Optimization of carotenoids production from *Paracoccus* sp BOG 006^{T}

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A thesis submitted in partial fulfillment of the requirement for the Degree of Master of Science in Food Science and Technology in the Department of Food Science, Nutrition and Technology.
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DECLARATION

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

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DEDICATION

To my family,
I affectionately dedicate this work to you. Your support has been a never ending spring of hope, your belief in me is a constant source of strength.

To Mark,
Without whom I would not have found the strength to soldier on. You are my inspiration.

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Interdependence is a higher value than independence; this is what the pursuit for my masters has taught me in depth. The research community is like a bee hive, each member great and small working together each playing his/her role for the benefit of all concerned.

This report is a synergistic product of many minds, each contributing a unique facet of my learning experience. For their accumulative and individual assistance I feel a deep sense of gratitude.

It would be futile on my part to name all persons I feel indebted to, for the list would be endless. I am especially grateful to the following persons for their guidance and patience to my questions:

- To Prof. Michael W Okoth
- To Prof. Francis Mulaa
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Your belief in me has been the greatest incentive. For this I thank you.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
BI	Beef infusion medium
BI_{opt}	Optimal Beef infusion medium
DAD	Diode array detector
DCM	Dichloromethane
HPLC	High performance liquid chromatography
LB	Luria Bertani
LB_{opt}	Optimal Luria Bertani medium
OCC	Open Column Chromatography
OD	Optical density
RPM	Rotation per minute
sp	Species
TLC	Thin Layer Chromatography
v/v	Volume per volume
w/v	Weight per volume
m.c	Moisture content
m.o	Micro-organism
λ	Wavelength

ABSTRACT

Carotenoids sources include plants, algae, and photosynthetic bacteria. A novel bacterium *Paracoccus* sp BOG 006^T which was isolated from the outflow of Lake Bogoria hot spring has also been identified to secrete carotenoids.

The main objective of the study was to optimize conditions for biomass production and to quantify the carotenoids secreted by *Paracoccus* sp BOG 006^T. The effects of pH, NaCl concentration, temperature, carbonate addition and acetate addition on the cell biomass production were studied. This was followed by a study of the effect of optimized growth media on cell biomass and carotenoids production. The effect of solvent; acetone, hexane: ethyl acetate 1:1, acetone: hexane 2:1 and dichloromethane: methanol 4:1, and condition variation on the carotenoid extraction process was also investigated.

The total carotenoids content was quantitatively evaluated spectrophotometrically and qualitatively using open column chromatography, thin layer chromatography and high pressure liquid chromatography.

The set of conditions stimulating the highest biomass growth were identified as: pH 9.0, 1% NaCl at 37°C. *Paracoccus* sp BOG 006^T had a higher growth rate in Beef Infusion (BI) than in Luria Bertani (LB) medium. The cell biomass was 67.3% higher in BI medium compared to the optimized LB medium (LB_{opt} medium). Total carotenoids and astaxanthin recovered from LB_{opt} medium was 3719.7 µg/g and 413 µg/g respectively. Hence higher carotenoid yields were obtained from cells grown in LB medium than from the BI medium which gave a lower yield of 2087.7 µg/g and 231.8 µg/g total carotenoids and astaxanthin respectively.

Total carotenoids recovered were highest in acetone. Extraction was found to improve with the increase in extraction time and centrifuge rotation speed. Extraction for 10 min was observed to achieve a better recovery than extraction for 5 min. Recovery was higher at 6000 rpm than at 4000 rpm. The highest carotenoid recovery was 3745.7 μg/g when using acetone at 6000 rpm for 10 min. The main carotenoids identified were lutein, β-carotene and astaxanthin.

Paracoccus sp BOG 006^T tolerated high pH of up to 10.0 and was capable of growth at relatively high temperatures of up to 45°C. This lowers its susceptibility to contamination, and indicates a good possibility for *Paracoccus* sp BOG 006^T application in industrial production of carotenoids. Since total carotenoids production was observed to be higher in LB medium, a sub-optimal medium for *Paracoccus* sp BOG 006^T growth, it was deduced that stress favours higher yields of carotenoids in *Paracoccus* sp BOG 006^T.

CHAPTER 1: INTRODUCTION

Carotenoids are a class of natural fat-soluble pigments found principally in plants, algae, and photosynthetic bacteria. They also occur in some non-photosynthetic bacteria, yeasts, and molds. Although animals appear to be incapable of synthesizing carotenoids, many animals incorporate carotenoids from their diet (Britton, 1995). Some 600 different carotenoids are known to occur naturally, and new carotenoids continue to be identified (Mercadante, 1999). The most abundant carotenoids are β-carotene, α-carotene, ɤ-carotene, lycopene, lutein, β-crpytoxanthin, zeaxanthin and astaxanthin. These carotenoids are considered commercially important.

The production of the carotenoids is mainly by chemical synthesis or by extraction from natural sources (Berry *et al.,* 2003). Microorganisms have been widely used for the commercial production of carotenoids. β-carotene is produced by fermentation with the alga *Dunaliella salina* (Borowitzka, 1999). Astaxanthin production has also been reported by fermentation using the red yeast *Xanthophyllomyces dendorous* and by the alga *Haematoccous pluvialis* (Lorenz and Cysewski., 2000).

Of the naturally occurring carotenoids, the xanthophyll astaxanthin (3, 3′-dihydroxy-β, β′-carotene-4, 4′-dione) has attracted increased interest. This interest has been attributed to the wide variety of its uses which include; antioxidant properties, ingredient of feed to enhance the pigmentation of animal flesh (Wathene *et al.,* 1998) and improvement of egg production and the general health of hens (Lignell *et al.,* 1998). The pharmaceutical and nutraceutical industries have also shown considerable interest in these compounds since they can enhance the human immune response and prevent or delay the onset of degenerative diseases and aging (Jyonouchi *et al.,* 1995).

However commercial astaxanthin is expensive (Lorenz and Cysewski., 2000). The international price approximated at US$2500 kg^{-1} with an annual worldwide market estimated at US$200 million in 2003 (Guerin *et al.*, 2003), is a reflection of the production cost. An additional challenge in the use of the carotenoid is that, although over 95% of the identified market consumes synthetically derived astaxanthin, mounting consumer demand for natural products and concerns regarding chemical additives in foods make the synthetic pigment less desirable. With this increase in concern there has been interest in improving astaxanthin production from its natural sources.

Astaxanthin is produced by a limited number of species of micro algae, marine bacteria and fungi (Johnson and Schroeder., 1996). Some microorganisms belonging to the genus *Paracoccus* also produce carotenoids with four species: *Paracoccus carotenifaciens* (Berry *et al.*, 2003), *Paracoccus marcusii* (Hirschberg and Harker., 1999), *Paracoccus zeaxanthinifaciens* (Tsubokura *et al.*, 1999) and *Paracoccus haeundaensis* (Lee and Kim, 2004) producing industrially important carotenoids.

In Kenya an investigation conducted by Osanjo *et al.*, (2009) to find new and robust biocatalysts and secondary metabolites from micro-organisms of Lake Bogoria a soda lake, resulted in the isolation of a carotenoid secreting Gram-negative obligate alkaliphilic bacterium, *Paracoccus* sp BOG 006^T (GenBank 16S rDNA nucleotide sequence accession number AJ580352). Astaxanthin has been reported as the predominant carotenoid produced by *Paracoccus* sp BOG 006^T. Being a novel strain, *Paracoccus* sp BOG 006^T growth requirements have not been defined.

Microbial growth and death rates are greatly influenced by a number of environmental parameters. Some conditions favor rapid microbial reproduction while others do not permit any growth. Each micro-organism has a specific tolerance range for specific environmental parameters (Atlas, 1984). The goal of this work was to identify the optimal growth conditions for *Paracoccus* sp BOG 006^T.

1.1 Purpose of the Study

To optimize carotenoid production of *Paracoccus* sp BOG 006^T by manipulating its growth conditions.

1.2 Justification for the Study

Due to the many uses of astaxanthin in the feed, food and nutraceutical industries a ready market is present. It is however observed that the price of astaxanthin, which is in part influenced by production cost, is a limiting factor in its use (Johnson and Schroeder., 1996). Traditionally astaxanthin production has been achieved by chemical synthesis (Britton *et al.*, 1996). However, consumer concerns regarding chemical additives in foods have stimulated research in biological systems for astaxanthin production (Johnson and Schroeder., 1996). The key limitations for industrial production by biological systems are the low yields in wild-type microbial strains to meet industrial demands, and costly extraction methods (Johnson and Schroeder., 1996). This demand for astaxanthin from natural sources has led to the need for optimization of its production and recovery from its commercially viable natural sources.

1.3 General Objective

To optimize conditions for *Paracoccus* sp BOG 006^T biomass production and quantify carotenoids secreted by *Paracoccus* sp BOG 006^T.

1.4 Specific Objectives

- To determine the optimal growth conditions for *Paracoccus* sp BOG 006^T.
- To develop an alternative medium for cultivation of *Paracoccus* sp BOG 006^T.
- To develop a method for recovery of carotenoids from *Paracoccus* sp BOG 006^T.
- To extract identify and quantify carotenoids secreted.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Carotenoids are fat soluble, colorful pigments that have numerous uses in humans and animals. Carotenoids represent one of the most widespread groups of naturally occurring pigments (Pfander, 1987). The majority of carotenoids are derived from a 40-carbon polyene chain, which could be considered the backbone of the molecule. The hydrocarbon carotenoids like β-carotene (Mangels *et al.*, 1993) are known as carotenes, while oxygenated derivatives of these hydrocarbons like astaxanthin, are known as xanthophylls (Weedon and Moss., 1995). The structures of β-carotene and astaxanthin are shown in Figure 2.1 and Figure 2.2 respectively.

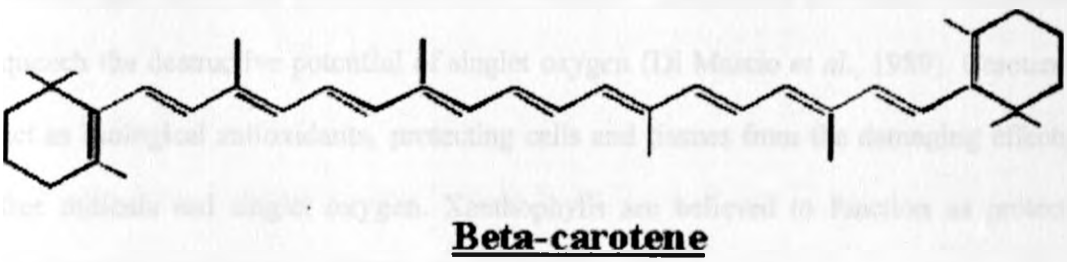

Figure 2.1: Structure of β- carotene. Source Melendez-Martinez *et al.*, (2007).

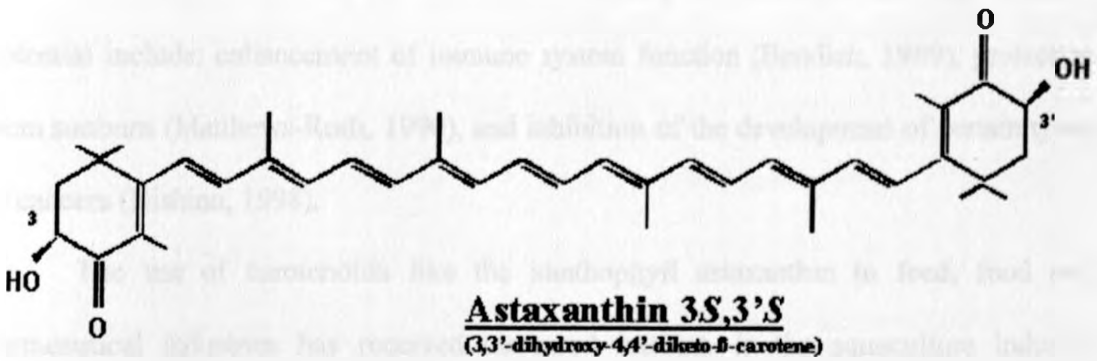

Figure 2.2: Structure of astaxanthin. Source Choi *et al.*, (2002)

The carotenoid structure is reported to ultimately determine the potential biological functions the pigment may have (Britton, 1995). The astaxanthin molecule is seen to be similar to that of β-carotene but the small differences in structure confer large differences in the chemical and biological properties of the two molecules. Astaxanthin structure exhibits superior antioxidant properties to β-carotene in a number of *in vitro* studies (Terao, 1989; Miki, 1991; Palozza and Krinsky., 1992; Lawlor and O'Brien., 1995).

2.1.1 Uses of Carotenoids

Carotenoids main function in organisms is related to light absorption. Carotenoids are involved in photosynthesis, photo protection, phototropism, photoreception and camouflage effects for concealment from enemies. Carotenoids have also been shown to quench the destructive potential of singlet oxygen (Di Mascio *et al.*, 1989). Carotenoids act as biological antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Xanthophylls are believed to function as protective antioxidants (Snodderly, 1995). The xanthophyll astaxanthin is an antioxidant that exhibits a strong free radical scavenging activity (Iwamoto *et al.*, 2000).

Other health benefits of carotenoids that may be related to their antioxidative potential include: enhancement of immune system function (Bendich, 1989), protection from sunburn (Matthews-Roth, 1990), and inhibition of the development of certain types of cancers (Nishino, 1998).

The use of carotenoids like the xanthophyll astaxanthin in feed, food and nutraceutical industries has received increased interest. In the aquaculture industry astaxanthin is used as a colorant (Fang and Chiou., 1996). Astaxanthin is utilized as an

ingredient of feed to enhance the pigmentation of animal flesh. It has been shown to be an effective pigment when incorporated into feeds for salmonidae and crustaceans (Omara *et al.*, 1985; Guillou *et al.*, 1995; Whatene *et al.*, 1998). Astaxanthin is thus commonly used world-wide to supplement fish feeds in order to obtain the desired pink to orange-red color. Due to astaxanthin potent antioxidant properties (Di Mascio *et al.*, 1991), it has been shown to prevent the oxidation of fats in rainbow trout during frozen storage thereby preventing rancidity (Liaaen-Jensen, 1978).

When included in poultry feeds, dietary astaxanthin was reported to improve egg production and the general health of hens (Lignell *et al.*, 1998). Astaxanthin-supplemented feed was found to increase the hatching percentage, resistance to *Salmonella* infection, and shelf life of eggs. Astaxanthin improved chick growth and feed utilization during the first 3 weeks after hatching, and reduced chick mortality due to yolk sac inflammation (Lignell *et al.*, 1998).

In pigs, astaxanthin increases boar semen volume and piglet litter size and survival rate (Lignell and Inborr., 1999). It is an active ingredient in several patented medications for mammals. In anti-stress formulations it is claimed to enhance the effect of anti-stress agents administered to farm animals to minimize weight loss due to crowding, extreme temperatures and other sudden environmental changes (Ito *et al.*, 1999). Astaxanthin is increasingly used in formulations for muscular dysfunction in horses, as well as for mastitis in dairy cows (Lignell and Inborr., 1999), and for gastrointestinal tract inflammation (Wadström and Alejung., 1998).

While the positive effects of astaxanthin in feeds have been recognized for years, the potential benefits to human health are only now being revealed. Owing to its

antioxidant activity, astaxanthin is recently used as a nutraceutical and a medicinal ingredient against degenerative diseases such as cancer (Chew *et al.*, 1999), skin-related illness and heart disease (Guerin *et al.*, 2003).

Several experimental studies have shown the effectiveness of astaxanthin as cancer prevention in rats and mice. Tanaka *et al.*, (1994a) showed that astaxanthin protected mice from urinary bladder carcinogenesis. They also concluded that the inhibitory effect of astaxanthin on cancer was even more pronounced than that of beta-carotene, which they had tested in a previous study (Tanaka *et al.*, 1994b).

Astaxanthin has been shown to significantly influence immune function in a number of *in vitro* and *in vivo* assays using animal models. Astaxanthin was shown to enhance *in vitro* antibody production (Jyonouchi *et al.*, 1991), and to also partially restore decreased humoral immune responses in old mice (Jyonouchi *et al.*, 1994). Studies carried out suggest that astaxanthin could be useful for prevention and treatment of neuronal damage associated with age-related macular degeneration. It may also be effective in treating Alzheimer's disease, Parkinson's disease, spinal cord injuries and other types of central nervous system injuries (Tso and Lam., 1996). It has also been indicated that astaxanthin is effective in protecting photoreceptors from degeneration (Tso and Lam., 1996).

Due to its function as a vitamin A precursor (Gobantes *et al.*, 1998), astaxanthin is one of the most important and economically valuable carotenoids. In view of its importance and numerous uses Guerin *et al.*, (2003) forecasted that the market for astaxanthin as a nutraceutical could reach several hundred million US dollars within 5 to 10 years.

2.1.2 Production of Carotenoids

Industrial production of carotenoids is either solvent-based extraction from natural sources or by chemical synthesis (Asker and Ueda., 2007). These methods are expensive (Domi'nguez-Bocanegra and Torres-Munoz., 2004). As a result of high production costs, commercial products command high prices (Lorenz and Cysewski, 2000). The commercial value of carotenoids like astaxanthin has created a need to develop methods to produce and isolate carotenoids more effectively (An and Johnson., 1991). Consumer and governmental concerns regarding chemical additives in foods has also stimulated the current trend to find alternative natural sources for effective production (Johnson and Schroeder., 1996).

2.2 Sources of Carotenoids

Most microorganisms synthesize carotenoids (Goodwin *et al.*, 1984). However, animals in general, do not synthesize carotenoids and those found in the bodies of animals are either a result of the direct accumulation of carotenoids from food or are partly modified through metabolic reactions. (Matsuno and Hirao., 1989; Liaaen-Jensen, 1978; Goodwin, 1984).

Effective microbial production of carotenoids like astaxanthin has been carried out using a number of microorganisms, such as the green algae *Haematoccous pluvialis* (Lotan and Hirschberg., 1995) and the red yeast *Phaffia rhodozyma* (Andrews and Starr., 1976). Only a few bacteria such as *Brevibaterium*, *Mycobacterium lacticola* (Neils and De Leenheer., 1991) and *Agrobacterium auratim* (Yokayama *et al.*, 1994) are known to synthesize the carotenoid. A novel bacterial strain *Paracoccus* sp BOG 006^{T} has also been shown to be capable of producing astaxanthin (Osanjo *et al.*, 2009).

2.2.1 *Paracoccus* sp BOG 006^T

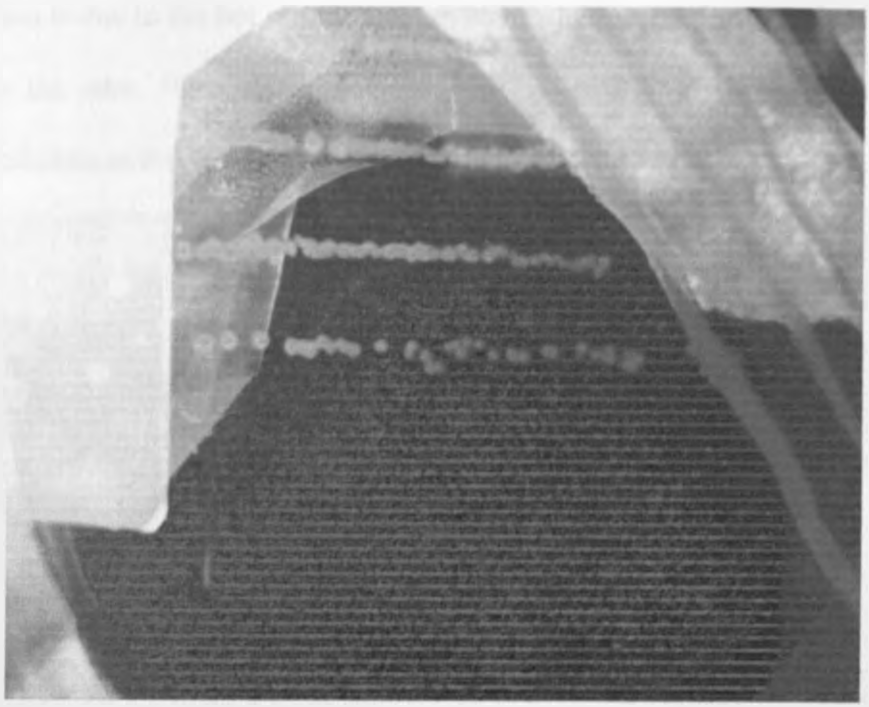

Figure 2.3: *Paracoccus* sp BOG 006^T colonies grown on Luria Bertani (LB) medium at 37 °C for 16 h.

Paracoccus sp BOG 006^T cells as shown in Figure 2.3 are Gram-negative, motile, aerobic, non-spore forming cocci to short rods 0.99-1.11 x 1.1 –1.5 μm in size. The genus *Paracoccus* currently comprises 24 species (Kelly *et. al.*, 2006). Four other species: *Paracoccus carotenifaciens* (Berry *et al.*, 2003), *Paracoccus marcusii* (Hirschberg and Harker., 1999), *Paracoccus zeaxanthinifaciens* (Tsubokura *et al.*, 1999) and *Paracoccus haeundaensis* (Lee and Kim , 2004) have also been reported to produce industrially important carotenoids. *Paracoccus* sp BOG 006^T was isolated from the hot spring outflows of Lake Bogoria

Lake Bogoria (Figure 2.4) is an alkaline-saline lake that has pH range from 9.0 to 11.0 while the lake temperature fluctuates between 30°C and 90°C. The alkalinity is due to its unique geochemistry and attributed to evaporation and leaching which causes

concentration of salts particularly sodium carbonate (Grant *et al.*, 1990). The large temperature variation is due to the hot springs and geysers that are at its shores and emit their outflow into the lake. *Paracoccus* sp BOG 006[T] isolated from the lake was classified as an alkaliphile as it was found to grow best in highly alkaline habitats.

Figure 2.4: Lake Bogoria in Kenya, (0°15'N, 36°06'E), the isolation site. Photograph provided by Leah Tsuma.

Research on biological systems for industrial carotenoid production is limited by the low yields of natural sources such as wild-type bacteria and mutant strains, and costly extraction methods (Johnson *et al.*, 1996; Canizares-Villanueva *et al.*, 1998; An and Johnson., 1991). To reduce production costs researchers are investigating the use of alternative substrates and optimizing culture conditions to increase yields of the pigment (Ramirez *et al.*, 2001).

2.3 Optimization of Growth Conditions

In nature, conditions for microbial growth are often unstable and rarely optimal. There may be periodic fluctuations in growth factors. Such environments rarely contain all the nutrients essential for cell synthesis in sufficient concentration to allow growth to proceed at its potentially maximum rate (Neijssei and Tempest., 1976). In the laboratory set-up it is possible to adjust growth conditions to achieve optimal growth. Growth conditions can be adjusted to optimize growth, thereby maximizing the accumulation of desired microbial metabolic products (Atlas, 1984).

To optimize microbial production of carotenoids growth, conditions are manipulated to ascertain the optimal growth conditions. Growth conditions of the culture medium must be controlled in order to achieve optimal growth (Atlas, 1984). This is attributed to the fact that growth of isolates causes environmental change. The changes in growth conditions lead to decline in growth. Each micro-organism has a specific tolerance range for specific environmental parameters. Outside the range of environmental conditions under which a given micro-organism can reproduce, it may either survive in a relatively dormant state or may lose viability (Neijssei and Tempest., 1976). From the factors inducing astaxanthin production in microbes reported in literature, pH variation, salt tolerance, growth temperature, carbonate requirement and the effect of NaOAC addition were examined and are discussed below.

2.3.1 pH Variation

Each organism has a pH range within which growth is possible and a well defined pH optimum. Most natural environments have pH values varying from 5.0 to 9.0 and only a few species, acidophiles, can grow at acidic pH of 2.0 or in alkaline pH greater than 9.0 (Atlas, 1984). Microbes that grow only in alkaline pH are referred to as alkaliphiles.

Alkaliphilic micro-organisms are typically found in habitats such as soda lakes and high carbonate soils (Atlas, 1984). These extreme environments are complex and are characterized by extreme variation in pH. Alkaliphiles are reported to have developed unique metabolic and physiological functions that ensure survival. Apart from survival, the development of the unique functions also offers potential for production of bioactive metabolites for exploitation (Grant and Jones., 2000). Lake Bogoria; the isolation site, has a high alkaline pH range of 9.0 to 11.0 attributed to a concentration of carbonates and carbonate complexes (Grant and Jones., 2000).

For optimal growth, the pH of the medium should be controlled. The pH value of the growth environment exerts a profound influence on growth, metabolism and death. Control is necessary for as cell growth proceeds, nutrients are taken up and end products excreted leading to change in the pH of the growth medium. The losses of ideal pH for growth results in a decline until the medium can no longer support growth. Fermentation pathways lead to changes in the pH. This is observed in seed and batch cultures. Thus in culture media and industrial fermenters, pH values must be controlled to achieve optimal growth rates (Atlas, 1984).

2.3.2 NaCl Tolerance

The salt NaCl, acts as an electrolyte when ionized in water providing Na^+ for cell membrane transport.

$$NaCl\ (s) \rightarrow Na^{1+}\ (aq) + Cl^{1-}\ (aq)$$

NaCl in the medium maintains osmotic equilibrium of the medium and the cells. This prevents destruction through lysis of the cell. Work with a *Paracoccus* isolate from a sea-sand sample Kim *et al.* (2006) reported an inverse relationship between NaCl concentration and cell growth.

2.3.3 Growth Temperature

Temperature influences growth of a cell by affecting enzymatic activity. The optimal temperature allows a faster reaction rate by initiating the greatest number of molecular collision without denaturing enzymes.

Up to a point, the velocity of an enzymatic reaction increases with temperature. This is due to the more frequent substrate collision with active sites. Beyond the optimal temperature however, the speed of the enzymatic reaction drops. The thermal agitation of the enzyme molecule disrupts the H-bond, ionic bond and other weak interactions that stabilize the active conformation and the protein molecule denatures. Proteins of most organisms denature at temperatures above 45°C. Few organisms can maintain a sufficiently active metabolism at very high or low temperatures.

Each micro-organism can grow only within certain limits of temperature. Growth temperature has a profound effect on the growth rate, macromolecular composition and levels of intracellular metabolites of an organism. It is of considerable importance to

determine the optimal temperature that gives the maximal cell yield for the production of a particular metabolite (Atlas, 1984).

2.3.4 Growth Medium

In media composition the rational point of departure for the preparation of media is to compound a mineral base, which provides all those nutrients that can be supplied to any organism in inorganic form. This base can then be supplemented with any required growth factors (Madigan and Martinko., 2006).

The design of a culture media should be based on scientific principles of nutrition. The chemical composition of cells which is broadly constant throughout the living world is used to indicate the major material requirements for growth. When the growth requirements of an organism are known it is possible to devise a set of conditions that will specifically favor the development of that particular organism. However, when the requirements are not known a complex media, which normally has a nutrient of unknown composition like yeast extract, is useful. Complex media are used for the cultivation of a wide range of microbes including those whose precise growth factor requirements are not known or are numerous (Madigan and Martinko., 2006).

The primary goal in the construction of culture media is to provide a balanced mixture of the required nutrients at concentrations that will permit good growth. However, many nutrients become growth inhibitors or toxic as the concentration is raised. Substrates such as salts of fatty acids like acetate, sugars and some inorganic constituents may also become inhibitory if in excess (Neijssei and Tempest., 1976).

2.3.5 Carbonate Requirements

The natural growth environment of the isolate *Paracoccus* sp BOG 006^T, Lake Bogoria, is a highly alkaline soda lake characterized by the presence of large amounts of soda, Na_2CO_3. Soda is usually present in the lake as natron $Na_2CO_3\,10H_2O$ or trona $Na_2CO_3.NaHCO_3.2H_2O$ and their derivatives. The concentration of soda results from evaporation in the closed basin and makes carbonate the predominant ion (Grant and Jones., 2000). $NaHCO_3$ and Na_2CO_3 addition has been successfully used to adjust the pH of media (Didier Alazard *et al.*, 2007).

2.3.6 Effect of Acetate on Growth

Isoprenoid precursors like acetate have been investigated with the aim of increasing biomass formation and carotenoid production (Meyer and Du-Preez., 1993). An *et al,* (1996) reported that the use of certain precursors like acetic acid increased cell growth and carotenoid production. This report is supported by Cifuentes *et al.* (2003) who also found that with increasing acetate concentration, a slight increment in growth occurred. However, Cifuentes *et al.* (2003) observed that although most work has found enhanced growth rates with acetate, it is also true that some studies have reported slow cell growth and low final cell densities. In these cases the acetate was considered as an inhibitor.

2.4 Carotenoids Recovery, Identification and Quantification

Extraction systems used for carotenoids must be adapted to the characteristics of the source of extraction (Houghton and Raman., 1998). When extracting carotenoids from wet samples, like freshly harvested cells, a water miscible organic solvent like

acetone, methanol, ethanol or a mixture should be used. This allows better solvent penetration (Rodriguez-Amaya and Kimura., 2004).

Carotenoids are qualitatively and quantitatively analyzed by chromatography, TLC, OCC and HPLC. The behavior of substances in chromatographic system is usually reproducible and this can yield information on their identity. Thin layer chromatography is efficient for identification purposes. Silica gel (SiO_2) is used as the stationary phase over. The solvent gradually moves up the stationary phase via capillary action, and it carries the deposited substances along with it at different rates. Carotenoids are identified from their R_f values. The R_f value for a substance is the ratio of the distance that the substance travels to the distance that the solvent travels up the plate (Houghton and Raman., 1998).

OCC is chromatography in descending, gravity flow and is good for quantitative analysis. The Separation of carotenoid pigments is followed visually, color enables monitoring of the different bands as they elute out of the column (Houghton and Raman., 1998). Quantification of carotenoids eluted is done spectrophotometrically using their tabulated adsorption coefficient ($A^{1\%}_{1cm}$).

High pressure liquid chromatograph is used for determination of pigment identity and content. HPLC main advantage over other chromatographic analysis is that reproducible separations can be performed over a shorter period using reusable columns without danger of degradation (Rodriguez-Amaya and Kimura., 2004)

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacterial Isolate

The bacterial isolate under study, *Paracoccus* sp BOG 006^T was cultured and maintained at University of Nairobi, College of Health Sciences, Department of Biochemistry.

3.2 Culture Conditions

The isolate *Paracoccus* sp BOG 006^T was revived from a glycerol stock by re-streaking on LB agar plates. The LB medium was prepared using NaCl 10 g/l (Sigma, Germany), bacto-tryptone 10 g/l (Merck, Germany), yeast extract 5 g/l (Merck, Germany) and bacto-agar 15 g/l (Merck, Germany). The medium was sterilized using an autoclave (3850 MLV, Tuttnauer, Germany) at 121°C for 30 min.

Sterile Na_2CO_3 solution was then prepared by dissolving 15 g of anhydrous Na_2CO_3 (Sigma, Germany) in 100 ml distilled water. The Na_2CO_3 solution was filter sterilized using a 0.20 µm disposable cellulose acetate syringe filter (Iwaki, Japan) and used to adjust the pH of the sterile LB medium to pH 9.5.The medium was then poured out on plates, and allowed to solidify.

The isolate *Paracoccus* sp BOG 006^T was streaked onto the solidified LB agar plates and incubated at 37°C for 16 h. A single 16 h colony was used to inoculate sterile 10 ml LB broth medium at pH 9.5 and incubated for 16 h with continuous shaking at 130 rpm at 37°C in an Orbital Shaker (Cooled, GallenKamp, Germany). Cell biomass was monitored by measuring OD at 600 nm. *Paracoccus* sp BOG 006^T was stored as glycerol stocks of 30% sterile glycerol and 70% *Paracoccus* sp BOG 006^T liquid culture

in a 1 ml epperndorf tube and stored at -20°C. From this culture a parent stock was revived on a monthly basis and was stored at -20°C.

3.3 Identification of *Paracoccus* sp BOG 006^T Growth Conditions

Unless otherwise stated the culture *Paracoccus* sp BOG 006^T was grown on LB medium or BI medium at 37°C. The pH was adjusted by the addition of 15% w/v filter sterilized Na_2CO_3 and the response variable, cell biomass, was monitored by measuring the OD at 600 nm using a UV-Visible spectrophotometer (U-2810, Digilab Hitachi, Japan) at intervals of 2, 4, 6, 8 and 24h. Culture conditions were varied according to the experimental design and all experiments performed in quadruplicate. To maintain sterility all inoculations were done in the microbiological safety cabinet (HS12, Heraelus, Germany).

3.3.1 Experimental design

The objective was to optimize astaxanthin production by *Paracoccus* sp BOG 006^T through the study of the influence of several growth conditions. A complete randomized design was used to investigate the influence of pH, NaCl addition, temperature, carbonate requirement, and acetate addition on the growth of the isolate. To evaluate the effect of the solvents: acetone, hexane: ethyl acetate 1:1, acetone: hexane 2:1 and dichloromethane: methanol 4:1, and condition variation on the recovery of carotenoids from *Paracoccus* sp BOG 006^T.a factorial design was used.

All experiments were carried out in quadruplicate (n=4). The results were presented as the mean values of the yield at a standard deviation of ±1.

3.3.2 Effect of pH on Growth

To test the pH range for growth and identify the optimal pH, a modification of Berry *et al.* (2003) method was applied. *Paracoccus* sp BOG 006^T culture was streaked on a sterile LB agar plate and incubated for 16h to ensure purity of the culture. LB broth medium was then prepared and 10 ml of the medium dispensed into 50 ml falcon tubes and sterilized using an autoclave (3850 MLV, Tuttnauer, Germany) at 121°C for 30 min. The pH of the sterile medium was adjusted to the experimental pH conditions using 15% w/v filter sterilized Na_2CO_3 (Sigma, Germany). The sterile LB broth medium with final pH values of 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5 were each inoculated with a single 16 h cell colony. The tubes were then incubated for 24 h with continuous shaking at 130 rpm at 37°C in an orbital shaker (Cooled, GallenKamp, Germany) and the cell biomass monitored during the incubation time.

3.3.2 Effect of NaCl on Growth

Paracoccus sp BOG 006^T tolerance to NaCl concentration was investigated using the methodology of Kim *et al.* (2006). LB broth medium was prepared using bacto-tryptone 10 g/l (Merck, Germany) and yeast extract 5 g/l (Merck, Germany); without the inclusion of NaCl. A 10 ml aliquot of LB broth medium was dispensed into 50 ml falcon tubes and the medium supplemented with NaCl (Sigma, Germany) to a final media concentration of either, 1, 2.5, 5, 7.5 or 10% w/v NaCl (Sigma, Germany). The LB broth medium in the falcon tubes was then sterilized and the pH adjusted to the optimal pH of 9.0.

A single 16 h cell colony was inoculated into the tubes containing the experimental NaCl concentrations. During the 24 h incubation period at 37°C in an

orbital shaker (Cooled, GallenKamp, Germany) with continuous shaking at 130 rpm, the biomass of the cells was monitored during the incubation time.

3.3.3 Effect of Temperature on Growth

The temperature range for growth for *Paracoccus* sp BOG 006^T was determined using a modification of Lee and Kim., (2004) method. Effect of temperature on cell growth was monitored by inoculating 10 ml of sterile LB broth medium with a single 16 h cell colony and incubating the tubes at 4, 25, 37, 45 or 55^{0}C for 24 h.

The sterile LB broth medium used to investigate the effect of temperature on growth had the optimal NaCl concentration of 1% w/v and an adjusted pH of 9.0. The optimal temperature condition was determined through monitoring the cell biomass in the course of the incubation time by spectrophotometry.

3.3.4 Effect of Carbonate on Growth

To determine the effect of carbonate on the bacteria, a modification of the method of Harker *et al.* (1998) was used to test growth of *Paracoccus* sp BOG 006^T in the presence and absence of Na_2CO_3. The LB broth medium was prepared with a 1% w/v NaCl concentration and sterilized at 121^{0}C for 30 min using an autoclave (3850 MLV, Tuttnauer, Germany). The pH of the sterile medium was then adjusted to pH 9.0 using either 15% w/v filter sterilized Na_2CO_3 (Sigma, Germany) or 15 % w/v filter sterilized NaOH (Sigma, Germany). A solution of NaOH was prepared by dissolving 15 g of the salt in 100 ml distilled water. The NaOH solution was filter-sterilized using 0.20 µm disposable cellulose acetate syringe filters (Iwaki, Japan).

A 30 ml aliquot of the sterile pH adjusted LB broth media was dispensed into 100ml conical flasks. Growth in LB broth medium pH adjusted using Na_2CO_3 was compared with growth in LB broth medium pH adjusted using NaOH by inoculating each of the media with a 16 h *Paracoccus* sp BOG 006^T colony. Incubation was carried out for 24 h at 37°C in an orbital shaker. Biomass accumulation was monitored for the incubation duration by measuring the OD at 600 nm.

3.3.5 Effect of Acetate on Growth

Cultivation of the isolate in the presence of acetate was done at 4 mM, 8 mM, and 12 mM NaOAC (Sigma, Germany).

The sterile LB broth medium used to investigate the effect of acetate on the biomass formation of *Paracoccus* sp BOG 006^T had a 1% w/v NaCl concentration and was pH adjusted to pH 9.0. 10 ml of the sterile growth medium was dispensed into 50 ml falcon tubes and supplemented with filter sterilized NaOAC to a final media concentration of either, 4 mM, 8 mM or 12 mM.

Each of the LB broth media at the above NaOAC concentrations were inoculated with a single 16 h colony and incubated for 24 h at 37°C in a orbital shaker (Cooled, GallenKamp, Germany) with continuous shaking at 130 rpm (Cifuentes *et al.*, 2003). Biomass was monitored during the incubation period. The biomass accumulation was monitored through the growth curve gradient as an indicator of cell growth rate.

The growth conditions that were observed to produce optimal cell biomass in LB medium were used to prepare the optimized medium denoted as LB_{opt} medium.

3.4 Development of an Alternative Media

3.4.1 Effect of Type of Media on Biomass Production

3.4.1.1 Growth of *Paracoccus* sp BOG 006^T in Different Media

A study of growth in LB_{opt} medium and Beef infusion medium (BI) was carried out.

To ensure purity of the isolate *Paracoccus* sp BOG 006^T, a loopful of the culture was streaked on a sterile LB agar plate and incubated for 16 h.

The optimized LB medium was prepared using 10 g/l NaCl (Sigma, Germany) 10 g/l bacto-tryptone (Merck, Germany) and 5 g/l yeast extract (Merck, Germany). Beef infusion was prepared according to the method of Atlas *et al.* (1984). A sample of 453.6 g of ground defatted beef was added to 1L of distilled water and left to stand overnight at 4°C. The mixture was then heated to 80-90°C for 60 min and left to stand for 2 h. The mixture was filtered through muslin cloth and 10 g/l Bacto-peptone and 5 g/l NaCl added.

A 10 ml aliquot of LB_{opt} medium and 10 ml of BI medium was dispensed into 50 ml falcon tubes and sterilized using an autoclave (3850 MLV, Tuttnauer, Germany) at 121°C for 30 min. The pH of the sterile media was adjusted to pH 9.0 using 15% w/v filter sterilized Na_2CO_3 (Sigma, Germany).

The tubes were inoculated with a 16 h cell colony and incubated for 24 h in an orbital shaker (Cooled, GallenKamp, Germany) with continuous shaking at 130 rpm at 37°C. Growth was monitored by measuring the OD at 600 nm.

3.4.1.2 Effect of Starch on *Paracoccus* sp BOG 006^T Growth

To assess the effect of starch addition on the growth of *Paracoccus* sp BOG 006^T, growth in beef infusion broth medium with a final starch concentration of 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5.0, 7.5 and 10.0% w/v starch was investigated.

Sterile beef infusion broth medium was prepared and 10 ml dispensed into 50 ml falcon tubes. The experimental starch concentrations were then dissolved in the BI medium by heating. The media with varying starch concentrations were sterilized and the pH adjusted to pH 9.0.

Each medium was inoculated with a single 16 h cell colony and incubated for 24 h at 37°C in an orbital shaker. The effect of starch addition on *Paracoccus* sp BOG 006^T biomass accumulation was monitored by measuring OD against blanks with complementing final starch concentrations at 600 nm.

The starch concentration that was observed to produce optimal cell biomass when added to BI medium was denoted as BI_{opt} medium.

3.4.2 Effect of Type of Media on *Paracoccus* sp BOG 006^T Growth and Carotenoid Production

The effect of the experimental growth media LB_{opt} medium and BI_{opt} medium on *Paracoccus* sp BOG 006^T growth and carotenoid production was investigated next. A 10 ml aliquot of sterile LB_{opt} medium and 10 ml of sterile BI_{opt} medium with pH adjusted to 9.0% was dispensed into 50 ml falcon tubes. Each of the tubes were inoculated with a single 16 h cell colony and incubated for 24 h at 37°C in an Orbital Shaker.

The growth rate in the different media was calculated as the change in OD divided by the change in time while the specific growth rate was deduced from the tangent of the angle (α) of the plotted curves.

Carotenoids from the *Paracoccus* sp BOG 006^T cells were extracted using acetone (Sigma, Germany) through mechanical lyses by vortexing in a rota-mixer (Deluxe, Hook and Tucker Ltd, UK) for 30 sec. Sand sterilized using an air oven at 80°C for 2 h was used for the mechanical lyses of cells. Total carotenoids from the cells were monitored by measuring OD at 450 nm.

3.5 Carotenoids Extraction

To ensure effective extraction, recovery using variations in solvent and conditions were studied using a factorial design. The factors and levels were; acetone, Harker *et al.* (1996), hexane: ethyl acetate 1:1, acetone: hexane 2:1, Miguel *et al.* (2000) and dichloromethane: methanol 4:1, Tsubokura *et al.* (1999) for solvent variation; rpm and time of centrifugation for condition variation.

The isolate was grown on sterile LB agar medium pH 9.0 for 16 h at 37°C and the plate checked for purity. When no contaminations were visible a seed culture was prepared by incubating for 16 h at 37°C, 10 ml LB_{opt} medium inoculated with a 16 h cell colony. A batch culture was then prepared by inoculating 250 µl of the seed culture in 250 ml LB_{opt} medium. After 24 h incubation at 37°C, the cells were harvested by centrifugation (Mikro 200R, Hettich, Germany) in 15 ml centrifugation tubes at either 4000 rpm or 6000 rpm for either 5 min or 10 min. The cells were then washed with distilled water and dried by inverting the centrifugation tubes on absorbent paper.

The experimental solvents were introduced to the cells and left overnight at 4°C in darkness. The cells were then mechanically lysed with sterile sand by vortexing using a rota-mixer (Deluxe, Hook and Tucker Ltd, UK) for 30 seconds and pellet extracted with the selected extraction solvent.

Following this, the supernatant was recovered by centrifugation and concentrated in a rotary evaporator (Heidolph R-200, Buchi, Switzerland) at a pressure of 400 mbars and temperature of 40°C. The samples were then stored at -20°C for further analysis (Miguel *et al.*, 2000).

Care was observed during extraction to limit contact with, light, heat, and acids to avoid alteration of the carotenoids. To avoid excessive heating, solvents with a low boiling point and rotary evaporator were used to extract and concentrate the extracts respectively. To protect from light degradation, operations were carried out under diffuse light where aluminum foil was used to cover the extract (Melendez-Martinez *et al.*, 2007).

3.6 Analysis and Quantification of Carotenoids

3.6.1 Spectrophotometric Methods

Spectrophotometry was used for quantitative determination of the total carotenoids concentration in the crude extract using the Beer-Lambert law ($A = \varepsilon c d$). The spectrophotometer was zeroed using 1 ml glass cuvets with the respective extraction solvent as the blank. An aliquot of the extracted carotenoid sample was then placed in the 1ml glass cuvet with a path length of 10 mm and analyzed using the UV-Visible Spectrometer (U-2810, Digilab Hitachi, Japan). Total carotenoids and astaxanthin concentration was calculated using an extinction coefficient (ε) of 130056 and 2100

respectively (Weber, 1990). Total carotenoids were monitored by measuring OD at 450 nm, then carotenoid concentration was calculated using the Beer-Lambert law ($A = \varepsilon c d$). The total carotenoids mass was obtained by dividing the OD by ε, multiplied with the molecular mass, multiplied by 1000 to get from mg/ml to µg/ml, multiplied by 10 to correct for the total volume and divided by the dry mass.

3.6.2 Chromatographic Analysis

Chromatographic analysis was used for the identification of carotenoids.

3.6.2.1 Thin Layer Chromatography (TLC) Analysis of Carotenoids

To isolate and purify the individual carotenoids in the extract a TLC analysis was carried out (Britton, 1991). 0.5 µl of the carotenoid extract was spotted on TLC aluminum sheets 5 × 10 cm silica gel 60 F_{254}(Merck, Germany).

The silica gel TLC plates obtained were 2 cm wide and 5 cm long. A straight line was drawn using a pencil 1 cm from the edge of the plates. This line was then subdivided using perpendicular lines to act as a guide for placing the spots. A second line was drawn about 1 cm from the other end of the plates to act as the solvent front guide. The marked TLC plates were then activated by placing in an oven at 60°C for 20 min to drive off water molecules that were bonded to the polar sites on the plate.

The crude carotenoids and the carotenoids standards were spotted using a glass capillary tube and air dried before developing the chromatogram. The TLC plates were spotted by placing the narrow end of the capillary tube into a vial containing the carotenoids extract and the solutions allowed to rise in the capillary tube. Once the capillary was loaded, it was allowed to touch the pencil mark on the surface of the TLC

plate and 0.5 µl of the extract deposited. The solvent was then evaporated from the spot. The spotted samples were then resolved using a mobile phase of hexane: acetone (9:1).

To develop the TLC plate 10 ml of the solvent was poured into a small wide-mouth glass bottle. The TLC plate was then introduced in the bottle using tweezers and the plate left in the chamber until solvent had advanced to the top pencil line on the slide. The slide was then removed from the developing chamber and visualized. The visualization was done by drying the TLC plates in air at 27°C followed by observation under visible and UV light. The retention time R_F was calculated and compared with the R_F of the standards.

3.6.2.2 Chromatographic Fractionation of Carotenoids

Open column chromatography (OCC) was used to separate the extract into fractions containing groups of similar polarity (Britton, 1991). A column with an internal diameter of 2 cm and length of 20 cm was packed with silica gel 60 F_{254} (Merck, Germany) and used in the analysis.

First the stationary phase was measured to fill half the column; this was then poured out of the column and weighed. The weight was used to calculate the amount of extract that could be analyzed without overloading the column or causing loss by irreversible adsorption to the stationary phase.

The stationary phase was equilibrated by suspending in the mobile phase by gently stirring until an even suspension was formed. Glass wool was introduced in the bottom of the column and the column clamped in an upright position with the tap firmly closed. The suspension was then slowly introduced in the column while tapping the walls of the column gently to allow air bubbles to rise to the surface. The suspension was then

allowed to settle and the tap opened leaving the mobile phase to run out slowly until the height of the supernatant liquid above the column packing was 2 cm.

The maximum weight of extract that the column could separate was determined as 1 g extract per 100 g dry stationary phase (Houghton and Raman., 1998). To prepare the sample, the extract solution was placed in an evaporating dish and mixed in 2.5% of the silica gel until an even dispersion was formed. The extract evenly coated on the stationary phase and formed a powder when the extract solvent dried.

The carotenoid extract sample was introduced as a powder to the top of the column. A fine spatula was used to sprinkle the extract-silica phase mixture evenly over the surface of the supernatant in the column up to a depth of 2 cm in order to achieve a good separation. The extract was left to settle on the surface of the stationary phase and more stationary phase was sprinkled into supernatant once the sample had been added preventing further dissolution of the extract into the mobile phase. The chromatogram was developed in a dark room to minimize the light's decolorizing effect on the carotenoids. The first aliquot of the mobile phase of acetonitrile: methanol: ethyl acetate in the proportion 88:10:2 was introduced and the tap opened to adjust the flow. A flow rate of 1 ml/min was used as recommended for conventional column chromatography by Houghton and Raman., (1998); the mobile phase was allowed to move down through the column under gravity.

The constituents of the extract moved at different rates through the column as bands. Separation of the carotenoid pigments was followed visually and glass test tubes used to collect 5 ml aliquots of elute which were analyzed spectrometrically. Fractions with the same profile were pooled and analyzed using TLC and HPLC.

3.6.2.3 High Pressure Liquid Chromatography (HPLC) Analysis of Carotenoids

Purity of individual carotenoid was carried out using HPLC analysis. Prior to each analysis, samples were filtered through 0.20 µm millipore disposable sterile syringe filters (Iwaki, Japan). The standard solutions were injected first followed by a 20 µl aliquot of the filtered extracted carotenoid sample. The aliquots were loaded in a HPLC instrument (Agilent 1100, Sigma- Aldrich, Germany) with a Kromasil C18, 250mm × 4.6 mm silica column, 5 mm guard column and a diode array detector. The flux rate was maintained at 1ml/min and the pressure at 0.30 psi. The mobile phase was a mixture of acetonitrile, methanol and ethyl acetate in the proportion 88:10:2 at 27°C. The pigments were monitored by measuring the absorbance at 450 nm. Peak identification and quantification were achieved by comparing the peak area and retention time of samples to the peak areas and retention times of the standard solutions.

3.7 Statistical Analysis

The data was subjected to statistical analysis utilizing either a completely randomized design or factorial design. Descriptive statistics; mean, confidence interval and standard deviation, were used to summarize the data on microbial growth at varying pH, NaCl concentration, temperature and media. The independent samples F-test was used to determine whether or not a significant difference existed between industrial basal media and the formulated media.

Analysis of variance and multiple comparisons were done using the computational program Genstat®. Differences were considered significant at a probability of 5%.

CHAPTER 4: RESULTS

4.1 Optimization of Growth Conditions in the Baseline Medium

Luria Bertani medium was used as the baseline medium for optimization in the growth of *Paracoccus* sp BOG 006^T. It was chosen as it is a standard and accessible commercial medium for growing non-fastidious bacteria in the laboratory set-up.

4.1.1 Growth under Different pH Conditions

The growth curves in Figure 4.1 show the growth of the culture in the lag and log phase expressed as OD at 600 nm. *Paracoccus* sp BOG 006^T growth was varied at different pH values. Growth at pH 7.5 and 8.0 was slow; while at lower pH values of 6.5 and 7.0 the growth was negligible. The pH range of growth was 8.0 to 10.0.

Figure 4.2 shows that after 24 h of growth, biomass content increased with pH up to a maximum level at pH 9.0 and thereafter declined. However, the level at pH 10.0 was still higher than that at pH 8.0. It was observed that growth in LB medium at pH 9.0 exhibited a 7.3 fold increase in cell biomass compared to that in the same medium at pH 6.5.

Figure 4.3 shows that the biomass level at pH 9.0 was higher than that at pH 10.0 The difference in biomass obtained at pH 9.0 was significantly different from the other experimental values ($p \leq 0.05$). All further experiments were carried out using this observed pH optimum.

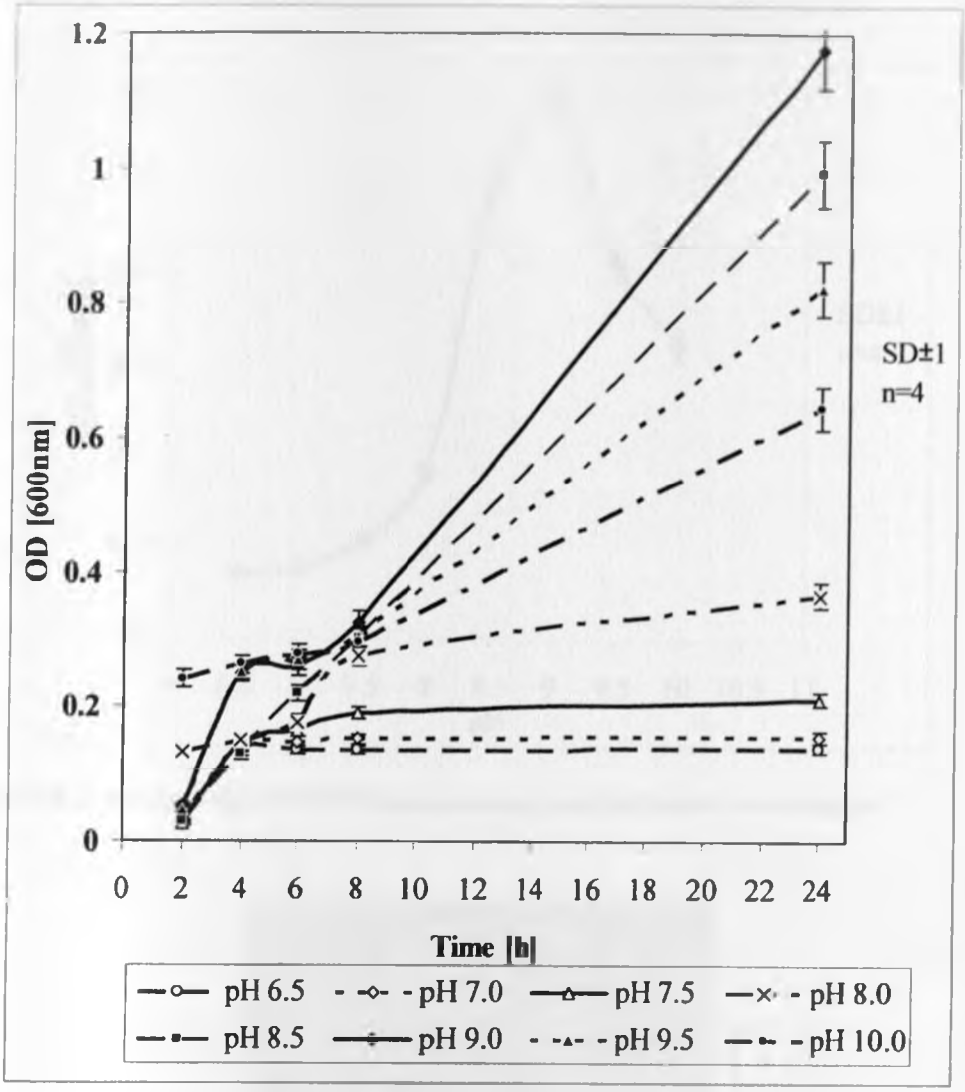

Figure 4.1: Effect of pH on growth of *Paracoccus* sp BOG 006[T] in LB medium for 24 h measured at λ 600 nm.

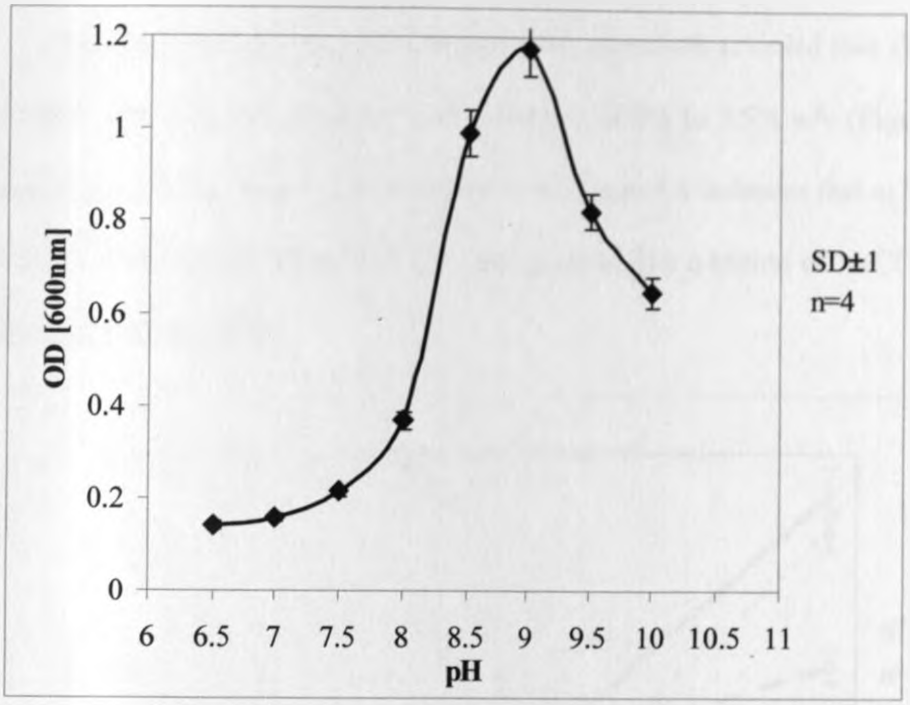

Figure 4.2: *Paracoccus* **sp BOG 006^T biomass content at 24 h of growth at varying pH.**

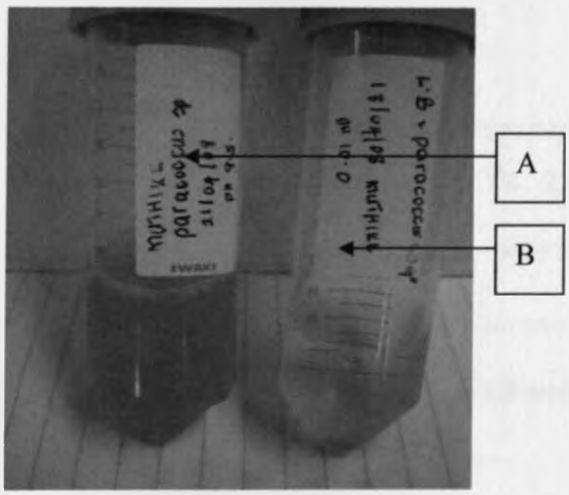

Figure 4.3: Distinctive biomass difference in *Paracoccus* **sp BOG 006^T grown in LB medium for 24 h at [A] pH 9.0 and [B] pH 10.0.**

4.1.2 Effect of NaCl on Growth of *Paracoccus* sp BOG 006^T

The effect of varying NaCl concentration on growth revealed that *Paracoccus* sp BOG 006^T had a narrow range of NaCl tolerance of 0% to 2.5% w/v (Figure 4.4), with maximal biomass at 1% w/v NaCl (Figure 4.5). Figure 4.4 indicates that at 5, 7 and 10% w/v NaCl concentration there was minimal growth. The addition of NaCl significantly affected growth ($p \leq 0.05$).

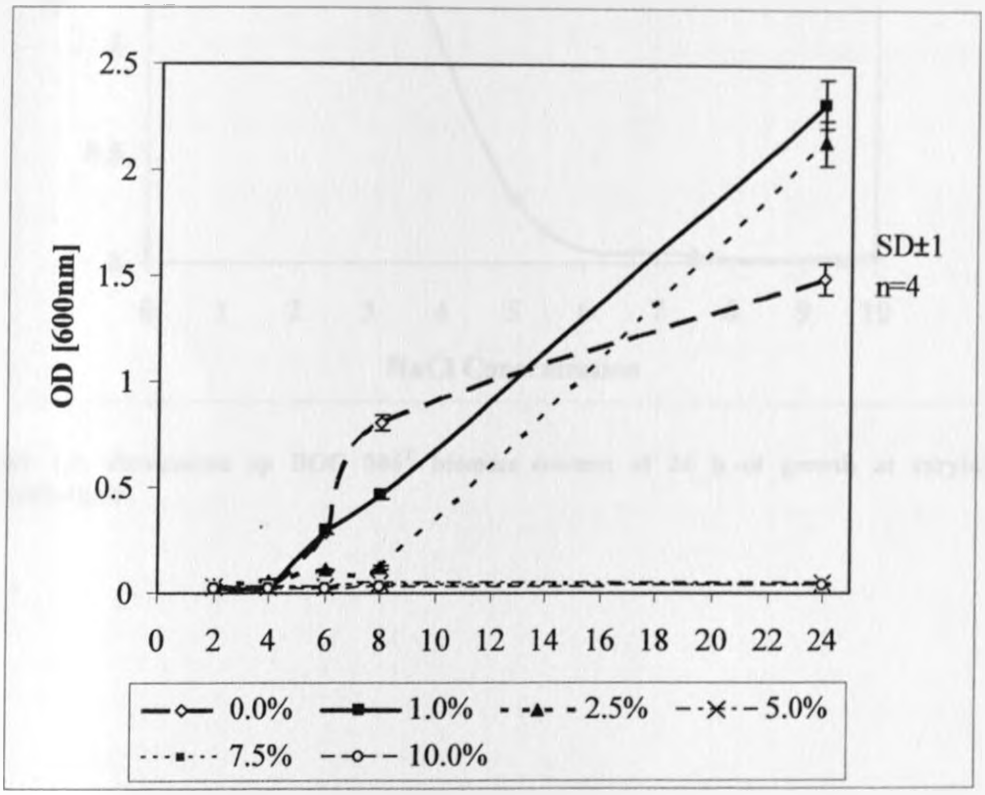

Figure 4.4: Effect of NaCl on growth of *Paracoccus* sp BOG 006^T in LB medium for 24 h measured at λ 600 nm.

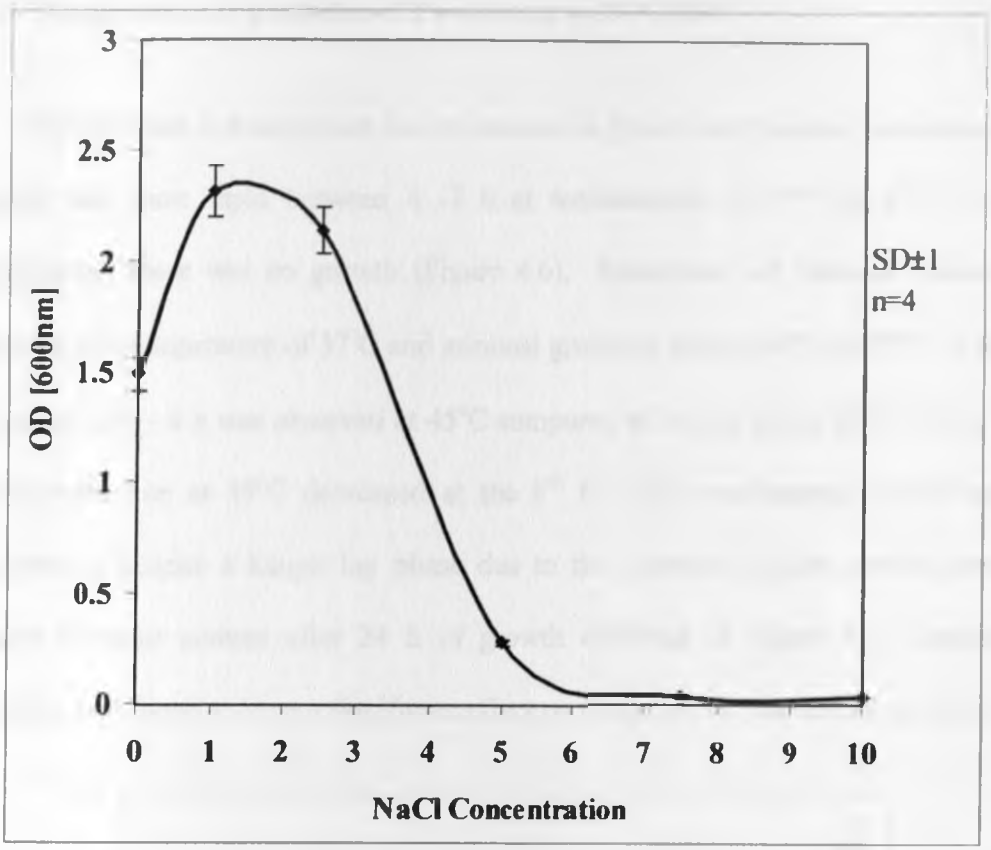

Figure 4.5: *Paracoccus* sp BOG 006[T] biomass content at 24 h of growth at varying NaCl concentrations.

4.1.3 Temperature Dependence of *Paracoccus* sp BOG 006[T]

The increase in temperature led to increase in growth and biomass production. The growth was most rapid between 4 -7 h at temperatures of 25°C to 45°C. At low temperature, there was no growth (Figure 4.6). Maximum cell biomass content was observed at a temperature of 37°C and minimal growth is seen at 4°C and 55°C. A shorter lag phase of 0 - 4 h was observed at 45°C compared to the lag phase of 0 - 6 h at 37°C. The growth rate at 45°C decreased at the 8[th] h, 37°C was selected as the optimal temperature despite a longer lag phase due to the relatively higher growth rates and highest biomass content after 24 h of growth observed in Figure 4.7. Temperature variation was found to have a significant effect on the growth of the culture ($p \leq 0.05$).

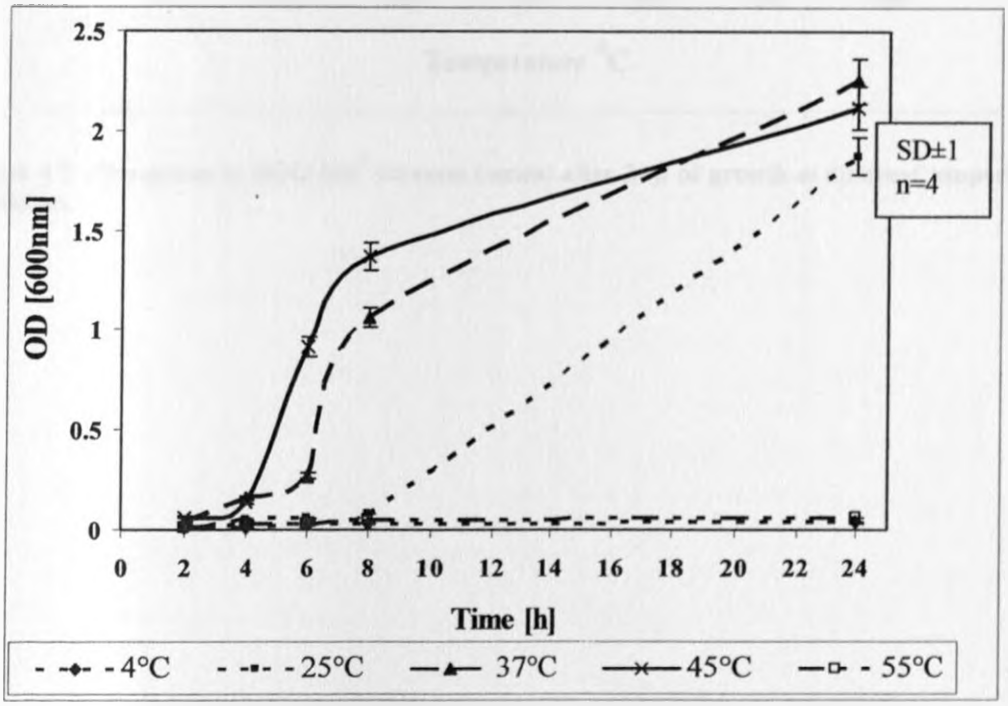

Figure 4.6: Effect of temperature on *Paracoccus* sp BOG 006[T] growth.

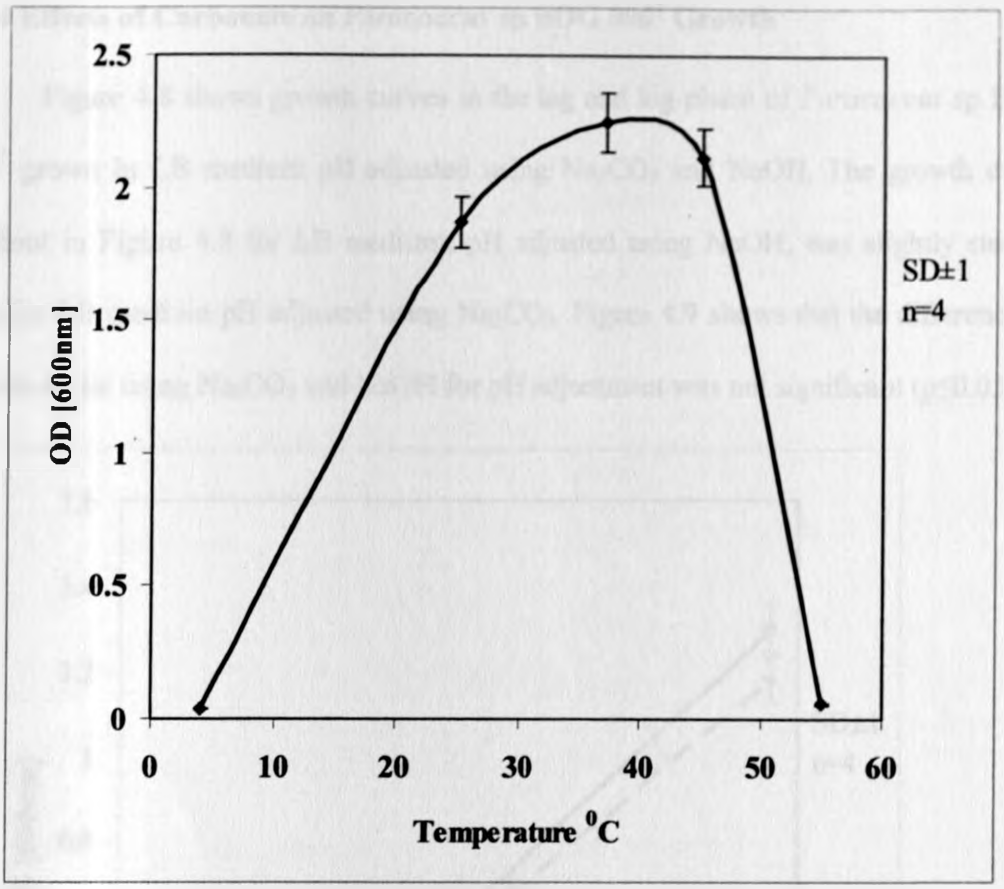

Figure 4.7: ***Paracoccus*** **sp BOG 006^{T} biomass content after 24 h of growth at different temperature conditions.**

4.1.4 Effects of Carbonate on *Paracoccus* sp BOG 006^T Growth

Figure 4.8 shows growth curves in the lag and log phase of *Paracoccus* sp BOG 006^T grown in LB medium pH adjusted using Na_2CO_3 and NaOH. The growth curve gradient in Figure 4.8 for LB medium, pH adjusted using NaOH, was slightly steeper than for LB medium pH adjusted using Na_2CO_3. Figure 4.9 shows that the difference in growth while using Na_2CO_3 and NaOH for pH adjustment was not significant ($p \leq 0.05$).

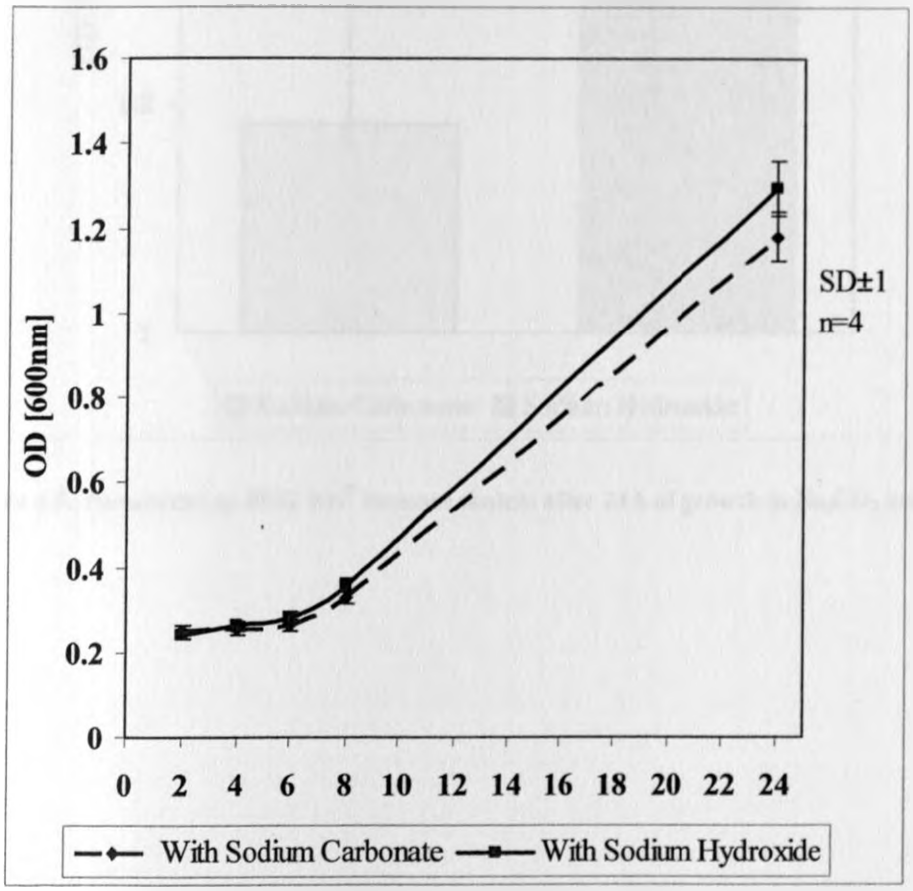

Figure 4.8: The effect of carbonate on *Paracoccus* sp BOG 006^T growth.

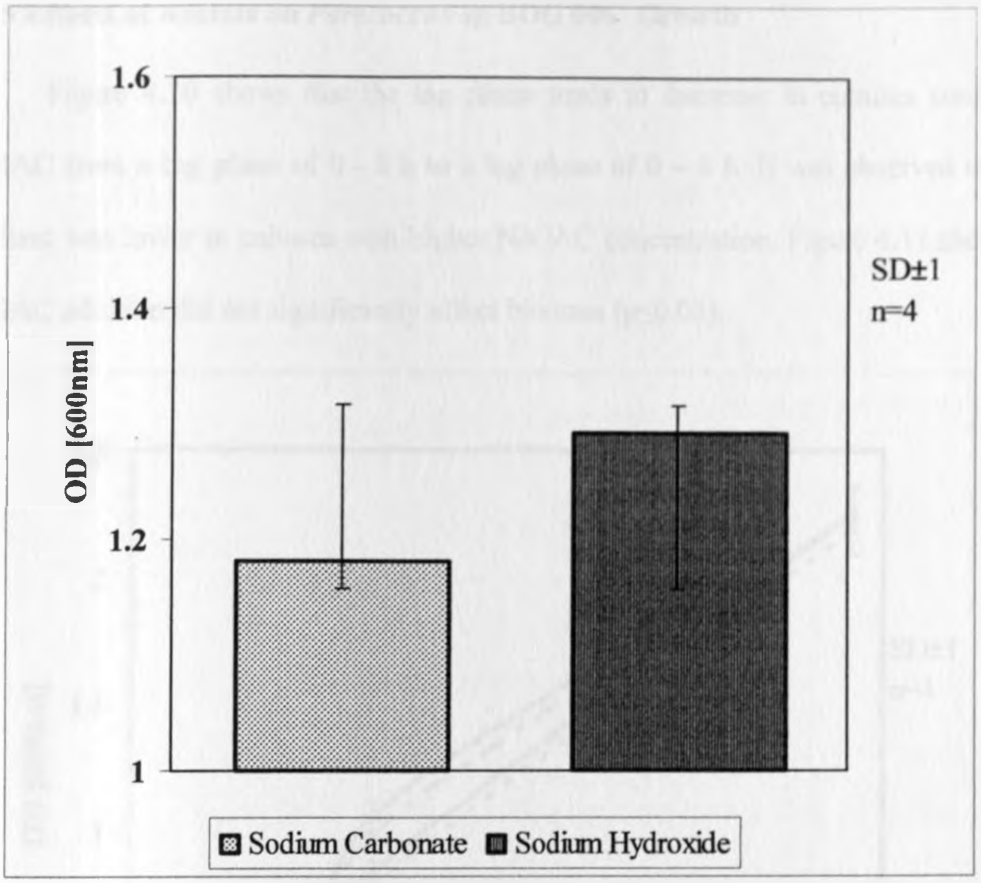

Figure 4.9: *Paracoccus* sp BOG 006^T biomass content after 24 h of growth in Na_2CO_3 and NaOH.

4.1.5 Effects of Acetate on *Paracoccus* sp BOG 006[T] Growth

Figure 4.10 shows that the lag phase tends to decrease in cultures containing NaOAC from a lag phase of 0 - 6 h to a lag phase of 0 – 4 h. It was observed that the gradient was lower in cultures with higher NaOAC concentration. Figure 4.11 show that NaOAC addition did not significantly affect biomass ($p \leq 0.05$).

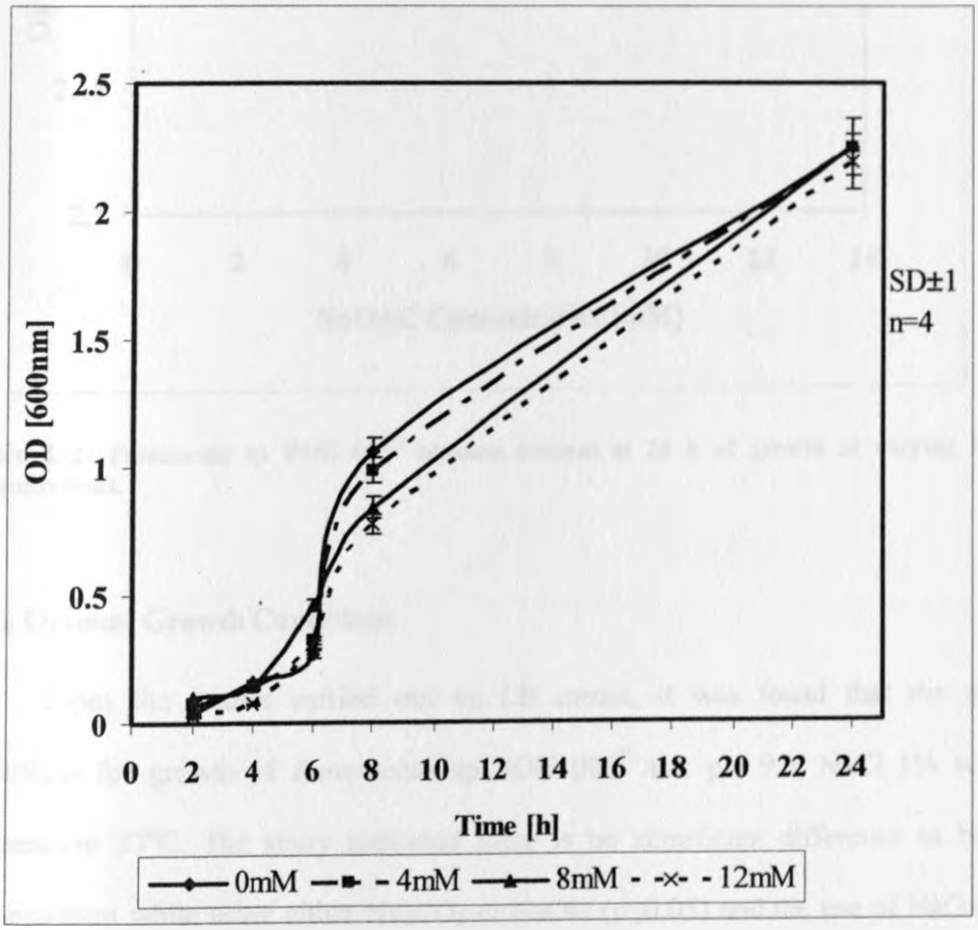

Figure 4.10: Growth of *Paracoccus* sp BOG 006[T] cultured in LB medium for 24 h with varying NaOAC concentration and biomass measured at λ 600 nm.

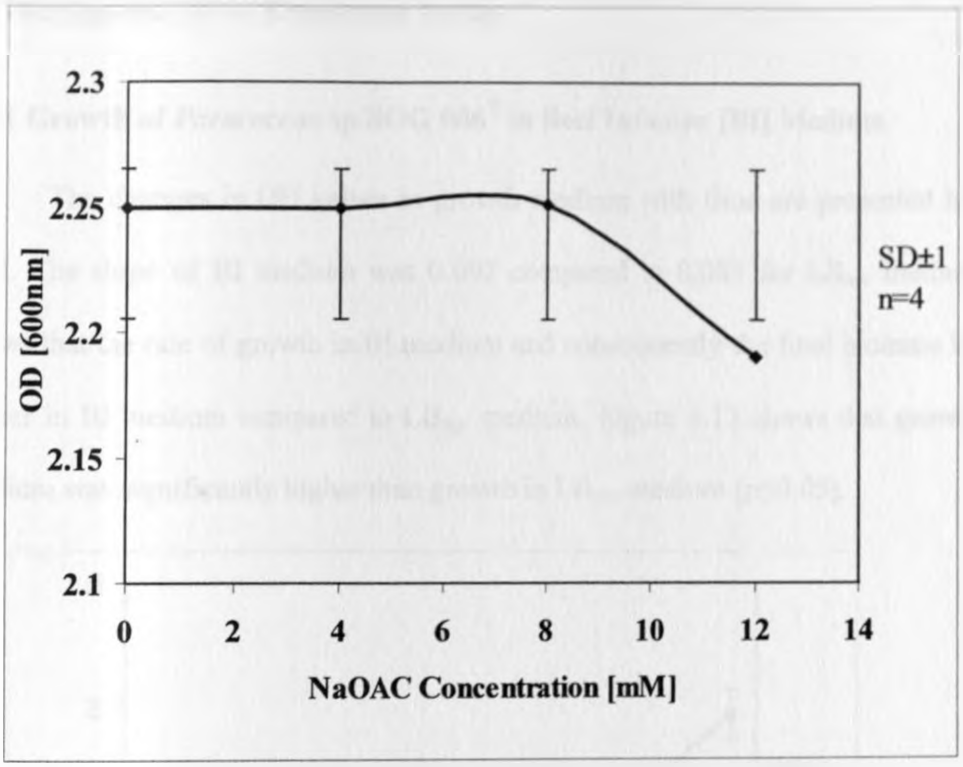

Figure 4.11: *Paracoccus* sp BOG 006^T biomass content at 24 h of growth at varying NaOAC concentrations.

4.1.6 Optimal Growth Conditions

From the studies carried out on LB media, it was found that the optimal conditions for growth of *Paracoccus* sp BOG 006^T are: pH 9.0, NaCl 1% w/v and temperature 37°C. The study indicated there is no significant difference in biomass accumulation while using either Na_2CO_3 or NaOH ($p \leq 0.05$) and the use of NaOAC did not lead to a significant variation in growth ($p \leq 0.05$). This medium utilized for *Paracoccus* sp BOG 006^T growth of under optimal conditions was termed LB_{opt}.

4.2 Development of an Alternative Media

4.2.1 Growth of *Paracoccus* sp BOG 006^T in Beef Infusion [BI] Medium

The changes in OD values in growth medium with time are presented in Figure 4.12. The slope of BI medium was 0.092 compared to 0.083 for LB_{opt} medium. This shows that the rate of growth in BI medium and consequently the final biomass is 67.3% higher in BI medium compared to LB_{opt} medium. Figure 4.13 shows that growth in BI medium was significantly higher than growth in LB_{opt} medium ($p \leq 0.05$).

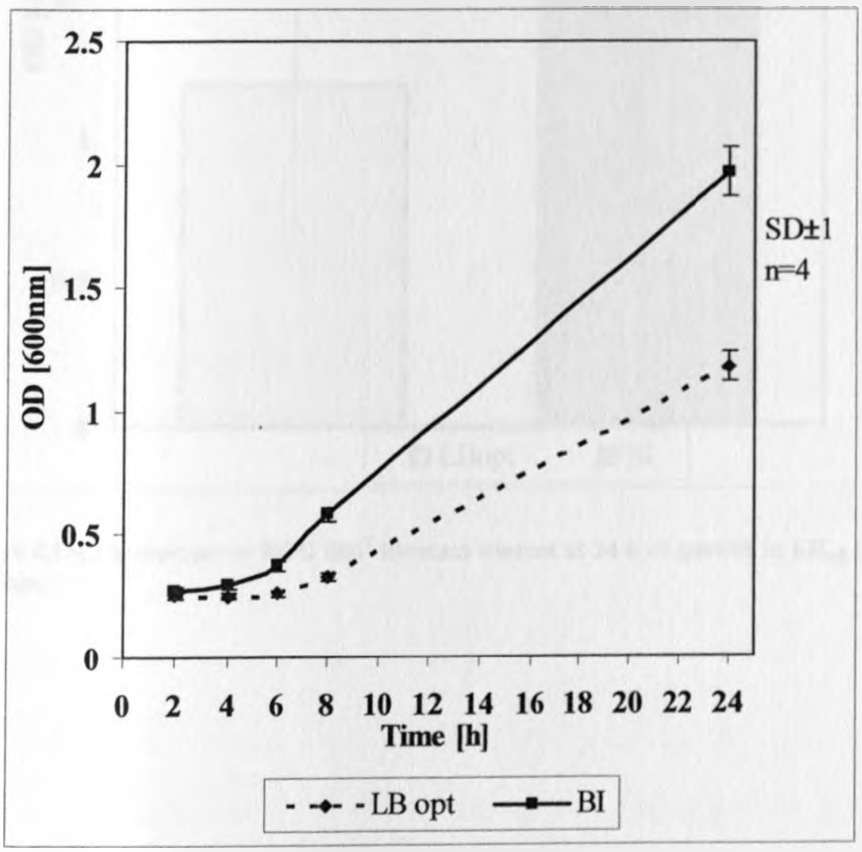

Figure 4.12: The effect of the LB_{opt} medium and BI medium on *Paracoccus* sp BOG 006^T growth.

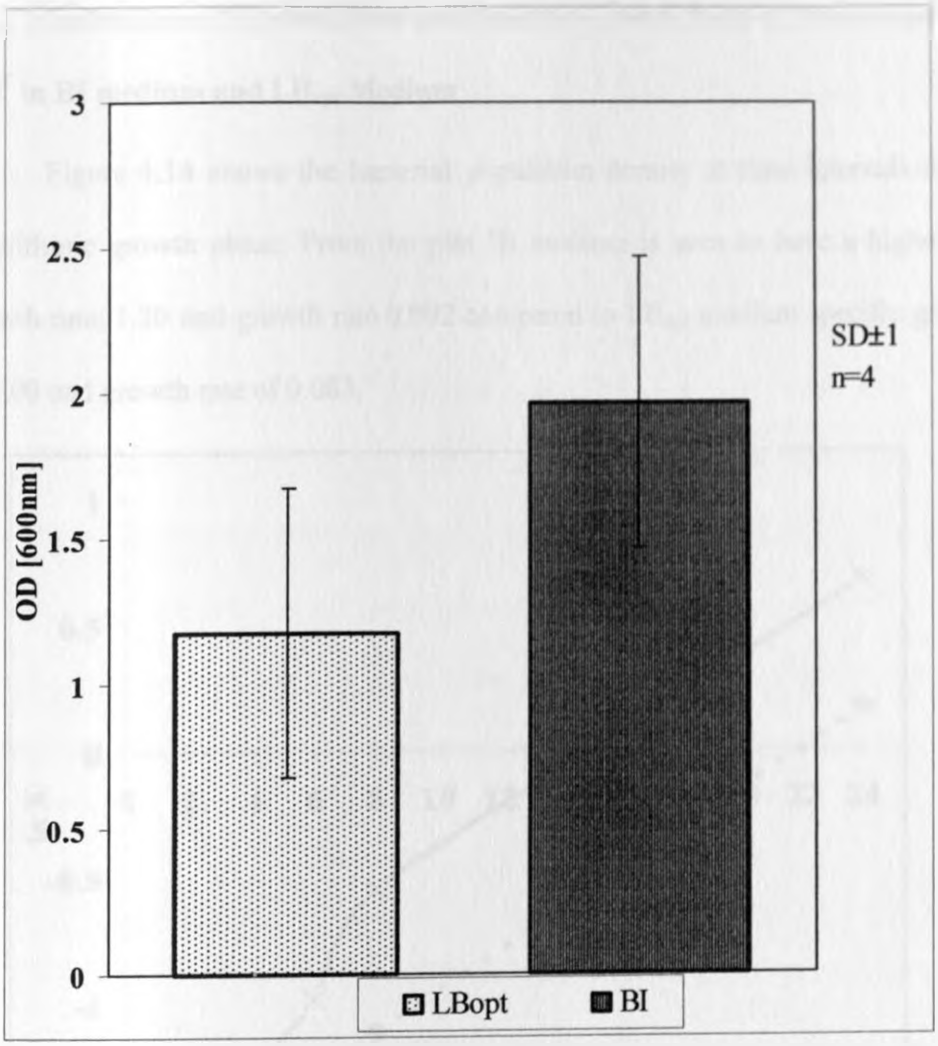

Figure 4.13: ***Paracoccus*** **sp BOG 006^T biomass content at 24 h of growth in LB_{opt} medium and in BI medium.**

4.2.1.1 Estimation of Growth Rate and Specific Growth Rate of *Paracoccus* sp BOG 006^T in BI medium and LB_{opt} Medium

Figure 4.14 shows the bacterial population density at time intervals during the logarithmic growth phase. From the plot BI medium is seen to have a higher specific growth rate; 1.20 and growth rate 0.092 compared to LB_{opt} medium specific growth rate of 1.00 and growth rate of 0.083.

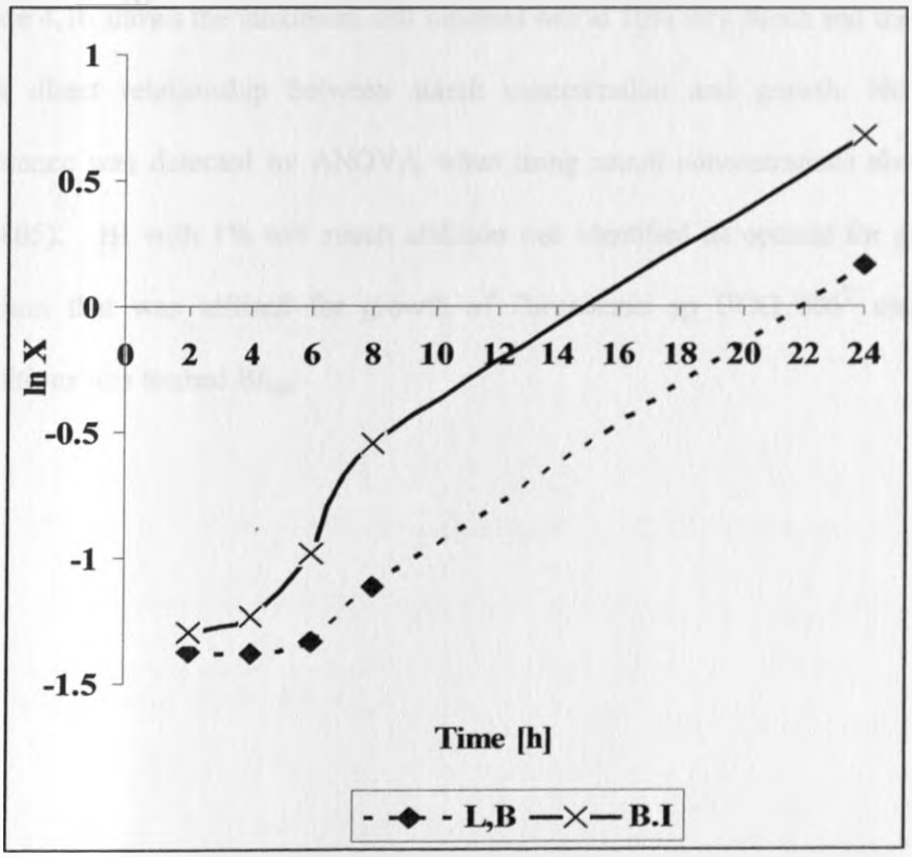

Figure 4.14: Specific growth rate of *Paracoccus* sp BOG 006^T cultured in BI medium and LB_{opt} medium for 24 h.

4.2.2 Effect of Starch on *Paracoccus* sp BOG 006^T Growth

Figure 4.15 shows the effect of 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10% w/v starch on growth. From the gradient of the growth curves in Figure 4.15 it was seen that above 1% w/v starch addition, biomass increased at a slower rate. The growth rate is seen to decrease from 0.098 at 1% w/v, 0.087 at 2.5% w/v, 0.062 at 5.0% w/v, and 0.045 at 7.5% w/v and ultimately reaches a minimal rate of 0.027 at 10% w/v starch. The plot in Figure 4.16 shows the maximum cell biomass was at 10% w/v starch and the occurrence of a direct relationship between starch concentration and growth. No significant difference was detected by ANOVA when using starch concentrations above 1% w/v ($p \leq 0.05$). BI with 1% w/v starch addition was identified as optimal for growth. This medium that was utilized for growth of *Paracoccus* sp BOG 006^T under optimal conditions was termed BI_{opt}.

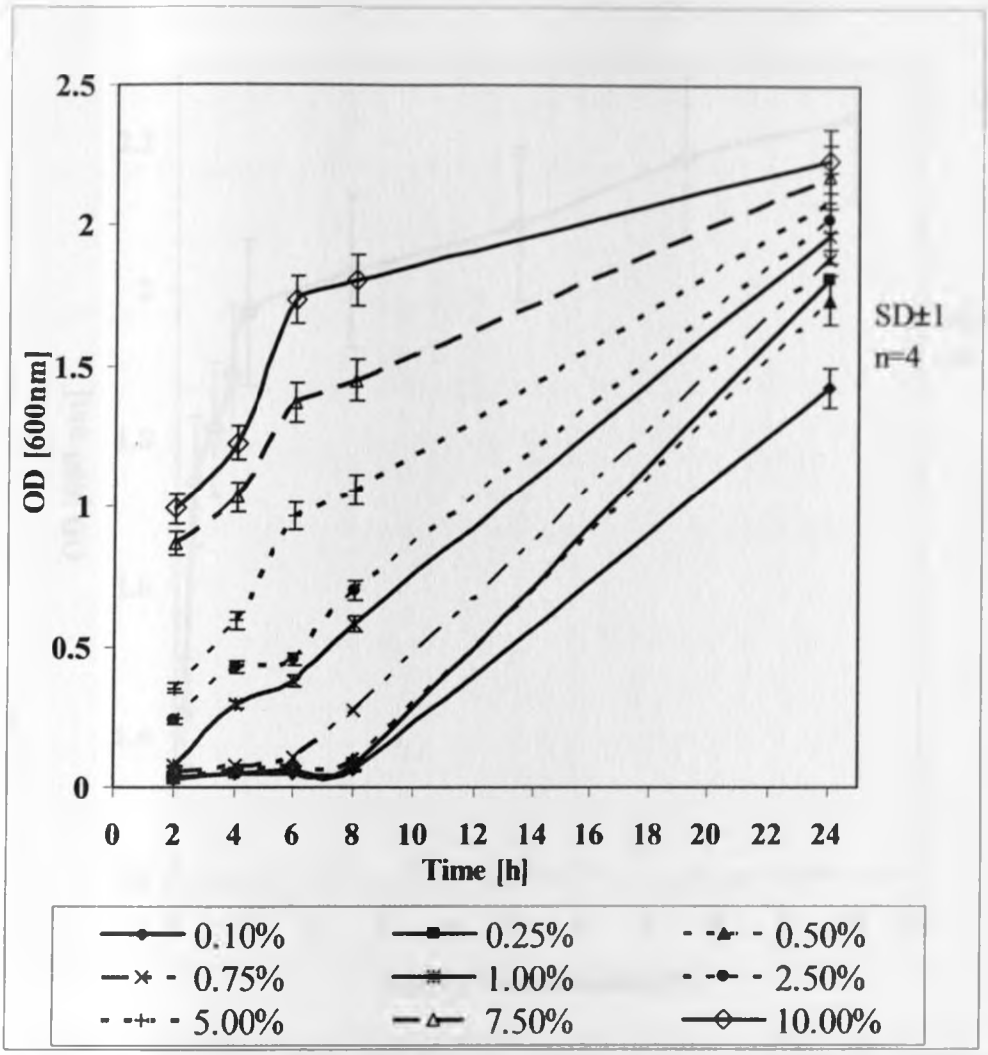

Figure 4.15: Effect of starch on *Paracoccus* sp BOG 006[T] cultured in BI medium for 24 h and the biomass measured as OD at λ 600 nm.

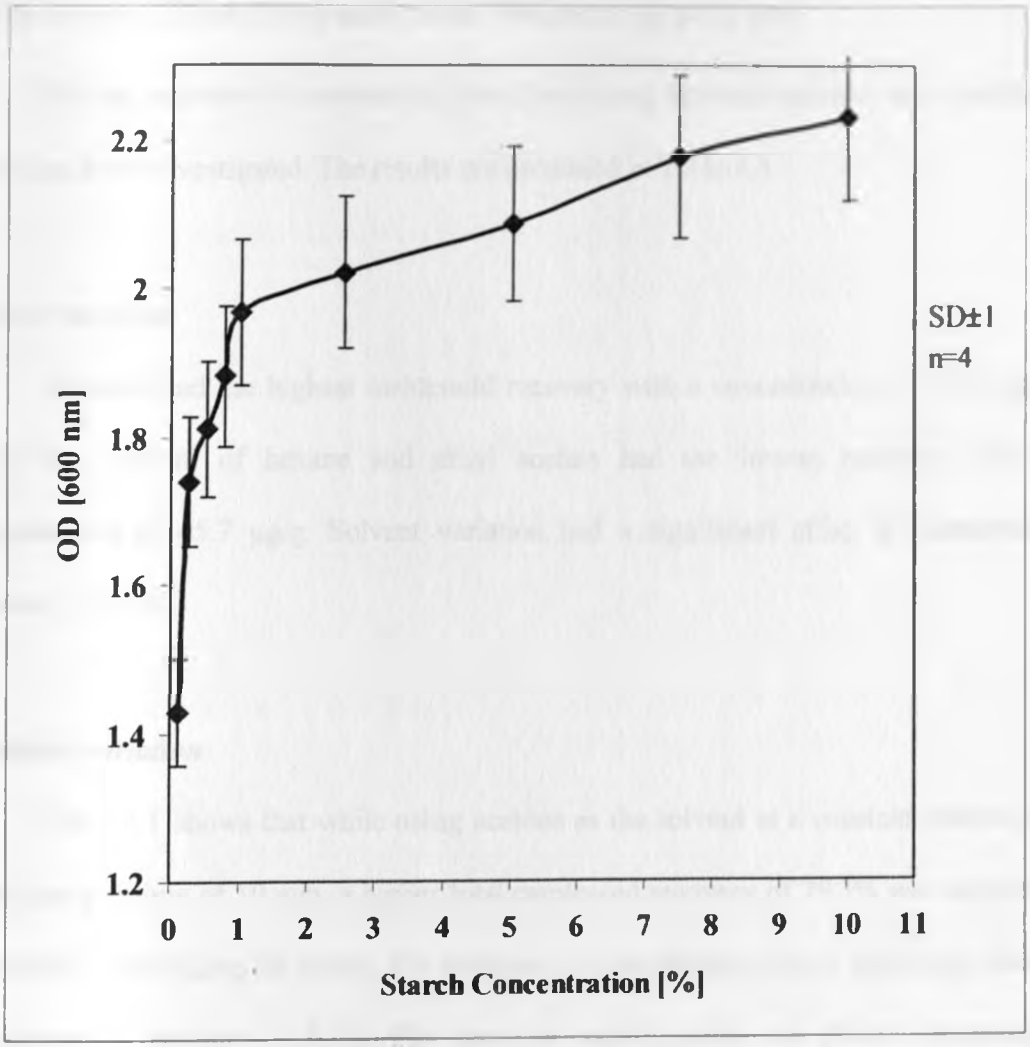

Figure 4.16: ***Paracoccus*** **sp BOG 006[T] biomass content at 24 h of growth at varying starch concentrations.**

4.3 Recovery of Total Carotenoids from *Paracoccus* sp BOG 006^T

For the recovery of carotenoids from the culture, different solvents and condition variations were investigated. The results are presented in Table 4.1.

Solvent variation

Acetone had the highest carotenoid recovery with a concentration of 3745 μg/g while the mixture of hexane and ethyl acetate had the lowest recovery with a concentration of 45.7 μg/g. Solvent variation had a significant effect in carotenoids recovery ($p \leq 0.05$).

Condition variation

Table 4.1 shows that while using acetone as the solvent at a constant rotation of 6000 rpm and time of 10 min, a higher total carotenoid recovery of 29.3% was achieved than when centrifuging for 5 min. The duration of centrifugation had a significant effect in carotenoids recovery ($p \leq 0.05$). The speed of centrifugation also affects carotenoids recovery significantly ($p \leq 0.05$).

Statistical analysis by ANOVA showed there was an interaction between solvent and centrifugation time, solvent and speed of rotation and solvent, rotation speed and time. The interaction between solvent and rotation speed was of greater significance than the interaction between solvent and time ($P \leq 0.05$).

Figure 4.17 shows the percentage of total carotenoids recovered from the *Paracoccus* sp. BOG 006^T cells harvested from BI_{opt} and LB_{opt} and total carotenoids recovered from the growth media. In LB_{opt}, 97% of the crude carotenoids were extracted

from the harvested cells and only 3% from the growth media. In BI_{opt}, 96% of the carotenoids were from the harvested cells and 4% from the growth media.

The plot indicated that total carotenoids recovered from the cells grown LB_{opt} was significantly higher than from cells grown in BI_{opt} ($p \leq 0.05$).

Table 4.1: Total carotenoid yields (µg) per cell wet weight (g) extracted from LB_{opt} using varying solvent and condition treatments.

Exp	Solvent	Rpm	Time [min]	Total Carotenoids Recovered µg/g
1	Acetone	4000	5	2767.3±0.007*
2	Acetone	4000	10	2864.5±0.010*
3	Acetone	6000	5	2896.4±0.002*
4	Acetone	6000	10	3745±0.074*
5	Acetone: Hexane [2:1]	4000	5	869.1±0.013*
6	Acetone: Hexane [2:1]	4000	10	814.7±0.028*
7	Acetone: Hexane [2:1]	6000	5	919.1±0.030*
8	Acetone: Hexane [2:1]	6000	10	963.4±0.014*
9	Hexane: Ethyl acetate [1:1]	4000	5	45.7±0.007*
10	Hexane: Ethyl acetate [1:1]	4000	10	84.3±0.007*
11	Hexane: Ethyl acetate [1:1]	6000	5	90±0.022*
12	Hexane: Ethyl acetate [1:1]	6000	10	121.5±0.024*
13	Dichloromethane: Methanol [4:1]	4000	5	1802.4±0.019*
14	Dichloromethane: Methanol [4:1]	4000	10	2567.2±0.010*
15	Dichloromethane: Methanol [4:1]	6000	5	2632.9±0.024*
16	Dichloromethane: Methanol [4:1]	6000	10	3276.1±0.110*

Note: * represent mean values ± 1 SD (n = 4).

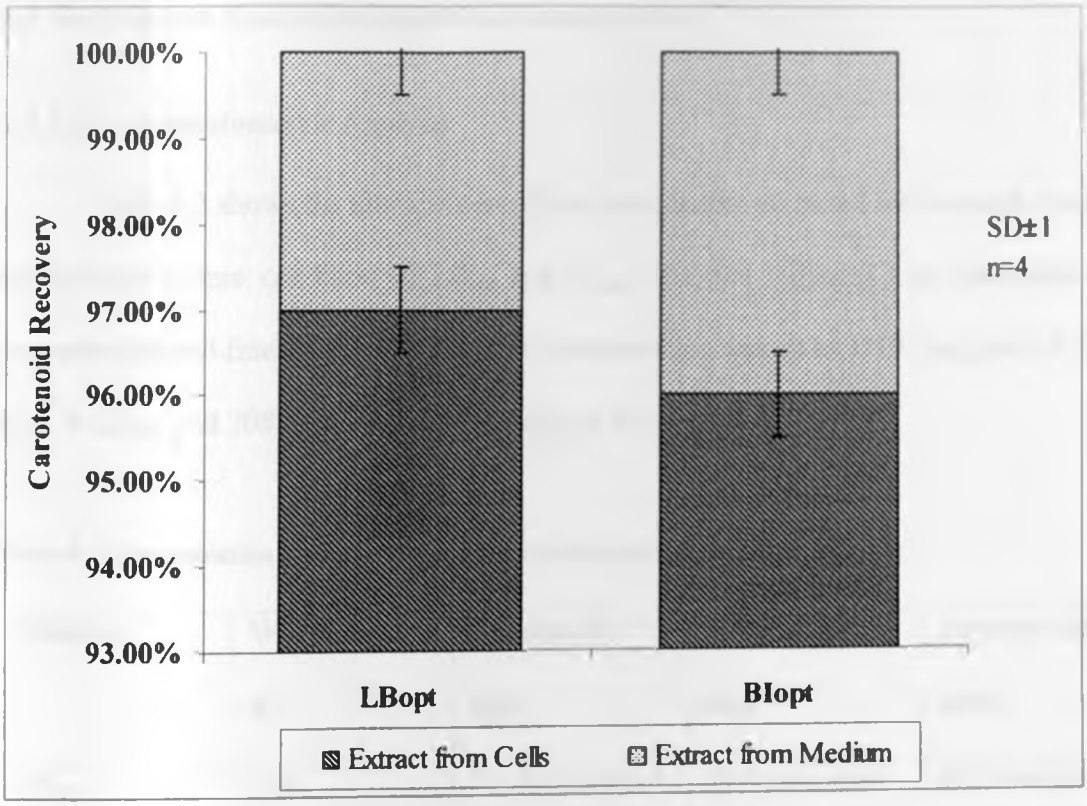

Figure 4.17: Carotenoids recovery from the *Paracoccus* sp BOG 006[1] cells and media.

4.4 Analysis and Quantification of Carotenoids

4.4.1 Spectrophotometric Analysis

Table 4.2 shows the total carotenoids and astaxanthin extracted with acetone from the optimal culture condition of LB_{opt} and BI_{opt}. The plot indicates total carotenoids concentration and free astaxanthin pigments concentration was up to 3719.7 µg/g and 413 µg/g in LB_{opt}, and 2087.7 µg/g and 231.8 µg/g in BI_{opt}.

Table 4.2: Concentration of total carotenoids and astaxanthin pigments.

Medium	Wet-weight g/l	Carotenoid µg/l	Carotenoid µg/g	Astaxanthin µg/g
LB_{opt}	3.00	11159.7±0.016*	3719.9±0.016*	413.1±0.016*
BI_{opt}	5.02	10480.2±0.010*	2087.7±0.010*	231.8±0.010*

Note: * represent mean values ± 1 SD (n = 4).

4.4.1.1 Absorbance Spectrum of *Paracoccus* sp BOG 006[T] Crude Extract

Analysis by spectrometer in Figure 4.18 revealed that the crude carotenoid extract absorbs UV radiation strongly at λ below 240 nm and absorbs visible light near 450 nm.

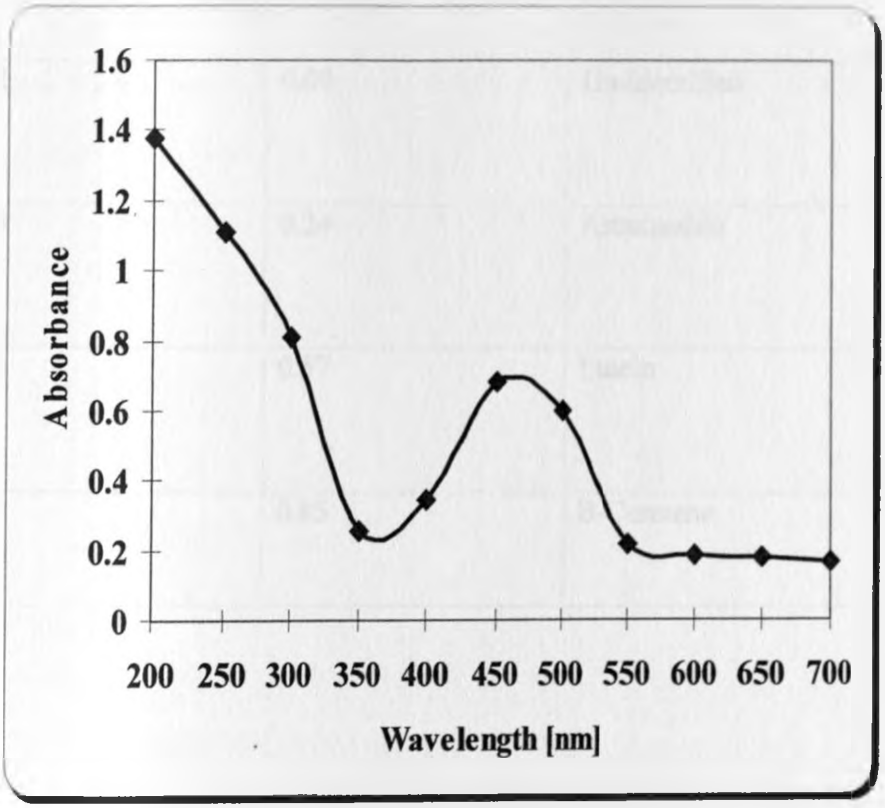

Figure 4.18: Energy absorbed by the crude extract as a function of wavelength.

Table 4.3: Identification of carotenoids.

Band	R_F	Carotenoid
B1	0.06	Un-identified
B2	0.09	Un-identified
B3	0.24	Astaxanthin
B4	0.57	Lutein
B5	0.85	B-Carotene

4.4.3 Chromatographic Fractionation of Carotenoids

Figure 4.20 shows the absorbance of fractions collected from OCC, measured at 450nm. The collected fractions were pooled into 7 fractions as represented in Table 4.4 and the fractions analyzed by TLC for qualitative analysis. It was observed that fraction 2 corresponded to B4 in Table 4.3, fraction 3 corresponded with B3 and fraction 4 corresponded with B5.

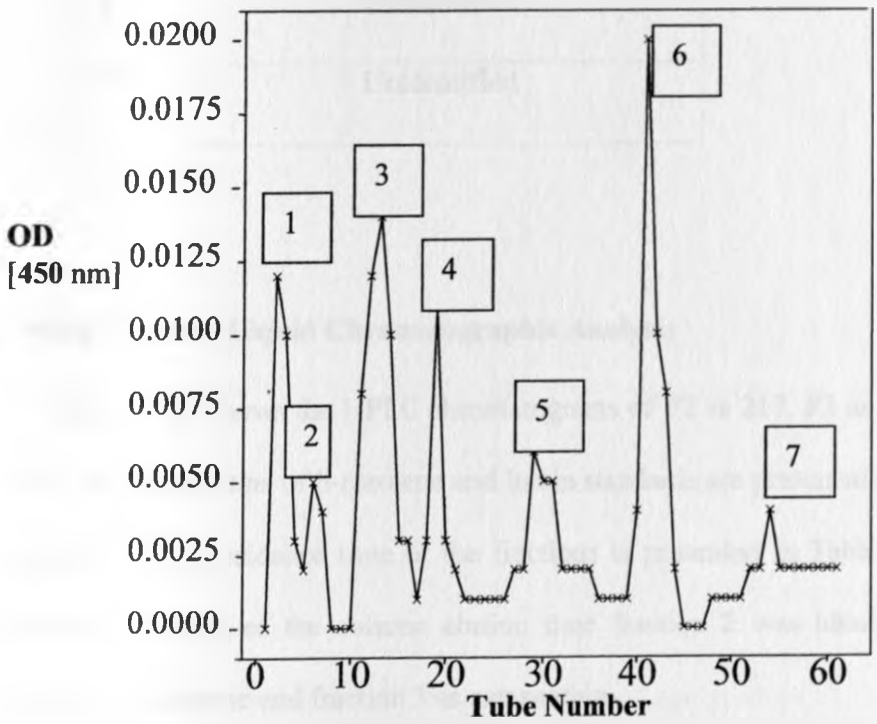

Figure 4.20: Fractions' chromatogram from OCC measured at 450 nm.

Table 4.4: Identification of fractions collected from OCC.

Fraction	TLC band
1	Unidentified
2	B4
3	B3
4	B5
5	Unidentified
6	Unidentified
7	Unidentified

4.4.4 High Pressure Liquid Chromatographic Analysis

Figure 4.21 shows the HPLC chromatograms of F2 as 217, F3 as 205, and F4 as 213. The chromatograms of β-carotene and lutein standards are presented as 202 and 210 respectively. . The residence time of the fractions is presented in Table 4.5. From the comparative analysis of the column elution time fraction 2 was identified as lutein, fraction 4 as β-carotene and fraction 3 as astaxanthin.

Quantification was done on the basis of peak area of the fractions. Peak areas were directly proportional to the concentration. The concentration of astaxanthin the major carotenoid of *Paracoccus* sp. BOG 006^T was observed as 400 µg/g.

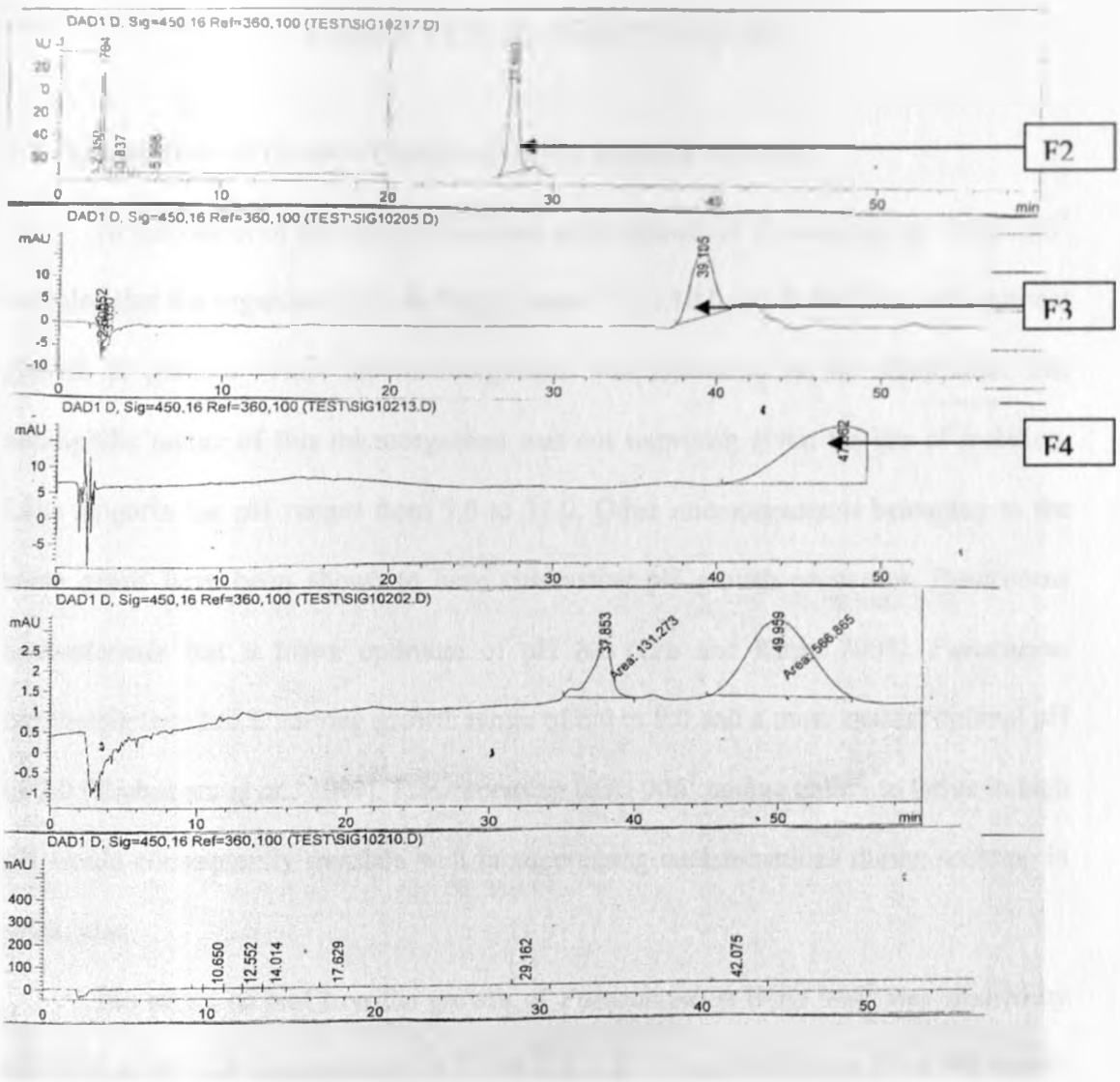

Figure 4.21: HPLC chromatogram.

Table 4.5: Fraction residence time in HPLC column and the identification of the carotenoid fractions.

Sample	Elution time	Carotenoid
F2	23.814 - 23.9	Lutein
F3	39.071 - 39.105	Astaxanthin
F4	47.642 - 47.662	B-Carotene

CHAPTER 5: DISCUSSION

5.1 Optimization of Growth Conditions in the Baseline Medium

At the outset of the study, evaluation of the growth of *Paracoccus* sp. BOG 006^T revealed that the organism grew in the pH range 7.5 to 10.0 in LB medium, with optimal growth at pH 9.0. Thus the microorganism was shown to be an alkaliphile. The alkaliphilic nature of this microorganism was not surprising given the site of isolation; Lake Bogoria has pH ranges from 9.0 to 11.0. Other microorganisms belonging to the same genus have been shown to have contrasting pH growth properties. *Paracoccus haeundaensis* had a lower optimum of pH 8.0 (Lee and Kim., 2004), *Paracoccus carotenifaciens* had a varying growth range of 6.0 to 9.0 and a more neutral optimal pH of 7.0 (Tsubokura *et al.*, 1999). *Paracoccus* sp BOG 006^T unique ability to thrive in high pH would consequently translate well in suppressing contaminations during scale-up in production.

The effect of NaCl on the growth of *Paracoccus* sp BOG 006^T was studied by doubling up the salt concentration; 0.0, 1.0, 2.5, 5.0, 7.5 and10.0% w/v. From the results of the concentrations investigated it was found that *Paracoccus* sp BOG 006^T had a NaCl tolerance range of 0.0 to 2.5% w/v. From the study 1% NaCl concentration was observed to produce optimal cell biomass. *Paracoccus* sp BOG 006^T growth was shown to have an inverse relationship with NaCl concentration. Upon comparison with other astaxanthin producing *Paracoccus* species, *Paracoccus* sp BOG 006^T was seen to exhibit a contrasting NaCl growth range to *Paracoccus haeundaensis* that grew at a broader and higher optimum of between 1.0% w/v and 6.0% w/v (Lee and Kim., 2004). However *Paracoccus* sp BOG 006^T was observed to be similar to both *Paracoccus haeundaensis*

(Lee and Kim., 2004) and *Paracoccus marcusii* (Harker *et al.*, 1998) in that they all had poor growth at NaCl concentrations above 7.0% w/v.

The effect of temperature was also analyzed on *Paracoccus* sp BOG 006^T growth. *Paracoccus* sp BOG 006^T was observed to grow best between 25°C and 45°C. A higher growth rate was observed at 45°C; although at 37°C a higher biomass concentration at the 24th hour of growth occurred. Growth of *Paracoccus* sp BOG 006^T was negligible at 4°C and 55°C; the reduced biomass production was attributed to dormancy of cell growth activities at the lowered temperature and the denaturation of the cell protein molecules at the higher temperature. *Paracoccus* sp BOG 006^T ability to thrive at 45°C without denaturing was recognised as a unique feature. Other *Paracoccus* species like *Paracoccus carotenifaciens* had no growth at temperatures above 37°C (Tsubokura *et al.*, 1999). Additionally, *Paracoccus carotenifaciens* (Tsubokura *et al.*, 1999), *Paracoccus marcusii* (Harker *et al.*, 1998) and *Paracoccus haeundaensis* (Lee and Kim, 2004) had lower optimal temperatures of 28°C, 25°C to 30°C and 25°C respectively.

Apart from pH, NaCl and temperature growth was also tested in the presence of different alkaline agents, *Paracoccus* sp BOG 006^T had 9.5% higher cell growth in LB medium with NaOH compared to growth in LB medium with Na_2CO_3. Statistical analysis showed that the samples with OH^- ions had no significant difference ($p \leq 0.05$) from the samples with CO_3^{2-} ions. The subsequent deduction from these results was that Na_2CO_3 role in the growth of *Paracoccus* sp BOG 006^T was in the adjustment of medium pH rather than in the provision of the actual cell substance as reported by Grant et al. (1990). This finding was similar to those of Atlas (1984) which, indicated that unlike

carbon, hydrogen and oxygen which are used structurally in the formation of cells, metal ions serve a functional role, like the control of pH, in biomass production.

As the final phase in optimization of *Paracoccus* sp BOG 006^T growth conditions, the effect of acetate on growth was investigated. A reduction of the lag phase from 0 - 6 h to 0 - 4 h was observed in the *Paracoccus* sp BOG 006^T growth curve when NaOAC was added to LB medium. It was also found that the addition of 4 mM, 8 mM and 12 mM acetate to LB medium lead to a slower cell growth of 0.091, 0.087 and 0.085 from 0.092 observed when there was no NaOAC. Using statistical analysis it was deduced that acetate addition caused no significant difference on cell mass growth ($p \leq 0.05$).

The findings in this study contradicted work that had found enhanced growth rates in the presence of acetate (Meyer and Du-Preez, 1993). However, the findings were concurrent with work of Borowitzka *et al.* (1991) which reported slow cell growth and low final cell densities when acetate was used for batch cell growth.

At the completion of growth condition optimization, it was concluded that optimal growth conditions were pH 9.0; adjusted using either Na_2CO_3 or NaOH, 1% NaCl and 37^0C. The LB medium utilized for growths of *Paracoccus* sp BOG 006^T under these conditions was termed LB_{opt}.

5.2 Development of an Alternative Medium

Evaluation of *Paracoccus* sp BOG 006^T growth in BI medium compared to LB_{opt} medium showed a 67.3% higher biomass in BI medium. This difference was considered significant ($p \leq 0.05$). The higher biomass production in BI medium was expected because of its rich nutrient base which provided a good environment for microbial growth (Neil *et al.*, 2004).

The growth rate and the specific growth rate in BI medium and LB_{opt} medium was determined as; 0.083 and 1.0 in LB_{opt} medium and 0.092 and 1.2 in BI medium. Beef Infusion medium was observed to have a steeper slope of 0.092; this indicated a greater rate of growth and a shorter doubling time. In the calculation of growth rate and specific growth rate the growing bacterial population was regarded as an autocatalytic system. The catalyzed reaction, growth, resulted in the production of the catalysts which is the living matter (Neijssei and Tempest., 1976). Due to the exponential nature of growth, growth was expressed in terms of the logarithm of the cells mass per unit time.

In the study of the effect of starch on *Paracoccus* sp BOG 006^T growth, maximal cell biomass was observed at 10% w/v starch. This indicated a direct relationship between biomass accumulation and starch addition. It was however observed that an increase in starch concentrations above 1% w/v starch did not translate to a significant increase in biomass ($p \leq 0.05$). Additionally, the rates of biomass accumulation were observed to decrease from 0.098 at 1% w/v, 0.087 at 2.5% w/v, 0.062 at 5.0% w/v, and 0.045 at 7.5% w/v to 0.027 at 10% w/v starch. Though maximal cell biomass was seen at 10% w/v, 1% w/v was selected as the optimal addition due to the reflected decline in the

rate of biomass accumulation at concentrations above 1% w/v starch addition. This decline in the growth rate was attributed to the effect of substrate inhibition.

It was therefore concluded that *Paracoccus* sp BOG 006^T growth in BI medium was optimal at 1% w/v starch addition. This BI medium utilized for growth of *Paracoccus* sp BOG 006^T under optimal conditions was denoted as BI_{opt}

5.3 Recovery of Total Carotenoids from *Paracoccus* sp BOG 006^T

The aim of the recovery experiment was to develop a process of carotene extraction that could effectively extract carotenoids from the *Paracoccus* sp BOG 006^T cells. When varying solvents were used the total carotenoid recovery was observed to be highest with acetone at 3745 µg/g and lowest with hexane and ethyl acetate (1:1) at 45.7 µg/g. The higher recovery while using acetone was attributed by Houghton and Raman., (1998) to acetone water miscibility that allowed better solvent penetration of freshly harvested culture cells. Other microorganisms that have previously used acetone for carotenoids extraction are *Haematoccous pluvialis* by Harker *et al.* (1996) and in *Paracoccus haeundaensis* by Lee and Kim., (2004).

Paracoccus sp BOG 006^T also showed an increase in total carotenoid recovery when the extraction time and agitation speed (rpm) were increased. It was observe that while using acetone as the solvent at a constant rotation of 6000 rpm, centrifugation for 10 min gave 29.3% better carotenoids yield than extraction for 5 min. Similarly, while using acetone and centrifuging for 10 min, 6000 rpm rotation gave 30.7% better carotenoid recovery than 4000 rpm rotation.

The effectiveness of the various extraction systems in the study were analyzed in relation to the total carotenoids in μg/g, recovered from the cell mass. An extraction system must be adapted to the characteristics of the source of extraction. The extraction system should release all carotenoids from the cell matrix and bring them into solution without alteration (Rodriguez-Amaya and Kimura., 2004).

5.3.1 Estimation of Total Carotenoid Recovery of *Paracoccus* sp BOG 006^T in BI_{opt} Medium and LB_{opt} Medium

Cell biomass and total carotenoid concentration extracted from 1L of LB_{opt} was 3 g/l and 3719.9μg/g. BI_{opt} yielded 5 g/l which was 67.3% higher than in LB_{opt} and 2087.7 μg/g total carotenoids which was 43.9 % lower than in LB_{opt}. Analysis found that the sub-optimal medium LB_{opt} had better carotenoid recovery than the optimal medium BI_{opt}. Though unexpected these findings were not surprising. Since carotenogenesis in microbes is a response to environmental stress like high irradiation, salt addition and acetate addition (Choi *et al.*, 2002) when the growth environment was not stressed there was an accumulation of biomass without subsequent carotenoid accumulation. These findings were similar to Cifuentes *et al.* (2003) were sub-optimal medium was used for biomass and carotenoid production.

5.4 Analysis and Quantification of Carotenoids

Analysis of *Paracoccus* sp BOG 006^T carotenoid crude extract deduced that the extract contained both apolar carotenes and the more polar xanthophylls. It was observed that the carotenoids extract appeared orange in color because the sample absorbed light at

the blue end of the visible spectrum 400 – 700 nm. This allowed light of higher λ like yellow orange and red to pass through.

Qualitative identification by thin layer chromatography analysis identified 5 bands. Three of the bands were conclusively identified as B3-astaxanthin, B4-lutein and B5- β-carotene. Two bands, B1 and B2 had inconclusive identification and would require further analysis for identification. The identification of the bands was done by comparing the R_F values of compounds with R_F of standards run on the same plate at the same time.

Thin layer chromatography analysis was reported as an efficient monitoring process for qualitative identification purposes (Houghton and Raman., 1998). However, the disadvantage observed in the present study was the degradation of the carotenoids on the plate because of atmospheric exposure.

Upon the identification of the thin layer chromatography bands, isolation and purification of the bands was carried out using an open column chromatographic technique. Several fractions with differing polarity were obtained from open column chromatography. Spectrophotometry at 450 nm was then used to group the fractions into 7 fractions. The reported results confirmed that fractions 2, 3 and 4 were pure fractions of lutein, astaxanthin and β-carotene respectively.

The drawback experienced in open column chromatography was the time involved for elution. This was attributed to the slow flow rate of elution. The long retention time in open column chromatographic was attributed to the water present in the stationary phase (Houghton and Raman., 1998).

The pure fractions from open column chromatographic were further quantified by high pressure liquid chromatography. The fractions monitored at 450 nm absorbance were

identified as astaxanthin, lutein and β-carotene by comparing the retention time of samples to the retention times of the standard solutions. Quantitative calculation of the main carotenoid of interest astaxanthin was done using Weber (1990); In *Paracoccus* sp BOG 006[T] the astaxanthin content was 400 µg/g. The production of astaxanthin by this isolate was found to be substantive enough to prompt its biotechnological application in the food and nutraceutical industry.

CHAPTER 6: CONCLUSION

The objective of this study was the optimization of *Paracoccus* sp BOG 006^T growth conditions for biomass production, identification and quantification of the carotenoids secreted. In order to achieve this objective the conditions considered for *Paracoccus* sp BOG 006^T growth were the pH, NaCl concentration, temperature, carbonate requirement and effect of acetate addition. For the optimization of carotenoids production, two media; optimized Luria Bertani (LB_{opt}) and Beef Infusion (BI_{opt}) were evaluated. The effect of solvent and condition variation on carotenoid recovery was evaluated to identify the variables that would give the higher carotenoids recovery.

The results obtained showed that optimal growth and recovery of *Paracoccus* sp BOG 006^T was pH 9.0, 1% NaCl and 37°C in both LB_{opt} and BI_{opt} media. *Paracoccus* sp BOG 006^T tolerance to high pH of up to 10.0 and its ability to grow at high temperatures of up to 45°C lowered susceptibility to contamination. This indicated a good possibility for *Paracoccus* sp BOG 006^T application in industrial production of carotenoids.

Total carotenoids production was observed to be higher in LB medium, a sub-optimal medium for *Paracoccus* sp BOG 006^T growth. Stress seemed to favour the production of carotenoids in *Paracoccus* sp BOG 006^T; indicating that carotenoid production by *Paracoccus* sp BOG 006^T is a reaction to environmental pressure. BI medium was seen to be unfavorable to carotenoid production. The richness of nutrients in BI medium translated to better biomass accumulation but lower carotenoids accumulation.

Carotenoid recovery was highest in acetone compared to hexane: ethyl acetate (1:1), acetone: hexane (2:1), and dichloromethane: methanol (4:1). Extraction was found

to improve with increase in extraction time and centrifuge rotation speed. Centrifugation for 10 min at 6000 rpm produced the best recovery of carotenoids from the cells. The penetrability of the solvent was attributed to its miscibility with water. Therefore extraction of carotenoids from samples with a water fraction is more efficient with solvent systems with water miscible organic solvents like acetone or methanol.

Chromatography was used to identify the carotenoids produced by *Paracoccus* sp. BOG 006^T.The carotenoids identified were lutein, β-carotene and astaxanthin. The main carotenoid produced by *Paracoccus* sp. BOG 006^T was identified as astaxanthin.

CHAPTER 7: RECOMMENDATIONS

From qualitative identification by thin layer chromatography, two bands B1 and B2 were not conclusively identified. Further analysis is recommended to identify the bands.

For the industrial application of the results from the study, more studies targeted on medium formulation for the up-scaling of carotenoid production should be undertaken. Though the investigated conditions are optimal for the growth of a seed and batch culture, these findings only represent the first step in optimizing carotenoids production of *Paracoccus* sp BOG 006^T. During the upscaling of carotenoid production other factors that affect carotenoid production like agitation, speed and air flow should be investigated.

Since optimization of carotenoid production and recovery of the carotenoids from *Paracoccus* sp BOG 006^T is expected to directly affect the production cost, further work capturing the economics and cost analysis of production should be done. This would assess the viability and profitability of industrial production of the carotenoid.

Because of the consumers and regulatory agencies requiring extracts that contain no residual solvents, alternative extraction methods to solvent extraction should be investigated. Supercritical fluid extraction that would use CO_2 should be investigated. CO_2 is recommended for carotenoid extraction because it is non toxic, non flammable, environmentally acceptable and has a low critical temperature of 31°C thus suitable for extraction of thermolabile compounds like carotenoids.

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4.4.2 Thin Layer Chromatographic Analysis

Figure 4.19 shows the 5 bands produced after TLC analysis of the crude extract. The calculated R_f of the bands are presented in Table 4.3 and from these R_f values calculated in Table 4.3 the carotenoids present in the crude extract were identified as; B4 as lutein, B5 as β-carotene and B3 as astaxanthin. The bands B1 and B2 were not identified.

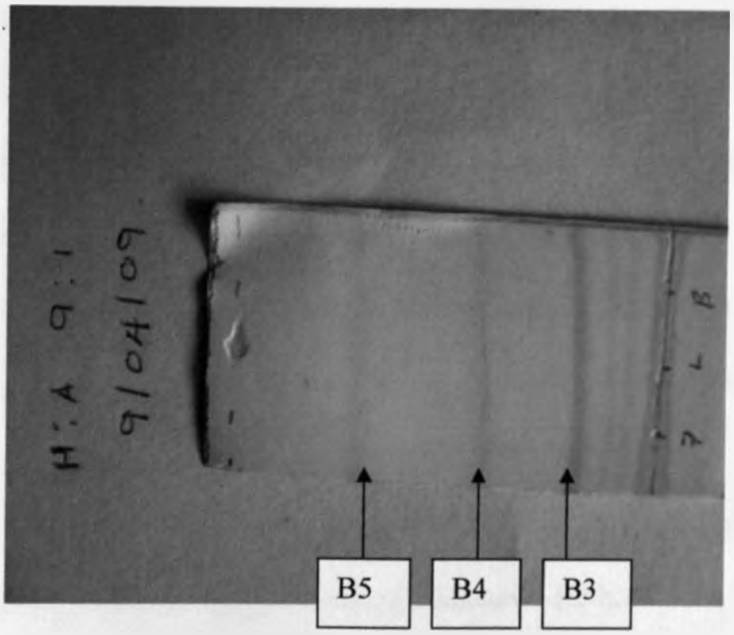

Figure 4.19: ***Paracoccus*** **sp BOG 006[T] crude carotenoid extract spotted and developed as a band.**

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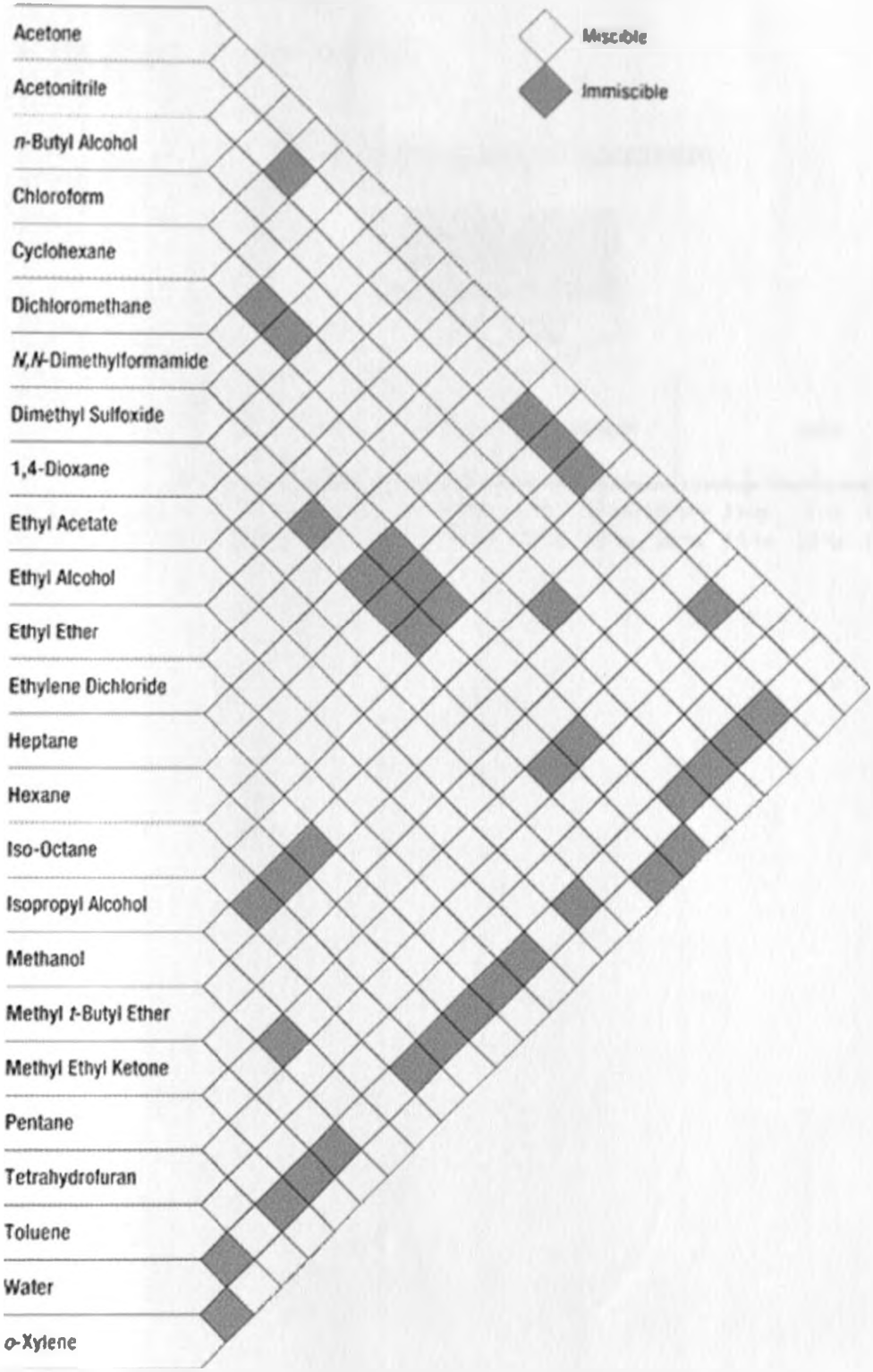
LIST OF APPENDICES

Appendix 1: Polarity Table



Appendix 2: UV-Visible Spectrum