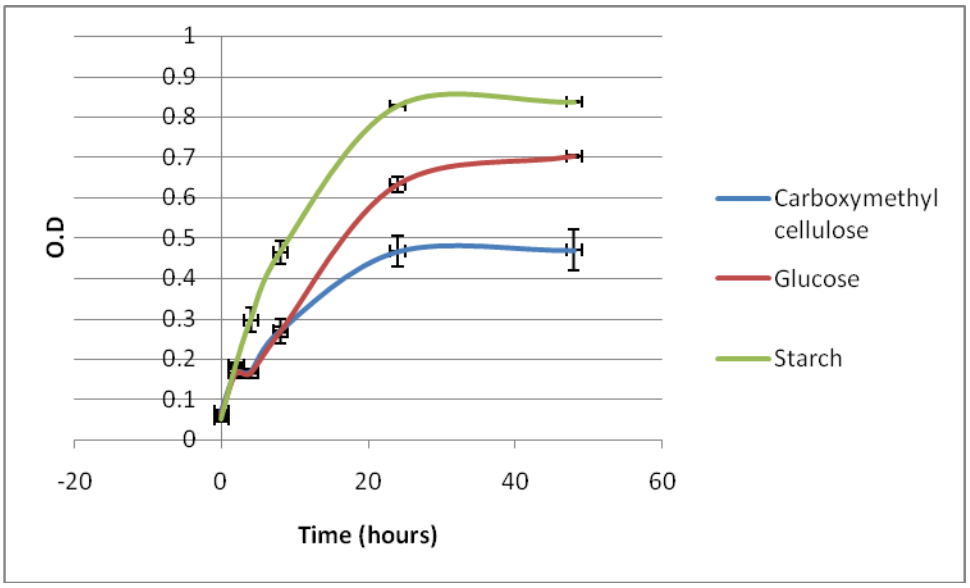

Figure 4.5: Optical density (absorbance at 600 nm) at different time interval after incubation at 54^{o}C in different sugar sources.

Although growth of the bacteria was supported in all the three carbon sources at 54^{o}C, the recorded growth was below 1.0 OD in all the three carbon sources. At this temperature more growth was observed in starch followed by growth in glucose. Growth in carboxymethyl was the lowest and was below 0.5 O.D. Growth in starch had no observable lag phase while in glucose and carboxymethyl cellulose there was a shortened lagphase.

On completion of temperature optimization, it was established that *Paracoccus baruchii* LBOG37growth was optimum at 40^{o}C. Thus, growth optimizations of *Paracoccus baruchii* LBOG37 at various pH values were carried out at 40^{o}C as indicated in Figure 4.6.

4.3

4.4 The pH Range for Growth

The results for pH optimization using various sugar sources were presented in figure 4.6.

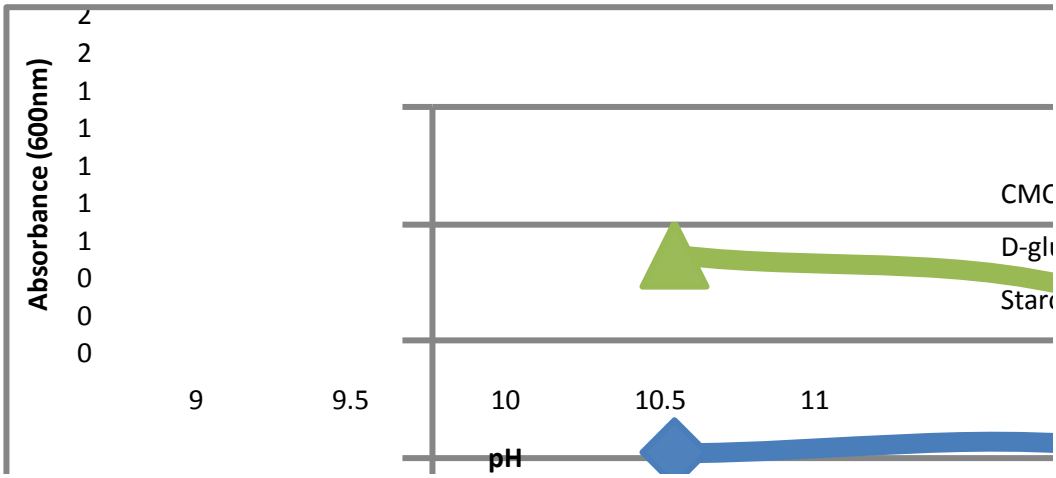

Figure 4.6: Growth at different pH values with different sugars

The results indicated that Paracoccus baruchii growth is supported at pH range of 9 to 11. However, growth of the bacteria in starch and in carboxymethyl cellulose was highest at pH of 9 and 9.5 after which the growth declined. On the other hand, growth in glucose was highest at pH of 10. This is indicative that this organism is alkalophilic.

4.5 Gel Electrophoresis

4.4.1 The 16S rRNA Gene Gel Electrophoresis

The 16S rRNA gene amplicon gel electrophoresis of *Paracoccus baruchii* LBOG37was recorded in form of a photograph (Figure 4.7).

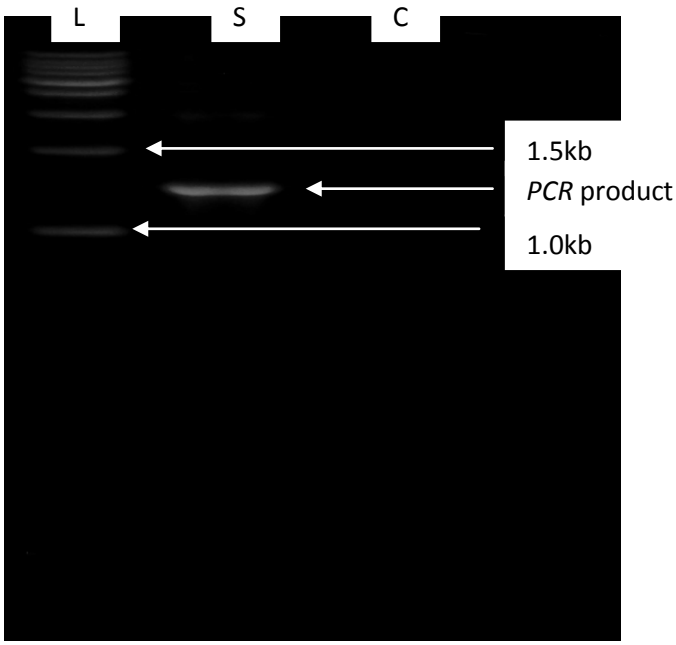

Figure 4.7: Agarose gel electrophoresis of 16S rRNA gene product of Paracoccus baruchii LBOG37 (L-molecular ladder; S-PCR sample; and C-control.

The 16S rDNA obtained had an approximate molecular weight of between 1.0 and 1.5 kb. To establish the actual size of the 16S rDNA product obtained, the PCR product was sequenced.

4.4.2 *phaAPCR* Product Gel Electrophoresis

The *phaA* gene amplicon gel electrophoresis of *Paracoccus baruchii* LBOG37was recorded in form of a photograph (Figure 4.8).

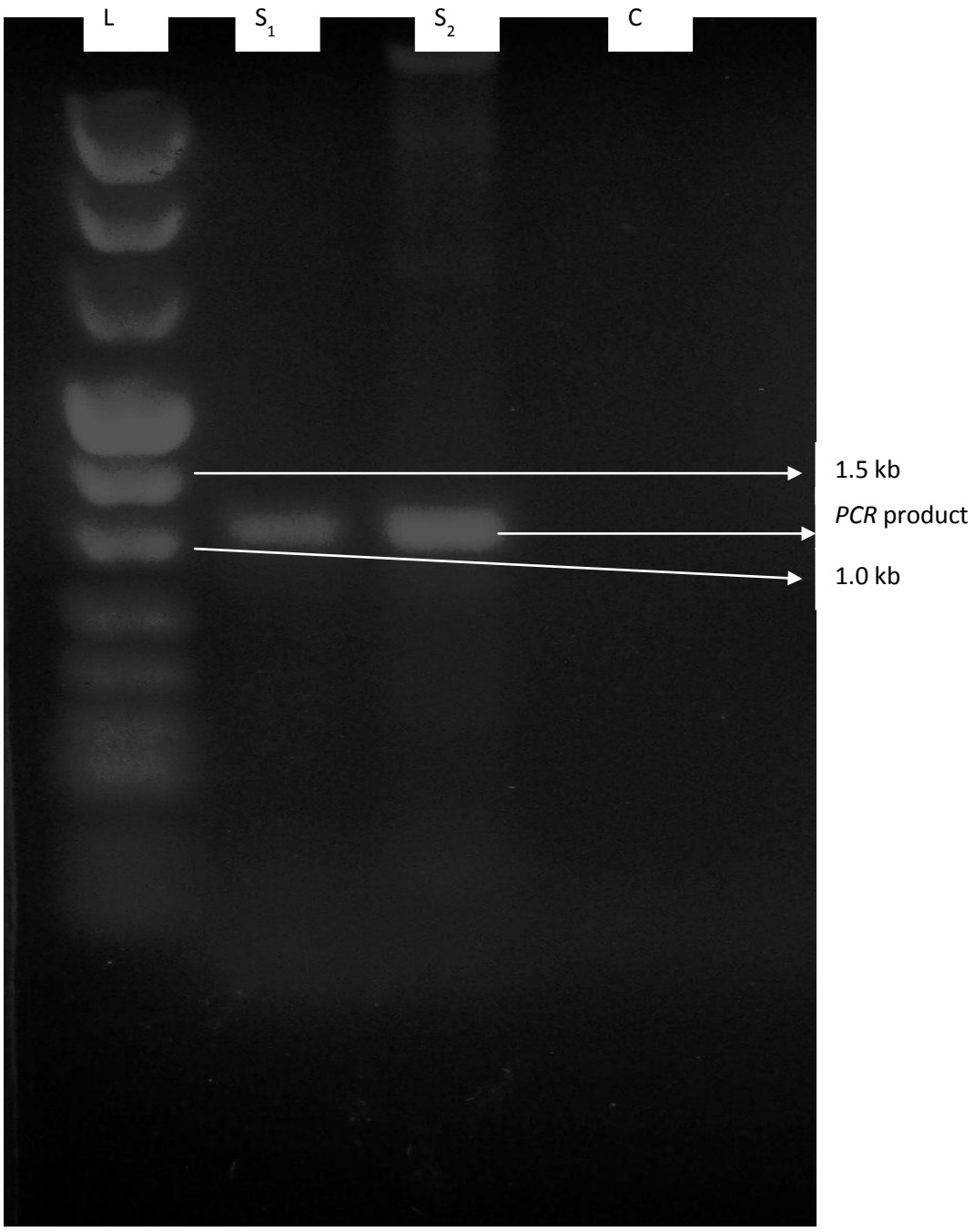

Figure 8: Gel electrophoresis of PCR products using degenerate primers targeting crtI gene (L-molecular ladder, S1- sample 1, and S2 – sample 2).

The PCR product obtained size was in the range of 1.0 to 1.5 k.b. The product was also prepared for sequencing.

4.6 Phylogenetic Analysis

The phylogenetic analysis was performed using the 16S rRNA gene sequences obtained upon sequencing of 16s rRNA *PCR* product and *crt*I gene sequences.

4.5.1 Phylogenetic analysis of 16S rRNA *PCR* sequences

After editing of the sequences using Chromas software, the FASTA sequences of the 16S rDNA are shown below.

>Isolate 16s rDNA

caccccctggcgcaggcctcgacatgcaagtcgagcgagatcttcggatctagcggcggacgggtgagtaacgcgtgggaac
gtgcccttctctacggaattgccccgggaaactgggagtaataccgtatacgccctttgggggaaagatttatcggagaaggat
cggcccgcgttggattaggtagttgccgtaccgatggcctaccaagccgacgatccactagctggcctgagaggatgattcag
ccacactgggactgagacacggcccagactcctacgggaggcagcagtggggaatcttagacaatgggggcaaccctgatc
tagccatgccgagggagtgaagaaggccctaagggttgtaaagctctttcagctgggaagataatgacggtaccagcagaag
aagccccggcctaactccgtgccagcagccgcggtaaaacggaaggggctagcgttgttaagaattactgggcgtaaagcg
cacgtaggcggacccgaaagttgggggtgaaatcccggggctcaacctcggaacggccttcaaaactatcagtctggagttc
gagagaggtgagtggaattccgagtgtagaggtgaaattcgtagatattcggaggaacaccagtggcgaaggcggctcactg
gctcgatactgacgctgaggtgcgaaagcgtggggagcaaacaagattagataccctggtagtccacgccgtaaaacgatga
atgccagtcgtcgggcagcatgctgttcggtgacacacctaacggattaagcatttccgcctggtttccaatgtcgcaagattaa
aacaaaaaggaattgacaggggcccgcacaagcggtggagcatgtgggttaatttcgaagcggcacgcagaaccttaccaa
cccttgacattacaggaccggaccgcagacgggcctttcacttcggcgacctgtggacaggtgcagcgatggcctgtacgtca
gctcgtgtcgtgagatgttcggttaagtccggcaacgagcgcaacccacgtccccagttgccagcattcagttgggcactctgt
ggaaactgccgatgataagtcggaggaagccccaaggaatcatggcccttacgggttgggctacacacgtgctacaatggtg
gtgacaatgggttaatccccaaaagccatctcagttcggattgtcctctgcaactcgagggcatgaagtccagatcgactagtaa
tcgggaacagcatctacccgacgacgctgcgctaacccgcaaaggtcaggactgg

The sequences obtained were 1293 bases in total. This was in agreement with the PCR band obtained which indicated that the 16S rDNA obtained was in the range 1.0 to 1.5

kb. The sequences were then subjected to phylogenetic analysis. Figure 4.9 shows a section of sequence alignment of the obtained sequences.

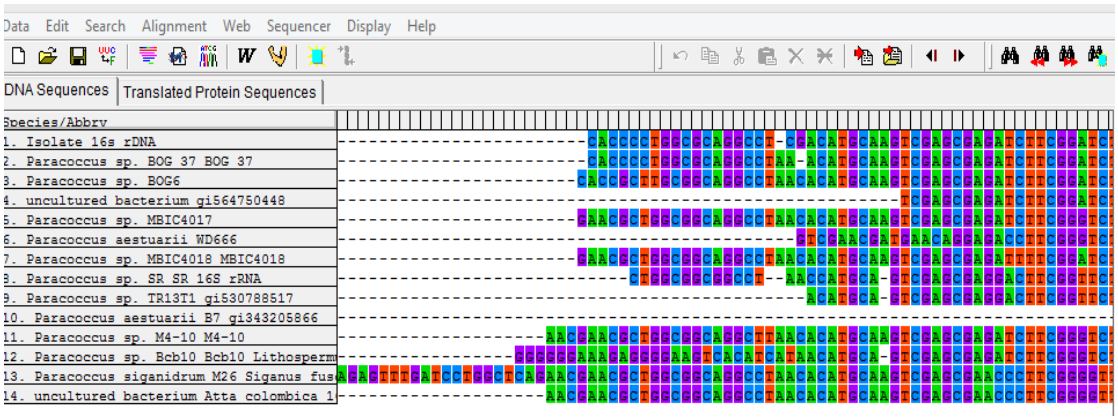

Figure 4.9: Sequence alignment of 16S rDNA

The results show that the 16S rDNA obtained aligned closely with other 16S rDNA in the gene bank obtained mainly from *Paracoccus*. This is an indication that the amplicon indeed was obtained from *Paracoccus* and that it is a 16S rDNA. Figure 4.10 is the Neighbor-Joining phylogenetic tree of the 16S rDNA sequences obtained from *Paracoccus baruchii* LBOG37 and that of the most related organisms.

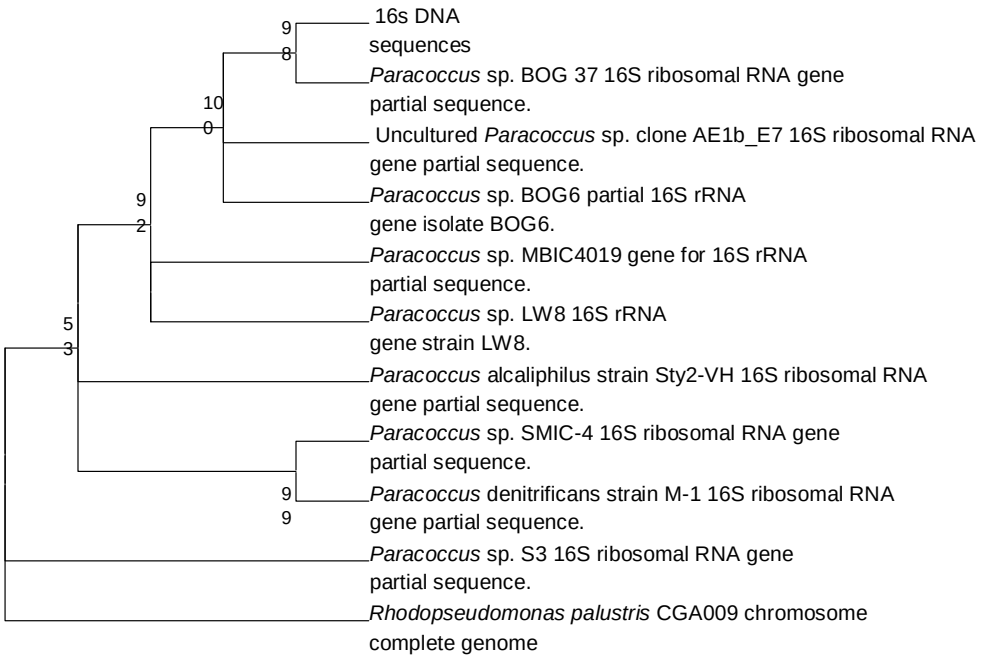

Figure 4.10: Neighbor-Joining phylogenetic tree of the 16S rRNA sequences obtained from Paracoccus baruchii LBOG37 and that of the most related organisms. Rhodopseudomonas palustris CGA009 sequences are used as outgroup

The results show that the obtained 16S rDNA sequences clustered close to the *Paracoccus* sp. BOG 37 16S rDNA partial sequences in the gene bank. This is indicative that the isolate that was being used in this study was similar to the one whose 16S rDNA sequences had been deposited in the GeneBank.

4.5.2 Phylogenetic analysis of *phaAPCR* sequences

The sequences obtained from PCR product *phaA* were translated using expasy software. Only one of the six ORFs which had the longest sequences returned viable hits in the BLASTp analysis at the NCBI website. The FASTA sequence of the open reading frame is shown below.

Translated phaA sequences from *Paracoccos baruchii*

MTKAVIVAAARTPVGSFLGSFANTPAHELGAAVLAEVVARAGIDPSEVSETIMG
QVLTAAQGQNPARQAHIKAGLPKEASAWLINQVCGSGLRAVALAAQQVILGDA
RVVLAGGQESMSLAPHAAYLRAGQKMGDMQMIDTMIRDGLWDAFNGYHMG
QTAENVARQWEITREQQDEFALASQHKAEAAQKEGR

The sequence had 193 amino acids which translates to 579 bases. The sequences were then subjected to phylogenetic analysis. Figure 4.11 illustrates a section of sequence alignement of the *phaA* sequences using MEGA 5.

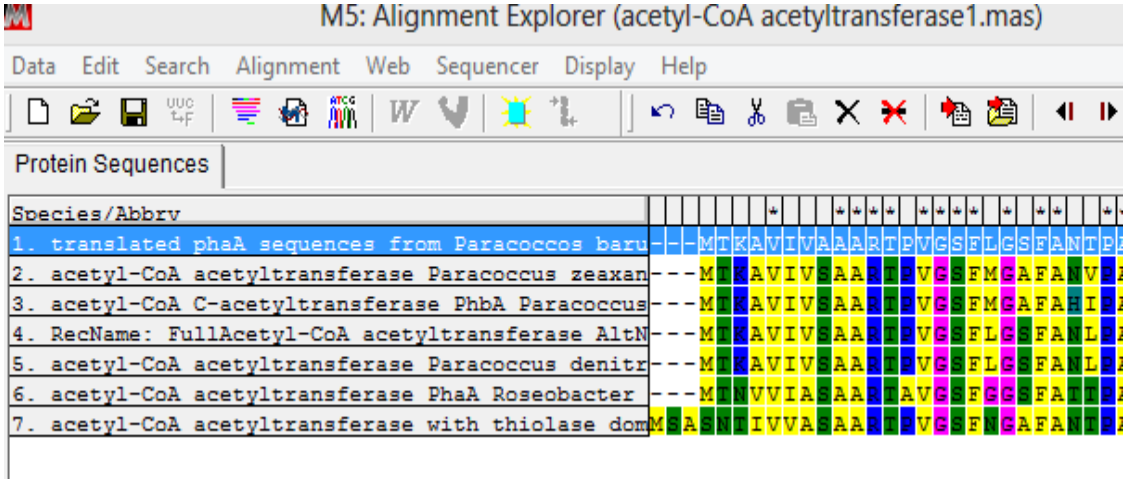

Figure 4.11: *phaA* sequence alignment

The results show that the obtained sequences aligned with acetyl-coA acetyltransferases from several *paracoccus* bacteria.

Figure 4.12 shows the Neighbor-Joining phylogenetic trees created by the MEGA software, version 5.2 (Tamura *et al*., 2011) using the first and second ORFs respectively.

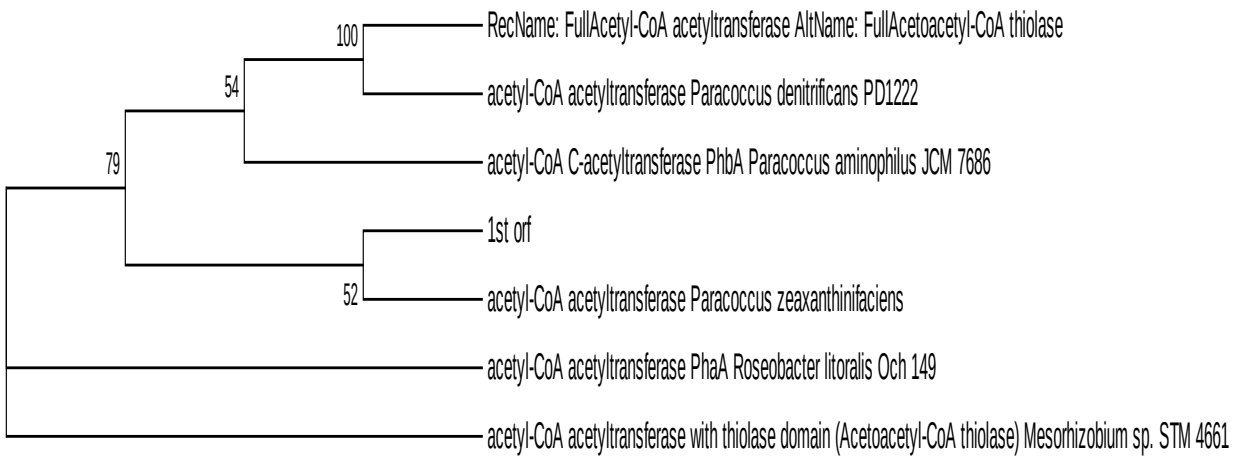

Figure 4.12: Neighbor-Joining phylogenetic tree of one of the open reading frame (1st ORF) obtained by translate tool in Expasy website using *PCR* product and the most closely related Acetyl-CoA acetyltransferase from other organisms, created using MEGA software, version 5.2 (Tamura *et al.*, 2011). Numbers reflect bootstrap support percentages (of 500 replications) generated in MEGA software, version 5.2. Only those bootstrap percentages of at least 50% have been shown as they demonstrate good support.

The sequences clustered close to those of acetyl-CoA acetyltransferase from *Paracoccus Zeaxanthinifaciens.* This indicative that *Paracoccus baruchii* could be having phaA gene which is responsible for synthesis of acetyl-CoA acetyltransferase.

CHAPTER FIVE

DISCUSSION

5.1 Revival *Paracoccus baruchii* LBOG37

The *Paracoccus baruchii* LBOG37 was successful revived and grown on modified Horikoshi (MH) agar media (Figure 4.1). The *Paracoccus baruchii* LBOG37 had a characteristic orange colour upon culturing on MH agar media. According to Amstrong (1997), colored bacteria often produce carotenoids. Carotenoids are natural pigments that have characteristic yellow, orange or red coloration (Namitha & Negi 2010). Other *Paracoccus* species that have similar orange colour are *Paracoccus carotinifaciens* Type Strain IFO 16121^{T}, and *Paracoccus marcusii* Type Strain DSM 11574T. These *Paracoccus* speciesare carotenoid producing organisms (Kelly, *et al*., 2006). The *Paracoccus bogoriensis* also has pigmentation which vary from red to orange and also produces carotenoids (Osanjo *et al*,. 2009). The *Paracoccus bogoriensis* was also isolated from Lake Bogoria where *Paracoccus baruchii* LBOG37 was isolated. Thus, the characteristic orange colour of *Paracoccus baruchii* LBOG37 could be indicative that the species is capable of producing carotenoids which are terpenoid pigments produced by various organisms such as bacteria, algae, fungi and green plants (Armstrong, 1997)**.** If so, the isolate could be of significance in various fields such as the pharmaceutical, chemical, food and feed industries. For instance, in the pharmaceutical industries the carotenoids produced by the organisms could be harvested and utilized in the manufacturer of anti-oxidants. The coloration produced by the carotenoids could be utilized in food industry for food coloration.

5.2 The growth conditions of *Paracoccus baruchii* LBOG37

Growth of the *Paracoccus baruchii* LBOG37 at all temperature had either a short lag phase or non at all. The exponential phase on the other hand was shortened at all

temperatures tested. The growth was initially slight after two hours. This was followed by an exponential growth and finally a decline. The initial slow growth could be attributed to the fact that the organism was trying to adapt to the growth conditions. On the other hand, the exponential growth could be attributed to the availability of enough and favorable growth conditions. Finally, the decline in growth could be attributed to the accumulation of waste products produced by the *Paracoccus baruchii* LBOG37and intra-competition for the limited resources in the media.

The growth of *Paracoccus baruchii* LBOG37 attained optimum growth rate after incubation for 24 hours for all temperatures irrespective of the carbon source used (Figure 4.1, 4.2, 4.3, 4.4 &4.5). A comparison of the optical density for various temperatures revealed that *Paracoccus baruchii* LBOG37 recorded an optimum growth rate at 40^{o}C irrespective of the carbon source. Thus, it was concluded that the optimum growth of *Paracoccus baruchii* LBOG37 is attained at 40^{o}C. According to Kelly, *et al* (2006b), the optimum temperature for growth of most of *Paracoccus* species is in the range of 25-37^{o}C. For instance, Kelly, *et al* (2006b) reported that *Paracoccus aminophilus* Type Strain JCM 7686T grows at 30^{o}C but not at 37^{o}C, *Paracoccus carotinifaciens* Type Strain IFO 16121T optimum growth temperature is 28^{o}C and *Paracoccus kocurii* Type Strain JCM 7684T optimum temperature is 25-30^{o}C. However, *Paracoccus kondratievae,* isolated from maize rhizosphere grows at 30-50 ^{o}C (Kelly *et al,*. 2006). This implies that *Paracoccus baruchii* LBOG37 is thermotolerant. This assertion is supported by the fact that the *Paracoccus baruchii* LBOG37 was able to grow at temperatures as high as 54 ^{o}C. The *Paracoccus bogoriensis* (Osanjo, *et al*., 2009)isolated from Lake Bogoria where*Paracoccus baruchii* LBOG37 was found to grow between 37^{o}C and 54^{o}C with an optimum growth at 40^{o}C. Based on these findings it can be postulated that *Paracoccus* species isolated from Lake Bogoria have an optimum growth temperature of 40^{o}C.

All the pH ranges, (9-11), that were tested in this study supported the growth of *Paracoccus baruchii* LBOG37 at 40^{o}C. However, the optimum pH for the growth of the

organism seems to be dependent on the carbon source used for inoculation. The data shows that the growth rate of the *Paracoccus baruchii* LBOG37is higher at pH 9.0 and 9.5 when carboxymethyl cellulose and starch are utilized as carbon sources (Figure 6). Thus, it can be asserted that if carboxymethyl cellulose and starch are utilised as carbon sources, the optimum pH for growth of *Paracoccus baruchii* LBOG37is 9.0-9.5. However, for glucose the optimum growth rate of the *Paracoccus baruchii* LBOG37 is attained at ph 10.0 (Figure 4.6). This indicates that the pH of the media might influence the carbon source utilized by the *Paracoccus baruchii* LBOG37. However, it should be noted that the *Paracoccus baruchii* LBOG37 optimum pH is between pH 9.0 to pH 10.0. This is an alkaline condition indicating that the *Paracoccus baruchii* LBOG37 is an alkalophilic bacterium. According to a review by Kelly, *et al*., (2006b), most *Paracoccus* good growth ranges between pH 6.5-8.5 except for *P. alcaliphilus* (which is moderately alkaliphilic with an optimum of pH 8–9 and grows at pH 9.5, but only weakly at pH 7.0). The consideration of *P. Alcaliphilus* as a moderate alkaphilic implies that *Paracoccus baruchii* LBOG37 which has an optimum pH range of between 9 and 10 is a strong alkalophilic. *Paracoccus bogoriensis* isolated from the same environment like *Paracoccus baruchii* LBOG37 has strong alkaliphilic characteristic (pH range 7.5 - 10.5) (Osanjo *et al*,. 2009). This is not a dominant trait among the *Paracoccus*. Most of the alkaliphiles such as *P. aestuarii* and *P. alcaliphilus* (pH range 7 - 9.5) have been isolated from marine sources. Thus, *Paracoccus baruchii* LBOG37 is among the few strains of alkalophiles to be isolated from a salt lake environment. According to Asker *et al* (2012), many carotenoid-producing bacteria have been isolated from various extreme environments, such as very low temperatures, high salinity, strong light, acidic and alkaline, and thermophilic conditions. Based on Asker *et al* (2012) hypothesis that that the oxidative stresses in extreme environments are selective factors associated with pigmented microorganisms, which are able to synthesize antioxidants (i.e., carotenoids) to protect their vital molecules (e.g., proteins and nucleic acids), it can be argued that since *Paracoccus baruchii* LBOG37 is thermophilic and alkalophilic, it may also be

capable of producing antioxidants that enable it to survive under such extreme conditions.

Like *P. Bogoriensis, Paracoccus baruchii* LBOG37 was isolated from Lake Bogoria using MH media with glucose as a carbon source. This study established that all the carbon sources tested in this study, (carboxymethyl cellulose, starch and glucose), support growth of *Paracoccus baruchii* LBOG37 at different temperatures (Figure 4.1, 4.2, 4.3, 4.4 &4.5). This finding supports the assertion of Kelly (2006b) that all species of *Paracoccus* grow aerobically on a wide range of organic substrates. The growth of *Paracoccus baruchii* LBOG37 recorded varied growth rates at different temperatures (Figure 4.1, 4.2, 4.3, 4.4, & 4.5). At 37^{o}C the growth of *Paracoccus baruchii* LBOG37was higher when starch was utilized as carbon sources than in other sugars (Figure 3). A study by Wawrik *et al* (2005) on the effect of different carbon sources on community composition of bacterial enrichments from soil reported that different enrichments selected for dissimilar communities. It is therefore likely that starch might be used to select for *Paracoccus baruchii* LBOG37from its natural sources when incubated at 37^{o}C as compared to glucose and carboxymethyl cellulose. The carboxymethyl cellulose recorded the lowest growth at this temperature.

At 400C the growth of the *Paracoccus baruchii* LBOG37 seemed to be supported more again by starch although glucose also seemed to support faster growth. However, at 40°C the growth of the *Paracoccus baruchii* LBOG37 is faster in starch than in glucose (Figure 4.2). The *Paracoccus baruchii* LBOG37 reached optimum growth in glucose at the point where growth was declining in starch. Based on these findings, it can be hypothesized that when the *Paracoccus baruchii* LBOG37 is grown in a media containing both glucose and starch, the *Paracoccus baruchii* LBOG37 might auto-regulate genes for utilization of glucose to favor those genes for starch utilization. According to Bruckner and Titgemeyer (2002) catabolite gene activator protein (CAP-) or catabolite control protein (CcpA)-dependent catabolite repression serves as an auto-regulatory device to keep sugar utilization at a certain level rather than to establish

preferential utilization of certain carbon sources. Thus, in this case it is likely that the *Paracoccus baruchii* LBOG37 might repress the expression of genes responsible for glucose utilization in favour of starch utilization gene activation if the *Paracoccus baruchii* LBOG37 is incubated at 40^{o}C in presence of both starch and glucose as carbon sources.

At 45^{o}C the growth in glucose was the lowest and a higher growth was in starch followed by caboxymethyl cellulose (Figure 4.3). This is interesting fact to note that as the temperature increases the *Paracoccus baruchii* LBOG37seems to switch the carbon sources being utilised. The growth recorded when glucose was used as carbon source at 45^{o}C dropped to lower than OD of 1.0 while that in carboxymethyl cellulose is higher than OD of 1.0 after 24 hours incubation period.

The growth of *Paracoccus baruchii LBOG37* at 50^{o}C is higher when carboxymethyl cellulose is utilized as a carbon source than when either starch or glucose is utilized after incubation for 24 hours (Figure 4.6). This supports our argument above that as the temperature increases, the *Paracoccus baruchii* LBOG37 seems to switch sugar preference. This might imply that if the three sugars, starch, glucose and carboxymethyl cellulose, are present in a media and the *Paracoccus baruchii* LBOG37is cultured at 50^{o}C utilization of carboxymethyl cellulose might be favored. Graphical representation of the results clearly illustrates how growth rate is higher after incubation of the *Paracoccus baruchii* LBOG37at 50 ^{o}C in carboxymethyl cellulose than in the other two carbon sources. It should, however, be noted that the growth of *Paracoccus baruchii BOG37* in all the three sugars reaches optimum after 24 hours.

The growth recorded at temperature 54^{o}C in all the three sugars is below OD of 1.0. This shows that the growth of the *Paracoccus baruchii* LBOG37declines at temperatures above 50^{o}C regardless of the carbon source utilized. It should, however, be noted that at this higher temperatures starch is again favored over other sugars although the growth rate is lower as compared to what was recorded at lower temperatures. It is worthy

noting that none of the *Paracoccus* reported in literature so far has been tested to establish whether they utilize carboxymethyl cellulose. Thus, even though other *Paracoccus* might be able to utilize this carbon source for their growth, they are yet to be tested.

5.3 The 16S rDNA

The results of gel electrophoresis of the *PCR* product that amplified the 16S rRNA gene indicated that the length of the product was between 1kb and 1.5kb. This was within the range of 16S rRNA gene products from other organisms. Thus, it was hypothesized that the band represented the 16S rRNA gene. To ascertain if this was true, the *PCR* product was subjected to gene clean and sequenced. Upon sequencing the sequences obtained were 1293 which was closer to the one deposited in GeneBank. The sequences were used to carry out phylogenetic analysis to help in accepting or rejecting the hypothesis that the *PCR* product that was obtained was indeed a 16S rRNA gene.

BLASTn analysis showed that the gene product was related to 16S rRNA sequences in the gene bank. This confirmed that indeed our *PCR* product was indeed a 16S rRNA gene amplicon. The BLASTn analysis also indicated that the *PCR* product sequences were related to *Paracoccus* species because most of the hits were from *Paracoccus* species. According to Shah *et al* (2010), the rRNA gene is the most conserved (least variable) DNA in all cells. Portions of the rDNA sequence from distantly related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16s rDNA sequence can show evolutionary relatedness among microorganisms. Carl Woese, who proposed the three Domain system of classification - Archaea, Bacteria, and Eukarya - based on such sequence information, pioneered this work (Shah *et al*., 2010). Thus, based on this

argument and finding of BLASTn analysis in this study, it was concluded that the species under study was indeed *Paracoccus* species. However, the organism

The 16S rRNA gene profiling has been applied to the analysis of the genetic diversity of complex bacterial populations since the middle 1990s, (Shah *et al*., 2010) and is one of the primary steps in any metagenomics project. In this study, 16S rRNA gene analysis was aimed at establishing the relatedness of the organism under study to the original strain that had been submitted to the Genbank as the *Paracoccus* sp. LBOG 37 but has since then been renamed *Paracoccus baruchii* LBOG37. To do this, the PCR sequences were subjected to phylogenetic analysis. The phylogenetic analysis generated a neighbour joining phylogenetic tree. From the phylogenetic tree it was interesting to note that the *Paracoccus baruchii* LBOG37 under study was a closely related to *Paracoccus* sp LBOG37 sequences in Genebank even though BLASTn results indicated that they have only 96% sequence identity. This was contrary to the expectation of the study since it was anticipated that the organism under study (*Paracoccus* sp. LBOG37) was *Paracoccus baruchii* LBOG37. However, the 16S rRNA gene sequence data has no defined "threshold values" above which there is a universal agreement of what constitutes a definitive and conclusive identification to the rank of species hence creating a widely used individually acceptable criterion for a species match. Most taxonomists, though not by consensus, have accepted a percentage identity score of ≥97% and ≥99% for any isolate to be classified under a genus and species, respectively. For example, it has been shown that the 16S rRNA gene sequence data of an unknown isolate with its nearest neighbor exhibiting a similarity score of <97% and ≥95%, is representative of a new species with the isolate in question being assigned to the corresponding genus (Janda & Abbott, 2007). Similarity values of >97% can also be representative of a new species or alternatively indicate clustering within a previously defined taxon (Janda & Abbott, 2007; Petti, 2007). However, when the similarity is placed at <95%, the unknown isolate is assigned to a family but with a possibility of claiming a novel genus (Asker *et al*., 2012).

The phylogenetic tree also indicated that *Paracoccus baruchii BOG37*was also related closely to *Paracoccus baruchii BOG6* which was also isolated from the same environment as *P.baruchii* LBOG37, that is, from Lake Bogoria (Osanjo *et al.*, 2009). Osanjo *et al* (2009) reported that *Paracoccus baruchii BOG6* produces carotenoids such as astaxanthin. According to Misawa (2011), *Paracoccus sp. MBIC4019, Paracocus alcaliphilus* and *Paracocus denitrifican* produce carotenoids. The *Paracoccus sp.* MBIC4019 was isolated from marine environment (Misawa, 2011). According to Osanjo *et al* (2009), marine environment share similar salinity levels as Lake Bogoria where *Paracoccus baruchii* LBOG37was isolated. The 16S rRNA genes of these organisms (*Paracoccus sp. MBIC4019, Paracocus alcaliphilus* and *Paracocus denitrifican*) formed a cluster with the 16S rRNA gene of *Paracoccus baruchii* LBOG37. This is indicative that *Paracoccus baruchii* LBOG37could be capable of producing carotenoids. The 16S rRNA based analysis of metagenomic samples is complicated by several artifacts, including chimeric sequences caused by *PCR* amplification and sequencing errors (Shah *et al*., 2010). Shah *et al*(2010) further reported that at least 1 in 20 16S rRNA sequences currently in public repositories contains substantial anomalies, and that some metagenomics projects may overestimate the species diversity because of the presence of sequencing errors and chimeric sequences. On the basis of this argument, the differences between *Paracoccus baruchii* LBOG37 in this study and *Paracoccus baruchii* LBOG37 could be due to sequencing errors and presence of chimeric sequences. Thus it is possible that *Paracoccus sp.* LBOG37and *Paracoccus baruchii* LBOG37 is one organism.

Previously, strains having <3% difference between their 16S rRNA genes were considered the same species (Stackebrandt, 1994). However, the difference between 16S rRNA gene sequence of *P. sp* LBOG37 and *Paracoccus baruchii* LBOG37 is about 6% and hence nullifies any possibility of the two organisms being the same species. Moreover, differences between 16S rRNA genes for some *Bacillus* species, such as *B. anthracis, B. cereus*, and *B. thuringiensis*, are <1% (Sacchi *et al*., 2002). Such small differences (e.g., one base between sequences or partial matches at a single nucleotide

position in the 16S rRNA gene) have not been used for species differentiation. Sacchi *et al*., (2002) study clearly demonstrated that such small differences might be important for species identification. DNA-DNA hybridization and 16S rRNA sequencing studies have shown that these three *Bacillus* species (*B. anthracis, B. cereus, &B. Thuringiensis)* are closely related and probably represent a single species (Sacchi *et al*., 2002). If the three were classified as a single species, 16S rRNA sequencing appears to have the potential to differentiate strains at the subspecies level. In this study, the *Paracoccus baruchii* LBOG37used was thought to be *Paracoccus baruchii* LBOG37. However, from the BLASTn analysis it was established that the *Paracoccus baruchii* LBOG37sequences of 16S rRNA gene were only 92% identical. Thus it is probable that the *Paracoccus baruchii* LBOG37used in this studyis not *Paracoccus baruchii* LBOG37. The fact that phylogenetic analyses indicate that the two organisms are closely related, imply that the two organisms have a common recent ancestor.

5.4 The *phaA*gene

The investigator was targeting to amplify *phaA* gene using polymerase chain reaction (*PCR*). The results of gel electrophoresis indicated that the *PCR* band was formed between 1.0 kb and 1.5kb. The BLASTp analysis analysis revealed that the sequences were related to Acetyl-CoA acetyltransferase gene (phaA gene). The highest hits were obtained from genes isolated from *Paracoccus* species. From the phylogenetic analysis of the open reading frame that was related to Acetyl-CoA acetyltransferase, it was established that it was closely related to Acetyl-CoA acetyltransferase from *Paracoccus zeaxanthinifaciens*. The protein and the one from *Paracoccus zeaxanthinifaciens* seem to share a close common ancestor with Acetyl-CoA acetyltransferase from *Paracoccus denitrificans* PD1222, and *P. Aminophilus* JCM7686.*Paracoccus zeaxanthinifaciens* was isolated from marine environment (Berry *et al* 2003). According to Osanjo *et al* (2009), marine environment has similar salinity as that of L. Bogoria where *Paracoccus baruchii* LBOG37 was isolated. The clustering of *Paracoccus baruchii* LBOG37 with *P. Zeaxanthinifaciens* isolated from marine environment supports Osanjo *et al* (2009)

hypothesis that *Paracoccus* species were introduced into the lake by the migratory birds that travel from the marine habitats to the lake. It should also be noted that *P. Zeaxanthinifaciens* has also been shown to produce carotenoids (zeaxanthin).

The protein is also distantly related to Acetyl-CoA acetyltransferase from Roseobacter litoralis Och149. This organism was also isolated from marine environment (Christie-Oleza*et al*., 2012). This implies that *Paracoccus baruchii* LBOG37 shares ancestry with marine organisms. This could be due to the similarity of salinity found in marine environment and that in L. Bogoria where the organism was isolated. All these proteins in turn share a common distant ancestry with Acetyl-CoA acetyltransferase from Mesorhizobium sp STM 4661.

Acetyl-CoA acetyltransferase is a thiolase II encoded by *phaA* gene. Acetyl-CoA acetyl-transferase is one of the four enzymes involved in the conversion of two acetyl-CoA molecules into butyryl-CoA in the acetone-butanol-ethanol (ABE) pathway that is used for natural production of 1-butanol in Clostridium species (Lamsen & Atsumi, 2012). Acetyl-CoA C-acetyltransferase catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA (Kocharin, *et al*., 2012). Lamsen and Atsumi, (2012) argues that 1-butanol is one of the higher chain alcohols that is not hygroscopic and which provides energy densities that are more comparable to gasoline and hence is said to be the best alternative to ethanol as a biofuel. They further argue that enzymes involved in the production of 1-butanol can be metabolically engineered to increase its production. The detection of this gene in this *Paracoccus* species implies that it might be possible to use the gene in the production of 1-butanol if engineered with other necessary enzymes in an appropriate organism. Given that the organism is an extremophile which grows at higher temperatures and pH values, it is a novel gene that can be utilised in industrial conditions that are known to be extreme.

It is also possible that the acety-CoA acetyltransferase is involved in the biosynthetic pathway of carotenoids in the *Paracoccus baruchii* LBOG37. The biosynthesis of

carotenoids begins with the conversion of acetyl-CoA to active isoprene unit, isopentenyl pyrophosphate (IPP). The initial steps of the pathway involve the condensation of three acetyl-CoA molecules to produce a C_6 compound, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The formation of HMG-CoA in non-photosynthetic eukaryotic cells is catalysed by two separate enzymes, acetyl-CoA acetyltransferase and HMG-CoA synthase (Rochaix, 2004). In cyanobacteria, algae, and vascular plants there is considerable evidence indicating that the two reactions are catalysed by a single enzyme that requires Fe^{2+} and a quinone as cofactors (Rochaix, 2004). The HMG-CoA is then converted to mevalonic acid (MVA). The MVA is then phosphorylated to form 5-pyrophosphomevalonate which is decarboxylated to form Δ^{3-}isopentyl pyrophosphate (IPP). IPP is used as a building block for long chain isoprenoids that are utilized in carotenoid biosynthesis. Even though *Paracoccus baruchii* LBOG37 is a bacterium, it is probable that the synthesis of HMG-CoA involves acetyl-CoA acetyltransferase like in eukaryotes. This would imply that *P. sp* LBOG37 indeed synthesizes carotenoids.

The detection of the *phaA* gene in this species of *Paracoccus* could also be an indication that the organism produces and accumulates Poly-(ß)-3-hydroxybutyrate (PHB). Kocharin, *et al* (2012) states that PHB is one of the many types of Polyhydroxyalkanoates (PHAs), the other common one is the poly-ß-hydroxybutyrateco-3-hydroxyvalerate (PHBV). PHB and PHBV are said to be natural polymer plasticisers from biological processes. Thus, detection of genes involved in production and accumulation of PHB and PHBV indicates a possibility of an organism to be utilised in the production of bio-plastics. The production of PHB requires three genes: *phaA*, *phaB* and *phaC* (Kocharin, *et al*., 2012). In this pathway acetyl-CoA C-acetyltransferase encoded by *phaA*, catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA. NADPH-dependent acetoacetyl-CoA reductase encoded by phaB in turn catalyzes the reduction of acetoacetyl-CoA to (D)-3-hydroxybutyryl-CoA. Finally, PHA synthase encoded by phaC, catalyzes the polymerization of (D)-3-hydroxybutyryl-CoA monomers to PHB. Thus, there is need for further investigation to be undertaken using this organism to determine whether the species indeed produces PHB. Production

of PHB in this species of *Paracoccus* would be of value in the industrial production of PHB given the versatility of the organism in extreme conditions. Kocharin *et al* (2012) argues that natural PHB production and accumulation occurs in organisms as a storage compound in response to nutrient imbalance caused by growth under conditions of carbon source excess but limitation in other essential nutrients. This implies that if this organism indeed accumulates PHB, then it can depolymerize it and utilize it for growth in absence of carbon source. Thus, to be of value to humans as a bio-plastic producer, the genes can be isolated from the organism and engineered in other organisms such as Escherechia coli for increased production of PHB.

Although PHB represents the archetypical form of a biodegradable PHA, its unfavorable physical properties (i.e., brittle, low thermal decomposition temperature, low impact strength) preclude significant use of the homopolymeric form. It would be interesting if indeed this organism produces this form of PHB since it would provide a more stable form of PHB. This is because the organism can grow at a considerably higher temperature implying that PHB produced by it could have a higher thermal decomposition. This will mean that scientists would have to deal with the two remaining disadvantages of the compound, that is, brittleness and low impact strength. It should however be noted that copolymers derived from various proportions of 3-hydroxybutyrate and 3-hydroxyvalerate monomers have enhanced physical properties (i.e., greater flexibility, higher thermal decomposition temperature, and higher impact strength) (Williams *et al.*, 1996). Such copolymers are synthesized when cells are grown in the presence of propionic acid, with the crystallinity of the polymer decreasing as the proportion of hydroxyvalerate increases (Williams *et al*., 1996). Therefore, PHB isolated from our *Paracoccus* could enhance this strategy and hence result in the production of a better bioplastic.

Williams *et al*., (1996) reported that much progress has been made in the cloning, analysis, and expression of genes involved in the production of PHB. It was asserted that these studies have provided an important foundation from which to consider new

approaches for production of useful PHAs. In addition, Williams *et al*. (1996) argues that high-level production of PHB has been demonstrated in plants by introducing the genes encoding acetoacetyl-coenzyme A (acetoacetyl-CoA) reductase (*phbB*), PHA synthase (*phbC*), and β-ketothiolase (*phbA*) into Arabidopsis thaliana. Thus, if these genes could be isolated from the *Paracoccus* species in our study, they could be cloned and expressed to provide more versatile PHB.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusion

The study established that 16S rRNA sequences obtained were related to other *Paracoccus* species. Moreover, phylogentic analysis indicated that the gene sequences clustered closely to those in Genebank even though BLASTn results indicated that they have only 92% sequence identity. Therefore, it can be assumed that the organism used in this study was indeed *Paracoccus sp* LBOG37.

The results indicated that *Paracoccus baruchiiLBOG37* can grow at 37^{o}C to 54^{o}C. However, higher growth was witnessed at 40^{o}C. The results further showed that the organism is capable of growing at pH ranges from 9-11. Nevertheless, the growth was higher at pH 9.0 and 9.5. Moreover, the organism was able to utilize carboxymethyl cellulose, starch and glucose. Thus it can be concluded that *Paracoccus baruchiiLBOG37* is a versatile bacteria capable of utilizing carboxymethyl cellulose, starch and glucose as carbon sources and which grows optimally at 40^{o}C and at pH 9.0 and 9.5.

The phylogenetic analysis indicated that the gene amplified was related to *phaA* gene from other organisms. Thus, it can be concluded that *Paracoccus baruchii* has *phaA* gene.

6.2. Recommendation

1. A study using a different methodology should be undertaken to ascertain whether utilization of carbon source by *Paracoccus baruchii* LBOG37 is dependent on the prevailing temperatures.

2. A study using different primers or different methodology should be carried out to isolate and characterize *phaA* gene.

REFERENCES

Altschul, S. F., Gish W., Miller W., Myers E. W., & Lipman D. J. (1990).Basic local alignment search tool.*Journal of Molecular Biology,*215(3),403-410.

Armstrong, G. A. **(**1997). Genetics of eubacterial carotenoid biosynthesis: a colorful tale. *Annual Review of Microbiology,51*(1),628-659.

Asker, D., Awad, T.S., Beppu, T. & Ueda, K. (2012) Isolation, Characterization, and Diversity of Novel Radiotolerant Carotenoid-Producing Bacteria in José-Luis Barredo (ed.), *Microbial Carotenoids from Bacteria and Microalgae: Methods and Protocols*, Methods in Molecular Biology, 892, 21-60

Baker, S. C., Ferguson, S. J, Ludwig, B., Page, O.M., Richter, H., &Van Spanning, R. J. M. (1998) Molecular Genetics of the Genus *Paracoccus*, Metabolically Versatile Bacteria with Bioenergetic Flexibility. *Microbiology and Molecular Biology*. Rev. 62(4),1046-1078.

Berry, A., Janssens, D., Humbelin, M., Jore, J. P. M., Hoste, B., Cleenwerck, I., Vancanneyt, M., Bretzel, W., Mayer, A. F., Lopez-Ulibarri, R., Shanmugam, B., Swings J., & Pasamontes, L. (2003).*Paracoccus zeaxanthinifaciens* sp. nov., a zeaxanthin-producing bacterium. *International Journal of Systemic and Evolutionary Microbiology, 53,*231-238.

Bohmert,. K, Balbo, I., Kopka, J., Mittendorf, V., Nawrath, C., Poirier, Y., Tischendorf, G., Trethewey, R. N., & Willmitzer, L. (2000). Transgenic Arabidopsis plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight. *Planta 211*(6),841–845

Bruckner, R. &Titgemeyer, F. (2002). Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiology Letters 209, 141-148

Carlson, R., Fell, D., & Srienc, F. (2002). Metabolic pathway analysis of a recombinant yeast for rational strain development. *Biotechnol Bioeng 79*(2),121–134

Carlson, R., &Srienc, F. (2006). Effects of recombinant precursor pathway variations on poly[(R)-3-hydroxybutyrate] synthesis in. *J Biotechnol 124*(3),561–573

Christie-Oleza, J.A., Piña-Villalonga, J.M., Bosch, R, Nogales, B &Armengaud, J. (2012). Comparative Proteogenomics of Twelve *Roseobacter* Exoproteomes Reveals Different Adaptive Strategies Among These Marine Bacteria.Molecular Cellular Proteomics.11(2)

de Almeida, A., Giordano, A. M., Nikel, P. I., & Pettinari, M. J. (2010). Effects of aeration on the synthesis of poly(3-hydroxybutyrate) from glycerol and glucose in recombinant Escherichia coli. *Appl Environ Microbiol76*(6),2036–2040

Dziewit, L., Czarnecki, J., Wibberg, D., Radlinska, M., Mrozek, P., Schlüter, A., Szymczak, M., Pühler, A.& Bartosik, D. (2014).Architecture and functions of a multipartite genome of the methylotrophic bacterium *Paracoccusaminophilus* JCM 7686, containing primary and secondary chromids.*BMC Genomics15*,124

Hankermeyer, C. R., & Tjeerdema, R. S. (1999). Polyhydroxybutyrate: Plastic made and degraded by microorganisms. In: Ware GW (ed) *Revi Environ Contam Toxicol*, 159, 1–24

Harker, M., Hirschberg, J., & Oren, A. (1998). *Paracoccus rnarcusii* sp. nov., an orange Gram-negative coccus. *International Journal of Systemic Bacteriology,48*,543-548

Hiroe, A., Tsuge, K., Nomura, C. T., Itaya, M. & Tsuge, T. (2012). Rearrangement of Gene Order in the *phaCAB* Operon Leads to Effective Production of Ultrahigh-Molecular-Weight Poly[(*R*)-3-Hydroxybutyrate] in Genetically Engineered *Escherichia coli. Applied Enviromental Microbiol, 78*(9), 3177–3184.

Janda J. M. & Abbott S. L. (2007) 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils and Pitfalls, *Journal of Clinical Microbiology*, 45(9), 2761-2764

Joseph, A .C., Yajun Y., & Mattheos A.G.K. (2006) *Biosynthesis of isoprenoids, polyunsaturated fatty acids and flavonoids inSaccharomyces cerevisiae. [online]*.Creative Commons Attribution License. Available from: http://creativecommons.org/licenses/by/2.0 [Date accessed 21.9.2009]

Kelly, D.P., Euzéby, J.P., Goodhew, C.F., &Wood, A.P. (2006a). Redefining *Paracoccus denitrificans* and *Paracoccus pantotrophus* and the case for a reassessment of the strains held by international culture collections. *Int. J. Syst. Bacteriol*. 56, 2495 - 2500.

Kelly, D.P., Rainey F.A., &Wood A.P, (2006b). The genus *Paracoccus*: In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, (Eds) *The Prokaryotes* Stackebrandt. Springer, New York. 5, 232–249.

Khan, S. T., Takaichi, S., & Harayama, S. (2008). Paracoccus marinus sp. nov., an adonixanthin diglucoside-producing bacterium isolated from coastal seawater in Tokyo Bay. *International Journal of Systematic and Evolutionary Microbiology,58*,383–386

Kocharin, K. Chen, Y. Siewers, V. & Nielsen, J. (2012).Engineering of acetyl-CoA metabolism for the improved production of polyhydroxybutyrate in *Saccharomyces cerevisiae. AMB Express Journal* 2(52),1-11

Lamsen, E.N. & Atsumi, S. (2012).Recent progress in synthetic biology for microbial production of C3–C10 alcohols. Frontiers in Microbiology, Review. 3(196),1-13.

Lee J.H., Kim Y.S., Choi T.J., Lee W.J.,& Kim, Y.T. (2004).*Paracoccus haeundaensis sp*.nov., a Gram-negative, halophilic, astaxanthin-producing bacterium. *Int. J. Syst. Evol. Microbiol.* 54, 1699-702.

Melchiors, M., Keul, H., & Höcker, H. (1994). Synthesis of highly isotactic poly[(R)-3-hydroxybutyrate] by ring-opening polymerization of (R, R, R)-4,8,12-trimethyl-1,5,9-trioxacyclododeca-2,6,10-trione. *Macromol Rapid Commun 15*(6),497–506

Mesbah, N. M., Abou-El-Ela, S. H. & Wiegel, J. (2007).Novel and Unexpected Prokaryotic Diversity in Water and Sediments of the Alkaline, Hypersaline Lakes of the Wadi An Natrun, Egypt. *Microbial Ecology*,*54*(4),598-617

Misawa, N. (2011).Carotenoid β-Ring Hydroxylase and Ketolase from Marine Bacteria—Promiscuous Enzymes for Synthesizing Functional Xanthophylls. Marine Drugs 9(5), 757-771

Misawa, N., Yoshiko S., Keiji K., Akihiro Y., Susumu K., Toshiko S., Takeshi O., and Wataru M. (1995) Structure and Functional Analysis of a Marine Bacterial Carotenoid Biosynthesis Gene Cluster and Astaxanthin Biosynthetic Pathway Proposed at the Gene Level. *Journal of Bacteriology*, 177(22),6575–6584

Namitha, K. K. & Negi, P. S. (2010). Chemistry and Biotechnology of Carotenoids, *Critical Reviews in Food Science and Nutrition*, 50(8), 728-760

Nawrath, C., Poirier, Y., & Somerville, C. (1994).Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation.*PNAS 91*(26),12760–12764

Nikel, P. I., Pettinari, M. J., Galvagno, M. A., & Méndez, B. S. (2006).Poly(3-hydroxybutyrate) synthesis by recombinant *Escherichia coli* arcA mutants in microaerobiosis. *Appl Environ Microbioly 72*(4),2614–2620

Osanjo G. O., Elizabeth W. M., Leah T., Michael W. O., Wallace D. B., Heinrich L., Wolf-Rainer A., Michel D., Kenneth N. T., Peter N. G. & Francis J. M. (2009). A salt lake extremophile, *Paracoccus* bogoriensis sp. nov., efficiently produces xanthophyll carotenoids. *African Journal of Microbiology Research*, 3(8), 426-433

Petrasovits, L. A., Zhao, L., McQualter, R. B., Snell, K. D., Somleva, M. N., Patterson, N. A., Nielsen, L. K., & Brumbley, S. M. (2012). Enhanced polyhydroxybutyrate production in transgenic sugarcane.*Plant Biotechnol J 10*(5),569–578

Petti, C. (2007).Detection and Identification of Microorganisms by Gene Amplification and Sequencing.*Medical Microbiology*, 44(8), 1108-1114

Rainey, F. A., Ward-Rainey, N., Kroppensted, R. M. &Stackebrandt, E. (1996).The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage; proposal of *Nocardiopsaceae* fam. nov. *Znt J Syst Bacteriol46*(4),1088-1092.

Rochaix, J.D. Goldschmidt-Clermont, M. and Merchant, S. (2004) *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas* Kluwer Academic Publishers

Sacchi, C.T., Whitney, A.M., Mayer, L.W., Morey, R., Steigerwalt, A., Boras, A. Weyant, R.S.& Popovic A. (2002)Sequencing of 16S rRNA gene: A rapid tool for identification of *Bacillus anthracis.* Emerging Infectious Diseases. 8(10), 1117-1123

Shah, N., Tang H., Doak T.G. & Ye, Y. (2010).Comparing bacterial communities inferred from 16S rRNA gene sequencing and shotgun metagenomics. *WSPC Proceedings* Sept. 20, 2010. 17(6),1-12

Stackebrandt, E. & Goebel, B. M.(1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species in bacteriology. *INTERNATIONAL JOURNALO F SYSTEMATIC Bacteriology, 44*(4),846–849.

Steinbüchel, A., & Hein, S. (2001). Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. In: Babel W, Steinbüchel A (eds) *Adv BiochemEng/Biotechnol*, Springer Berlin, Heidelberg, 71,81–123

Steinbuchel, A., Hustede, E., Liebergesell, M., Pieper, U., Timm, A., & Valentin, H. (1993).Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria.*FEMS Microbiol Rev 10*(3–4),347–350

Sun M, Luo Y, Teng Y, Christie P, Jia Z & Li Z (2013). Tenax TA extraction to understand the rate-limiting factors in methyl-beta-cyclodextrin-enhanced bioremediation of PAH-contaminated soil.*Biodegradation, 24(*3),365–375.

Suzuki, Y., Kurano, M., Arai, Y., Nakashita, H., Doi, Y., Usami, R., Horikoshi, K., & Yamaguchi, I. (2002).Enzyme Inhibitors to increase poly-3-hydroxybutyrate production by transgenic tobacco.*Biosci Biotechnol Biochem 66*(12),2537–2542

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Method.*Molecular Biology and Evolution* 28(10), 2731-2739

Trotsenko, Y. A., & Belova, L. L. (2000). Biosynthesis of poly(3-Hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and its regulation in bacteria. *Microbiol 69*(6),635–645

Tsubokura A, Yoneda H, Takaki M. & Kiyota, T. (1999).Bacteria for production of carotenoids.*US Patent* 5,858-761.

Uchino, K., & Saito, T. (2006). Thiolysis of poly(3-hydroxybutyrate) with polyhydroxyalkanoate synthase from *Ralstonia eutropha*. *J Biochem 139*(3),615–621

Urakami, L.T., Tamaoka, J., Suzuki, K. & Komagata, K. (1989).*Paracoccus* alcaliphilus sp. nov., an Alkaliphilic and Facultatively Methylotrophic Bacterium. *International Journal of Systematic Bacteriology* 39(2),116-121

van Wegen, R. J., Lee, S-Y., & Middelberg, A. P. J. (2001). Metabolic and kinetic analysis of poly(3-hydroxybutyrate) production by recombinant *Escherichia coli*. *Biotechnol Bioeng 74*(1),70–81

Wawrik, B., Kerkhof, L., Kukor, J., & Zylstra, G. (2005) Effect of Different Carbon Sources on Community Composition of Bacterial Enrichments from Soil. *Applied and Environmental Microbiology*. 71 (11), 6776-6783

Williams, M.D. Rahn, J.A & Sherman, D.H (1996) Production of a Polyhydroxyalkanoate Biopolymer in Insect Cells with a Modified Eucaryotic Fatty Acid Synthase. *Applied And Environmental Microbiology* 62(7), 2540–2546

Zheng, Q., Wang, Y., Chen, C., Wang, Y., Xia, X., Fu, Y., Zhang, R., & Jiao, N. (2011).*Paracoccus beibuensis* sp. nov., Isolated from the South China Sea. *Current Microbiol 62* (3),710-714.

APPENDICES

Appendix I - DNA extraction buffer

Solution A was prepared by mixing 50 ml of 0.1M Tris (Sigma) at a pH of 8.5, 50 ml of 0.1M EDTA (Lab tech chemicals) at a pH of 8.0 and 25 g of sucrose (Merck Eurolab).

Solution B was prepared by mixing 10 ml of 0.1M Tris (Sigma) at a pH of 8.5, 5 ml of 0.1M EDTA (Lab tech chemicals) at a pH of 8.0, 1g sodium dodecyl sulphate (s.d.Fine-chem limited Mumbai) and distilled water to make 100 ml solution.

Lysozyme used was 20 mg/ml, RNAse A (Sigma) 20 mg/ml, proteinase K (Novabio chem. Corporation CA USA) 20 mg/ml, 3M NaCl and chloroform:isoamylalcohol (LOBA CHEME limited, Mumbai) (24:1)

Appendix II- Preparation of agarose gel

Preparation of 10x Tris Acetic EDTA (TAE) buffer

A sartorius analytic weighing balance was used to weigh 48.4g Tris base. This was placed in a 1 liter volumetric flask. Exactly 11.42 ml glacial acetic acid was added and 20 ml 0.5M EDTA added. The mixture was made up to 1 liter with distilled water. The solution was stored at room temperature.

Preparation of X1 Tris Acetic EDTA (TAE) buffer

One volume of X10 TAE was added to nine volumes of distilled water.

Preparation of X6 gel loading buffer

Three volumes of glycerol were added to seven volumes of distilled water. To this, 0.25% bromothymol blue and o.25% xylene cyanol FF was added. The solution was then stored at $+4^{o}C$.

Preparation of 1% Agarose gel and Gel electrophoresis

Unless stated otherwise 1% Agarose gel was used for Agarose gel electrophoresis and was prepared as follows: Casting gates and combs were placed in gel casting tray on a level surface. A sartorius analytic weighing balance was used to weigh 1g Agarose. This was placed in a conical flask and 1x TAE added to make 100ml. The mixture was heated in a microwave for about 2 minutes. The solution was allowed to cool under running tap water to hand-warm ($50\text{-}60^{o}C$). To this 1 µl of ethidium bromide was added. The gel was poured into the casting tray. Any bubbles in the gel were removed and the gel was allowed to set for at least 40 minutes under undisturbed condition. The combs were removed followed by removal of casting gates.

Appendix III - Extraction of DNA from agarose using Qiaex gel extraction kit

The tubes and the gel were weighed again to determine the weight of the gel. Buffer PB (3 volumes) was added to 1 volume of gel (1g/ml). The mixture was incubated at 60^{o}C for 10 minutes to dissolve the gel. The color of the mixture (pH indicator I and Buffer PB) was checked to ensure that it was yellow. The QIAquick spin column was placed in a provided 2 ml collection tube. The sample was applied to the QIAquick column and was centrifuged for 30–60 seconds to bind the DNA. The flow-through was discarded. The QIAquick column was placed back into the same tube. Buffer PE, 0.75 ml, was added to the QIAquick column and centrifuged for 30–60 seconds to wash the QIAquick column. The flow-through was discarded and the QIAquick column was placed back in the same tube. The column was centrifuged for an additional 1 min.QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 30 µl elution buffer was added to the center of the QIAquick membrane. The column was let to stand for 1 minute, and then it was centrifuged for 1 minute. The eluent in the 1.5 ml microcentrifuge tube was the purified DNA and was used for further molecular analysis.

Appendix IV - Extraction of DNA from agarose using Micro spin gel extraction kit

The tubes and the gel were weighed again to determine the weight of the gel (1g/ml). DNA binding buffer (3 volumes) was added to 1 volume of the gel. The mixture was incubated at 60^{o}C in a water bath for seven minutes until the gel had completely melted. The mix was vortexed every two minutes during incubation. The micro spin column was placed in a provided 2 ml collection tube. Exactly 650 µl of DNA Agarose solution was applied to the micro spin column and was centrifuged for 1 minute at 10,000xg to bind the DNA. The flow-through was discarded. For volumes over 650 µl, the remaining solution was added to the column and centrifuged for 1 minute. The flow through was discarded. The column was washed by adding 300 µl of binding buffer and centrifuging at 10,000xg for 1 minute. Wash buffer (600 µl) diluted with absolute ethanol was added into the column. This was allowed to stand at room temperature for 3 minutes and then it was centrifuged at 10,000xg for 1minute. The flow through was discarded and the washing step was repeated. Once the flow through was discarded, empty column was centrifuged for 1 minute to remove all alcohol and dry the column matrix for better yields. The column was placed into a clean 1.5 ml micro-centrifuge tube. DNA elution buffer (30 µl) was added directly onto the column matrix and allowed to stand at room temperature for 2 minutes. This was followed by centrifugation at 10,000xg for 1 minute. The flow through in the micro-centrifuge tube was stored at -20^{o}C for further analysis after 5 µl being used to run gel electrophoresis to confirm successful purification of *CRTIPCR* product.