

NOVEL POLYHYDROXYBUTYRATE (PHB) PRODUCTION USING A WASTE DATE SEED FEEDSTOCK

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List of abbreviations and acronyms

<i>ACSSF</i>	Adsorbed carrier solid state fermentation
<i>CHCl₃</i>	Chloroform
<i>Da</i>	Dalton
<i>DCW</i>	Dry cell weight
<i>DSC</i>	Differential scanning calorimetry
<i>FTIR</i>	Fourier transform infrared
<i>h</i>	Hour(s)
<i>HPLC</i>	High performance liquid chromatography
<i>g</i>	Gram(s)
<i>GC</i>	Gas chromatography
<i>GA</i>	Arabic Gum
<i>kg</i>	Kilogram(s)
<i>L</i>	Litre (s)
<i>MCM</i>	Mixture chloroform: methanol
<i>ml</i>	Millilitre(s)
<i>mm</i>	Millimetre(s)
<i>M_w</i>	Molecular weight
<i>Nm</i>	Nanometre(s)
<i>NMR</i>	Nuclear magnetic resonance
<i>OD₆₀₀</i>	Optical density at 600 nanometres
<i>PA</i>	Polyamide
<i>PBAT</i>	Polybutyrate adipate terephthalate succinate
<i>PC</i>	Polycarbonate.
<i>PCL</i>	Polycaprolactone
<i>PHA</i>	Polyhydroxyalkanoates
<i>PHB</i>	Polyhydroxybutyrate
<i>PE</i>	Petroleum ether
<i>PEG</i>	Polyethylene glycol
<i>PET</i>	Polyethylene terephthalate
<i>PLA</i>	Polylactic acid
<i>PUR</i>	Polyurethane
<i>rpm</i>	Round per minute
<i>P</i>	Product
<i>PUF</i>	Polyurethane foam
<i>S</i>	Substrate
<i>scl-PHAs</i>	Short-chain-length
<i>sec</i>	Second(s)
<i>SSF</i>	Solid-state fermentation
<i>TEM</i>	Transmission electron microscope
<i>T_g</i>	Glass transition temperature
<i>TN</i>	Total nitrogen
<i>T_m</i>	Melting temperature
<i>Y_{x/s}</i>	Yield of biomass
<i>Y_{p/s}</i>	Yield of product
<i>μm</i>	Micrometer (s)
<i>Y_{x/s}</i>	Yield of biomass

Novel polyhydroxybutyrate (PHB) production using a waste date seed feedstockRawa Yousuf. The University of Manchester, 8th December 2017,

Submitted for the degree of Doctor of Philosophy

Abstract

Polyhydroxybutyrate (PHB) is a biodegradable, linear polyester that has potential as a promising alternative to petrochemical derived plastics as it possesses the same properties as several current and widely used synthetic, non-biodegradable petrochemical-based plastics. PHB is a natural polyester which is accumulated by many bacteria as an intracellular store of energy and carbon, under stress conditions; limited in one or more essential nutrient, with the carbon source in excess. Currently the PHB production cost is far greater than that of petroleum based plastics. Recent research, therefore, has focused on improving the cost-effective synthesis of PHB from different substrates and microorganisms. The improvement of fermentation processes and strains allowing for PHB to be produced from an inexpensive carbon source is required to compete with synthetic plastics and to mimic their desired properties.

The goal of the work reported in this thesis is to assess the suitability of using waste date seed as a feedstock for PHB production under various stress conditions. Date seeds have a high nutrient content, are available in large quantities and are relatively cheap. The novelty of this study lies in the fact that waste date seed can be used as the feedstock for biopolymer production, based on the development of various techniques to make these nutrients bioavailable for the bacterium, *Cupriavidus necator* for PHB accumulation.

The results include fructose hydrolysis from date seeds and the development of a mass transfer model to describe the process, demonstrating that the high nutrient content of date seeds makes them a promising raw material for microbial growth and that a meaningful amount of PHB can be produced. Using fructose rich waste date seed derived medium, with an initial fructose concentration of 10.8 g/l, maximum dry cell weight and PHB concentrations of 6.3 g/l and 4.6 g/l, respectively, were obtained, giving a PHB content of 73%. An investigation into the suitability of using waste date seed oil extract as an alternative carbon source for PHB synthesis was also carried out. This date seed oil was used as the sole carbon source in a series of microbial fermentation experiments, and the results demonstrate that date seed oil is a feasible substrate for PHB production. A maximum dry cell weight (DCW) of 14.35 g/l was obtained, with a PHB content of 82%, using 20 g/l of date seed oil. Subsequently, the effect of using mixed-substrate (date seed hydrolysate media and date seed extracted oil) on PHB synthesis was investigated using various ratios of substrate feeding. A ratio of 1:1 fructose to oil produced the highest biomass and PHB concentrations of 15.22 g/l and 12.36 g/l, with PHB content 84.1%, respectively. Solid state fermentation using polyurethane foam (PUF) as inert solid support also proved to be a successful alternative for traditional SSF method for PHB production with ease. The maximum PHB production was 0.169 ± 0.03 g/g_{PUF} and biomass was 0.4 ± 0.003 g/g_{PUF}. This work results demonstrate that the use of a generic waste date seed medium as a feedstock for PHB synthesis is technically feasible.

It is shown that waste date seed provides a novel approach to produce value added products, in this case biopolymer (PHB). The specific studies carried out lead to the wider outlook that a general feedstock derived from date palm by-product, seeds, has potential to be utilised to synthesise a wide range of products based on the microorganism used. More improvement of this process to develop the efficient production of nutrients as well as improve product yields and subsequently, integration of the process into a broader biorefining process would be an essential contribution in the improvement of the sustainable bio-products industries.

Declaration

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Signed:
Rawa Yousuf

Date:
8th December 2017

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Rawa Yousuf

Associated Publications

- Yousuf, R. G. and Winterburn, J. B. (2016) 'Date seed characterisation, substrate extraction and process modelling for the production of polyhydroxybutyrate by *Cupriavidus necator*', *Bioresource Technology*. Elsevier Ltd, 222, pp. 242–251

- Yousuf, R. G. and Winterburn, J. B. (2017) 'Waste date seed oil extract as an alternative feedstock for Poly(3-hydroxybutyrate) synthesis', *Biochemical Engineering Journal*. Elsevier B.V., 127, pp. 68–76.

- Yousuf, R. G. and Winterburn, J. B. "Process optimisation of Poly (3 - hydroxybutyrate) (PHB) synthesis by *Cupriavidus necator* using fed-batch and two-stage batch fermentation strategies ". **(under preparation)**.

CHAPTER

ONE

1 Introduction

Plastics are a ubiquitous part of the modern life, and their molecular structure can be manipulated to meet the requirements of almost any imaginable application including construction, health care, transportation, food packaging, consumer goods and communications. Currently the vast majority of plastics in use are manufactured using a petrochemical route, derived from materials of fossil oil and gas origin. About 4 to 6% of the world's annual petroleum production is used for the production of plastics and more 4% is utilised to power manufacturing processes (British Plastics Federation, 2017). Plastics are produced as long chains of repeating molecules, known as monomers, and while alternative production routes exist, via plants such as sugarcane and corn for instance, only a small quantity of plastics are produced through these routes.

Celluloid plastic was first invented in the 1860s but not improved for wider industrial scale use until the 1920s when it began to be replaced by cellulose-acetate. Plastics production rapidly increased in the 1940s, becoming one of the fastest-increasing worldwide industries. The global plastics industry has grown continuously over the last 50 years, from an annual production of 1.7 million tons in 1950, increasing to 300 million tons in 2015. This equates to an average growth in plastic production of 9% per year, based on non-renewable resources, as plastics gradually replaced materials such as metal and glass. (Shen et al., 2009). Figure 1.1 shows the world and European plastics production over the period 2005 to 2015. From Figure 1.1, it can be seen that plastics production increased globally but was almost stable in Europe for this period.

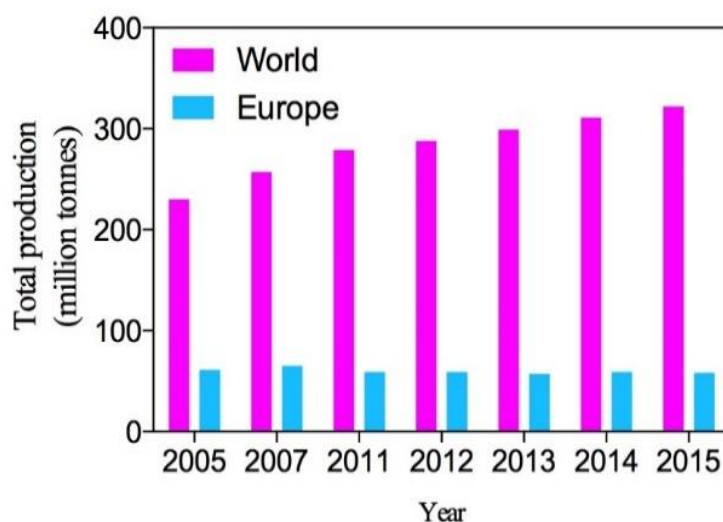


Figure 1.1. World and European total production of plastics over the period 2005 to 2015 with 250 million tonnes capacity, (PlasticsEurope, 2017).

plastic consumption reached 100 kg/person/year in North America and Western Europe, while currently plastic consumption in Asia is about 20 kg/person/year, though this value is expected to increase rapidly. Because of the availability of relatively cheap labour in many countries such as Cambodia, Indonesia, Vietnam, Laos, and Myanmar, renders a competitive feature. Also Asia's growing user base, widening of plastic export and import markets and extending foreign trading powers presents external investors significant opportunities (Plastics and Manufacturers, 2016).

In general, plastics manufacturer is moving to Asia where 45.6% of the world plastic production occurred in 2015, with China alone manufacturing almost a quarter of global production (Figure 1.2). In 2010, Chinese manufacture exceeded European production. India has also undergone a strong increase in plastic production because of increases both in the manufacturing sector and population.

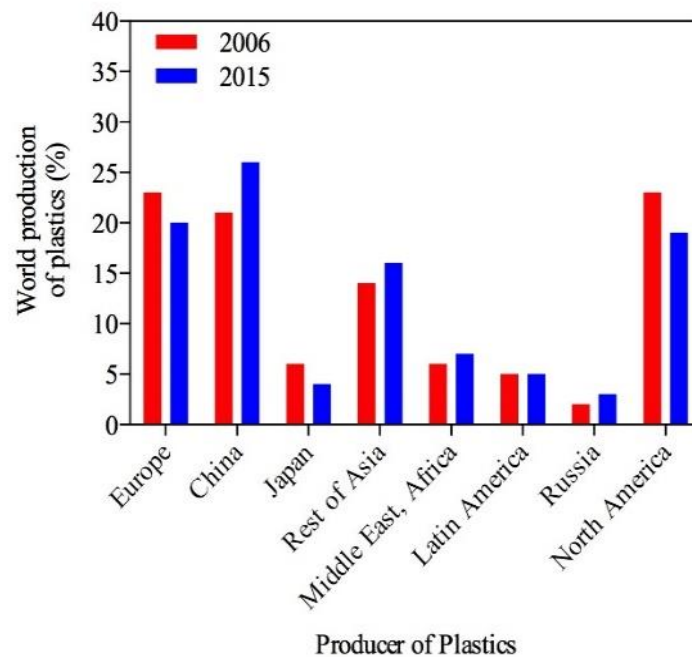


Figure 1.2. Comparison of world plastics materials production in 2006 and 2015 with total demand about 260 million tonnes capacity, (Plastic et al., 2015).

Annually the world plastic industry generates revenue of around \$600 billion, and with a market driven by convenience and consumerism, along with the relatively low price, demand for plastic materials is increasing. Recycling and recovery, however, the insufficient, and each year, millions of tons of plastic end up in oceans and landfill area. Plastics demand remained strong across all industry sectors in 2016, although actual growth was limited, reflecting an overall downturn in the world's economy. The relative size of end-use applications continued to be stable compared with previous years, with packaging

remaining the largest application of plastics, representing about 39.9% of the total plastics demand. Figure 1.3 shows the distribution of European plastics demand in 2016 by end use, with a total demand about 49 million tonnes.

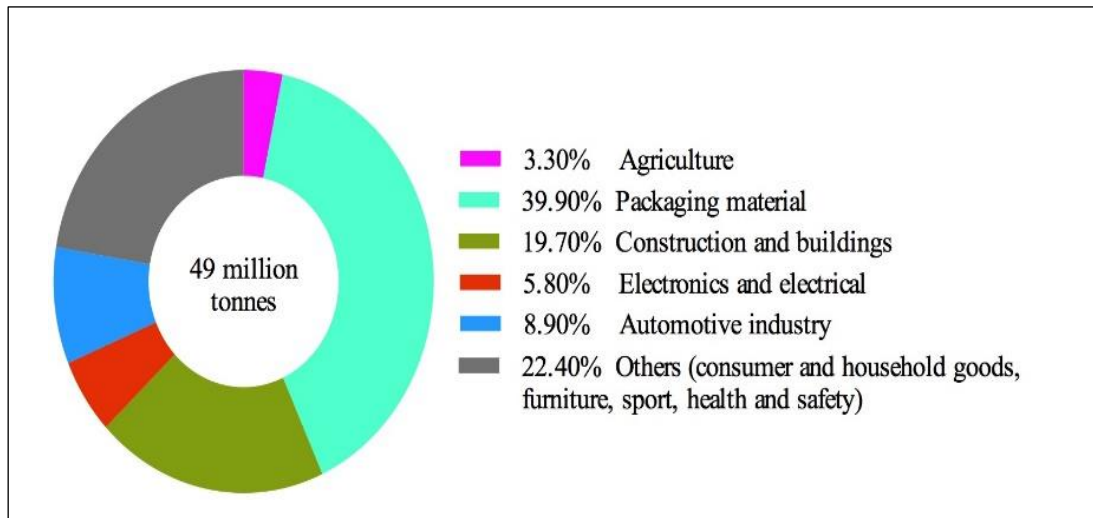


Figure 1.3. Distribution of European plastics demand by segment in 2016 with total demand about 49 million tonnes, (EcoSphere, 2014).

Plastics are extremely versatile materials and have been essential in the development of society since the 20th century, though they have environmental impact associated not only with their manufacture, but also at their disposal. This is because plastics have a slow degradation rate, making them environmentally persistent and hence presenting a serious pollution issue (Castilho et al., 2009 ; Marshall et al., 2013). A wide range of plastic end up in the oceans every year, causing financial losses by tourism and fisheries also time spent for cleaning beaches. A recent study from 2015 estimated that 5.26 trillion plastic particles weighing 268,940 tons in total are floating in the world's oceans (Plastic et al., 2015). Because plastics shift with currents and wind, extremely few areas in the ocean have escaped plastic pollution. Approximately \$13 billion is spent each year to product the environmental destruction by plastics to marine ecosystems. Marine wildlife such as whales, seabirds and dolphins are vulnerable to plastic pollution, which can kill marine animals by either them becoming entangled in non-degradable plastics debris or choking on polymers they mistake as food, (Figures 1.4 and 1.5).



Figure 1.4. Turtle caught in plastic debris,
 (“sea turtle plastic,” 2017)



Figure 1.5. Seabed pollution.

Current levels of plastic usage and disposal generate various environmental problems. Each year a major part of plastic manufactured is utilised to make different disposable items of packaging and/or other short-lived goods, which are discarded within a short time of manufacture, hence use of plastic in this manner is not sustainable. Due to the durability of the plastics involved considerable quantities of disposed of end-of-life plastics materials are accumulating as debris in landfills. Available plastic disposal methods include incineration, landfills, source reduction, recycling, and bio or photo-degradation. Both incineration and recycling divert a substantial part of plastic refuse away from landfill. Incineration is an expensive method and, in some situations, produces harmful chemical substances, such as hydrogen cyanide and hydrogen chloride. Another option is recycling however the difficulty with this method is that it is time consuming to sort a wide variety of plastics, because the presence of coatings, pigments and fillers which limit the use of recycled materials. The landfill is the other alternative way of practice which is the easier and most common method of waste management as different types of waste materials can be disposed easily.

For waste polymer produced as a result of household use contact with cleaning products, fatty foodstuffs, cosmetics and fecal matter which make polymer bind chemically with different substances, making the recovery difficult. Urbanisation has also contributed to plastic pollution, especially in big cities. The plastic that is thrown on the land will enter drainage lines and choke them, consequently increasing the risk of local flooding in cities, as experienced in 1998 in Mumbai and 2007 in South Asian (Bangladesh, India, Bhutan, Pakistan and Nepal). Because of these materials are not biodegradable, our environment and ecosystem are polluted and disturbed by the accumulation of waste plastic.

The molecular weights of plastics range from 50 to 1,000 kDa, and these high molecular weights are one of the main reasons for the resistance of plastics to biodegradation, and hence their environmental persistence. The disposal of plastics has threatened the environment with an accumulation rate of around 25 million tons per year (PlasticsEurope, 2013). In 2014, 25.8 Mt of post-consumer plastic waste finished up in the main waste streams. In Europe about 69.2% of plastic was recovered through energy recovery and recycling processes while 30.8% still send to landfill. Within the various plastic applications, the highest recycling rate was obtained from packaging which reached 39.5% and comprised more than 80% of the total quantities of recycling. (Melorose et al., 2015). In response to increasing concern about the harmful effects of petrochemical-based plastic materials in the environment, several countries are conducting different solid-waste management programs such as incineration, landfill and recycling.

One option to overcome the disposal problems of petrochemical based plastics is to find alternative polymers with a high degree of biodegradability which can be produced from renewable resources via the application of modern biotechnology. These alternative types of plastic must retain the desired properties of traditional synthetic plastics and, ideally, should biodegrade completely without leaving any undesirable remains once discarded. Biopolymers can be mainly classified into four groups based on their biodegradable characteristics (Mudgal and Bain., 2011),(Figure 1.6):

- Biodegradable and petrochemical-based: biopolymers have biodegradable or compostable properties, but they are not produced from renewable resources such as polybutylene terephthalate glutarate (PBTG), polybutylene terephthalate adipate (PBTA), polybutylene terephthalate succinate (PBTS), polycaprolactone (PCL), and poly (butylene succinate) (PBS).
- Biodegradable and from renewable resources: including PHAs, polylactide (PLA) and its blends, cellulose-based materials, and starch-based materials.
- Non-degradable and from renewable resources: such as bio- PET30 (consist of 30% PET which made from the renewable source).
- Non-degradable, petrochemical-based: such as Polyethylene (PE), Polypropylene terephthalate (PET) and Polypropylene (PP).

		Renewable resource			
Non-biodegradable	Bioplastic (1) Bio-PE Bio-PP Bio-PET30		Bioplastic (2) PHA PLA Starch Cellulose		Biodegradable
	Conventional plastic PET PE PP PVC PS EPS		Bioplastic (3) PCL PEG PBS PBTG PBTS		
		Petrochemical resource			

Figure 1.6. Biopolymers groups classification based on their biodegradable characteristics and raw material used for production, renewable or petrochemical, modified from (Mudgal and Bain., 2011).

In addition, biopolymers can be classified into four groups depending on their components:

- Biopolymers that can be synthesised directly by living organisms such as PHAs, silk, cotton, oil proteins wool, natural fibres, natural rubber, cellulose, starch and lignin.
- Biopolymers synthesised via monomer polymerisation, which are either already present in nature or derived from materials which exist in nature such as PLA, soy-based polyols and their derivatives and polytrimethylene glycol.
- Blending of petrochemical-derived monomers and monomers synthesised from renewable resources including soy-based urethanes and isosorbate-containing polycarbonates.
- Polymers produced from combinations of petroleum-based materials and renewable resources such as starch-polyvinyl alcohol.

One microbially produced biopolymer of particular interest is poly-3-hydroxybutrate (PHB). PHB is microbial storage polyester which is synthesised by several microorganisms and has a dual function as an energy reserve substance and as a metabolite accumulating in response to stress conditions, limitation of an essential nutrient such as nitrogen, phosphorous or oxygen in the presence of excess carbon.

When nutrient supplies are an imbalanced, it is useful for bacteria to store excess nutrients intracellularly (Gironi and Piemonte, 2011).

The properties of PHB are similar to many synthetic thermoplastic polymers, such as polypropylene, making PHB a promising candidate as a drop in replacement for petroleum-based plastics in wide range of applications such as paper coatings, packaging and manufacturing of plates and bottles (Batcha et al., 2014). Previous research on PHB has focused on making production economical and improving the quality of biopolymers such that they are able to compete with the physical and mechanical properties of petrochemical-derived polymers, allowing biopolymers to be utilised as direct replacements for commonly used synthetic polymers (Mohidin Batcha et al., 2014).

Industrial production of PHB by fermentation has not been widely applied because of the high costs of production. The substrate costs for production and subsequent downstream recovery are high, currently about 10 times more than for traditional synthetic polymers (Sharma-shivappa et al., 2012). Therefore, using PHB is currently economically unattractive in spite of the advantages of PHB being biodegradable. For example, the cost of petroleum based polymers, Polyethylene (PE) or Polypropylene (PP), are \$0.25-0.5US\$/kg, whilst PHB costs \$6-16 US\$/kg (Reddy et al., 2003) (L. L. Madison and Huisman, 1999). This cost differential is the main obstacle to the replacement of traditional polymers with bio-based alternatives in the market. The cost of raw materials accounts for over 50% of the total biopolymer production cost, with around 70-80% of the raw material costs being the carbon source used as a substrate for both microorganism growth and biopolymer production (Wang et al., 2013).

In order to reduce the cost of biopolymer production a cheap source of carbon and nutrients is required. Thus, using agricultural waste residues such as dairy waste, date seeds, grain crops and starch could substantially decrease substrate and hence production costs. Recent research has focused on using food and agriculture waste streams to obtain a growth media and currently these waste streams are the by-products of different food processing industries that are not used or recycled for any other purpose.

These materials often have an economic value less than the cost of recovering and reusing them; therefore, they are thrown away as waste. There is, however, an opportunity to add value to these waste streams through the production of biopolymers. More than 200 types of microorganisms are able to accumulate PHB, such as photosynthetic bacteria, archaeobacteria, and Gram negative and Gram-positive bacteria.

C. necator H16 is a Gram-negative bacterium that is a model PHB producing microorganism because it is able to accumulate PHB to a high level of 90 % of dry cell weight (Chee et al., 2015). Unfortunately, *C. necator* cannot grow on glucose, the main sugar that is obtained from cellulosic material, which limits the use of this strain for PHB production using sources of non-edible, lignocellulosic biomass. There is potential to use fruit waste for *C. necator* growth and PHB production because these types of waste contain high levels of fructose and this bacterium can metabolise fructose and convert it to bioproducts (Fukui et al., 2014a).

Date palm, also known as *Phoenix dactylifera L.*, is one of the oldest known fruits in the arid and semiarid areas of the world which has been cultivated in North Africa, Arabian Peninsula and the Middle East for at least 5000 years. The total annual world production of dates has now reached 7 million tons distributed across 30 countries (Al-Shahib and Marshall, 2003). Date fruit consists of two parts; a soft, edible, fruity pericarp and a hard seed, with each fruit containing one seed that accounts for around (10 – 15 %) of the total date weight. This means the annual production of waste date seed is in excess of 1 million tons. Normally the edible date fruit is consumed by humans and the seeds thrown away as a waste. However, the seed waste also has a high nutrient content, comparable to that of the fruit, and contains a large amount of energy that could be used for various value added purposes (Basuny, 2011). Date seeds are a waste product of many industries, and are composed of 5-6% protein, 20–40% dietary fibre, 50-70% carbohydrates and about 10-12% oil, and also contain some nutrients such as magnesium, calcium, potassium and phosphorus (Abdul Afiq et al., 2013).

Recently, much attention has been focused on the utilisation of date seeds as an important waste that could be used as value-added products such as dietary fibres, biofuel or cooking oil, coffee, and medicinal products (Besbes et al., 2004a). Researchers have determined the chemical composition and nutritional value of the pericarp or flesh part of the dates while the available information is limited regarding the chemical composition and nutritional value of the date seeds. The main target of this project is to shed light on the most chemical composition of the date seed type (Zahide) which are commonly grown in Iraq and also investigate their use as a renewable, sustainable, rich media and inexpensive carbon source for bacterium growth to produce biopolymer (PHB) using various types of fermentation systems by *C.necator*.

This thesis is organised in the following way: **Chapter 1** gives a generic idea about the effect of synthetic plastics on the environmental pollution and the how importance the improvement of a biodegradable plastic, PHB, production strategies using waste and by-products materials. The challenges of the production process, as well as an analysis of the biopolymer market are also presented.

In **Chapter 2** a critical literature survey is highlighted in this chapter and provides a detailed review of biopolymer types and their production, properties and industrial applications. PHB production utilises from agro-industrial wastes and works conducted by other researchers is included. Different fermentation techniques which are used for PHB production also described in this chapter including SmF, SSF and ACSSF. A description of the dates cultivation and their importance to producing various value-added products through microorganisms is also given.

The scope of the thesis and the aim of PhD research are presented in **Chapter 3**.

All the materials and methods that are used in this study are listed in **Chapter 4**. As well as a kinetic model for describing PHB production. The experimental work conducted in this study is detailed in **Chapters 5** through **8**. The results of a study demonstrating the utilisation of date seed hydrolysate media on PHB production are presented in **Chapter 5**. The process development using different fermentation methods also can be found in this chapter. The results of using different techniques to extract the oil from date seed and optimisation process are presented in **Chapter 6**, as well as investigate the ability to utilise oil extracted to produce PHB. The results obtained from studying the effect of using mixed-substrate (hydrolysed media and date seed oil) on PHB productivity under different feeding ratio are reported in **Chapter 7**. In addition, it describes the strategy of a solid-state fermentation for biopolymer production using polyurethane foam as an inert support material are also included in this chapter. Both characterisation and thermal properties investigate for all PHB samples that obtained from all the results reported previously, are presented in **Chapter 8**. Finally, conclusion and the future work are described in **Chapter 9**.

CHAPTER

TWO

2 Literature review

2.1 Introduction

In this chapter of the thesis a thorough critical review of information regarding to the many aspects related to this project is reviewed, providing the context for the subsequent interpretation of the experimental results presented, as well as identifying benchmarks as a basis for comparison. The purpose of this literature review is to give the general understanding of the field of biopolymer production from renewable resources, specifically focusing on the production of Polyhydroxybutyrate (PHB) from agricultural and food waste. Consideration is given to the various effective strategies for waste management required to add value to waste streams which could contribute to the competitiveness of bioplastics compared to petroleum-based plastics. Polyhydroxyalkanoate (PHA) production, characterisation, and natural biological function are discussed. General background understanding of plastics and biopolymers including markets, applications, types and classification, and costs are presented in sections 2.1, 2.2 and 2.3. A description of the metabolic pathways and enzymes involved in PHB accumulation can be found in section 2.4, while the information about date palm and date seeds including production, application and their anatomical structure is reviewed in section 2.5. Section 2.6 reviews reports of the different waste materials utilised for PHA production, with section 2.7 containing a summary of the previous production, microorganisms, and feedstock. Finally, the current challenges hindering widespread use of PHA are outlined in section 2.8.

2.2 Synthetic polymers and the necessity for biodegradable polymer

In order to avoid misunderstandings, the terms plastic, polymer and biopolymer should be defined. According to the American Chemistry Council (ACC), “plastic” is a generic expression used to describe synthetic man-made organic polymeric materials, whilst “polymers” are defined as chemicals made of many repeating units. The International Union of Pure and Applied Chemistry (IUPAC) defines “biopolymers” as polymers that are produced by living organisms. This includes a range of materials from starch and cellulose to polyhydroxyalkanoates. The word “Polymer” is a Greek word, consists of two parts: “poly” and “mer” meaning several and portion or unit, respectively.

Plastics play an essential role in almost every aspect of modern life and can be modified to meet the material requirements for almost any application because of their low cost, excellent performance, lightweight, properties ranging from soft rubber to stiff fibres,

tensile strength and the ease by which they can be fabricated into complex shapes. Crude petroleum is a key, unsustainable resource and a considerable percentage of annual petroleum production, 8%, is utilised in the production of polymer products (Énergie et al., 2014). In 2005, about 18 million barrels of crude petroleum-equivalent were utilised to produce approximately 2 million polyethylene terephthalate (PET) bottles. This percentage represents the largest application field for petroleum outside the transport and energy sectors (Panda et al., 2010).

The use of synthetic plastics has grown significantly over the last decades, and in the coming years the public demand for petroleum derived materials could exceed the availability of natural resources, due to the increasing global population as well as increasing wealth and growing middle classes in many countries including India and China. Plastic pollution impacts oceans, waterways, and land by increasing the solid waste streams that cause negative environmental effects. Marine animals can be affected by plastic waste through direct ingestion, entanglement or through exposure to chemical substances which disrupt biological functions. Humans can also be affected by fluctuating levels of sex hormones or by disruption of the thyroid hormone (Canesi and Fabbri, 2015). A best possible solution for combating the effect of plastic waste in the environment is to find alternative, non-petroleum-based processes to produce biodegradable polymers from renewable resources, to reduce the environmental impact of plastic and polymer production, use and disposal. The PHA family of biopolymers can be produced from renewable resources and are capable of biodegrading to carbon dioxide and water after a maximum of one year.

2.3 Bioplastics an outlook

Green or biodegradable plastics are naturally occurring and are often derived from renewable resources, being widely publicised as a possible solution for concerns regarding the using of traditional petroleum-based plastics. Biopolymers are polymers of biological origin and in our daily life, we encounter different biopolymers provided by nature, including cellulose in trees and plants, starch in potatoes, maize-cassava, natural rubber like poly-cis-isoprene, proteins and DNA. Another example is “hair” which consists of protein filaments (L. L. Madison and Huisman, 1999). As a major source of energy carbohydrates are one of the most common examples of naturally occurring polymeric materials. Carbohydrates are comprised of long repeated units of sugar and can also call polyhydroxy aldehydes depending on their structure (Philip et al., 2007). Figure 2.1 summarises the different types of natural biopolymers and their function.

The level of sophistication which exists in nature and enables the production of polymers is far greater than that of man, for example the amino acids programme polymerisation into proteins at ambient pressure and temperature. Nature does not synthesise polymers for serving mankind accompanied by engineering materials but to serve its purposes including energy reserve materials. The main advantage of synthetic polymers is that they are more heat stable compared to their natural counterparts, as also have relatively simple chemical structures (Keshavarz and Roy, 2010).

The term biopolymer is used in various ways, based on their applications. The accepted definition includes polymers which belong to the above-mentioned groups (Figure 2.1); they are either biodegradable and/or renewable. Also, biopolymer material can be synthesised by a wide range of plants and microorganisms; natural rubber, for example, is a plant derived biopolymer that can be isolated from the tree *Ficus elastica* by traditional techniques. Microbial biopolymers are accumulated either by fermentation directly or via chemical polymerisation of monomers, which are in turn synthesised by fermentation. Biopolymer accumulating microorganisms need particular nutrients to grow as well as controlled environmental conditions (Marjadi and Dharaiya, 2013). It is believed that microbial biopolymers are accumulated as a way of storing carbon intracellularly or as a result of a defence mechanism. In both cases, the synthesised biopolymers can be recovered and utilised. These biopolymer materials have various interesting properties and functionalities such as biocompatibility, making them of interest in the medical field for instance (Endres and Siebert-Raths, 2011)

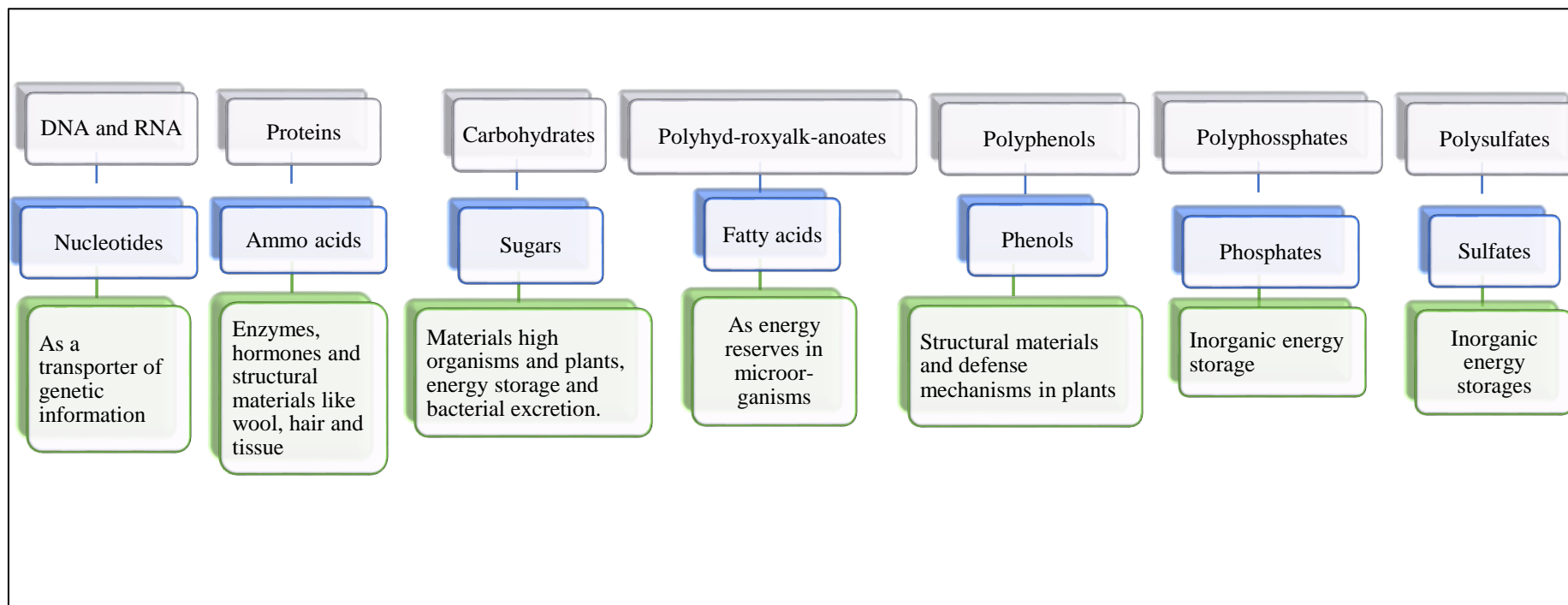
Figure 2.1. Nature polymers, monomer, and their function (modified from Bugnicourt et al., 2014).

Table 2.1. Examples of bioplastics by production processes, Modified from (Matsuura et al., 2008)

<i>Process</i>	<i>Source</i>	<i>Example</i>
Fermentation	Bacterial polyester	Polyhydroxyalkanoate (PHA), Polylactic acid (PLA)
Chemical Polymerisation	Crude oil	<i>biodegradable</i>
		Polybutylene (PBS) Aliphatic polymers (Polyglycolic acid (PGA)) Aliphatic-aromatic (Polycaprolactone copolymers) Polybutylene succinate (PBS) Polyethylene glycol (PEG) Polycaprolactone (PCL)
		<i>Non- biodegradable</i>
		Polyethylene PE Material composed from 30% PET made from renewable resource (PET30) Bio-polypropylene (PP)
Extraction/Polyme risation	Natural polymers from plants	Starch , rubber and Cellulose acetate (CA)

Moreover, Amache et al., (Amache et al., 2013) similarly classify biodegradable polymers into three groups, based on their biodegradability:

1. Polymers synthesised chemically which are susceptible to microbial and enzymatic attack. Commercially, they are not workable because they do not possess the corresponding properties of petroleum-based polymers;

2. Starch-based biodegradable polymers which are a blend of polymer and starch. Soil microorganisms are able to degrade the starch but, due to the non-biodegradable polymer portion they do not degrade fully and remain in the environment for a quite long time. Furthermore, the required starch production conflicts with food markets.

3. Polyhydroxyalkanoate (PHA) is the only family of biopolymers which is fully biodegradable. They are polyesters of different hydroxyalkanoates (HAs), which accumulate intracellularly by various microorganisms.

Biopolymers can be further classified into four groups depending on their components, (Chanprateep, 2010a):

1. Biopolymers synthesised directly by living organisms such as PHAs, silk, cotton, oil proteins wool, natural fibres, natural rubber, cellulose, starch and lignin.

2. Biopolymers synthesised via polymerization of monomers which are either already present or derived from materials which exist in nature, such as PLA, soy-based polyols (and their derivatives) and polytrimethylene glycol.

3. Blending of petrochemical-derived monomers with monomers synthesised from renewable resources, including soy-based urethanes and isosorbide-containing polycarbonates.

4. Polymers produced from combinations of petroleum-based materials and renewable resources such as starch-polyvinyl alcohol.

The global production of bio-based plastics and their respective percentages are presented in Figure 2.2.

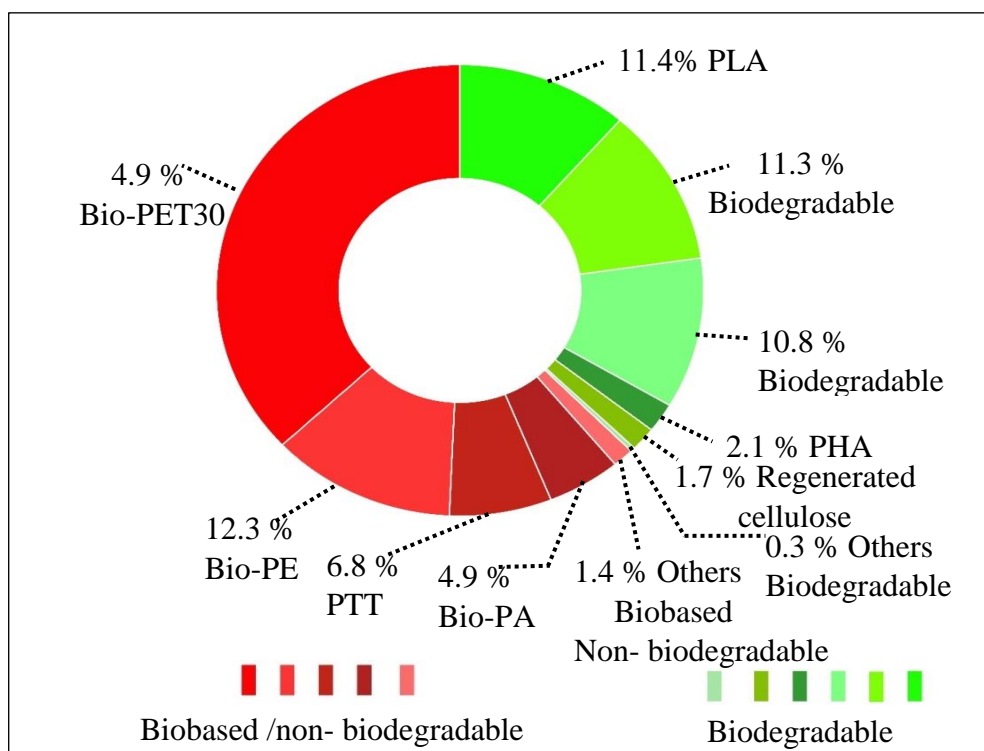


Figure 2.2. Global bio-based plastics production modified from (Bioplastic, 2014).

Furthermore, many types of biopolymers are already manufactured by industry at large scale, polylactic acid and starch based polymers are produced in quantities of about 185,000 tonnes and 183,000 tonnes annually respectively, for example (Ates, 2014). Some other classes of biopolymers are still to be manufactured at commercial scale, however, including polyhydroxyalkanoates (PHA). PHAs have considerable potential applications in various sectors, with production levels of just 34,000 tonnes in 2014, far below the production level of other types of biopolymers. Mainly the restrictions applicable to commercial processes are because of production issues including expensive carbon substrates, low productivity, lack of scalable recovery methods for intracellular biopolymers and utilising of co-substrates for copolymer production (like co-feeding of valeric acid and butyric acid) as well as technical challenges.

Hence, producing a biopolymer utilising renewable resource is not currently the perfect answer for inexpensive biopolymer production (Wang and Lee, 1997).

Bioplastics are more sustainable than traditional type (synthetic polymer) as they can be synthesised from renewable resources and degrade rapidly, hence providing one possible to solution to the growing global problem of plastic waste. The successful production of biodegradable plastics will lessen the burden placed on the environment by consumers. (Berkesch, 2005). Table 2.2 shows an overview of some synthesised biopolymers, their discovery and applications.

Table 2.2. Overview of some synthetic biopolymers, discovery, and their applications.

<i>Types of biopolymer</i>	<i>Definition</i>	<i>Properties</i>	<i>Discovery</i>	<i>Applications</i>	<i>Reference</i>
Bio-Polyethylene	<ul style="list-style-type: none"> • Thermoplastic polymer • Composed of ethylene subunits 	Highly chemical resistant, melting point 105 -115 °C.	≈40 years ago.	Bottles, plastic bags.	(Aligned and Nature, 2012)
Cellulose	<ul style="list-style-type: none"> • Organic polysaccharide • Composed of D-glucose. 	Insoluble in water, odourless and biodegradable.	1838	Paper, energy crops, medical applications toys, and car interiors.	(Flores-Hernández et al., 2014)
Cyanophycin	<ul style="list-style-type: none"> • A branched polymer, accumulates in cyanobacteria. • Consists of equimolar amounts of aspartic acid and arginine 	Insoluble under physiological conditions.	Discovered in France about 100 years ago by Borzi.	Drug carriers, a safety agent for ultrasound,	(Tokiwa et al., 2009a)
Polyhydroxalkanoate (PHA)	Linear polymers, accumulate naturally in bacteria under unbalanced conditions.	Consists of hydroxycarboxylic acid. Biodegradable, non-toxic thermoplastics	1925 by Lemoigne	Biomedical and industrial applications.	(Krehenbrink et al., 2002)
Polyisoprene	<ul style="list-style-type: none"> • Natural rubber produced by many plant species. • Building blocks isopentenyl pyrophosphate. 	Good flexibility and tensile strength characteristics. It forms crystals at room temperature with melting point 54.5°C.	1736	Sponges, sporting materials, motor mounts, and mostly in tires.	(Anderson and Dawes, 1990)
Polylactic acid (PLA)	<ul style="list-style-type: none"> • Thermoplastic polyester. • Formed from lactic acid monomers. 	High strength, stiff, melting point is 200°C	1948	Moulded industrial products, plates, cups and pins.	(Threadingham et al., 2011)
Starch	Natural thermoplastic polyesters stored in tissue of plants. Not present in animal tissues.	Composed of glucose softening and transformation at high temperatures, biodegradable with spherical granules insoluble in water	back to 30,000 years ago in Europe.	Papers, cosmetics and paints.	(Södergård and Stolt, 2002)

2.4 *History of plastics and bioplastic development*

Most petroleum-based plastics are made of chain monomer of polyethylene, methylene (CH₂). The chain is usually as long as from 10³ to 10⁵ of repeated methylene. Because of long chain, consequently, it is spoken of as high macromolecules or polymers and this molecular structure contributes to non-biodegradability of it (J. Brydson, 1999). In soil, plastics will take up to 10³ years to degrade while in water, they will take around 450 years. through 1930s, petro-plastic had reached the commercial status: poly(methyl methacrylate), polystyrene, poly(vinyl chloride), low density nylon and polyethylene (Pasquini, 2005).

Plastics are typically chain of monomers, polymers, of high molecular weight, or may contain other substances to develop their performance or to reduce costs. The word plastics derives from the Greek (plastikos), "fit for molding", from (plastos) "molded".

And it refers to their plasticity or malleability over manufacture, that permit them to be pressed, cast, or extruded into a massive variety of shapes including films, boxes, fibres, tubes, bottles, plates and much more, nowadays, the most used plastic is polypropylene and polyethylene. Polypropylene is a polymer type that was a offshoot of low molecular weight and it discovered in 1950s (Pasquini, 2005).

There are two types of polymers: thermoplastic and thermosets and the most of polymers are thermoplastics. Thermoplastic plastics are defined as they can be heated and reformed time and time again and this is essential for easy recycling and processing. Unlike thermoplastic, thermosets can't be re-processed, which is if re-heated, they will scorch.

In addition, plastics can be moulded into various things such as bottles or anything else or mixed with solvents to be paint or an adhesive as well as they can deteriorate but never decompose totally. Synthetic polymers are typically prepared of monomers that derived from gas or oil, and plastics are typically made from these by addition of different chemical additives. There are currently more than 20 various groups of plastics, each with varieties materials and massive grades; they are cheap, strong, durable, lightweight and high electrical and thermal insulation properties. The diversity of polymers and the (Source: Absolute Astronomy, (Source: American Chemistry).

The polymers diversity and versatility of their properties promote the production of an enormous array of plastic products which bring energy savings, technological advances and vast other societal benefits (Andrady & Neal 2009). The first actually synthetic polymer, was improved by Belgian chemist Leo Baekeland during 1907s, and many other synthetic

plastics were subsequently improved during the next few decades. It was not till the 1940s and 1950s, nevertheless, that production mass of everyday items of plastic really started (Thompson, 2009).

The history of synthetic plastics, produce from non-petroleum resources, begins in 1868 when John Hyatt invented first synthetic plastic material known as Celluloid. Celluloid was made from different materials: cellulose, wood pulp a or by treating cotton fibres with nitrogen and camphor. Soon both rayon and cellophane were invented, based on treating cellulose with other solvents and acids. In 1907 Leo Baekeland invented the first petroleum-based plastic called Phenol Formaldehyde (Bakelite). Since these discoveries were made interest in bioplastics was side-tracked by the rapid growth of the petroleum-based plastics market.

In the 1920's effort was made by Henry Ford to find applications for agricultural surplus, resulting in experiments with synthesising automobile parts from plastic made from Soya beans (Kuruppilil, 2011). The soy plastics resin was not fully plant based, as part of it consisted of phenol formaldehyde. The idea of soy plastic failed due to several different issues such as lack of moulding technology for complex parts and the strong formaldehyde odour, After the second world war, the only the non-petroleum-based plastic the consumption of which was increasing was cellophane.

Interest in biopolymers grew during the 1970's when biodegradable alternative materials were beginning to receive more attention because of the rapid depletion of crude petroleum. This, combined with the adverse effects that synthetic products have on the environment, accompanied by their growth rates of consumption are the driving forces to find alternatives to the petroleum-based products. During the 1980's green materials became more common. Therefore, biodegradable materials were brought out such as sheets, films and model-forming (Chee et al., 2015) (Al-Farsi and Lee, 2008).;(Tokiwa et al., 2009a). Table 2.3 shows the historical development of plastics.

Table 2.3. The main aspects of the development of plastic production modified from (Lee et al., 2005; Pathak et al., 2014; Razer Evans, 2015; Chassenieux et al., 2013; Osswald et al., 2011).

Year(s)	Polymers improvement
1800's	1862 The first ever human made plastic was a bio- based plastic. It was made from various materials including wascallees Parkesine, cellulose and nitrate.
	1869 <ul style="list-style-type: none"> • Development of the most utilised form of plastic, celluloid, that is commonly applied in film • Bioplastics are introduced for commercialization
	1924 <ul style="list-style-type: none"> • Henry Ford used food stock to produce a bioplastic for cars construction
1930 's	Discovery of poly γ -glutamic acid (γ -PGA) in <i>Bacillus sp.</i>
1941	Henry Ford produced the first bioplastic cars
1950 's	1950 Discovery of Xanthan
	1957 Cellulose produced from <i>Acetobacter Xylinum</i>
	1958 Development (γ -PGA) in E medium
1960 's	Bacterial cellulose (BC) was cultivated
1970 's	Pullulan was produced commercially Increased demand for non-oil based plastic due to oil crisis
1980 's	1983 Medium chain-length polyhydroxyalkanoate (MCL-PHA) discovered in <i>Pseudomonas oleovorans</i>
	1988 Poly (3HB) was produced from <i>Ralstonia eutropha</i>
	1989 Both short chain length (scl) and mcl monomers were discovered in PHA
1990 's	1990 British Company, Imperial Chemical Industries, launched a biodegradable bioplastic called Biopol
	1992 Cheap carbon source (whey) was used to produce poly (3HB) by recombinant <i>Escherichia coli</i>
	1994 Investigating <i>Aeromonas</i> strains accumulated poly (3HB-co-3HHx)
	1997 <ul style="list-style-type: none"> • Cultivation of <i>Alcaligenes latus</i> using fed-batch under nitrogen limitation • Poly (3HB) and poly (3HA) process was estimated economically by bacteria fermentation
	1998 <ul style="list-style-type: none"> • Cloning of poly (3HB) was synthesized from <i>A. latus</i> • Poly (3HB) was produced in chemical media using fed-batch cultivation of recombinant <i>E. coli</i> harboring <i>A. latus</i>
	1999 <ul style="list-style-type: none"> • Fed- batch cultivation of recombinant <i>E. coli</i> harboring <i>A. latus</i> was used to produce poly (3HB-co-3HV) using chemical media • Poly (3HB-co-3HV) was synthesised by recombinant <i>C. necator</i>
2000 and beyond	2000 <ul style="list-style-type: none"> • Strategy for the production of γ-PGA was developed in fed-batch cultivation and PHB was synthesised from whey <i>E. coli</i> in lab-scale • Poly (3HB-co-3HHx) was produced in the fed-batch by <i>A. hydrophila</i>
	2001 Cell recycle fed-batch cultivation strategy for poly (3HB) was developed which produced from whey by recombinant <i>E. coli</i> in the lab-scale fermenter.
	2002 Poly (3HB) was produced from whey by the fed-batch cultivation recombinant <i>E. coli</i> in the large-scale fermenter.
	2004 PHB can accumulate in <i>A. faecalis</i> by providing nitrogen deficiency in the lab-scale fermenter.
	2006 <ul style="list-style-type: none"> • The growing size of the petroleum-based plastics market becomes a concern. • Daniel Burd discovered how the plastic decomposes in three months by two types of bacteria: <i>Sphingomonas</i> and <i>Pseudomonas</i>.

Alternative sources of raw materials. However, the intensive utilisation of mineral resources including oil, gas and coal, results in their continuing and significant diminution and contributes to climate variations on the planet. Substitution of petroleum-based plastics with plastics obtained using renewable resources is a significant strategy to control the emissions of greenhouse gases and to contribute to the development of a more sustainable society. The intensive interest for this material is documented by an exponentially growing range of 'Green plastic materials' being announced on the web. Bio-based plastics can be synthesised from a wide variety of renewable resources and last a few years the most utilised raw materials are products of carbohydrate-rich obtained from the agricultural section (Mudgal et al., 2013). The utilisation and hence diversion of agricultural commodities into non-food applications has increased debate about the tension between food and chemicals/fuel, whilst there is an opportunity to use agro and food waste materials as a starting point for biopolymer production. In the future, based on the recent market forecast, worldwide capacities of bioplastics production will increase by 50% between 2016 to 2021 from 4.2 to 6.1 million tonnes (European Bioplastics, 2016).

2.5 Polyhydroxyalkanoates (PHA)

Biopolymers are mainly produced by the assimilation of carbon and are utilised by microorganisms as energy storage molecules to be metabolised when other energy sources are not accessible. PHAs are the most promising intracellular reserve polymers that are synthesised by living microorganism using renewable sources, being fully biodegradable and with properties comparable to synthetic plastics. This makes PHAs convenient candidates for the substitution of petroleum derived plastics (Sudesh, 2013), (Singh Saharan et al., 2014) and (Kourmentza et al., 2017). PHAs are a family of polyesters which consist of hydroxyalkanoate, HA, units that are utilised by bacteria as intracellular energy and carbon reserves (PingTan, 2012) and (Poli et al., 2011). Although various types of PHAs have been discovered, as shown in Figure 2.3, the poly-3-hydroxybutyrate (PHB) homo-polymer is the best known and characterised.

PHAs family are natural polyesters that different microorganisms produce and accumulate as energy and carbon storage materials or as a sink for excess decreasing power, under stress conditions of limiting essential nutrients (N, O, P, S, or Mg) and the carbon source presence in excess. Once the limiting nutrient is supplied again, the PHA start degrading via intracellular PHA-depolymerase enzyme, after which they are metabolised. To date, about 100 types of microorganisms are known to synthesise these microbial polyesters, with production levels in the range of 50-90% of dry cell weight being

achievable. These microbial polymers are accumulated in the bacterial cytoplasm as defined granules, and these granules can be observed easily by transmission electron microscopy (TEM) (Kawaguchi and Doi, 1990). Figure 2.3 shows where PHA granules in *C. necator* cells were imaged. The sample was taken from this study using a media derived from waste date seed.

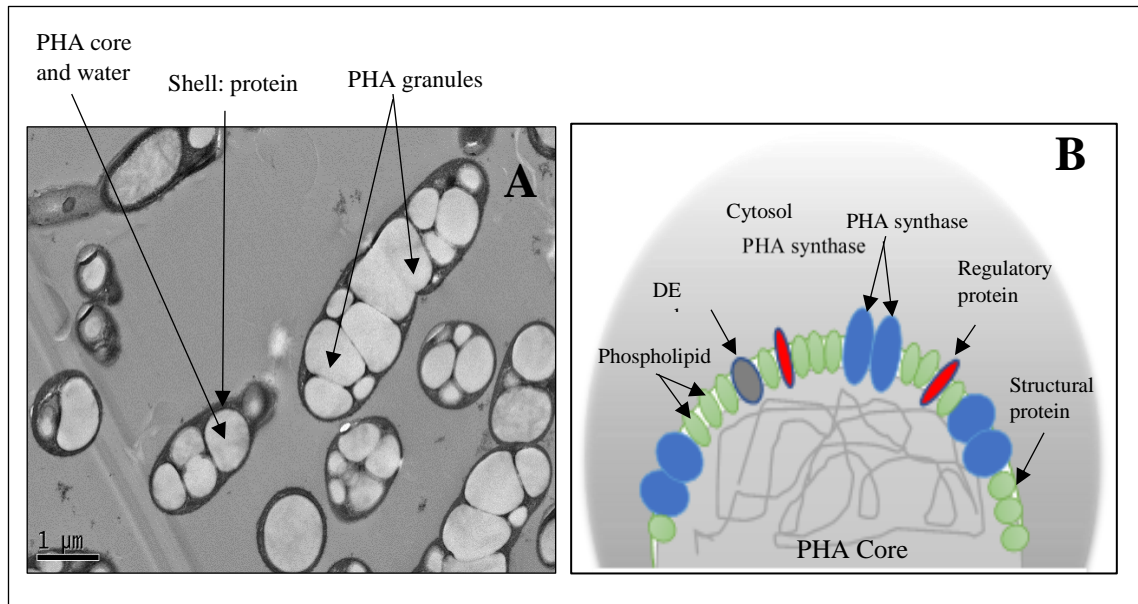


Figure 2.3. Transmission electron microscopy (TEM) picture of *C. necator* cells containing large amounts of PHB granules using waste date seed as a media; 1 μm; (B) Scheme of PHA granule updated from (Parlane et al., 2016).

PHAs can be synthesised via fermentative process mainly under internal/external growth-limiting conditions (Anderson and Dawes, 1990) and (Bengtsson, 2009) or alternating aerobic/anaerobic conditions (Dai et al., 2008). Using a wide range of substrates including pure substances, glucose, cellulose starch, sucrose, chemicals, 4-hydroxybutyric acid and propionic acid, by-products, molasses, syngas, glycerol and whey, and carbon dioxide (Mundus et al., 2014). Various types of bacteria and several archaea such as *Cupriavidus necator*, *Alcaligenes latus*, some *Methylophilus*, *Azotobacter vinelandii*, and recombinant *Escherichia coli* are capable of producing PHAs as energy reserves (L. Madison and Huisman, 1999). In addition, advanced research has been conducted to synthesise PHAs autotrophically utilising hydrogen as an energy source with carbon dioxide (CO₂) as a carbon source (Volova et al., 2011) and (Sudesh et al., 2000).

PHAs are attractive polymer materials not only due to their mechanical properties being comparable with conventional polymers, but also their biodegradation rate is high, breaking down into water and carbon dioxide, by various microorganisms. Thus; their carbon cycle is entirely closed (Akaraonye et al., 2010a), (Leja and Lewandowicz, 2010)

and (Rydz et al., 2014). Many biological plastics could meet the biodegradability requirement that our communities require. This work focuses on PHAs due to their price competitiveness with petroleum-based plastics (as shown in Figure 2.4).



Figure 2.4. PHA bottle biodegradation, type BiopolTM, by incubation in mud for 80 days (Sudesh et al., 2000).

PHA homopolymers, block and random copolymers, with more than 150 various PHA monomers being found, PHAs with quite different mechanical and thermal properties can be formed. Such diversity has allowed the improvement of materials for various applications, including environmentally friendly, biodegradable polymers for packaging purposes, biocompatible implant fibres and drug delivery. As additionally, PHA monomers can be utilised to improve biofuels, chiral intermediates, or drugs (Khanna and Srivastava, 2008).

In spite of the advantageous features of PHAs, currently, they have two crucial disadvantages hindering their large scale application (Leroy et al., 2012):

- Their price is still quite high compared to commodity synthetic polymers about 6-16 \$/kg (Khanna and Srivastava, 2005).
- They have a quite low melting temperature which, because of thermal degradation at the melting temperature, results in complex processing.

PHA production from mixed cultures that make use of a wide range of cheap and renewable carbon sources such as volatile fatty acids present in fermented waste streams would increase the chances for PHA to be considered as a commodity bioplastic as well as reducing the environmental impact (Gurieff and Lant, 2007). Consequently, increasing efforts to maximise PHA content, to control PHA chemical structure and to fully characterise physical properties of mixed culture PHAs are being carried out.

Another feature is based on the fact that PHA can be synthesised from renewable biomass sources, such as carbohydrates, amino acids and fatty acids as main sources of carbon and energy. These renewable materials are produced via CO₂ and H₂O conversion through sunlight fixation, therefore; PHA is a renewable material (Madison and Huisman, 1999).

In general, PHAs can be classified into three categories according to the number of carbon atoms in the monomer units. When the PHA monomer consists of 3–5 carbon atoms, the polymer is known as a short-chain-length (SCL) (including PHB and P3HB4HB), while medium chain-length (MCL) is composed of 6–14 carbon atoms (including P3HBHHx), family and, finally, long chain-length (LCL) are formed of 14 or more of carbon atoms (like poly(3-hydroxy-7cis-tetradecanoate). These biopolymers have a range of molecular weights from 2×10⁵ to 10×10⁶ KDa, while monomers number between 1,000 and 30,000 depending on growth conditions, the type of PHA and the producer microorganism (G. Q. Chen, 2010). The chemical structure of PHAs and some of the identified monomers are shown in Figure 2.5:

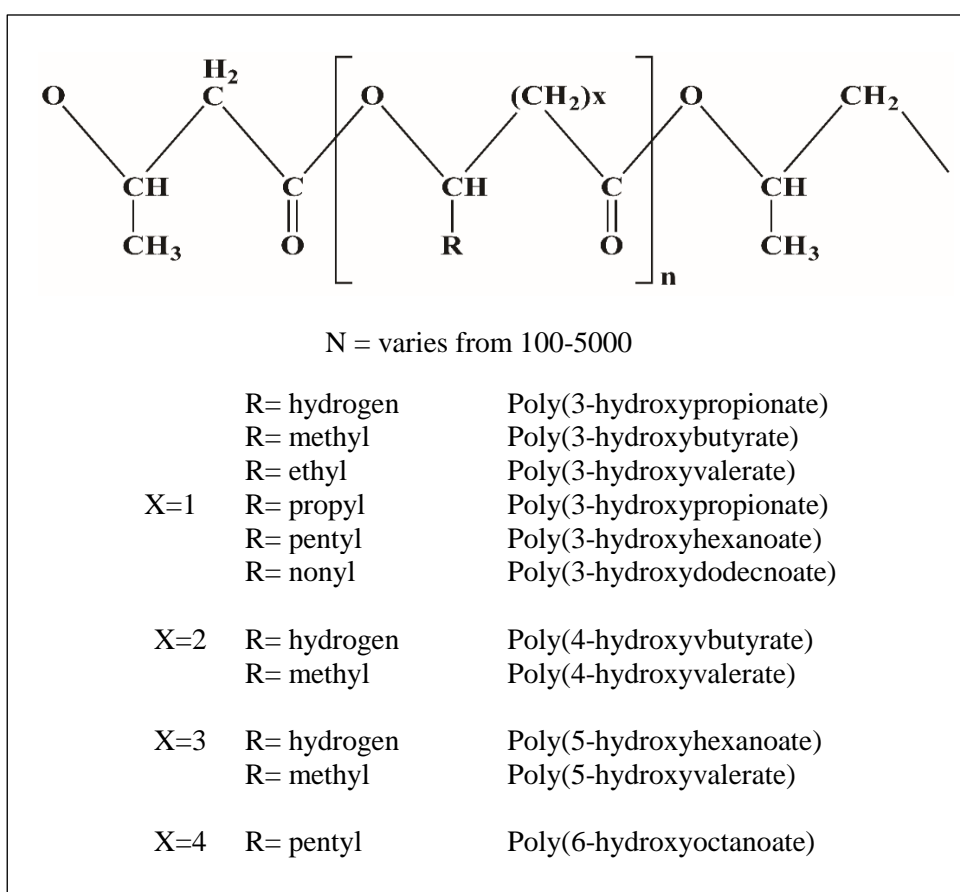


Figure 2.5. The general structural formula of PHAs and some identified co-polymers (Lee et al., 1995).

The biopolymers shown in Figure 2.5 can be divided into two categories; short chain length (scl-PHAs) in which the polymer repeat units are hydroxy fatty acids with chain length corresponds to 4-6 carbons atoms and medium chain length (mcl-PHAs) where the repeat unit of a polymer has chain length more than six carbon atoms. The first category is characterised by its thermoplastic behaviour and crystallinity whilst the second one possesses adhesive properties and elastomeric behaviour (Solaiman et al., 2006c). In general, the PHA family are non-toxic, fully biodegradable, biocompatible and can be synthesised from renewable resources that decrease their environmental effect. The melting temperature (T_m) is in the range 60-180°C (Sudesh et al., 2000). Usually, PHAs plastics have a high polymerisation degree (polydispersity index in the range 2-4), are optically active, highly crystalline, insoluble in water as well as isotactic (possessing stereochemical regularity in repeated units). The main range of applications run from food packaging to chemical synthesis, agricultural, disposable items and medical uses including implants, biodegradable carriers, tissue scaffolding and non-woven patches (Lo et al., 2001).

In PHB production by fermentation, the costs of the substrate for production and subsequent downstream recovery are high, therefore using PHB is currently economically unattractive in spite of it being biodegradable and biocompatible. The cost of raw materials accounts for over 50% of the total biopolymer production cost, with around 70–80% of the raw material costs being the carbon source, which used as a substrate for both microorganism growth and biopolymer production (Wang et al., 2013). In order to reduce the cost of biopolymer production, a cheap source of carbon and nutrients is required. Thus, using agricultural waste residues such as dairy waste, date seeds, grain crops and starch could substantially decrease substrate and hence production costs.

Recent research has focused on using food and agriculture waste streams to obtain a growth media and currently these waste streams are the by-products of different food processing industries that are not used or recycled for any other purpose. These materials often have an economic value less than the cost of recovering and reusing them; therefore, they are thrown away as a waste. There is, however, an opportunity to add value to these waste streams through the production of biopolymers. Unfortunately, *C. necator* H16 cannot grow on glucose, the main sugar that is obtained from cellulosic material, which limits the use of this strain for PHB production using sources of non-edible, lignocellulosic biomass. There is potential to use fruit waste for *C. necator* H16 growth and PHB production because these types of waste contain high levels of fructose (Fukui et al., 2014b).

2.5.1 Polyhydroxybutyrate (PHB)

Polyhydroxybutyrate is a linear polyester of the homopolymer, 3-hydroxybutyric acid which is the most extensive and the best-characterised member of PHAs family. PHB is accumulated as granules intracellularly by a variety of Gram-positive and Gram-negative organisms under unbalanced growth conditions which a nutrient limitation and excess of the carbon source (Valdés-García et al., 2017). It is produced by various microorganisms such as *C. necator* and *Bacillus megaterium* in response to physiological stress conditions and can be produced either by pure culture or mixed cultures of bacteria.

PHB has a wide range of molecular weight depending on the microorganism, growth conditions and extraction methods, and can vary from 50000 to over a million. The biopolymer has the essential properties of thermo-plasticity and biodegradability in compost and various environments. As a consequence, PHB has attracted significant commercial interest (Angelini et al., 2016).

Microbial synthesis of PHB starts with the two molecules of acetyl-CoA being condensed to give acetoacetyl-CoA, then subsequently is reduced to hydroxybutyryl-CoA. This latter compound is used as a monomer to be polymerised to form PHB (Straathof, 2014). The first mention of PHB in the literature was as early as 1901, but the details were reported by the beginning in 1925 by Maurice Lemoigne (Suriyamongkol et al., 2007). He observed a different size of granule like inclusions inside the bacterial cytoplasmic fluid, which were not soluble as lipids normally are. By using microscopic observation, acid numbers, optical activity, melting point, the variation of molecular weight, autolysis and solubility, he found this material was polyester with empirical formula $(C_4H_6O_2)_n$. Because of difference in the polymerisation degree, he accounted for the difference between two isolated fractions (Lee et al., 1995). Also, he used Lipids β -hydroxybutyryques as a name for the product and determined the melting point to be 157°C. Over the next 35 years, as a result of academic curiosity, these inclusion bodies were studied. By 1952, Peaud and Kepes noticed that isolated fractions of the polyester were linear an alcohol group at one end and a carboxylic acid at the other and had a high molecular weight and a melting temperature of 180°C, (Matias and Filomena De Andrade Rodrigues, 2011). Weibull created the correlation between the presence of intracellular lipid granules in the cytoplasm of several bacterial strains and PHB (Weibull, 1957). The first data regarding the molecular weight and properties of these polyesters were reported by Williamson and Wilkinson. Wilkinson and Macrae observed that the accumulation of PHB granules increased with limiting the concentration of nitrogen in the growth medium (Williamson and Wilkinson, 1958).

Furthermore, Doudoroff and Merrick studied the polymer degradation process in both enzymatic and via biosynthesis within the bacterial cells. After that, scientists concluded that bacteria store the PHB granules as an energy reserve material similar to the way glycogen and starch are accumulated by other living organisms (Dawes and Senior, 1973). By the late 1950s and the early 1960s, both Baptist (Grace) and Werber (in the USA) started producing pound quantities of PHB for commercial assessment, therefore; they got patents for both production and isolation processes and improved different articles including sutures and prosthetic devices (Doudoroff and Merrick, 1961). Their inventions extended to the utilising of un-purified high yield product of fermentation in a laminate of plastic. However, their yield of PHB from the fermentation was relatively low (20-60%) (Wang and Liu, 2014; Aslan et al., 2016; Solaiman et al., 2006a) as well as their isolation of the product by solvent extraction process being quite expensive. In addition, the product of polymer was fully contaminated with bacterial remains, making it tough to melt process. However, this project was pioneering not only in terms of reporting on PHB production as a plastic potential but also in proposing the utilisation of PHB as a biocompatible material (Bugnicourt et al., 2014).

During the 1970s, by combining the experience of the Agriculture Division in large scale fermentation and skills in the processing of polymer and from the Plastics Division, Imperial Chemical Industries (ICI) was prepared to tackle PHB commercialisation. The oil crisis motivated to search for natural substitutes for synthetic plastics (Vasile and Zaikov, 2009). Then, ICI applied particular conditions to produce PHB up to 70% of biomass by *Alcaligenes eutrophus*. However, the PHB mechanical properties did not show any particular advantages over oil-based plastics such as polypropylene, due to extreme brittleness. As the price of oil stabilised, PHB production costs stayed higher than those for oil-based plastics, therefore; the original idea of improving PHB was put on hold (Chee et al., 2010). However, ICI had achieved a significant advance in PHB production by patenting a procedure to synthesise β -hydroxybutyrate and β -hydroxyvalerate copolymers (PHBV) (Masani et al., 2008).

2.5.1.1 Polyhydroxybutyrate properties and application

Even though PHB is the most known member of the PHAs family, to date more than 150 various monomeric units have been known as constituents of this polyesters family, giving rise to a massive versatility regarding potential applications and physical properties. Because of the diversity of PHA copolymer monomers, various physical properties which imply several applications are presented (Panchal et al., 2013). Such variety has allowed the

improvement of different applications, such as packaging, fibres, biocompatible and biodegradable implants and drugs and fine chemicals. The physical properties and chemical structure are quite similar to those of petroleum-based synthetic plastics and the physical state can determine the properties of PHB, which depend primarily on distribution of monomer units, molecular weight and distribution of these molecules in the chain. PHAs have the common features of biocompatible, non-toxic, biodegradable, and recyclable thermoplastics. These features give them advantages over synthetic polymers, especially polypropylene, and are presented in Figure 2.6.

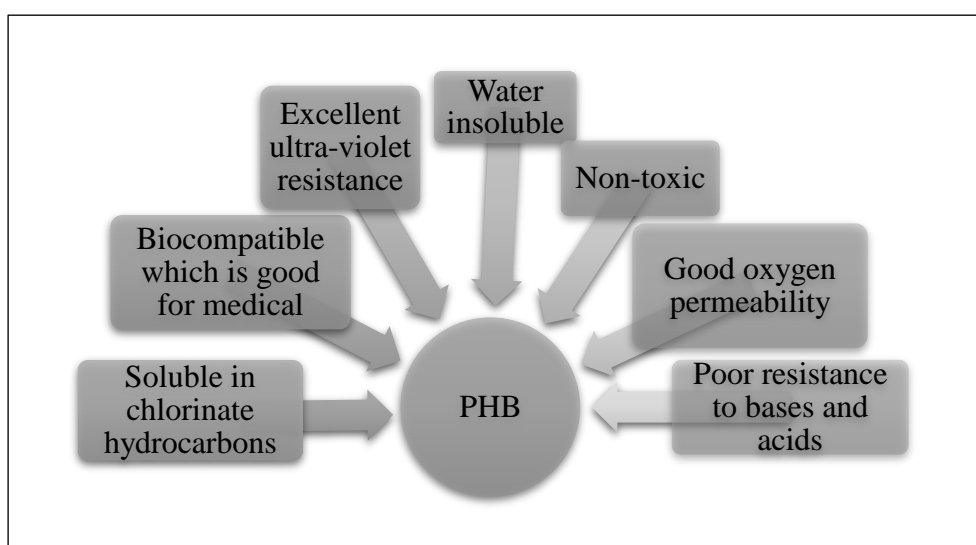


Figure 2.6. Some useful properties for PHB biopolymer adapted from (Ojumu et al., 2004).

PHB is a right-handed helix, optically active and R-configuration with central chiral carbon, melting point at 180°C and resistance to UV and solvents. It is a fully degradable thermoplastic polymer, partially crystalline material, brittle and stiff and microstructure, glass temperature and level of crystalline impact on the degree of brittleness (Gumel Y. Chisti, 2013; Baei et al., 2009). Because PHB is stiff, brittle and highly crystalline, it has quite poor mechanical properties, such as a low elongation at break, which limits its extent of industrial applications. Until now, there is no significant commercial synthesis of PHB products due to the higher production cost compared commercial plastics, difficult processing as well as high brittleness. Copolymer formation can alter the properties of PHB, such as increasing its toughness, and this can be changed in response to commercial requests. PHB is biodegradable even though it is not soluble in water.

Two different monomers can be combined to give materials with extremely different properties (Batcha et al., 2014). They are optically active, piezoelectric, highly crystalline and insoluble in water. As well as, the large variety of PHA monomers found provides a wide polymers spectrum with different physical properties. PHAs are thermoplastic polymers which they vary in their properties depending on their chemical compositions (homo, co-polyester or contained hydroxyl fatty acids). The general properties of PHA are presented in Table 2.4.

Table 2.4. Summarising the average properties of PHA's (Verlinden et al., 2007).

<i>Property (units)</i>	<i>Value</i>
Glass transition temperature, T_g (°C)	2
Melting temperature, T_m (°C)	160-175
Crystallinity degree, X_c (%)	40-60
Young's modulus, E [GPa]	1-2
Tensile strength, σ [MPa]	15-40
Elongation at break, ϵ (%)	1-15
Water vapour transmission rate; WVTR [$\text{g}\cdot\text{mm}/\text{m}^2\cdot\text{day}$]	2.36
Oxygen transmission rate. OTR [$\text{cc}\cdot\text{mm}/\text{m}^2\cdot\text{day}$]	55.12

Since PHB polymers have properties not available in other synthetic polymers, it is used in tissue-engineered cardiovascular products. It can be used as a system for drug delivery for prolonged therapy. PHB monomer is used in medical applications such as a surgical implant without any side effects such as inflammation, including some PHB nanoparticles were specific to certain cells such as cancer cells in the breast. PHB is used in pharmacology to deliver medicines as tablet packing and also has applications in fast foods, agriculture, and bottles (Verlinden et al., 2007). Figure 2.7 shows the most common PHB applications in the various field.

PHB polymers can be used to replace petrochemical plastics in numerous applications such as performance additives, foils and moulded goods. The main applications of the PHA family include substitute petrochemical base plastics currently in use for coating and packaging, also disposable items such as feminine hygiene products, razors, diapers, utensils, as well as disposable containers including cups and shampoo bottles. In addition to their potential uses as plastic materials, the PHA family are also useful as stereo-regular compounds which can use as chiral precursors for synthesising of optically active compounds. Including compounds are particularly utilised as biodegradable transporters for the long-term dosage of medications, drugs, hormones, herbicides and insecticide, see Figure 2.7. They are also broadly employed as osteosynthetic materials, vascular grafts, bone plates, surgical sutures, and heart valves (Reddy et al., 2003) and (Philip et al., 2007).

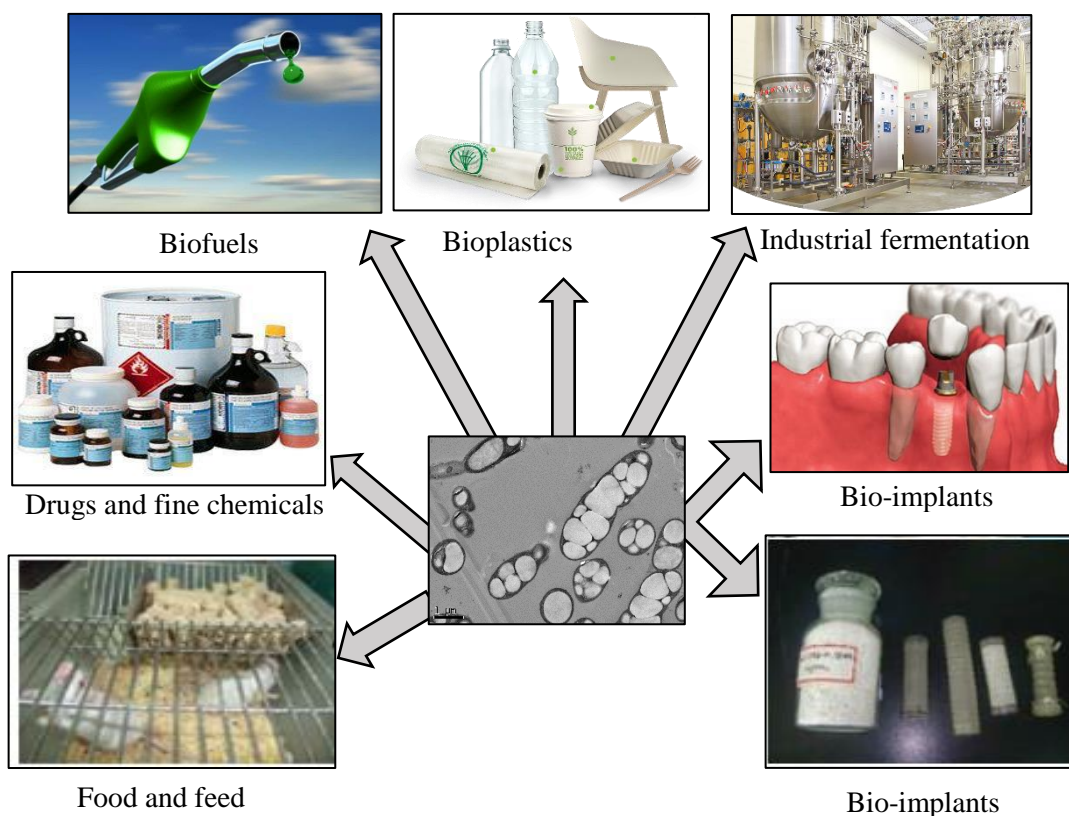


Figure 2.7. Polyhydroxyalkanoate (PHA) applications in different fields.

2.5.1.2 History of commercialisation and industrial production of PHA

In order to obtain a successful implementation system not only for PHB synthesis but also for commercialisation, all the fermentation conditions should be standardised. Ultimately, The PHB production price depends on the cost of a substrate, the yield of PHB on the substrate, and the product formulation efficiency in downstream processing (Tsuneo Yamane et al., 1996a). This implies the high level of PHB production as a percentage of dry cell weight and high productivity (gram of product (PHB) per unit volume and time). Wide use and commercial applications of PHB is obstructed because of its price.

Although PHAs can be utilised in a wide variety of applications, industrial production by fermentation has not been adopted because of the high costs of production. The current cost of PHB 10 times greater than that of synthetic polymers. For example, the cost of petroleum-based polymers, Polyethylene (PE) or Polypropylene (PP), is 0.25-0.5 \$/kg, whilst Biopol™ costs 6-16 \$/kg (Khanna and Srivastava, 2005a(Choi and Lee, 1997)). Furthermore, the cost production of PHB utilising the natural producer *A. eutrophus* is about \$16/ kg which is almost 18 times high-priced than the production cost of polypropylene. While by using recombinant *E. coli* as the producer, the PHB price can be

decreased to \$4/ kg, which is almost similar to other biodegradable plastics prices including aliphatic polyesters and PLA, and the commercial price should be in the range \$3–5/ kg (de Koning and Witholt, 1997). The reasons for this have been because of the using of pure substrates and cultures, the need to preserve sterile conditions and subsequent downstream recovery and purification. Among these, over half of the total production cost is accounted for by recovery and purification which have been regarded as essential factors for greatly reducing the cost. Furthermore, both the plant capacity and PHB yield were found to impact the overall production cost significantly, while the carbon substrate cost affected the total economics of PHB production (Mudliar et al., 2008; Bhattacharyya et al., 2012; Jung et al., 2005).

Although the improvement of both fermentation and purification technologies, along with the genetic engineering utilise, can help reduce biodegradable plastics production cost, the price is still quite high. Currently, just four types of PHBs and copolymers, PHB, PHBV, PHBHHx and P3HB4HB, have been synthesised on a large scale for commercial purposes, and the annual production of 70,000 tonnes/year. Despite several types of bacteria have been identified to synthesis PHAs, not all of them are appropriate for polyesters production on a large scale. Several factors affect the bacteria selection for an industrial process, such as accumulation and growth rates, achievable cell densities, bacteria safety and stability, a range of usable carbon sources, costs of the nutrients especially carbon source and PHA contents, polymer extractability, molecular weights of the product and finally by-products occurrence (Kessler et al., 2002).

The industry of biodegradable polymers lacks the features of economies, the large-scale production that is easily accomplished in the case of petroleum-derived polymers. For example, the capacity of polyethene production is about 300,000 tonnes per year, while biopolymers capacity reaches the range of 1,000 to 20,000 tonnes per year (Chanprateep, 2010b). The first company produced PHB for commercial applications was W. R. Grace and Company (W.R. Grace & Co., New York, USA), in 1959, the company shut down because of lacking purification methods and low efficiency of production (Baptist, 2002). Later on, during 1976, Imperial Chemical Industries (ICI Ltd., Billingham, UK) produced both PHB and P3HB/3HV, the latest was commercialised under Biopo^l™ as a trade name (Holmes, 1985). However, these biopolymers were highly cost compared to petroleum-based plastics, and the expected rise in oil prices, biopolymers production was not economically until the 90's. Table 2.5 shows both PHB and copolymers of PHB synthesising companies worldwide (Chen, 2010; Guilbert et al., 2005; Rudnik, 2008 ;Posada et al., 2011).

Table 2.5. Worldwide PHAs family commercial production, Current and potential big scale production plants of polyhydroxyalkanoates, (Chen and Chen, 2001; Page et al., 1992; Corporation, 2009; Pessoa-Jr et al., 2005b; Hänggi, 1990; Chanprateep, 2010a).

<i>PHAs types</i>	<i>Substrate</i>	<i>Microorganism</i>	<i>Company</i>	<i>Trade name</i>	<i>productivity tonnes</i>	<i>Time</i>	<i>Price (\$/kg)</i>
Several PHAs	Transgenic plants	Not known	ADM, USA (with Metabolix)	Not known	50000	2005- present	2.2
Several PHAs	Unknown	Not known	Procter & Gamble, USA	Tepha	Not known	1980s-2005	–
PHA copolymer	Unknown	<i>Aeromonas caviae</i> and <i>C. necator</i>	Meridian, USA	Nodax™	10000	2007-present	Unknown
PHB	Unknown	<i>Al. latus</i>	Biotechnology, Germany	Biomer®	50	1990s-present	3.75-6.25
PHB	Unknown	Unknown	Chemie Linz, China	Unknown	20-100	1980s	Unknown
	Unknown	Unknown	Jiangsu Nan Tian, China	Unknown	Not known	Unknown	Unknown
PHB, PHBV	Unknown	<i>Alcaligenes sp.</i>	Biocycles, Brazil	Biocycles	10000	1990s-present	2.5-3
PHB P3HB 4HB	Unknown	Unknown	Tianjin Northern, China	Not known	Pilot plant scale	1990s	Not known
PHB	Methanol	Unknown	Mitsubishi Gas Chemical, Japan	Biogreen™	30-60000	2010	2.75
PHB	Unknown	Unknown	BTF, Austria	Unknown	20-100	1990s	Unknown
PHV ¹	Unknown	Unknown	Zhejiang Tian An, China	Unknown	Unknown	Unknown	Unknown
PHA	Unknown	Unknown	Good fellow	Unknown	Unknown	Unknown	Unknown
PHBV	Unknown	Unknown	ICI, UK	Biopol	Unknown	Unknown	Unknown
PHB, PHV	Transgenic plants	Unknown	Monsanto, USA	Unknown	Unknown	1990s	Unknown
PHB, PHV	Unknown	Unknown	Wacker, BASF, Germany	Unknown	Pilot plant scale	1980s-2005	Unknown
PHA/PHB/PHO	Unknown	<i>Recombinant E. coli K12</i>	Metabolix, USA	Metabolix PHA	Unknown	Unknown	2.2-5

<i>PHAs types</i>	<i>Substrate</i>	<i>Microorganism</i>	<i>Company</i>	<i>Trade name</i>	<i>Productivity tonnes</i>	<i>Time</i>	<i>Price (\$/kg)</i>
PHB/PHV	Glucose-	<i>C. necator</i>	Metabolix, USA	Biopol™	Unknown	2005	4
PHB and P(3HB/3HV)	Sugar cane	<i>Alcaligenes sp.</i>	PHB Industrial Company (Brazil)	Biocycle	50	Unknown	Unknown
PHBH	Unknown	Unknown	P&G (US)	Nodax™	20,000-50,000	Unknown	2.50
PHBH	Unknown	Unknown	Lianyi Biotech (China)	Nodax™	2000	Unknown	3.70
PHBH	Unknown	Unknown	Kaneka Corporation (Japan)	Kaneka PHBH	1000	Unknown	Unknown
P(3HB/4HB)	Unknown	Unknown	Tianjin Gree Bio-Science	Green Bio	10,000	2004-present	Unknown
PHB & P(3HB/3HV)	Glucose	<i>Alcaligenes</i>	ICI / ZENECA BioProducts (UK)	Biopol™	20,000	Not known	Unknown
PHA from P&G	Not known	Not known	Meridian (US)	Meridian	272,000	2013	Unknown
PHB	Sucrose	<i>Alcaligenes latus (DSM1124)</i>	Biotechnologische (Austria)	Not known	Not known	2004-present	Unknown
PHB	Unknown	Not known	Telles (US)	Mirel™	50,000	2005-present	1,50
PHB & P(3HB/3HV)	Glucose	<i>C. necator H16</i>	ICI / ZENECA Bio-Products (UK)	Biopol™	Unknown	Unknown	Unknown
P(3HB/3HV)	Unknown	Unknown	Tianan Biologic, Ningbo (China)	Enmat(R)	10,000	Unknown	3.26
PHBV	potato starch	<i>Recombinant Escherichia Coil</i>	Zhejiang (China)	Econgen	2000	1990s-present	Unknown
Eastar-Bio Copolymer	Starch	Not known	Novamont S P A (Italy)	Minery PHA	60,000	2008-present	Unknown

During 1975-1976, the Imperial Chemical Industries (ICI) was begun to improve the PHB production as a response to the increase in the oil price. As early as in 1983, ICI began synthesising 'Biopol' by *C. necator* H16. Also in Japan, both Nippon Gohsei and Cargill Dow Polymers are marketing their 'Mater-Bi' and 'EcoPla', respectively. Mater-Bi biopolymer has been used in transmission packaging for electrical materials, agricultural mulch films, composting trials also (Percy, 2012).

In 1990, the German company Wella produced a new packaged of bottles shampoo made of Biopol™. ZENECA Bio Products at Billingham (UK) produced PHB and P3HB/3HV at scales up to 200,000 litres for 48h and a two-step of fed-batch fermentation process using a mutant *Ralstonia eutropha* H16 and utilising the technology improved by Schlegel et al. (Schlegel et al., 2003). Through the first step, a mineral salt medium was used for biomass growth using glucose and an amount of phosphate. Then, the second step was carried out, under phosphate limiting, until reaching 100 g/l of total dry weight. While the copolymer P3HB/3HV was produced by mixing feed of glucose and propionic acid (the ratio was selected according to the desired content of 3- hydroxyvalerate in the copolymer unit) in the stage of polymer accumulation (Byrom, 1990). Meanwhile, the bio-technologist for schungsgesellschaft company in Austria improved a one-step. Industrial process using *Alcaligenes latus* DSM1124 strain which accumulates PHB during balanced growth up to 85% of DCW. In spite of the Company produced a biomass of 60 g/l utilising sucrose and a mineral salts medium, in 1993 the company stopped the production (Kessler et al., 2002). During 1992 (in Brazil) a joint venture began between alcohol producer (the Balbo Group) and a sugar producer (Irmaoes Biagi) to produce PHB Industrial S.A. which produce 50 tonnes per year of PHB under Biocycle™ as a trade name (Pessoa-Jr et al., 2005). In the same year, the German Company Biomer Inc. gained microbial strains for PHB commercial production and the technology from Austrian company Petrochemical Danubia to release Biomer™ as the commercial name of PHB in 1995.

In 1996, ZENECA Bio-Products (UK) Company was sold to Monsanto Company (USA) which started producing transgenic plants process (like rapeseed and soybean) for PHB and PHB/ HV agricultural production, expecting a lower cost for the product (Kessler et al., 2002). Then finally was sold to the biotech-company Metabolix, Inc., Massachusetts (USA) Company. In 2004, Both Metabolix, Inc. and Archer Daniels Midland Company (ADM), agricultural processing world leader and fermentation technology and one of largest processors of wheat, ethanol soybeans (soy meal and oil), corn (sweeteners and flour), cocoa in the world, announced a new strategy to commercialise the PHA production. (USA) was

the selected place for the first commercial PHA that produce from the plant (corn) with an initial capacity reaching to 50,000 tonnes per year (Midland, 2006). In March 2010, Telles Company (the joint venture and Metabolix, Inc.) started producing the first commercial-scale PHA utilising a corn syrup resin under the trade name of Mirel™. It was expected to start shipping in April of 2010, but because of commercial and technical problems, by January 2011, the product (Mirel™) was not commercialised yet (Schut, 2011). Up to date, Mirel™ plastic is being commercialised as film and bag plastics by both UCA and Lakeside companies. By 2008, Metabolix, Inc. announced Company the structure of a second technology platform to produce PHA production directly from non-food crop plants, switch grass, and the associated biomass being utilised for energy production after the polymer is recovered (Thomas G. Auchincloss, 2008).

The main target of this process was to get PHA using switchgrass at a percentage of 20% of total dry cell weight and about 75% could be recovered. So, if the yields of switchgrass are in the range 10 to 15 tonnes per acre, each acre yield about 1.5 to 2.25 tonnes of PHA plastics or other derived chemicals, hence one million acres yield approximately 3.3 to 5 billion pounds (Somleva et al., 2008). The Nodax™ technology was released PHB and PHBH (Poly-hydroxybutyrate-co-hydroxyhexanoate) for a wide range of applications by the multinational corporation Procter and Gamble, Tsingta University in China the Riken Institute in Japan, but it was sold by 1993 (Chanprateep, 2010b).

In Takasago City, Hyogo, Japan (2010), Kaneka Corporation Company announced for launching a new soft polymer derived from plant- called Kaneka PHBH, with the capacity of 1,000 tonnes per year (Corporation, 2009). In addition, in Japan, Mitsubishi Gas Chemical Company made progress on the PHB production using fermentation of methanol under the trade name BioGreen™ (Edward Kosior et al., 2006). In Canada, company Biomatera Inc. specialises in the manufacture released a very interesting inexpensive biopolymer production via agricultural residues fermentation. This biopolymer had applications in creams and gels manufacturing which utilised slow-release agents in the manufacturing of cosmetic, tissue matrix regeneration and drug (Chanprateep, 2010b). At the beginning of PHAs family were the introduction to the market, a wide range of industrial, medical and commercial applications such as pencils, bottles, diapers, golf t-shirts and cosmetic cases (Baptist, 2002). But the largest applications for these types of bioplastics are those where large quantities of residues are accumulated, such as bottles, food-films, hygiene products and plastic bags, etc (Zinn et al., 2001).

Because of PHAs are biocompatibility materials, they can be utilised by human bodied without any risk for the health, human blood has 3-hydroxybutyric acid. Thus, they have a wide spectrum of medical applications. In this sense, PHA has been widely utilised for knitting, drugs delivery and base for many medical products like thread, syringes, etc. Another interest application for PHA is the various producing molecules through hydrolysis. PHAs enzymatic and chemical hydrolysis release several monomers which can be converted into high commercial molecules such as 3-hydroxyacids-esters, 2-alkanesm, 3-hydroxyacids, acid lactones, β -amynoacids and 3- hydroxyalkanols, (Woodford., 2017).

Mitsubishi and Nippon Shokubai under the trade names LUNARE ZT and Lunare SE market EnviroPlastic. Bionolle, a thermoplastic aliphatic polyester, is manufactured by Showa Highpolymer and Denko of Japan. It is produced by the polycondensation of glycol with dicarboxylic acids. Lacea is another type of bioplastic manufactured by Mitsui Chemicals (Japan) from fermented starch, derived from a variety of renewable resources, including tapioca, corn and beet. Lacea is comparable to polyethylene in terms of transparency and similar to polystyrene or polyethylene in terms of processability. Also, it claims excellent mould resistance, low heat of combustion that is similar to that of paper, biodegradability superior to that of earlier polylactic acid-based materials and superior stability in processing use (Pouton and Akhtar, 1996). Japan, Daicel Chemical Industries, has developed biodegradable blends of two different types of material, acetyl cellulose and polycaprolactone resin with the brand name 'Celgreen'. Shimadzu improved a fermentation process for lactic acid and collaborated with Mitsubishi Plastics Industries to develop poly-L-lactic acid. The resins are marketed with the trade name Lacty. While the other bioplastics manufactured by Japan-based firms are Eco-Ware and Eco-Foam (which are starch-based) and Cardoran and Pulluran which are based on polysaccharides, (Ahn et al., 2000).

2.5.1.3 Utilisation of waste materials for PHA production

Recently the issue of effluent from operation processing and their disposal has acquired common recognition. In various areas of the world, particularly the developing countries, the environmental problems are the same (Omezuruike Okonko et al., 2006). Human beings produce huge amounts of wastes as we go around our daily life. Wastes come from different sources such as junk mail, washing machines, toilets, packaging food preparation, baths, newspapers, hobbies, the landscape and auto and home maintenance projects. As well as, wastes are created in producing the services and goods we use. In general, waste is defined as any material, that has not yet been completely utilised such as

the leftovers from consumption and production. However, waste is an expensive and sometimes inevitable results of human activities.

It includes plant materials, agricultural (cellulose, hemicellulose and lignin), industrial (industrial activities), and municipal wastes and residues (domestic waste: household, institutional and public cleansing) and (Commercial waste: shops, restaurant, offices and markets).

On the other hand, waste also refers to solid or liquid discharged from business premises, residences, institutions and small-scale industries. Waste can be characterised according to its organic contents or bulk, specific contaminants and physical characteristics Omezuruike Okonko et al., (Omezuruike Okonko et al., 2006) reported that each waste contains its unique characteristics and quality, that then offers the treatment type required. The two divisions of waste (industrial effluent and domestic) have various make-ups and usually require different treatment processes. Generally, the waste treatment process is classified into various levels: primary, secondary, tertiary, and quaternary treatment and with each level targeted at removing a more particular class of contaminants (Choubey et al., 2250) and (Omezuruike Okonko et al., 2006). Although several disposals and clean-up options exist, no single treatment process can be utilised to all types of waste streams and the trend in the world is to convert wastes into various useful products including fine chemical products and fuels, through the microorganism's manipulation or by recycling waste products as much as possible and the microorganism's role in waste utilisation has been investigated extensively by many authors. Waste utilisation is an economically efficient method and ecologically safe for the waste management since; the wastes are not treated either spending money nor disposed of off in the landfill causing pollutions.

Organic origin materials are known as biomass, the term that describes energy material that originates from biological sources, and are of major significance to sustainable development because these materials are renewable as opposed to fossil carbohydrates and non-organic materials (van Wyk, 1997). Examples of farm organic wastes: maize cobs, maize chaff, pawpaw fruit peels and banana peels. Also, it was shown that the short life-cycle and production of large quantities of off springs could be harnessed for the raising of feed for livestock/fish and in many cases human consuming. This invertebrates culture offered economic advantages to the farmer and it developed on the environmental quality by converting waste materials into beneficial products. Surveys on the potential for waste to reduce energy issues, in Tanzania using of agro-industrial residues for anaerobic transformation into biodiesel, sisal industry and biogas. The largest producer of agro-

industrial residues has a possibility to produce energy that might considerably supplement the current shortfall of generation of hydropower

(Kivaisi and Rubindamayugi, 1996). During 2004, (Martin et al., 2004) reported that the utilise the substrate obtained from agricultural by-products for enzyme production was inexpensive and can facilitate scale-up production of enzymes industry in the tropics. The raw material cost, mainly the carbon source, is considered to be one of the main factors that affect the overall economics of PHA manufacture, particularly for large-scale processes (Castilho et al., 2009). Consequently, the economic feasibility of PHA production is intrinsically combined with developing efficient fermentation processes using inexpensive carbon sources.

The utilisation of waste products as carbon sources presents a double opportunity for the concomitant production of value-added materials and reduction of waste disposal costs (Du et al., 2012). The appropriate waste materials utilised for PHAs synthesis can be classified into six media groups: cellulosic and hemicellulosic media, whey-based media, sugar-based media, oil- and glycerol-based and starch based media (Amache et al., 2013). As shown in Table 2.6 which present different microbial strains have been evaluated for their ability to accumulate PHAs from using various type of waste.

Table 2.6. The cost of both substrate and PHB yield used on PHB production (modified from Tsuneo Yamane et al., 1996b).

<i>Substrate types</i>	<i>Substrate price (\$/kg)</i>	$y_{p/s}$	<i>Production cost (\$/Kg_{PHB})</i>
<i>Waste-based substrate</i>			
<i>Cheese whey</i>	0.071	0.33	0.22
<i>Can molasses</i>	0.22	0.42	0.52
<i>Hemicellulose hydrolysate</i>	0.069	0.2	0.34
<i>Hydrolysed corn starch</i>	0.22	0.185	0.58
<i>Pure substrate</i>			
<i>Acetic acid</i>	0.595	0.38	1.56
<i>Ethanol</i>	0.502	0.5	1
<i>Glucose</i>	0.493	0.38	1.3
<i>Methanol</i>	0.18	0.43	0.42
<i>Sucrose</i>	0.29	0.4	0.72

As mentioned previously, the carbon cost represents up to about 50% of the PHB production cost (Solaiman et al., 2006b), therefore, using of inexpensive substrates can reduce the production cost. A report from Lee SY (Lee, 1996) summarised the impact of substrate price and production yield on the final PHB production cost. Table 2.7 indicates that for the same yield, the final product cost can be more than seven times high-priced depending on the substrate utilised. Historically, fatty acids were the preferred carbon source for mcl-PHAs production while last a few years, using of by-products from industries or waste streams as fermentative carbon sources have led to real interest.

Consequently, other various substrate sources were applied to synthesise mcl-PHAs including a combination of pure glycerol and soybean oil-based biodiesel. Soy molasses was also investigated to produce mcl-PHA but just low productivities were attained (Ashby et al., 2005) (Ashby et al., 2004).

On the other hand, utilisation of cheese whey, starch hydrolysate xylose, bagasse and molasses, which are cheap and renewable substrates, has been tested for the production of PHB. According to Table 2.7, hemicellulose hydrolysates and cheese whey show the least expensive carbonaceous compounds for microorganism biopolymer synthesis even when compared to quiet inexpensive substrates such as methanol. Comparing the benefit obtained by using hemicellulose hydrolysate instead of glucose, the production cost reduces by four, in spite the conversion yield is less for hemicellulose hydrolysate. A maximum PHB yield of 25 g/l was achieved using *Bacillus sp. JMa5* was growing in media containing sucrose sugar and cane molasses, making the production process economically feasible (Solaiman et al., 2006b).

Table 2.7. Various types of raw materials utilised in biopolymer industries.

<i>Waste-medium types</i>	<i>Microorganism</i>	<i>Gram stain</i>	<i>PHA type, PHA concentration(g/l) and PHA content (%)</i>			<i>Fermentation Scale</i>	<i>Reference</i>
<i>Cellulose and hemicellulose-based waste</i>							
Bagasse hydrolysate	<i>Burkholderia sacchari</i>	–	2.73	PHA	–	10 L	(Silva et al., 2004)
	<i>Cupriavidus necator</i> H16		3.9	PHA	54	Shake flask	(Yu and Stahl, 2008)
		–	–	Poly-(3HB-co-3HVco4HV)	56	Shake flask	(Lopes et al., 2014)
Wheat Bran hydrolysate	<i>Halomonas boliviensis</i>	–	4	P(3HB)	–	1.3 L	(Van-Thuoc et al., 2007)
Soy bean	<i>Recombinant E.coli</i>	–	4.4	PHA	–	Shake flask	(Yup Lee, 1998)
Orange peel	<i>Cupriavidus necator</i> H16	–	–	P(3HB)	–	Shake flask	(Guzman Lagunes and Winterburn, 2016)
Poplar wood from Hemicelulosic fraction	<i>Pseudomonas hydrogenovora</i>	–	6.57	P(3HB)	–	Shake flask	(Bertrand et al., 1990)
Xylose with propionic acid	<i>Burkholderia cepacian</i> Atcc 17759	–	1.6-3.7	PHBV	–	Shake flask	(Cesário and de Almeida, 2015)
Xylose and glucose from sugar cane bagasse	<i>Burkholderia cepacian</i> IPT 046	–	–	P(3HB)	34.8	2 L	(Li et al., 2007)
Hemicelulosic hydrolysed	<i>Burkholderia cepacian</i> Atcc 17759	–	2	P(3HB)	–	Shake flask	(Keenan et al., 2006)
Cellulose in tequila bagasse	<i>Saccharophogus degradaus</i> ATCC	–	1.5	PHA	–	1.5 L	(Alva Munoz and Riley, 2008)
Malt & soya waste	<i>Alcaligenes latus</i> DSM	–	–	P(3HB)	71-33	Shake flask	(Yu et al., 1999)

<i>Waste-medium types</i>	<i>Microorganism</i>	<i>Gram stain</i>	<i>PHA type, PHA concentration(g/l) and PHA content (%)</i>			<i>Fermentation Scale</i>	<i>Reference</i>
Oils and glycerol based							
Canola oil	<i>W. Eutropha</i>	–	18.27	P(3HB)	–	3 L	(López-Cuellar et al., 2011)
Linseed oil	<i>Pseudomonas aeruginosa</i>	–	1.8	PHA	50.2	Shake flask	(Bassas et al., 2008)
Olive oil mill was	<i>Recombinant P.putida</i>	–	0.13	P(3HB)	–	Shake flask	(Javers and Karunanithy, 2012)
Oleic acid	<i>Pseudomonas putida</i>	–	19	PHA	–	Shake flask	(Ward et al., 2005)
Lauric acid		–	25		–	Shake flask	
Unsaponified olive oil	<i>Aeromonas caviae</i>	+	–	mcl-PHA	96	1.5 L	(Cromwick et al., 1996)
Soybean oil	<i>C.necator</i>	–	38.1	PHA	–	Shake flask	(Kahar et al., 2004a)
	<i>Pseudomonas stutzeri</i>	–	1	PHA	–	Shake flask	(He et al., 1998)
	<i>Wild strain of C. necator and recombinant</i>	–	2.5-6	P(3HB-co-3HHx-co-3HO)	–	10 L	(Tsuge et al., 2009)
Waste Glycerol	<i>Osmophilic wild type strain</i>	–	16.2	mcl-PHA	–	5 L	(Koller et al., 2015)
	<i>Cupriavidus necator DSM 545</i>	–	–	P(3HB)	50	8 L	(Cavalheiro et al., 2009a)
	<i>Pseudomonas putida GO16</i>	–	–	P(3HO/HD)	33	5 L	(Follonier et al., 2012)
Waste frying oil (from rapeseed)	<i>Cupriavidus necator</i>	–	1.2	mcl-PHA	–	Shake flask	(Xu et al., 2010)
	<i>Pseudomonas aeruginosa</i>	–	2.3	mcl-PHA	–	Shake flask	(Fernández et al., 2005)
		–	5.4		–	Shake flask	(Haba et al., 2007)
		–	3.43		–	Shake flask	(Costa et al., 2009)
	<i>Cupriavidus necator H16</i>	–	–	P(3HB) Poly-(3HB-co-3HV)	35-76	3L	(Verlinden et al., 2011)

Waste-medium types	Microorganism	Gram stain	PHA type, PHA concentration(g/l) and PHA content (%)			Fermentation Scale	Reference
Spent palm oil	<i>C.necator</i>	–	4.4	P(3HB-co-4HB),	–	8 L	(Rao et al., 2010)
Waste vegetable oil	<i>Pseudomonas sp.</i>	–	–	PHA	23.5	4L	(Song et al., 2008)
Butter oil, soybean oil and coconut oil	<i>Pseudomonas aeruginosa</i>	–	2.1	mcl-PHA	–	Shake flas	(Ashby and Foglia, 1998)
Coconut oil tallow	<i>Pseudomonas saccharophila</i>	–	0.8	mcl-PHA	–	Shake flask	(Solaiman et al., 1999)
Palm kernel oil	<i>C.necator</i>	–	6.79	P(3HB-co-3HV-co-3HHx)	–	3 L	(Bhubalan et al., 2008)
		–	2.5	PHA	–	1.5 L	(Lee et al., 2008)
Jatropha oil	<i>Marine bacteria, SM-P-3M</i>	–	0.306	PHA	–	Shake flask	(Shrivastav et al., 2010)
<u>Euphorbia and Castor</u>	<u><i>Pseudomonas aeruginos</i></u>	–	–	PH	20-30	Shake flask	(Tan et al., 1997)
Starch based							
Wheat	<i>C.necator</i>	–	–	PHA	51.1	Shake flask	(Koutinas et al., 2007)
Soluble starch	<i>Azotobacter chroococcum</i>	–	–	P(3HB)	–	3.5 L	(Kim, 2000)
	<i>Bacillus cereus</i>	+	0.48	P(3HB)	–	Shake flask	(Prakash, 2008)
Waste potato starch	<i>Ralstonia eutropha</i>	–	–		94	5 L	(Haas et al., 2008)
Rice straw	<i>Bacillus cereus</i>	–	–		57.5	2 L	(Smith, 2006)
Beet molasses	<i>Recombinant E.coli</i>	–	9	mcl-PHA	–	2.5 L	(Liu et al., 1998)
	<i>Bacillus cereus</i>	+	0.16	PHA	–	5 L	(Yilmaz and Beyatli, 2005)
	<i>Azotobacter vinelandii</i>	–	23	mcl-PHA	–	30 L	(W. Page, 1992)
Sugar cane	<i>Pseudomonas fluorescens</i>	–	22	PHA	–	1L	(Jiang et al., 2008a)

Waste-medium types	Microorganism	Gram stain	PHA type, PHA concentration(g/l) and PHA content (%)			Fermentation Scale	Reference
Date syrup	<i>Bacillus megaterium</i>	+	1.5	P(3HB)	–	Shake flask	(Omar et al., 2001a)
	<i>Bacillus SA</i>	+	5.8	P(3HB)	–	1L	(Khiyami et al., 2011)
Corn syrup	<i>Cupriavidus necator</i> DSMZ 545	–	–	P(3HB)	65	Shake flask	(Kumar et al., 2013)
Hydrolysed starch	<i>Halomonas boliviensis</i>	–	–		56	Shake flask	(Van-Thuoc et al., 2007)
Molasses							
Sugar beet molasses	<i>Azotobacter vinelandii</i> UWD	–	19-22	P(3HB)	–	Shake flask	(W. J. Page, 1992)
		–	–	P(3HB)	36	4 L	(Chen and Page, 1997)
Sugar cane molasses	<i>Bacillus Jma</i>	–	–	P(3HB)	25-35	Shake flask	(Chen and Chen, 2001)
	<i>Bacillus megaterium</i> ATCC6748	+	2.2	P(3HB)	43	2 L	(Liu et al., 1998)
			30.5		42	Shake flask	(Kulprecha et al., 2009)
Molasses	<i>Bacterial consortium</i>	+	0.37-0.5	PHAs	–	Shake flask	(Zakaria Gomaa, 2014)
	<i>Bacillus sp. JMa5</i>	+	–	P(3HB)	35	Shake flask	(Mahishi et al., 2003)
	<i>Cupriavidus necator</i> H16 DSM 545	–	–	P(3HB)	31– 44	Shake flask	(JK et al., 1998)
Soy molasses	<i>Pseudomonas corrugate</i>	–	–	mcl- PHA	5-17	Shake flask	(Solaiman et al., 2006d)
	<i>Bacillus sp.CL1</i>	+	–	mcl-PHA	90	Shake flask	(Full et al., 2006)
	<i>Pseudomonas Corrugate</i>	–	–	PHA	17	Shake flask	(Solaiman et al., 2006d)
Waste water							
Alhechin (olive oil mill wastewater)	<i>Azotobacter chroococcum</i> H23	–	–	PHA	70	10 L	(Ceyhan and Ozdemir, 2011)
Wastewater	<i>Enterobacter aerogenes</i>	–	5.2	P(3HB)	–	Shake flask	(Martinez-Toledo et al., 1995)

Waste-medium types	Microorganism	Gram stain	PHA type, PHA concentration(g/l) and PHA content (%)			Fermentation Scale	Reference
Whey Based							
Whey	<i>Recombinant E.coli</i>	–		P(3HB)	35.5	5L	(Ahn et al., 2001)
		–	5.2	P(3HB)	–	1.5 L	(Ahn et al., 2000)
		–	21.00	PHA	96.2	220 L	(Thirumala et al., 2009)
	<i>Haloferaz mediterranei</i>	–	7.2	P(3HB)	–	10 L	(Koller and Braunegg, 2015)
	<i>Pseudomonas hydrogenovora</i>	–	1.27	PHA	–	Shake flask	(Koller et al., 2008a)
	<i>Hydrogenophaga pseudoflava</i>	–	3.00	P(3HB)	–	Shake flask	(Koller et al., 2007)
	<i>Thermus thermophilus HB8 ATCC 27634</i>	–	–	mcl-PHA	35.6	Shake flask	(Pantazaki et al., 2009)
Hydrolysed soy and malt	<i>Bacillus sp.</i>	–	18.42	PHAs	–	Shake flask	(Law et al., 2001)

In general, the most common, cheap substrate utilised as an industrial waste is molasses, either from beet or sugarcane. So far, many articles have been published on the PHAs production utilising various raw materials as carbon sources as well as different strains have been assessed for their ability to produce PHA from date syrup, sugar cane and beet molasses. By using molasses, the highest PHA concentration reported is 23 g/l (Page et al., 1992) and 22g/l (Jiang et al., 2008b) by *Azotobacter vinelandii* and *Pseudomonas fluorescens*, respectively using submerged fermentation. Within the current technology and regarding other strains, it has been found that *Bacillus sp.* is less feasible to produce PHAs due to the low levels of production (Omar et al., 2001b) Khiyami et al., 2011; Yilmaz and Beyatli, 2005).

Different microorganisms are able to utilise starch-based waste media efficiently and produce PHAs (Haas et al., 2008) found that the PHA yield of 94 g/l using *Ralstonia eutropha* as the producer microorganism and potato starch as substrate. Glycerol, acetic acid and sucrose have been utilised to produce scl-PHAs, but the most efficient substrate for scl-PHAs production is glucose. Several microorganisms can be used some petroleum-derived materials as carbon sources such as aldehydes, alkanes and alkenes that act as precursor substrates for the mcl-PHAs production (Francis, 2011). When the process is scaled-up, the raw materials part to rising fraction of overall PHAs production costs, and these raw material costs being the carbon source. The economic feasibility of PHA manufacturing is intrinsically related to improving efficient biotechnological processes by using a cheap carbon source (Castilho et al., 2009).

Therefore, use of waste and by- products as carbon sources offer the feature of simultaneously enabling both reduce the disposal costs and the production of value-added materials (Du et al., 2012). One group of waste media that can be used easily by different microbes is starch based waste media. The results of producing 94 g/l PHA by *C. necator* utilising potato starch as a substrate while was 51.1 g/l of *C. necator* by using wheat (Koutinas et al., 2007). Based media for and production high value-added materials. Also, cellulose and hemicellulose-based waste materials have been studied extensively. By 2011, Brazil, as a top county for producing sugar, produced about 625 Mtons of sugarcane and approximately 280 kg of wet bagasse was generated from each tonne of sugarcane. (Chandel et al., 2011) and (Silva et al., 2004) reported the possibility of using of bagasse as a substrate for production PHA by *Burkholderiasacchari* and they obtained about 2 g/l PHB.

Glycerol is one of the most common substrates for biological processes, about 500,000 tonnes of crude glycerol annually is produced as a side product of biodiesel production process (Koller and Braunegg, 2015). Both (Cavalheiro et al., 2009b; Koller et al., 2008b) successfully used this product for PHA synthesis, Cavalheiro et al. obtained 38 g/l of PHA by *C.necator*, while Koller et al., achieved 16 g/l of PHA production using an *osmophilic* wild-type strain. By using oil-based waste materials as a carbon source, various concentrations of PHAs have been reported in the literature, getting the highest concentration of 85-95 g/l, using *C. necator*, reported by (Kahar et al., 2004a). Among the waste and by-product materials discussed, the most promising one emerged as whey. Whey is considered as an attractive substrate and is one of the most common studied waste raw materials because of its availability, high nitrogen content about 14-16% N in protein (Robert, 2006) as well as, it is the major waste material of casein and cheese production. Annually the European Union produce around 40 Mtons of cheese whey, and approximately 13 Mtons of that remains unused (Koller et al., 2005).

As can be seen from Table 3.7, the majority of the study achieved utilising whey as a substrate is of the recombinant strain of *E.coli*. Moreover, whey was utilised in the largest bioreactor scale (220 L), and the highest concentration of PHA observed was 96.2 g/l by (Ahn et al., 2000). It is noted from the examples given in table 3.7 that PHB can be accumulated using wide range types of waste and by-product materials as well as fermentations has so far been centred on small laboratory scale of no more than 1 L of working volume. Another expectation can be observed that the PHB content might be got in this study in the PHB in the range 60-80%. These tables are used as a basis for comparison when explanation the fermentation experiment results reported in this thesis.

2.5.1.4 Bacteria producing polyhydroxyalkanoate (PHA)

Various microorganisms under unbalance growth conditions are capable of producing PHB such as photosynthetic bacteria, archaeobacteria, as well as gram-negative and positive bacteria. An overview of PHAs accumulating by an extensive range of bacterial species with their substrates (carbon sources) is outlined in Table 2.8. As well as this table presents several bacteria species are capable of accumulating a broad range of polymers such as co and ter-copolymers with different functional categories applying a wide range of substrates.

Table 2.8. An overview of PHA accumulating by different bacterial stains and carbon sources.

<i>Strains</i>	<i>PHAs Types</i>	<i>Substrates</i>	<i>% PHA content</i>	<i>References</i>
<i>Gram-negative bacteria</i>				
<i>A. latus DSM 1123</i>	P(3HB)	unknown	47-88	(Wang and Lee, 1997)
<i>Bacillus mycoides strain RLJ B-017</i>			47.7	(Thakur et al., 2001)
<i>Azohydromonas lata</i>			95	(Penloglou et al., 2012)
<i>Bacillus megaterium</i>	P(3HB)	unknown	62.4	(Naranjo et al., 2013)
<i>Zobellella denitrificans MW1</i>			87	(Ibrahim and SteinbÄchel, 2010)
<i>M. rhodesianum MB 126</i>	P(3HB)	unknown	42	(D. Kalaiyezhini and Ramachandran, 2015)
<i>H. mediterranei ATCC 33500</i>	Poly-(3HB-co-3HV)	unknown	55.6	(Han et al., 2015)
<i>Pseudomonas aeruginosa IFO3924</i>	mcl-PHA	unknown	36-39	(Gumel et al., 2012)
<i>Azohydromonas australica ATCC 29713</i>	P3HB	unknown	76.5–79.4	(Gomez et al., 1996)
<i>Azotobacter beijerinckii, NCIB 11292</i>	P3HB	unknown	24.8	(Lasemi et al., 2013)
<i>Burkholderia cepacian, ATCC</i>	P3HB	Xylose	58.4	(Pan et al., 2012)
<i>Burkholderia cepacian, DSM 50181</i>	P3HB	Glycerol	31.3	(Zhu et al., 2010)
<i>Burkholderia cepacian, NCIB9085</i>	P3HB	Fructose, glucose, sucrose	50.4–59	(Gomez et al., 1996)
<i>OC1aulobacter vibrioides, DSM 4727</i>	P3HB	Glucose	18.3	(Rehm and Qi, 2001)
<i>Methylobacterium extorquens ATCC</i>	P3HB	Methanol	40–46	(Bourque et al., 1995)
<i>Methylobacterium extorquens</i>	P3HB	Methanol	35–62.3	(Mokhtari-Hosseini et al., 2009)
<i>Novosphingobium nitrogenifigens Y88 DSM 19370, ICMP 16470</i>	P3HB	Glucose	81	(Smit et al., 2012)
<i>Pseudomonas oleovorans ATCC 8062,</i>	scl-mcl-PHA	4-Hydroxyhexanoic acid	18.6	(Valentin et al., 1994)
<i>Paracoccus denitrificans ATCC</i>	P3HV	<i>n</i> -Pentanol	22 –24	(T Yamane et al., 1996)

<i>Strains</i>	<i>PHAs Types</i>	<i>Substrates</i>	<i>% PHA content</i>	<i>References</i>
<i>Pseudomonas mendocina</i> ATCC 25411,	scl-mcl-PHA	octanoate	13–19.3	(Lee et al., 1995)
<i>Pseudomonas putida</i>	mcl-PHA	Terephthalic acid from polyethylene	27	(Kenny et al., 2008)
<i>Pseudomonas putida</i> GPo1	mcl-PHA	Alkenes, <i>n</i> -alkanes	2–60	(Lageveen et al., 1988)
<i>Pseudomonas putida</i> mt-2 NCIMB 10432	mcl-PHA	citric acid, glucose, glycerol and octanoic.	4–77	(Nikodinovic et al., 2008)
<i>Pseudomonas putida</i> F1 ATCC 700007, DSM 6899	mcl-PHA	Benzene, ethylbenzene and toluene	1–22	(Hori et al., 2002)
<i>Seudomonas putida</i> KT2440 ATCC 47054	mcl-PHA	4-Hydroxyhexanoic acid	26–75.4	(Jiang et al., 2013)
<i>Sphaerotilus natans</i>	PHB	Glucose	40	(Takeda et al., 1995)
<i>Alcaligenes eutrophus</i>	PHB	Propionate	26-36	(Park et al., 2014)
<i>Bacillus megaterium</i> QMB 1551		Gluconate	46-85	(Liebergesell et al., 2008)
<i>Pseudomonas oleovorans</i>	PHB	Gluconate	1.5-5	(Liebergesell et al., 2008)
<i>Bacillus megaterium</i> QMB 1551	PHB	Glucose	20	(Floccari et al., 1995)
<i>Methylobacterium rhodesianum</i>	PHB	Fructose/Methanol	30	(Ackermann and Babel, 1997)
<i>Pseudomonas frederiksbergensis</i> GO23 a NCIMB 41539	mcl-PHA	Terephthalic acid from polyethylene terephthalate pyrolysis	24	(Kenny et al., 2008)
Gram positive				
<i>Bacillus megaterium</i> DSM 90	P3HB	Citric and succinic acid Glucose and glycerol.	9–50	(Shahid et al., 2013)
<i>Bacillus megaterium</i> CCM 1464, DSM 509,	P3HB, sclmcl-PHA, mcl-PHA	octanoic acid	3–48	(Shahid et al., 2013)
<i>Corynebacterium hydrocarboxydans</i> ATCC 21767	3HB, 3HV	Acetate, glucose	8–21	(Haywood et al., 1991)

<i>Strains</i>	<i>PHAs Types</i>	<i>Substrates</i>	<i>% PHA content</i>	<i>References</i>
<i>Corynebacterium glutamicum</i> ATCC 15990, DSM 20137, NCIB 10337	P3HB, mcl-PHA	Acetic acid, citric acid, glucose, glycerol, succinic acid	4–32	(Shahid et al., 2013)
<i>Microbunus phosphovorius</i> DSM 10555, JCM 9379	3HB, 3HV	Glucose	20–30	(Akar et al., 2006)
<i>Nocardia lucida</i> NCIMB 10980	3HB, 3HV	Acetate, succinate	7–20	(Valappil et al., 2006)
Archaea				
<i>Haloferax mediterranei</i> ATCC 33500, CCM 3361, DSM 1411	P3HB3HV	Vinasse	50–73	(Bhattacharyya et al., 2012)
	P(3HB)	Glycerol and crude glycerol from biodiesel production	72.8–76	(Nakazawa et al., 2015)
<i>Various archaeal strains</i>	P3HB, P3HB3HV	Fructose, glucose, glycerol	0.8–22.9	(Chee et al., 2010)
<i>Pseudomonas putida</i> GP _{p104}	P(3HB)	Octanoate	14–22	Liebergesell <i>et al.</i> , 2008)
<i>Cupriavidus necator</i> H16	Glucose	P(3HB)	24	(Du et al., 2000)
<i>Cupriavidus necator</i> NCIMB 11599	Glucose+propionic acid	<i>P(3HB-co-3HV)</i>	74	(Lee et al., 1994)
<i>C. necator</i> NCIMB 11599	Glucose	P(3HB)	70–80	(Ryu et al., 1997) and (Shang et al., 2007).
<i>Cupriavidus necator</i> B-10646	Glucose+valerate,hexanoate,propionate, γ -butyrolactone	P(3HB)	85	(Volova et al., 2014)
		P(3HB/3HV/4HB)	83	
		P(3HB/3HV/3HHx)	83	
<i>Cupriavidus necator</i> H16	Orange peel	P(3HB)		(Guzman Lagunes and Winterburn, 2016)
<i>Cupriavidus necator</i>	Fructose+ valerate	<i>P(3HB-co-3HV)</i>	86	(Du et al., 2001)

<i>Strains</i>	<i>PHAs Types</i>	<i>Substrates</i>	<i>% PHA content</i>	<i>References</i>
<i>Recombinant Cupriavidus necator</i>	Palm olefin, crude, palm acid oil and palm kernel oil	P(3HB-co- 3HHx)	40-90	(Loo et al., 2005)
<i>Cupriavidus necator ATCC 17699</i>	Fructose	P(3HB)	26.7	(Seo et al., 1998)
<i>Cupriavidus necator NRRL B-14690</i>		P(3HB)	34	(Patwardhan and Srivastava, 2008)
<i>Cupriavidus necator B-5786</i>		P(3HB)	82	(Volova and Kalacheva, 2005)
<i>Cupriavidus necator ATCC 17699,</i>	Acetate, butyrate, lactic acid, propionic acid	P(3HB)	79.0–82.0	(Chakraborty et al., 2009)
<i>Cupriavidus necator KCTC 22496,</i>	Fructose, Glucose	P(3HB)	67.0–70.5	(Gomez et al., 1996)
<i>Cupriavidus necator NCIB 10442</i>	Corn oil, oleic acid, olive oil, palm oil	3HB, 3HV	3.9–40.7	(Fukui and Doi, 1998)
<i>Cupriavidus necator DSM 545</i>	Glucose, propionic acid	P(3HB3HV)	80.0	(Du et al., 2001)

From the Table 2.8 can be seen that most strains used for PHAs accumulation are Gram-negative which have been isolated from a board range of environments such as contaminated soil, activated sludge and waste water. Generally, these types of bacteria are preferred for PHA accumulation because of their ability to utilise a wide range of carbon sources with high productivities. *C. necator* is a Gram-negative bacterium which considered a model PHB producing microorganism, because it is able to accumulate PHB to a high level of 90% of dry cell weight. *C. necator* can metabolise a wide range of carbon sources to accumulate PHB, such as carbohydrates, fatty acids as well as carbon dioxide (Chee et al., 2010). It has been reported that the *C. necator* genus is the most versatile producer of PHA. Some potential novel methods of producing PHA are using genetic engineering. Slater *et al.* cloned the genes required for PHB production from acetyl-CoA in *C.necator*. The genes introduced successfully into *E.coli* wild type, where the new *E.coli* recombinant strain was capable of synthesising PHB in large quantities reached to 80 %. Genetic engineering has been applied to optimise bacterial accumulation of PHAs.

E.coli, a Gram-negative bacterium, is widely utilised for gene transfer applications and genetic modification to obtain high PHAs producing recombinant strains. However, the presence of lipopolysaccharide (LPS) endotoxins in the Gram-negative bacteria's outer cell membrane, is considered the main concern with this bacteria that may lead to co-purify with PHA granules during the extraction process (Tan et al., 2014). LPS endotoxin causes an intense inflammatory response (because is a pyrogen material) hence making the PHA inadequate for medical applications (Ray et al., 2013). However, these methods increase the overall price and complexity of PHA manufacturing as well as changes in PHA properties such as poly-dispersity and reduction in molecular mass.

On the other hand, Gram-positive bacteria are mostly found to accumulate scl-PHA polymer with low productivity, therefore; have yet to be utilised for commercial production of PHA. In spite of producing smaller amounts of scl-PHA, Gram-positive bacteria are pore over Gram-negative bacteria having to their lack of LPS material hence making PHA is better sourcing for biomedical applications (Valappil et al., 2008). However, several Gram-positive bacteria are identified to synthesis lipidated macro amphiphiles such as lipoteichoic acids (LTA) and lipoglycan that have the same immunogenic properties of LPS (Ray et al., 2013).

2.5.1.5 Challenges of PHA production

Synthetic plastics are based on petroleum, a non-regenerative and finite resource. It is hard to predict precisely how long petroleum will be obtainable in large quantities, but estimates see a depletion of the petroleum sources for the actual century. It is thought that because of the increased expenditures the petroleum price is going to raise on long-term. (Owen et al., 2010) and (Miller et al., 2015). In order to compete with synthetic plastics, bio-based and biodegradable plastic materials must mimic the desired chemical and physical properties of their chemical homologues. PHA family offer a promising alternative due to having similar properties to synthetic elastomers and thermoplastics. Interest in PHAs as replacements for traditional plastics began to grow in the 1970's, since then PHAs have been synthesised at different fermentation scales, starting from shake flasks to industrial fermenters, as environmentally friendly with properties similar traditional plastics making them appropriate candidates for their substitution (Schlegel et al., 2003).

Different types of strains, including wild strains and recombinant, have been used in batch, fed-batch and continuous fermentations utilising various carbons sources, feeding strategies and operation conditions. Despite several undisputed advantages of PHA, mainly wider use of PHA is limited, and only a few companies are commercialising PHA, and its production cost account for less than 0.02% of the plastic market (Bioplastic, 2014). Therefore, today PHAs products are serving niche markets only. However, wide use of PHA and commercial applications is hampered because of its price. The Table 2.9 summarises the major drawbacks are leading to restrict the widespread of PHA use with their expectations.

The raw materials costs and products purification are determined as the crucial cost factors for a considerable large-scale production. The most important obstacles affecting the overall production process are: production of PHAs are still a complex process with a lower efficiency, leading to high PHAs production cost; glucose produced from hydrolysis of starch, which is considered a major feedstock for PHA production, has grown its price quite fast. PHA does not have incredibly consistent properties or structures compared with its competitors, conventional plastics; processing of PHA is more complicated than petrochemical plastics because their crystallisation process is slow; as well as the variation in material properties that leads to impediments in plastic processing. Other problems regard the PHAs processing including the homopolymer (P3HB) begins to degrade at temperatures close to the PHA high melting point (180°C) thus random chain scission reactions cause a decrease of the number-average of polymerization degree within minutes about 50 percentage.

Table 2.9. Summarises the major drawbacks are leading to restrict the widespread of PHA use with their potential solution.

<i>PHAs challenges</i>	<i>Potential solution</i>
High cost of extraction	<ul style="list-style-type: none"> • Decrease the substrate cost that use for bacteria growth coming from waste and by products material. • Bioprocesses development based on activated sludge or kitchen waste as substrates also be a significant choice for the competitive industrial biotechnologies. • Use mixed culture to increase PHAs production or modified the bacteria strains. • Optimisation the production processes.
Producing PHAs with mechanical properties equivalent to plastics.	<ul style="list-style-type: none"> • In processing, PHAs are sensitive to water but using a proper drying natural additive and a suitable choice of compatibilisers is promising for the preparation of blends for PHAs and others natural polymers such as starch and proteins which can produce the plastics items with high degree of biodegradability. • Understanding the kinetics of crystallization of PHAs and suitable choice of additives to improve the elongation at break and obtain the stability in the mechanical properties. • Optimisations the PHAs use in blends with other biodegradable polymers to reduce the cost of the final with consider the product still preserving the outstanding properties.
Quality and consistency of microbially produced PHA	<ul style="list-style-type: none"> • Similarity of PHAs produced in mixed culture and Optimisation of the quality.

Therefore, higher temperature causes an exponential growth of the rate of degradation thus the application of melt extrusion technology's hard to apply. However, this mechanical problem can be overcome by synthesising copolymers of 3-hydroxybutyrate with 4-hydroxybutyrate, 3-hydroxyhexanoate, or 3-hydroxyvalerate, that possess lower melting temperature (Volova et al., 2013). On the other hand, the PHA copolymers production in almost every case requires adding precursors to the media and these again, increase the production costs. With the aim of commercialising PHAs, substantial efforts have been devoted to decreasing the production cost out of the development of bacterial strains, more efficient fermentation strategy or recovery processes (Choi and Lee, 1999).

2.6 *Cupriavidus necator*

Cupriavidus necator or *Wautersia eutropha* as scientific community was renamed in 2004, is a non-pathogenic gram-negative bacterium, which found in both soil and water. Formerly, *C. necator* classified as *Alcaligenes eutrophus* and *Ralstonia eutropha* according to German Collection of Microorganisms and Cell Culture (DSMZ). The *C. necator* name derives from Latin words “cuprum” which mean (copper) and “avidus” (loving) while “necator” refers to “slayer” (Vandamme and Coenye, 2004), it can be placed under the following parentage see Figure 2.8.

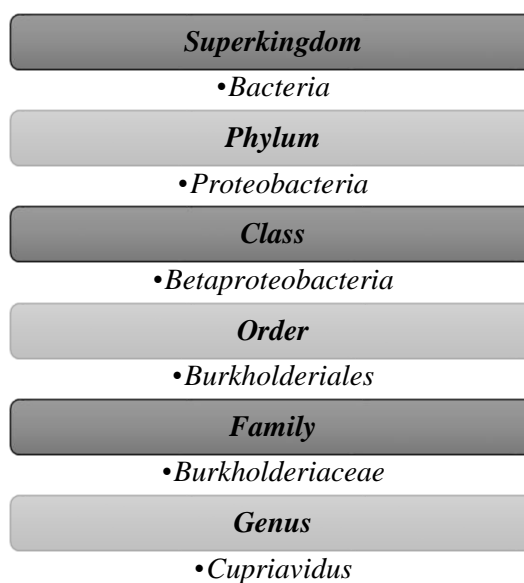


Figure 2.8. The lineage of *C. necator* .

C. necator is coccoid rod-shaped bacterium which reproduces by binary fission (the rods decrease in size and become rounded). The colonies of *C. necator* are off-white, smooth, snotty and convex with the entire edge with diameter around 2-4 μm when cultivated at 30°C for two days on the nutrient agar plate. See 2.9. *C. necator* is aerobic and also can grow under anaerobic conditions and the optimum growth conditions under is 30°C, and the pH range is between 7.0 and 8.0 (Casida, 1988).

Various chemicals such as acetate, mannose, lactate, succinate, fructose, amino acids and yeast extract can be used as carbon/nitrogen sources for cell growth. *C. necator* strain N-1, are not capable of utilising several chemicals such as glucose, lactose, glycerol, rhamnose and xylose as a carbon source for their growth. However, many strains such as DSM4058 and DSM545 have been reported to be utilised glucose, mannitol mannose and glycerol as carbon sources while H16 is not able to metabolise glucose (Sharma et al., 2016). The most interesting characteristics of *C.necator*, which attracted biotechnological field, is used as an excellent producer of PHB in inclusion bodies as granules via the fermentation process. Moreover, it can play an important role in the microbial degradation of aromatic compounds such as phenol and biphenyl in the environment (Salehizadeh and Van Loosdrecht, 2004).

C. necator serves as the model of the microorganism to produce PHB as a storage of carbon and energy source in excess of carbon resources (carbohydrates, lipids and amino acids) and limitation of nutrients such as nitrogen, oxygen and phosphate. It has the ability

to accumulate a large amount of PHB biopolymer up to 90 % dry cell weight as granules under unbalanced growth conditions (Muhammadi et al., 2015).

Various types of *C. necator* produces PHA with short chain lengths (SCL) such as PHB, which contains only 3-hydroxybutyrate (3HB) or 4HB monomer, P (HB- co -HV), containing 4HV monomer or 3HB and 3-hydroxyvalerate (3HV). Because of availability and easily of genetic tools to manipulate *C.necator*, strains are composed in the way that can produce short chain length (SCL) and mixed chain length (SCL and MCL) of PHA polymers (Volodina et al., 2016).

Although *C. necator* is capable of utilising a wide range of organic compound produce PHB such as carbohydrate, fatty acid, aromatic compounds as well as organic acids, researchers have engineered *C. necator* to extend the substrate utilisation range to synthesis PHB in more quantities and valuable because *C. necator* metabolism pathway tractability. Another aspect of that, *C. necator* has ability to choose the tracking of the cell growth: heterotrophic growth (which means utilizing organic compounds as carbon source for growth), or autotrophic (use H₂ or CO₂ as a carbon source) which fixes CO₂ via the Calvin Benson-Bassham (Park et al., 2011).

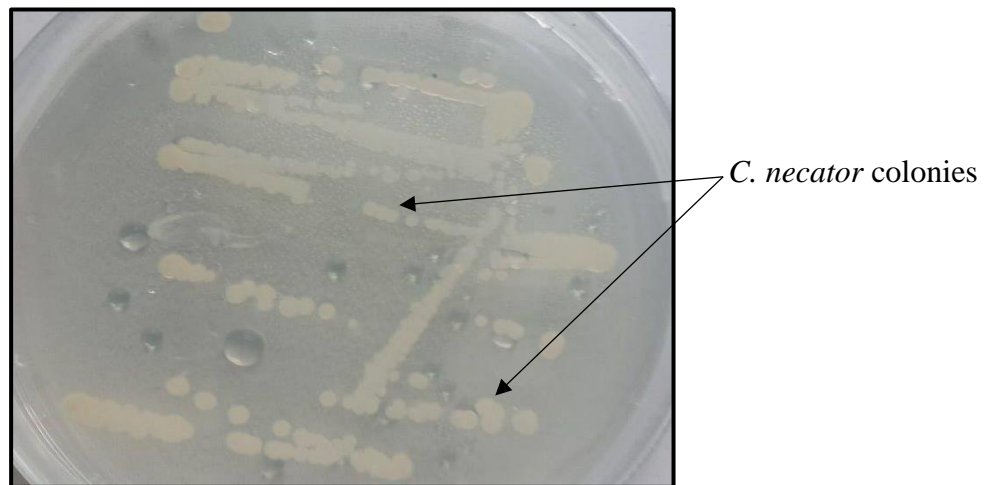


Figure 2.9. *C. necator* colonies grown on nutrient broth agar plate.

2.6.1 PHB synthesis in *C. necator* cells

PHAs are accumulated by various living microorganisms. The main candidates for the large-scale synthesis of PHA are bacteria and plants. The cells of the plant can only deal with low yield ($\leq 10\%$ of DCW) of PHA production. High levels (10-40% DCW) of polymers inside the plants have a negative growth effect and plant improvement. At present, these problems have not been overcome. In contrast, PHA produced to levels as high as more than 80% (w/w) of DCW (Steinbüchel and Lütke-Eversloh, 2003).

PHA accumulating is a natural way for bacteria to store energy and carbon when essential nutrient supplies are unbalanced. These polymers are produced when the growth of bacterial is restricted by depletion of phosphorous, nitrogen or oxygen and excess of the carbon source is still existing. While the most common restricting is nitrogen for many types of bacteria, such as *C. necator* and *Azotobacter* spp. The most efficient limitation is oxygen.

As PHA granules are insoluble in water, the polyesters are produced in intracellular granules inside the bacteria cells. It is worth for bacteria to storage excess nutrients inside their cells, particularly as general physiological fitness of the bacteria is not impacted. By polymerising intermediates molecules from soluble into insoluble, the cell does not undergo modification of its osmotic state. Thus, the leak of these valuable materials out of the cell is limited and the nutrients store will stay securely available at a small maintenance cost (Peters and Rehm, 2005). The surface of PHA granule is coated with a layer of phospholipids and proteins. A class of proteins, Phasins, are the dominant materials in the interface of the granules. The phasins not only affect the number of PHA granules but also the size (Pötter and Steinbüchel, 2005) and (Pötter et al., 2002).

As mentioned earlier, *C. necator* is considered the most effective and extensively studied microorganism for PHB synthesis due to capable of accumulating PHB granules in the range of 80-90% of total dry cell weight being reached, with cultures up to 6100 mg/l. American company Imperial Chemical Industries were the first of use *C. necator* to begin worldwide by the late of the 1980s. *C. necator* can utilise a wide range of carbon sources as a substrate such as sugars, vegetable, and plant oils (Belgacem and Gandini, 2008) and (Patwardhan and Srivastava, 2004). Holmes (Holmes, 1985) reported that the mutant strain accumulated up to 80% (w/w) of PHB by utilising glucose as a carbon source, whereas accumulated PHB content of 70% of dry weight on glycerol utilising. The PHB synthesis is the simplest biosynthesis pathway by involving three enzymes (β -ketoacyl-CoA thiolase enzyme, NADPH-dependent acetoacetyl-CoA dehydrogenase and PHA polymerase) with their encoding genes (phbA, phbB and phbC, respectively). The PHB biosynthesis consists

of three main steps, as shown in Figure 2.10. The first step in this process starts when two molecules of acetyl-coenzyme A (acetyl-CoA) is condensed into form acetoacetyl-CoA molecule by aiding the reversible binding of β -ketoacyl-CoA thiolase enzyme.

This step is inhibited when CoASH is available in a high concentration, which is released when acetyl-CoA enters the TCA cycle. When NADPH-dependent acetoacetyl-CoA dehydrogenase enzyme reduces acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA, is called the second step.

Finally, the monomers of (R)-3-hydroxybutyrylCoA are polymerised into PHB via PHA polymerase (Steinbüchel and Fuchtenbusch, 1998),(Gamal et al., 2014) and (Doi, 1990). PHA synthase, the key enzyme for PHB biosynthesis, in *C. necator* reacts with substrates containing three to five carbon atoms, with C4- substrate as its preference. As a consequence, PHB polymers obtained from this pathway contain short-chain-length monomers ((Akiyama et al., 2003a) and (Chien and Ho, 2008). Apart from monopolymer, *C. necator* can also synthesise PHB copolymers including poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), poly(3 hydroxybutyrate-co-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3 hydroxyvalerate). Currently, a glucose-utilising mutant of *C. necator* is used industrially by Metabolix (USA) to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate). This copolymer is sold under the trade name of Biopol (Denis et al., 1998), (Vigneswari et al., 2015) and (Chai et al., 2013)

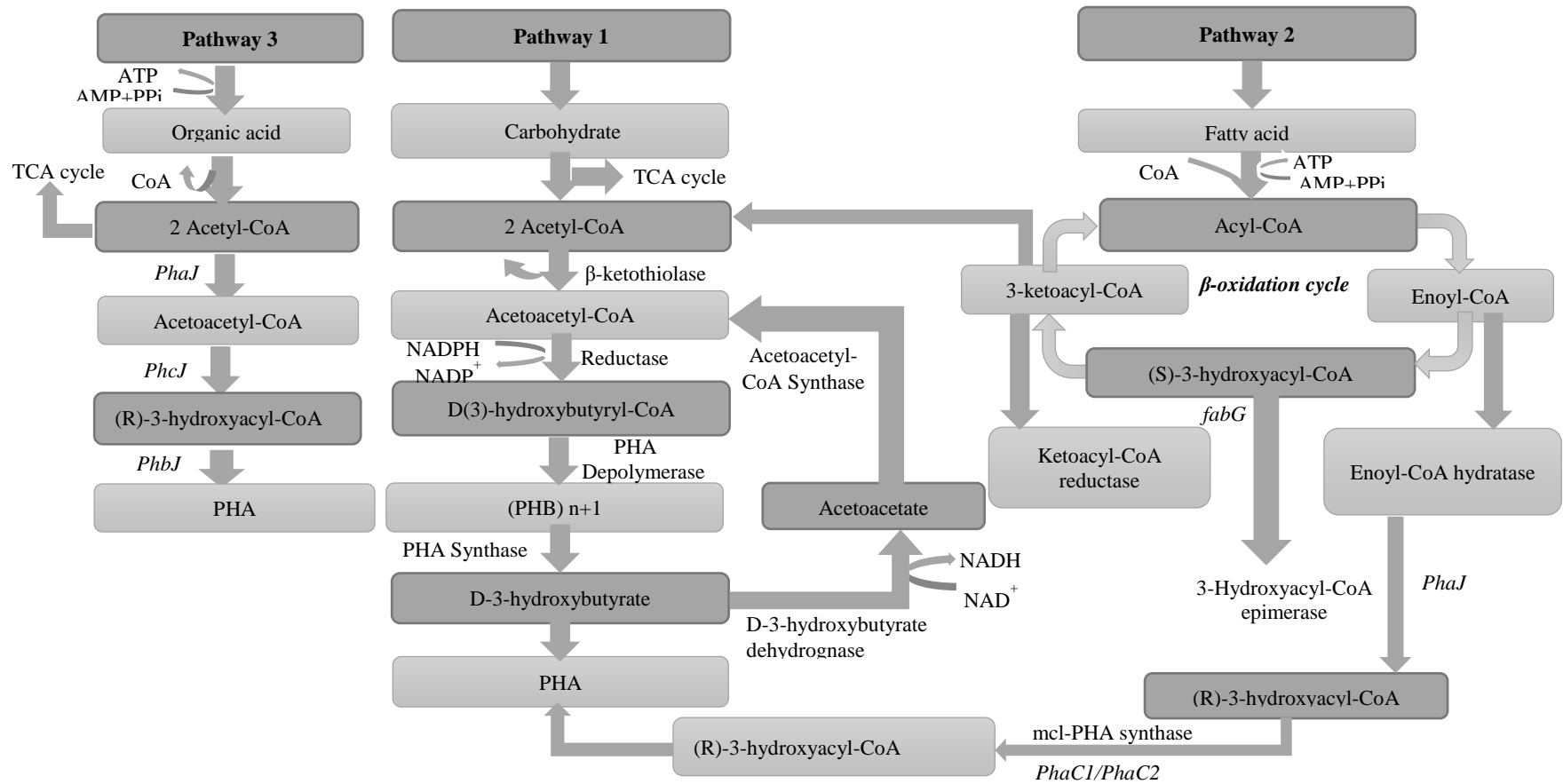


Figure 2.10. Metabolic pathway for PHB synthesis by *C. necator*.

2.7 Dates and date seeds palm

Date palm, also known as *Phoenix dactylifera* L., which consists of around 200 genera and more than 2,500 species, is one of the oldest known fruits in the arid and semiarid areas of the world, having been cultivated in North Africa, Arabian Peninsula and the Middle East for at least 5000 years. During the past three centuries, the United States, South America, Mexico, Australia, India/Pakistan and Southern Africa were introduced as new production areas for the date palm. The term of *Phoenix dactylifera* L. derives from the genus "Phoenix" of the legendary bird of Ancient Greece and *dactylifera* "daktulos" which mean date palm and finger shape, respectively (Al-shahib and Marshall, 2003). Date palm has always played a significant part in the economy and society of these countries because dates are an important income source and an essential food for local populations in many countries in which they are cultivated. The total world production date has increased exponentially over the last decades, in 1985 the production was 1.85 million metric tons (MMT), while in 1990 raise to 3.43 MMT, this amount almost increased about 120% to reach 7.51 (MMT) in 2015 million tonnes distributed across 30 countries. Figure 2.11 presents the worldwide production date in 2015 (Amir, 2015).

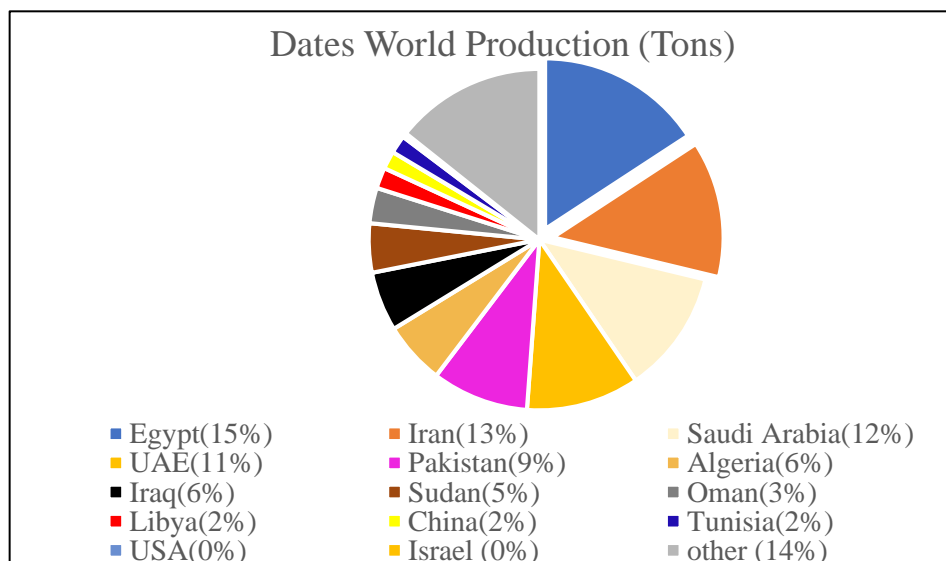


Figure 2.11. Worldwide production of date in 2015 (Amir, 2015).

The top three date producing countries are Egypt, Iran then Saudi Arabia with 15%, 13% and 12% of world production respectively, while India is the largest importer date country (El Hadrami et al., 2015; Sarah et al., 2014). Iraq is considered one of the important date producing countries (6% of date's world production); annually, around 420,000 metric ton (MMT) of dates are produced from the number of trees that are estimated no more than 8-9 million. According to the Iraqi Ministry of Agriculture and Fisheries, date palm tree cultivation accounted for 51% of the total cultivated area and date fruit representing about 78% of the total fruit production. In 2004, dates excess were 55000 metric tonnes, and about 33% of which were from low-quality types such as Mabseeli, Umsellah. Therefore, utilisation of such excess is quite important to maintain the cultivation of dates and to boost the income of that sector (Shabani et al., 2012).

Date trees length around 15-25 m and a cross-sectional radius about 20-40 cm with strong stalk are made of cellulose fibres. Various types of the cultivated date palm are public for their high quality. Dates are an excellent source of vitamins, fibres, energy, nutrients, macro and micro-elements and at least 15 minerals, including magnesium, calcium, potassium and phosphorus. Therefore, the date palm is known as the "tree of life") (Augstburger et al., 2012), Figure 3.12 a. as well as dates help to delay wrinkle formation, ameliorate a cough, and firm the infant's' gums in teething stage As well as, dates have a high content of crude fibre, therefore; they support the digestion and food absorption process. Hence avoid the human body from certain health conditions including diabetes, colon cancer and heart disease (Mohamed et al., 2014; Lee et al., 2005; Bauza et al., 2002; Al-orf et al., 2012; Silvera et al., 2005).

In General, the date fruit categories into many maturity stages, Figure 3.12 b. The first stage is the "hababouk" stage, in this stage, the fruits are completely covered with the calyx leaves with pea-size. The fruit in a second stage known as "kimri" with green colour and oblong shape. The third stage called "khalal" or "besser" stage, the fruit colour changes from green to red through yellow with a maximum weight and size. The fourth stage, the "rutab" stage, the date flesh become softer, darker in colour while the weight decreases because of loss of water. The fifth stage called the "tamar" stage, the fruit in this stage reaches full-grown, the colour changes into darker and looks dehydrated (Adeosun et al., 2016).

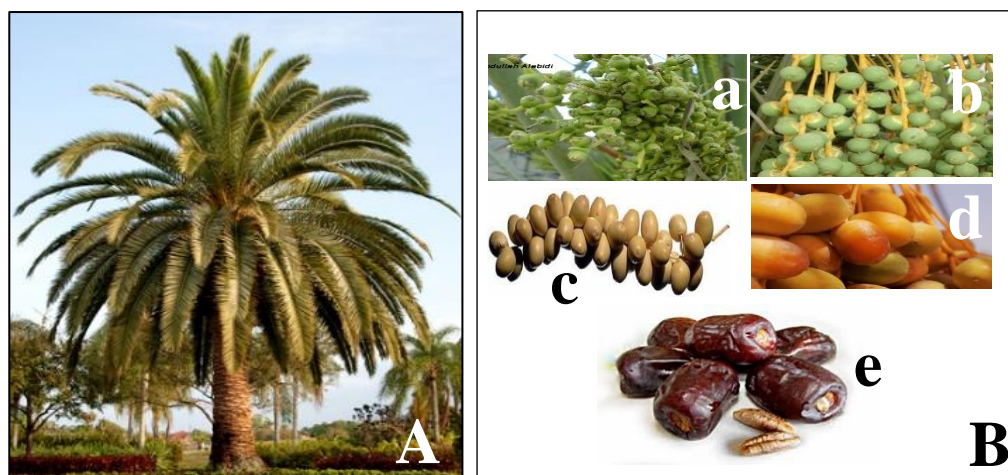


Figure 2.12. (A) presents the date palm tree and (B) different growth and maturity stages of date fruit. (a) hababouk stage. (b) Kimri stage. (c) Kalala stage. (d) Rotba stage. (e) Tamar stage.

Date fruit consists of two parts; a soft, edible, fruity pericarp and a hard seed, with each fruit containing one seed that accounts for around (10-15%) of the total date weight. This means the annual production of waste date seed is in excess of 1 million tonnes. Normally the edible date fruit is consumed by humans and the seeds thrown away as a waste. However, date seeds also have a high nutrient content, comparable to that of the fruit, and contain a large amount of energy that could be used for various value-added purposes.

Date seeds are a waste product of many industries, date fruit is rich in various nutrients and provides an excellent source of rapid energy due to its high level which in the range of as follows:

Table 2.10. The range date seeds composition (wt %) of different varieties (Abdurhman and Akasha Bsc, 2014; Najafi, 2011)

<i>Date seed compositions</i>	<i>Percentage (%)</i>
cellulose	40%
lignin	15%
water-soluble hemicellulose	19%
water-insoluble hemicellulose	4%
water-insoluble pectin	9%
carbohydrates	70–80%
protein	5–6%,
oil	10–12%

Most date's carbohydrates are in the form of glucose and fructose, which are absorbed easily by the human body. In last a few years, Remuch attention has been focused on the utilisation of date seeds as an important waste that could be used as value-added products such as dietary fibres, biofuel or cooking oil, coffee, and medicinal products (Wakil et al., 2015; Jose Romeno Faleiro, 2015).

Fibres of date seed are the solid insoluble part, mainly consists of lignin, cellulose, hemicellulose and insoluble proteins. The fibres amount is higher, however, during the extraction process, pectinase enzymes and cellulase break down from insoluble polymers into smaller soluble molecules (Shafiei et al., 2010).

Researchers have determined the chemical composition and nutritional value of the pericarp or flesh part of the dates while the available information is limited regarding the chemical composition and nutritional value of the date seeds. Date seeds, which are the main waste product of the date fruit, are odorless and light to dark brown with slight bitterness and bland taste. Date seeds, also known as pips, pits, stones and kernels from the portion of the integral date, which consists of 6–12% of total date weight at the Tamar stage).

They become obtainable in large amounts when pitted dates are obtained through industrial date processing for paste or/and syrup or by packing plants (Hossain et al., 2014). The high content of fibre might indicate the possibility of utilising the seeds as a material of useful food for health interest and they were utilised as a partial replacement of corn in the diet of domestic animal and as supplements in the ruminant's diet. As well as organic fertiliser, caffeine-free product to replacement coffee and making activated charcoal. Danish et al., (Danish et al., 2014) studied of using activated and raw date seeds as possible adsorbents for pigment containing waters while Al-Omair tried date waste to recover heavy metal cations such as Zn_2^+ , Cd_2^+ , and Cu_2^+ , applying batch adsorption process from simulated artificial wastes water. Activated carbon was synthesised using date seeds via zinc chloride as an activator material compared to utilising H_3PO_4 (Al-Omair and El-Sharkawy, 2007).

In the Middle East, several studies suggested the existence of hormonal impact in the date seeds, such as: (Aldhaheeri et al., 2004) studied date seeds extracts to prompt contraction of rat uterus in a way similar to oestradiol. Over the last few years and at the University of United Arab Emirates, some researchers reported that feed containing fungi degraded date seeds decreased the population density in colony formation units for total bacteria counts of *Escherichia coli*, *Shigella spp.*, *Cambylobacter spp.*, and *Salmonella spp.*, in chicken gut. In Tunisia by Zribi et al set up an osmotic dehydration process to form osmo-dehydrated fruits

from date by-products and lemon. Microbiologically, these products were stable for three months at 48°C and can be used or incorporated as a component in food formulations (Zribi et al., 2017).

2.7.1 Anatomical structure of the seed

Normally dates contain one seed which is solid and has a rectangular shape, pointed at the ends and occupies the fruit centre, weighing between 0.5 - 4 g, length 12 - 20 mm, width 6 - 15 mm. The length of the is three times the width of the seed which represents 10-20% of the total weight of the fruit, See Figure 2.13. The seed of the fruit is covered by a dark brown coat, consisting of two different sides, one of which is convex and is known as the dorsal side. The other side, ventral side, has a furrow or groove which extends along the seed and is considered as a feature of Phoenix is often occupied by a white fleshy tissue called a wick. On the convex surface, about one-third along the length of the seed, there is a speck. A slightly reduced small and round determine the position of the fetus and are called Microphyle is the first leaf to be released at germination (Mathew et al., 2014).



Figure 2.13. Date seeds with their structure.

In general, the date seed consists of, (Sghaier-Hammami et al., 2009):

- Seed Coat or Testa: It is a thick solid wall with a light brown or dark colour according to the variety, covers the fetus and the tandemosphere.
- Endosperm (Swaida): is fabaceous of half-cellulose or semi-transparent solid, Hemicellulose, which is considered the seed bulk and good examples of storage tissue. The endosperm is the largest part of the seed during a dormant phase.

During the dormancy period, functional and structural studies showed that the endosperm is characterised, (Sakr et al., 2010): as shown in Figure 2.14.

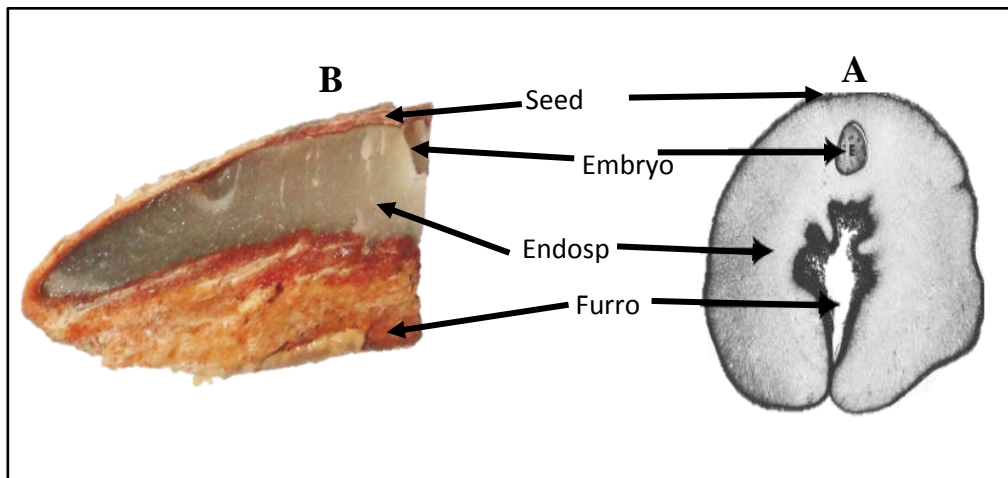


Figure 2.14. (A) cross section and (B) Longitudinal section which present anatomical structure of the date seed.

1. the endosperm consists mainly of one type of living cells that are identical in their contents while different in their shapes depending on where they are located. The cells close the edges are long and inviting while the interior is identical to the shape diameters. Endosperm cells are alive and breathe airborne but at a lower rate than the embryo.

2. Cells contain a small amount of cytoplasm filled with several storage organelles which are different in their volume. It has been found that protein is the predominant component of large organelles while fat is the main component in small organelles.

3. The walls of the cells are quite thick which considered the place of the carbohydrates is stored.

4. All endosperm cells also contain nuclei and bodies surrounded by a double membrane believed to be plastids and Mitochondria while ribosomes were not observed yet.

2.8 Solid state and Adsorbed Carrier Solid-State fermentation development

Solid state fermentation has been used as an alternative fermentation to submerge for biopolymer (PHB) production. Generally, solid state, substrate, fermentation is known as micro-organisms growth on a non- soluble material that acts as the source of nutrients and physical support or sometimes act only as physical support in the absence or nigh - the absence of free liquid (Pandey, 1992). The solid substrate should be utilised to define just those processes in which the substrates themselves act as energy/carbon source, taking place in the near-absence or absence of free liquid (water).

SSF has many advantages over submerge fermentation (SmF), including simpler media for fermentation, higher quality for product recovery, smaller space requirement, decrease energy requirements, no foam formation, lower waste water production, easier aeration. As well as lower levels of catabolism and absence of strict control parameters, reduce bacterial contamination (Ramana Murthy et al., 1993). In recent year, this type of fermentation has given much promise in the improvement of many products and bioprocesses. Many authors have reviewed the history, scientific elaboration and improvement of SSF from time to time. Evidently, enzymes production and food fermentation were the areas wherever SSF originated, Table 2.11 history and development of SSF products.

Table 2.11 History and development of solid state fermentation system products (Soccol et al., 2017).

<i>Time</i>	<i>Products</i>
2000 B.C.	Vinegar, bread making by Egyptians
1000 B.P. (before of Christ in Asia)	Cheese making by <i>Penicilium roquefortii</i> .
2500 B. P	Fish fermentation/preservation with sugar, starch and salts.
2500 B. P	Sauce and koji process
550 B.P.	Koji acid
7 th century	Koji acid was introduced from China to Japan
16 th century	Tea
18 th century	Vinegar from Pomace, use of gallic acid in tanning, printing.
1860-1900	Sewage treatment
1900-1920	Production of fungal enzymes.
1920-1940	Citric acid, Gluconic acid, development of drum-type fermenter
1940-1950	Development in fermentation industry and Penicillin production.
1950-1960	Steroid transformation.
1960-1980	Protein enriched feed and Mycotoxins production.
1980-1990	Different primary and secondary metabolites production, work on kinetics and modelling aspects of SSF, development of column of the fermenter.
1990-present	Improvements on fundamental aspects of SSF and products/bioprocesses development.

SSF fermentation showed several problems such as the sterilisation of substrate, controlling of temperature and pH, as well as the contamination of the system which mainly detected at long process period. Usually, the substrates in SSF are by-products of agro-industry, and these materials have many disadvantages, including the excessive thickness of the layer, low porosity thus the inadequate internal structure that obstructs the aeration, inefficient nutrient uptake and heat removal. In addition, most of these systems the direct estimation of microbial cell growth is not attainable because it cannot separate solid substrate residual from biomass; therefore, there are indirect methods based on ATP, glucosamine, protein or concentration of O₂ or CO₂.

Using inert support allows direct biomass measurement, cleaner product extractions and increase homogeneous aeration. On the other hand, disadvantages of SSF fermentation are the nutrients availability to reach for the microorganism may increase, decrease, or relatively remains constant during the fermentation period, while in the, they are readily accessible (Technology and Education, 2005). In spite of, PHB is an attractive alternative replacement for conventional polymers, until a short time ago, only SmF submerged processes had been used for PHB production. Several reports have been found on the effective utilisation residues of agro-industrial including sugar cane bagasse, cassava bagasse, sugar beet pulp, apple pomace, and coffee pulp/husk by different microorganisms. Many processes have been improved that make the microorganisms to use these residuals as raw materials for the production of fine value-added products and bulk chemicals such as enzymes, ethanol, organic acids, single cell protein (SCP), mushrooms, amino acids and biologically active secondary metabolites.

Because PHB is accumulated as intracellular inclusion bodies, the bacterial biomass retrieval from the medium, which contain solid agro-industrial residues as the nutrients substrate, during fermentation period is a highly challenging task. Therefore, only a few cases were reported on PHB synthesis using SSF as a model of the fermentation process. Unfortunately, a few reports have been published about PHB synthesis using SSF. De Oliveira et al., (de Oliveira et al., 2011) found that *Ralstonia eutropha* produced PHB by SSF fermentation by using soy cake and soy cake supplemented with sugarcane molasses (2.5 %). The major inherent problem that associated PHB production using SSF is the difficulty in restoring bacterial cells from the solid substrate after the ending of the fermentation.

To overcome this problem an inert support can be used to carry the media such as ion exchange resins (like Amberlite) and polyurethane foams (PUF) where the liquid media nutrients are absorbed. The substrates that used in SSF should absorb water thus the microorganism can stimulate growth and do metabolic activities. In this context, PUF foam has several advantageous characteristics, such as low density, high porosity and relatively high capacity to absorb water. Moreover, PUF foam has an adequate pore size that can provide a satisfactory environment condition for bacteria growth. PUF permits cell adsorption to a large range because it enables mobilisation of a high number of microbial cells within a short period. By using a defined liquid media and an inert support material with a homogeneous physical structure not only develops the monitoring and controls the SSF system but also improve the reproducibility of the fermentation process. Sindhu et al., (Sindhu et al., 2009) reported that PUF provides a homogenous, continuous aerobic

environment until the end of the fermentation period. In this working, the polyurethane foam (PUF) was utilised as the inert support in SSF medium.

The PUF has several physical properties including low density, relatively high absorption capacity for water and high porosity. PUFs allow the cells to adsorb to a large extent since they allow a large number of cells to immobilise in a short fermentation period. It has been reported that using of nutritionally inert materials in SSF system helps to monitor of process parameters, scaling-up strategies, designing of media and different other engineering aspects. The PUF can provide a continuous homogenous aerobic condition till the end of incubation period (Hesseltine, 1987).

2.9 Summary

Industrial of biotechnology is gaining significance in modern society including antibiotics, steroids Amino acids, Industrial enzymes and Therapeutic Proteins/Antibodies. With a growing interest, not only in sustainable means of producing materials but also in shifting from production based on raw materials of fossil origin to renewable resources and also from non-biodegradable to fully biodegradable products. PHB has been attracting much attention as a replacement for traditional petrochemical plastics because it has material properties similar to various thermoplastics and elastomers, in addition to being fully biodegradable upon disposal under particular environmental conditions. The increasing cost of oil and volatility of the crude oil market have led to a thrust to decrease our dependence on oil and oil-based products. Together with a growing environmental awareness, this has encouraged the utilisation of alternative, environmentally-friendly, renewable products, including biomass. The increasing interest in replacing oil-based products using renewable, inexpensive and natural materials is essential for sustainable improvement in the future and is going to have a significant effect on the environment and polymer industry. However, the utilisation of PHB in a broad range of applications is hindered by their expensive production cost, including the expensive raw materials and complexity of the production process. One approach to decreasing PHB production costs is utilising waste derived from non-food, food and agro-industrial crop, developing added value routes for these wastes. In order to realise this potential, the technical feasibility of such a bioprocess to transform waste to PHB must be established, requiring significant experimental study before scale-up and industrial production can be achieved. From all the information that mentioned in this chapter, it can be concluded that it is worth to investigate the ability to use waste date seed as a rich,

inexpensive media for biopolymer production after pre-treatment them to extract the all the necessary compounds for microbial growth. In spite of many researchers have used waste date seed to produce various products (as mentions in detail in section 3.7), no one has been used them for biopolymer production especially waste date seed contains a large amount of carbohydrate (60-70%) as well as oil. The scope of this thesis and objectives of the research are outlined in the next chapter.

CHAPTER
THREE

3 Scope and objective of the thesis

3.1 Aim of the research

In view of the innumerable of plastics applications, biodegradable plastics are urgently required to decrease the adverse worldwide economic and environmental effects of traditional plastics. As well as, since global oil reserves are limited, there is required for new resources of durable materials (Akaraonye et al., 2010a). Renewable materials that produced from microorganisms can provide resources of the sustainable alternative to petroleum-based chemicals such as polymers. According to Landucco and Bozell study, integrating renewable materials into the economy can reduce crude oil demand, hence restricting economic downturns in the chemical industries because of oil price volatility (Bozell and Landucci, 1993). Also, it would extend the options of the chemical industry by raising raw materials flexibility and expand the spectrum of potential chemical products. Consequently, this would provide a convenient answer to the current issues with petroleum derived chemicals. The improvement of biodegradable polyhydroxyalkanoates as an alternative to petrochemical-based plastics, hence, has the potential to provide a global solution and permanent to solve the issues associated with conventional plastics. Among biopolymers, PHB has attracted much attention in last few years due to their varied physical/chemical properties, biodegradability, and biocompatibility. A total of 6,844 patents and 4,613 publications have been published between 2000 and 2015 on PHA (Sukan, 2015). However, the high production cost of PHB in terms of raw material, extraction method, making the market price is significantly higher than polymer from the petroleum-based product.

In order to reduce this cost, a cheap carbon source and nutrients are required. Thus, using agricultural waste residues could substantially decrease substrate and hence production costs. Recent research has focused on using food and agricultural waste streams to obtain a growth media and currently these waste streams are the by-products of different food processing industries that are not used or recycled for any other purpose. These materials often have an economic value less than the cost of recovering and reusing them; therefore, they are thrown away as a waste. There is, however, an opportunity to add value to these waste streams through the production of biopolymers and it is from this concept that the original idea for this PhD thesis came. The novelty of this research lies in the development of a strategy for the pre-treatment of the waste date seed and sequential bioconversion to produce value added products, in this case biopolymer (PHB).

The main target of the work reported in this thesis is to investigate the feasibility of utilising waste date seed to produce value-added chemicals, making PHB as a target molecule using *Cupriavidus necator*. In order to achieve these goals, the experimental work in this project was divided into three key sections: characterisation of the waste date seed to determine if it contains essential compounds for microbial growth and the subsequent development of a method for substrate extraction (nutrient-rich media and oil). Then, investigation of bioconversion (*C.necator*) of this waste into biopolymer (PHB) utilising these extracted substrates. Characterisation of the biopolymer product (PHB samples produced from the all previous methods), and improving their properties was also investigated as a second part of this study. To achieve these aims, some additional specific objectives are set:

- To characterise the nutrient content of waste date seeds.
- To optimise the substrate extraction process.
- To study the feasibility of using a waste date seed derived substrate (hydrolysate media and oil) for PHB production.
- To investigate bioprocess modelling describing the PHB synthesis system.
- To establish the effect of using mixed-substrate derived media for *C. necator* growth and PHB productivity.
- To assess the potential of PHB synthesis utilising adsorbed carrier solid state fermentation technique, with polyurethane foam as carrier.
- To extract and characterise PHB samples produced by *C. necator* grown on different types of carbon source derived from waste date seed and fermentation system.

3.2 Structure and scope of the thesis

The cost of biopolymer production is one of the most important factors restricting the industrial application of PHB. The main target of the research presented here, therefore; production of microbial PHB via fermentation utilising waste material (date seeds), along with investigation the biopolymer properties. The work presented in this thesis is separated into nine chapters., ***Chapter one, two and three*** as described previously, and the rests are follows as:

Chapter four describes all materials, methods and techniques that have been used in this study. The results of experimental work conducted are explained in ***Chapters five to eight*** as following:

The growth of *C. necator* on date seed derived media and optimisation of the growth using various techniques were investigated and the results are shown in **Chapter five**. Partial characterisation of the date seeds and a kinetic study of media extraction is also included in this chapter. Most of this chapter is published as a first paper.

Chapter six the present chapter highlights various techniques used for date seed oil extraction and optimisation, as well as the growth of the bacterium on defined salts media using this oil as a substrate. The first part of this chapter is published as a second paper.

Chapter seven shows the results of the development of a solid-state fermentation strategy for PHB production using polyurethane foam as an inert support material impregnated with date seed derived medium. As well as the effect of using mixed-substrate (hydrolysed media and date seed oil) on *C. necator* growth and PHB productivity in various feeding ratio are presented in this chapter.

In **Chapter eight** a characterisation and thermal properties study of all PHB samples produced from all the results reported previously is presented. Most of this chapter is published in the second paper.

Chapter nine contains a general discussion, conclusion, and suggestions for future work.

CHAPTER

FOUR

4 Materials and methods

4.1 Introduction

All materials and methods used in this study were listed and described in this chapter. In general, this chapter is classified into three main sections: experimental, analytical and theory/calculations. The first section begins with describing bacterial strains reactivation, and maintenance in Section 4.2.1, then waste date seed preparation and characterisation were discussed in Section 4.2.2. Furthermore, it was interesting to study cultures media and inoculum preparation for bacterial growth and PHB production including date seeds derived media, the effect of pre-treatment method, the effect of supplementing nutrients, mineral media and the effect of nitrogen type and concentration. PHB production using shaken flask scale was presented in Section 4.2.4.1 which covered feeding nitrogen, batch fermentation with two-stage nitrogen limited, nitrogen limited fed batch fermentation, effect of oxygen mass-transfer coefficients and determination of oxygen transfer coefficient. PHB concentration from emulsified oil culture as well as mixed-substrate fermentation media is described in Section 4.2.4.3.7, while solid state fermentation system including effect of incubation volume, effect of the shape, effect of container size, effect of incubation time as well as repeated solid-state fermentation are presented in Section 4.2.4.3.2 Biomass recovery from fermented PUF and scanning electron microscopy is also presented in this section. Polymers identification and characterisation are presented in Section 4.3.4. Theory and calculation covering mathematical modelling of fructose extraction from date seed powder, mathematical modelling of cell growth and PHB accumulation and kinetic parameters of date seeds oil extraction process is presented in Section 4.4.

4.2 Experimental methods

4.2.1 Microorganism

4.2.1.1 Bacterial strains, reactivation, and maintenance

The bacterial strain used in this study was *C. necator* H16; obtained from American Type Culture Collection (ATCC17699, US). In order to create master stock, the following procedure was followed: *C. necator* lyophilised cells were re-activated by cultivation in 10 ml of nutrient broth 2 and incubated at 30°C for 24 h. After that the culture was transferred aseptically into 500 ml shake flask containing 100 ml of nutrient broth, incubated for 24 h at 30°C. Cryobank bed system vials (Copan Italy) were prepared and kept at -80°C in ultra-low temperature freezer (U101 Innova, New Brunswick Scientific, Canada).

One bed of the working stock vials was used to inoculate a nutrient agar petri dish. The plate was incubated at 30°C for 48 h then stored in a cold room, at 4°C, for use in experiments.

4.2.2 Date seeds

4.2.2.1 Preparation

The date seeds used in this study were obtained from a local Iraqi supermarket, free of charge, soaked in water for three hours, washed to remove any remaining date flesh and then dried overnight at about 60°C. The date seeds were milled in a heavy-duty grinder (UMA PHARMAE) to obtain different fractions (≤ 4 mm) by using sieving (Fisher Scientific test sieve). The fine powder preserved at -20°C for subsequent use.



Figure 4.1. Date seeds were purchased from a local Iraqi supermarket, after washed and dried overnight at 60°C.

4.3 Analytical Methods

4.3.1 Microscopic observations

C. necator cells morphology and membrane were observed under microscopy after fermentation broth samples (10 μl) were taken, placed on the slide of the microscope. The slide moves slowly over the burner flame several times, then some drops of fuchsin staining were added and left for 5 min then washed with tap water.

4.3.2 Date seeds characterisation

4.3.2.1 Determination of pH

To determine the PH, 2 gm of date seed powder was suspended in 10 ml of distilled water, then the mixture was stirred for one hour, centrifuged at 10000 rpm for 5 min and finally the pH was measured using a standard pH probe (Mettler Toledo, USA) (Salakkam, 2012).

4.3.2.2 Determination of ash

Ash is an inorganic material remaining in the residue after burning the material. The ash content was calculated, according to (AOAC, 1990) by different in the weight before and after burning. A cubic container contains 10 g date seeds powder put in a furnace (Muffle furnace) overnight at 550 °C to burn off all impurities; then the container left in the desiccator to cool for 2 h. Ash calculated from equation 3:

$$\text{Ash (\%)} = \frac{\text{weight of ash}}{\text{weight of samples}} \times 100 \quad (3)$$

4.3.2.3 Determination of moisture content

Moisture content is the amount of water that the material contains at any given time. Moisture content was measured by weighing the empty container after drying in the oven at 60 °C for 12 h. Then 10 g of date seeds powder was placed into the container then dried at 105 °C for 24 h. After that, the container was transferred to the desiccator to cool down and this method was reported by Nennich and Chase (Nennich and Chase, 2007). Reweighting the container, and moisture content was calculated according to equation 4:

$$\text{Moisture (\%)} = \frac{w_1 - w_2}{w_1} \times 100 \quad (4)$$

Where W_1 = weight (g) of the sample before drying, W_2 = weight (g) of the sample after drying.

4.3.2.4 Determination of dry matter

Dry matter is defined as the material remaining after water removing by heating up to the constant weight. Dry matter was determined by weighing the empty container; after that 10 g of date seeds powder was placed into the container, drying in the oven at 60°C for 24 hrs. Pre-weight, the container with the powder, subtract the weight of the container to get the weight of the dry sample. Dry matter was calculated according to Equation 5:

$$\text{Dry matter \%} = \frac{w_1}{w_2} \times 100 \quad (5)$$

Where W_1 = weight (g) of the sample after drying (dry sample weight), W_2 = weight (g) of the sample before drying (wet sample weight) (Nennich and Chase, 2007).

There is a relationship between dry matter and moisture given by the equation 6:

$$\text{Dry Matter (\%)} = 100 - \% \text{ Moisture} \quad (6)$$

4.3.2.5 Quantification of total sugar

Fructose concentration was measured using the phenol-sulphuric acid method (Giannoccaro et al., 2006). Eight standard curves of fructose were prepared at concentrations from 0.02 to 1 mg/ml. Duplicate samples of 1 g date seeds powder were placed into universal tubes containing 10 ml of 80 % ethanol, boiling the tubes in a water bath for 10 min at 95°C. The mixture centrifuged, and the supernatant filtered through a 25 µm syringe filter. 1 ml of filtrate samples and standards were mixed with 1 ml (5% w/v) phenol solution and after that quickly mixed with 5 ml of sulphuric acid 98% by vortex, then cooled at room temperature for 30 min. After that the mixtures were vortexed again, and the absorbance was measured at 490 nm using a UV-visible spectrophotometer (UV min-1240, UV-VIS spectrophotometer, Shimadzu). The amount of soluble fructose was calculated as a percentage of the sample on a dry basis.

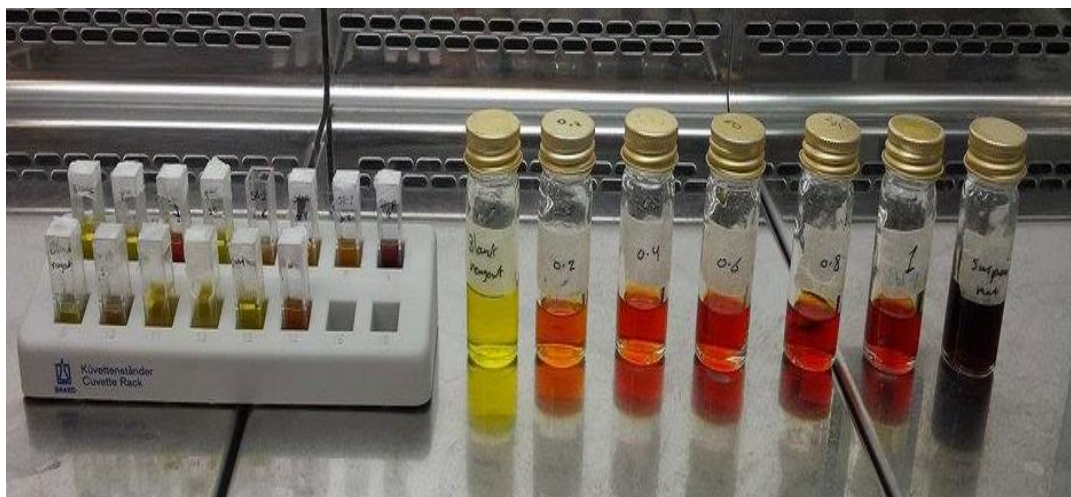


Figure 4.2. Phenol-sulphuric acid method for measuring fructose concentration in date seed powder sample following (Giannoccaro et al., 2006) method.

4.3.2.6 Determination of crude fibres

Crude fibre was determined by a method described by Borchani et al., (Borchani et al., 2010). The principle of this method is treatment the sample with acid and subsequent alkali and the oxidative hydrolytic reactions will be degraded the cellulose and lignin in the sample. After final filtration, residual weighted, then ignite, cool and weight again, the

difference between the weight consider the crude fibre content. A 2 g of dried date seeds powder after extract the oil was placed into tube contain 200 ml (0.25 N) of concentrated sulphuric acid, boiling in water bath at 95°C for 30 min, centrifuged at 6500 rpm for 15 min. The supernatant was discarded, and the residual was washed with the boiling water for seven times until the residue was free of acidic.

Subsequently, the residual then boiled with 200 ml sodium hydroxide solution (0.3 N) for 30 min, centrifuged at 7000 rpm for 15 min, washed with 25 ml of 1.25 % boiling sulphuric acid (38%), then centrifuged and placed the residual into pre-weighed ceramic plate, heated at 130 °C for 2 h. Finally, the plate was cooled using desiccator and weighted again. The residual was ignited at 600°C for 30 min and cool again in a desiccator and weighted. Then equation 7 was used to calculate the crude fibre:

$$\% \text{ crude fibre in ground sample} = \frac{\text{the loose weight on ignition } (W2-W1)-(W3-W1)}{\text{weight of the sample}} \times 100 \quad (7)$$

4.3.2.7 Determination of protein

Proteins consist of a large, complex molecule called amino acid which is connected to each another by a bond (peptide bond) in the long chain and play different roles in the body. The date seeds protein is extracted by solubilising date seeds powder in the water at 60 °C, 10:1 ratio (water: powder) and pH 8.5 using (2N) NaOH for 45 min. The mixture was centrifuged to remove the insoluble fibre. Then, the protein was precipitated by adjusting the pH to 4.5 (2N HCl), the protein crude was removed from the soluble sugars by centrifuging. The protein crude was washed with water and centrifuged again. This washed protein was neutralised to pH 6.8 using 2N NaOH, the product was centrifuged and the supernatant was removed and the precipitation was dried until the constant weight was achieved (Wang et al., 2004).

4.3.2.8 Determination of reducing sugar

Reducing sugar is a sugar type that contains free aldehyde and serves as a reducing agent because it has the ability to donate electrons to another molecule.

Total reducing sugar concentration was measured using the DNS method described by Gusakov et al., (Gusakov et al., 2011) which conclude by mixing 3 g of date seeds powder in 5 ml distilled water using a vortex mixer, then centrifuged at 7000 rpm for 4 min to separate the supernatant. A 3,5-dinitrosalicylic acid (DNS) reagent was prepared by dissolving 1 g of DNS reagent in 30 ml distilled water after mixing. A 30 g of sodium potassium tartarate was added to the solution to get a milky- yellow solution. A 20 ml of 2N

4.3.2.10.2 Boiling with reflux system

The second method used for extraction fructose was boiling with reflux system. To study the effect of the time and temperature on fructose extraction from date seed powder, a 500-ml a round bottomed flask containing 25 g of date seeds and 200 ml (1:8 w/v ratio) of water and heated for 10 h using oil bath (Fisher Scientific) with a reflux system to prevent water evaporation at different temperatures (90°C, 120°C and 150°C).

The first sample (1 ml) was taken after 30 min, with samples subsequently taken every hour. Another aqueous extraction ratios were used with different temperatures and time to extract the fructose. 3 g powder of date seeds mixed with tap water at ratios of 5:1, 10:1, and 15:1 (v/w) and extraction temperatures of 25 °C, 50 °C, and 80 °C for 15 and 30 min.

4.3.2.10.3 Acid and alkaline treatment

Two types of acid (H₂SO₄ and HCl) and one base (NaOH) were used as solvents for fructose extraction from date seeds. A powder sample was mixed with each solvent with a solvent-to-sample ratio 2:3 (v/w) and at extraction temperatures of 100 °C. The first sample was taken after 15 min and then every half-hour intervals for 3 h.

4.3.2.10.4 Sugar analysis using high-performance liquid chromatography (HPLC)

Fructose concentration was quantified using high performance liquid chromatography (HPLC) with a refractive index detector. An UltiMate 3000 Dionex HPLC system, Biorad Aminex HPX-87P column, and Refractomas 520 ERC unit used. An isocratic method was used with 5mM H₂SO₄, as an eluent, a flow rate of 0.4 ml/min, the column temperature of 50°C and 20 µl of sample injected for analysis. A fructose calibration curve was prepared with five points from (0.25-3 g/l) and samples were prepared by centrifuging 2 ml of sample, recovering the supernatant and filtering with 0.22 µm syringe filters into HPLC vials prior to analysis.

4.3.2.10.5 Determination of Total Nitrogen (TN) in date seed media

TN concentration was determined using a total nitrogen analyser unit (TNM-1, TOC-control V). Standards of five different concentrations (1-50 mg/l) using KNO₃ as a source of nitrogen, were prepared to cover the range of nitrogen concentrations in the media. For analysis 15 ml of each sample was transferred to a universal tube after being filtered using 0.2 µm syringe filter.

4.3.3 Cell growth measurement

4.3.3.1 Optical density

The optical density of the samples (1 ml) taken from date seed hydrolysate media was measured at wavelength 600 nm by using a UV-visible spectrophotometer (UV mini-1240, UV-VIS spectrophotometer, Shimadzu). While the sample taken from mineral media, the optical density was measured by taking 1 ml of the broth centrifuged for 6 min. Then removed the supernatant and washed with (0.7%) NaCl, then re-suspended in 1 ml distilled water using vortex, after that the OD_{600 nm}.

The optical density was measured by taking 1 ml of the broth centrifuged for 6 min. Then removed the supernatant and washed with 0.7% NaCl then re-suspended in 1 ml distilled water using vortex, after that the OD_{600 nm} using a spectrophotometer, UV-mini1240 (Shimadzu, USA).

4.3.3.2 Dry cell weight (DCW)

A 5ml of the culture sample was transferred to a dry, pre-weighed tube and centrifuged at 7000 rpm for 15 min at room temperature. The supernatant was decanted and refrigerated for further analysis whilst the cell pellet was washed and re-suspended twice in distilled water and (0.7%) NaCl respectively. The tube was then dried at 60°C until a constant weight was obtained. Then the DCW was determined gravimetrically.

4.3.3.3 PHB extraction from the bacterial biomass

Solvent extraction is one of the oldest methods to recover PHB from bacterial cells. The solvent action can be divided into two steps, first it modifies membrane permeability of the cells and then it solves PHB. For that purpose, many chlorinated hydrocarbon solvents were used such as chloroform, methylene chloride and 1,2-dichloroethane, or some cyclic carbonates including propylene and ethylene carbonates and about 95% of the PHB was extracted from the cell mass by this way, (Baptist and Grace, 1962).

PHB was recovered following the method reported by Hahn et al., (Hahn et al., 1995). A certain volume of the fermentation broth was centrifuged at 7000 rpm for 15 min, the supernatant discarded, and the pellet was dried at 60 °C for 48 h, grinded to obtain a fine powder using liquid nitrogen. The powder was mixed with 50 volumes of chloroform at 30°C for 48 h, then the solution was centrifuged at 7000 rpm for 10 min to remove the most of the non-PHB cell while the clear polymer solution was recovered using 0.45 µm filters. The ratio 7:3 (v/v) of methanol to distillate water was mixed to prepare the non-solvent solution that mixed with a clear polymer solution (five times the volume of chloroform) in order to precipitate the polymer (PHB). Purified PHB was obtained by filtering and drying at 60°C. The purified PHB was stored in a universal bottle at the room temperature. Equation 10 was applied to recover PHB from *C. necator* biomass and calculate the maximum yield:

$$PHB\ yield = \frac{\text{Weight of polymer recovered}}{\text{Total amount of product}} \quad (10)$$

4.3.3.4 PHB identification and quantification

After the pellets were dried at 60 °C for 48 h, then grinded until a fine powder was obtained using liquid nitrogen. The PHB was extracted by following Riis, and Mai et al., method (Rohini et al., 2006). The principle of this method is alcoholysis and transesterification of PHB by hydrochloric acid and propanol to obtain esters of hydroxybutyric acid, then 1,2- dichloroethane (DCE) is used as a solvent and extractive. See Figure 4.5.



Figure 4.3. Schematic diagram for PHB extraction from *C. necator* cells using Riis, and Mai et al., method.

Initially, a reagent mixture was prepared by mixing 5 volumes of DCE, 4 volumes of 1-propanol and 1 volume of concentrated HCl (37% purity). A 40 mg of the powdered pellet was added in a universal bottle contained 4 ml of reagent mixture. Then, bottles were placed in a boiling water bath for 2 h, and shaken from time to time. After the boiling time, they were cooling at room temperature about half an hour, then 4 ml of distilled water was added to each bottle and shaken for 20-30 s. The mixture was left to settle for 5 min to get two phases, the heavier (lower) phase, DCE-propanol layer, contained the hydroxybutyric acid esters was filtered using 0.45 µm syringe filter into chromatography vials.

The standards prepared by dissolving 10 mg of Poly[(R)-3- hydroxybutyric acid] (sigma, Germany) in 10 ml of reagent mixture, heated at 150°C until the solution became transparent, then the stock solution of PHB was ready. The stock solution was added to various amounts of reagent mixture to a final volume of 4 ml. The standards bottles were placed in a boiling water bath for 2 h and treated as broth samples. A 7820A Gas Chromatography system with flame ionisation detector (FID), 7679A Headspace sampler (Aligent Technologies, USA) with CP7556 Varian poraplot Q-HT 10 m x 0.32 mm x 10 um column (Aligent Technologies, USA) was used. The column operating conditions were: injection volume 1 µL at 230°C. The initial temperature was 120°C, increased gradually over 3 min to 230°C, and the detection temperature was 200°C using Helium as the carrier gas. The PHB concentration was calculated by comparing peak areas to those of standards, using [(R)-3-Hydroxybutyric acid, ≥ 98%] (sigma-Aldrich, Germany) of known concentration. The PHB concentration was calculated by comparing peak areas to those of standards of known concentration.

4.3.3.5 Date seed oil extraction

Date seed oil was extracted with a Soxtec system HT 1043 extraction unit (Velp Scientifica, Europe) equipped with six thimbles as described by Obruca et al., (Obruca et al., 2014) method. Different solvents, Petroleum ether (PE), a mixture of chloroform and methanol (MCM), and hexane were tested. The dry date seed powder (20 g) was transferred to the thimbles (4 g each) and 250 ml of each solvent type was added. The extracted oil was obtained after removing the solvent using rotavapor apparatus at 60 °C. The extracted oil was stored in a freezer (−30 °C) for subsequent physico-chemical analyses. The amount of date seed oil extracted was determined as from the difference in dry weight.



Figure 4.4. A Soxtec system HT 1043 extraction unit (Velp Scientifica, Europe) equipped with six thimbles.

4.3.3.6 Date seed oil analysis

In order to evaluate the fatty acids in date seeds oil, esterification reaction (preparation of fatty acid methyl esters (FAME)) are required and subsequent analysis by GC-MS (Cert et al., 2007). Several methods were found to prepare the FAME from oils. For this study, the AOCS method Ce 2-66 (Page, 2012) was followed to obtain FAME from date seeds oil extracted and all samples were run in duplicate. The GC injector port was set at 230°C and the detector temperature was 270°C. However, the GC oven was programmed with the increase starting temperature from 155°C up to 250°C with 10°C min of the temperature rate and splitting ratio 1:100. The polar capillary column [BPX 70, SGE Company (ID: 0.25 mm, capillary: 0.25 mm and length: 30 m)] was used for the separation the compounds of FAME. The peaks were recognised based on their retention times using standard FAMEs (Akbari et al., 2012).

4.3.4 Cultures media and growth conditions

4.3.4.1 Inoculum preparation for all experiments

After media prepared according to each case, adjusting the pH to 7 (using NaOH and HCl (1 N)), the single colony of *C. necator* from the Petri dish was used to inoculate 10 ml of nutrient broth media, which was grown for 24 h at 30°C and 200 rpm. Cells were then harvested and re-suspended in 10 ml of working media. After a 24 h adaption stage, 5 ml of the inoculum culture were used to inoculate each shake flask; growth conditions for all experiments were 30°C and 200 rpm.

4.3.4.2 Culture media and inoculum preparation for PHB production

In this study, a complex media was derived from date seed to examine the effect of using this media on bacterial growth and PHB production under various conditions, as described in the following sections:

4.3.4.3 Date seeds derived media

Various media were prepared, as a rich media derived from waste date seed, to examine the effect of using this media on bacterial growth and PHB production under different stress conditions. The incubation conditions, for all experiments, were 30 °C and 200 rpm and applied at least in duplicate. The preparing conditions for each case was described as following:

4.3.4.4 Effect of pre-treatment method

After the media was obtained using two different methods (boiling and steam), further sterilisation was achieved using 0.2 µm sterilisation unit in order to avoid microbial contamination. A 100 ml of sterile media of each type was placed in 500 ml shake flasks, incubation and then the fermentation system was started.

4.3.4.5 Effect of supplementing nutrients

Three different types of media were prepared by supplementing each one with different types of nutrients in each case, as shown below:

Media A: 100 ml of date seeds derived media using steam-treatment method without any nutrients supplemented.

Media B: 100 ml of date seeds derived media using the steam-treatment method supplemented with all salts (except nitrogen) that required for *C. necator* to grow. The nutrients (per L) were reported by Aramvash et al., (A. Aramvash et al., 2015):

Media C: the composition of this media is similar to the media B in terms of main salts but without trace elements solution.

Table 4.1. Mineral media composition used for *C. necator* growth reported by (Aramvash et al., 2015)

<i>Media solution</i>	<i>Chemical compound</i>	<i>Concentration (g/l)</i>
<i>Main salts</i>	MgSO ₄ 7H ₂ O	1.2
	(NH ₄) ₂ SO ₄	2.0
	KH ₂ PO ₄	1.54
	Citric acid	1.7
		<i>Concentration (mg/l)</i>
<i>Trace elements salts (10 ml/l)</i>	ZnSO ₄ 7H ₂ O	2.25
	Na ₂ B ₄ O ₇ (7H ₂ O)	0.23
	FeSO ₄ 7H ₂ O,	10
	CaCl ₂ (2H ₂ O),	2
	(NH ₄) ₆ Mo ₇ O ₂₄	0.1
	CuSO ₄ (5H ₂ O),	1
	MnSO ₄ (5H ₂ O),	0.6
	(35 %) HCl,	10

4.3.4.6 Effect nitrogen type and concentration

To investigate the effect of nitrogen sources on *C. necator* growth and PHB production, different nitrogen types and concentrations were tested. The nitrogen sources included (NH₄)₂SO₄, yeast extract and urea and each type were tested in three various concentrations (10, 5 and 2.5 g/l). Samples were taken periodically, and each sample was

centrifuged for 15 min at 7000 rpm. The supernatant was used for measuring TN, fructose concentration while the cell pellets were washed twice with 5 ml 7% NaCl and distilled water respectively, then used for DCW and PHB quantification.

4.3.4.7 Mineral media

The composition of the mineral medium which used to grow *C. necator* (per L) was prepared with the same composition that reported by Aramvash et al., (A. Aramvash et al., 2015) (the composition was mentioned in Section 4.2.3.5). Various types of oils (date seed, vegetable, rapeseeds, and sunflower) were tested as carbon source at different concentration (5 to 25 g/l) in order to compare them. The pH was adjusted to 7 (using NaOH and HCl (1N)) and the sterilising of the media achieved using the autoclave at 121°C for 20 min and the oil was sterilised separately. After preparing all media that mentioned above and during the first 12 h, samples were taken every 3 h, after that every 12 h, the OD_{600nm}, DCW, TN and PHB concentration were measured at each point and experiments were carried out at least in duplicate.

4.3.5 Fermentation and PHB Production PHB production using shaken flask scale

4.3.5.1.1 Feeding nitrogen

Experiments were conducted using two different techniques of fermentation: fed-batch and two-stage batch fermentation, to investigate optimal conditions for PHB accumulation by inducing the *C. necator* with nitrogen limitation. The effect of introducing limited nitrogen on PHB accumulation, either by transferring the cells to nitrogen limited media after growth in unlimited nitrogen concentration or by feeding media of nitrogen limited at three different time points established depending on growth measurements, was tested. Parameters including OD_{600nm}, DCW, PHB concentration and content were measured. The started time of stationary phase (t_0) was determined previously from growth measurements (OD_{600nm} and DCW). Based on the growth measurements results, t_0 was measured to be 16 h after inoculation time.

4.3.5.1.2 Batch fermentation with two-stage nitrogen limited

PHB fermentation investigates on introducing nitrogen limitation by changing nitrogen rich media, media obtained from date seed hydrolysis using autoclave method and supplemented with 2.5 g/l urea as an extra nitrogen source, at (t_0) h were conducted with 200 ml in 500 ml shake flasks. Each flask was inoculated with 10 ml inoculum prepared by one colony from nutrient agar plate, and incubated at 200 rpm and 30°C, in an incubator shaker. Samples were taken up to (t_0) and the parameters (OD_{600nm}, DCW, TN

concentration and PHB content) were calculated. At the introducing nitrogen limitation time point, the remaining broth, after sampling, was centrifuged at 7000 rpm, for 20 min and the residual was decanted and the same volume of date seeds derived media without any supplementation was added. Three flasks were incubated at 30°C and 200 rpm in the incubator shaker for up to 120 h from the start of the second step. The samples were taken periodically from each flask every 3 h for the first 12 h and then every 12 h for the first time of fermentation period.

4.3.5.1.3 Nitrogen limited fed batch fermentation

Three 500 ml shake flasks containing 200 ml of nitrogen rich media were inoculated with 10 ml *C. necator* inoculum and incubated at 30 °C and 200 rpm in the incubator shaker. Samples were taken at 3 h intervals until t_0 then 50 ml date seeds derived media without any supplementation was fed starting at t_0 h, with two more feeds being added at 12 h intervals. From the start of feeding, samples were taken from the three flasks periodically, as same as in two stage batch fermentation for determining all parameters including OD_{600nm}, TN concentration, DCW and PHB content.

4.3.5.1.4 Effect of oxygen mass-transfer coefficients

A ratio inoculum to working volume of 10 % (v/v) was placed in flask experiments with 500 ml volume. Four various volumes of culture media were used: 50, 100, 150, and 200 ml in order to investigate various oxygen mass-transfer coefficients (k_{La}). The pH was adjusted at 7 and the culture media was sterilised by autoclave. Media were carried out at least in duplicate using an incubator shaker at 30 °C and a rotatory shaking of 200 rpm.

4.3.5.1.5 Determination of oxygen transfer coefficient (k_{La})

At shake flask scale, the oxygen transfer coefficient (k_{La}) was estimated using the correlation obtained from Liu et al., (Liu et al., 2006), equation 1.

$$k_{La} = 0.141 \cdot N^{0.88} \cdot \left(\frac{V_L}{V_0}\right)^{-0.8} \quad (1)$$

Where, V_L and V_0 are the liquid and flask volume respectively, and N is the shaker speed, min^{-1} .

4.3.5.1.6 Determination of DCW and PHB concentration from emulsified oil culture

The DCW and PHB content of samples from salts media containing date seed oil as a sole carbon source were measured as described previously by Budde et al., (Budde et al., 2011). DCW of samples from oil fermentations was determined by taking 10 ml samples in

25 ml pre-weighed centrifuged tubes, centrifuging for 15 min at 7000 rpm, washing with 5 ml distilled water and 2 ml hexane and centrifuged again. Then re-suspending the cells in 2 ml distilled water, freezing at -20°C , and lyophilizing. The hexane was used in the washing step in order to remove remained oil.

The method that reported by Kahar et al., (Kahar et al., 2004a), which used for quantifying oil concentration, was applied with emulsified date seed oil medium but could not achieve good results for oil recovery. Therefore, a method for measuring the oil concentration in emulsified oil medium was tested. 10 ml medium was taken in a 15ml centrifuge tube and centrifuged for 10 min, at room temperature and 7000 rpm. The supernatants were transferred to 50 ml centrifuge tubes, and the pellets were washed with 5 ml distilled water and again centrifuged to recover the remained oil that associated with the cell pellets. Then, these supernatants were mixed with the previous centrifugation supernatant (to be 15 ml total). A 20 ml of chloroform and methanol mixture (2:1 v/v) were added to the tube, vortexed for one min.

Then, sample was centrifuged for 5 min and 5 ml of the bottom phase (organic phase) was transferred to a pre-weighed aluminium dish. The solvent was removed by leaving dishes in vacuum hood overnight at room temperature, and more 24 h in the oven at 60°C . After drying, dishes were weighed to determine the mass of recovered oil and equation 2 used to calculate the oil concentration in the medium:

$$\text{Oil concentration} = \text{Mass retrieved} \left(\frac{V_{org}}{V_{trans}} \right) \left(\frac{1}{V_{sample}} \right) \quad (2)$$

$V_{organic}$ is the organic phase volume after the extraction. $V_{transferred}$ was 5 ml and V_{sample} was the volume of medium taken from the culture, 10 ml.

4.3.5.1.7 Mixed-substrate fermentation media

The experiments were carried out in shake flasks scale under varying of feeding ratio between date seeds derived media and mineral media containing 11.2 g/l fructose and 20 g/l of date seeds oil, respectively as a carbon source. Three ratios of 1:1, 1:3, and 3:1 (fructose to oil) were applied to explore the effect this mixed-substrate on *C. necator* growth and PHB accumulation. Furthermore, another feeding strategy was tested by feeding 25 ml of date seed oil (without mineral media) at 48 h of the fermentation period. Fermentation conditions were as same as the conditions of previous experiments, in terms of pH, temperature and working volume.

4.3.5.1.8 Preparation of emulsified oil medium

A 2% date seed oil was emulsified with different concentration of Arabic Gum 0.7, 0.5, 0.3 and 0.1%. In order to study the effect of Arabic Gum on oil emulsification, cell growth and PHB production, as followed: firstly, a 10× of Arabic Gum solution was prepared in distilled water, then centrifuged at 7000 rpm for 20 min to separate insoluble particles. The clarified Arabic Gum solution and date seeds oil were mixed and emulsified using homogenising for two minutes. After emulsifying the date seed oil, the media was autoclaved, cooled. Pre-culture conditions *C. necator* was grown for 24 h in 2 ml of nutrient broth culture tubes, inoculated from a single colony. A 2 ml of culture was used to inoculate flasks contained 200 ml salts, 2% dates seeds oil and 0.1% Arabic Gum. The temperature kept constant at 30°C. The initial pH was adjusted to 6.9 ± 0.1 .

4.3.5.2 Fermentation equipment

All fermenter experiments, both date seeds derived media and date seeds oil with mineral media, was conducted in a 1.7 L autoclavable benchtop laboratory bioreactor utilised for fermentation and cell culture (Electrolab, model 351 equipped with 300 stirrer control) and 1L working volume. The probes of DO (Broadley James Corporation, Oxyprobe D140) and pH (Sentek, Sterprobes) were connected to the fermenter and calibrated previously to sterilisation at 121°C for 20 min. All probes, including the probe of temperature, were connected to the controller of the fermenter immediately after sterilisation. The fermenter was left overnight to be ready for inoculating after the sterile medium was transferred aseptically. The medium was inoculated with 100 ml inoculum. Specific air flow rate was maintained at 1 vvm. DO set point was 30% while agitation speed (200-600 rpm) which controlled by the level of DO. The pH adjusted to be 7 and controlled at this value using NaOH (2 N) and HCl (1N). The air inlet and outlet were sterilised using 0.2 µm membrane filter (Sartorius, Midisart 2000). Around 10 ml sample was taken every 3 h during the first 12 h and every 12 h periodically until the fermentation stopped at 96 h. Samples were utilised to measure OD_{600nm}, as well as to determine DCW, PHB concentration and TN concentration.



Figure 4.5. Autoclavable benchtop laboratory bioreactor utilised for fermentation and cell culture

4.3.5.3 Solid state fermentation

4.3.5.3.1 Polyurethane foams (PUF)

The hydrophilic polyurethane foams were cut into various shape based on the experimental condition and each foam was weighed. The porosity was 0.95 and with 0.2-0.5 mm as pore size while the apparent density was around 0.04 g/ml. They were used as inert support materials for adsorbed carrier solid state fermentation. They were thoroughly washed with distilled water and dried in an oven at 60°C for 48 h.



Figure 4.6. Image shows the shape of polyurethane foams.

4.3.5.3.2 Date seeds culture medium for *C. necator* growth and PHB accumulation

The fermentation was carried out in 1000 ml conical flasks, and each flask contains four grams PUF (each of them weight approximately 0.203 ± 0.002 g). They were sterilised at 121°C for 20 min. PUF was impregnated with the specific volume of date seeds derived medium. The initial pH of the medium was adjusted to 7 and temperature maintained at 30°C for 5 days. The media was inoculated with (10 % v/v) pre-culture of one colony from the Petri dish using nutrient broth medium for 24 h, 200 rpm at 30°C . During the fermentation period, the samples were taken periodically, and each experiment was carried out in triplicates for each condition.

4.3.5.3.3 Biomass recovery from fermented PUF

Distilled water was used for the biomass extraction from the fermented PUF. At each time, two pieces of PUF were placed in the conical flask and 50 ml of distilled water was added then, agitated for 20 min at 250 rpm. This method was repeated for four times to ensure that bacterial cells were extracted completely. The resulting, a pool of the four times filtrates, was centrifuged at 7,000 rpm for 20 min and the pellets were washed twice: one with (0.7%) NaCl and the second using distilled water.

After that, the biomass was lyophilised at 60°C until the constant weight was obtained to determine the dry matter weight and subjected to PHB quantification. The biomass and PHB data were expressed as a total biomass ($\text{g}_{\text{biomass}}$) or PHB (g_{PHB}) / PUF weight (g_{PUF}).

4.3.5.3.4 Optimisation the fermentation process

To study the effect of inoculation volume and time, the foams volume and shape, as well as the container volume on *C. necator* growth and PHB accumulation, various fermentation experiments were achieved. After sterilisation, the flask of (3 g) of PUF was inoculated with 75 ml (1:25 w/v) for 5 days at 30°C . By the end of the fermentation time, the samples were withdrawn as the whole flask in duplicate and the cell mass collected from each flask was agitated with 50 ml distilled water for 25 min, then centrifuged at 7,000 rpm for 20 min. The washing and centrifuged were repeated for four times before the final pellets were collected and lyophilised.

4.3.5.3.5 Effect of incubation volume (solid: liquid ratio)

The effect of moisture content on the biomass production and PHB accumulation was studied using different ratios (1:5, 1:10, 1:15, 1:20, 1:25, 1:30 and 1:35 foams: media volume). The fermentation conditions were same as the conditions used in section 4.2.4.3.4.

4.3.5.3.6 Effect of the shape (surface area and depth)

Various volumes of foam (A: 5×5×5 mm, B: 10×10×5 mm, C: 15×15×5 mm, D: 20×20×5 mm) and depth (A: 5×5×5 mm, B: 5×5×10 mm, C: 5×5×15 mm, D: 5×25×20) were tested, in order to study the effect of foams dimensions on cell growth and PHB production. The fermentation conditions were same as the conditions used in section 4.2.4.3.4.

4.3.5.3.7 Effect of container size

In order to investigate the effect of flask volume (oxygen supply) on cell growth and PHB accumulation, three different sizes of shake flasks, 250 ml, 500 ml and 1 L, were studied. The foams were in small cubic shape with weight equal to 4 g. The fermentation conditions were same as the conditions used in section 4.2.4.3.4.

4.3.5.3.8 Effect of incubation time

In order to study the effect of incubation time on growth and PHB accumulation, a range of incubation time (3 days, 5 days and one week) were studied using fermentation conditions similar to the conditions used in section 4.2.4.3.4.

4.3.5.3.9 Repeated solid-state fermentation

For the repeated batch fermentation condition: after 24 h of cultivation, the culture broth was replaced by a new fresh medium and this replacement was achieved periodically, every 12 h to the end of fermentation time. The method used for this experimental was plotted Figure 4.3.

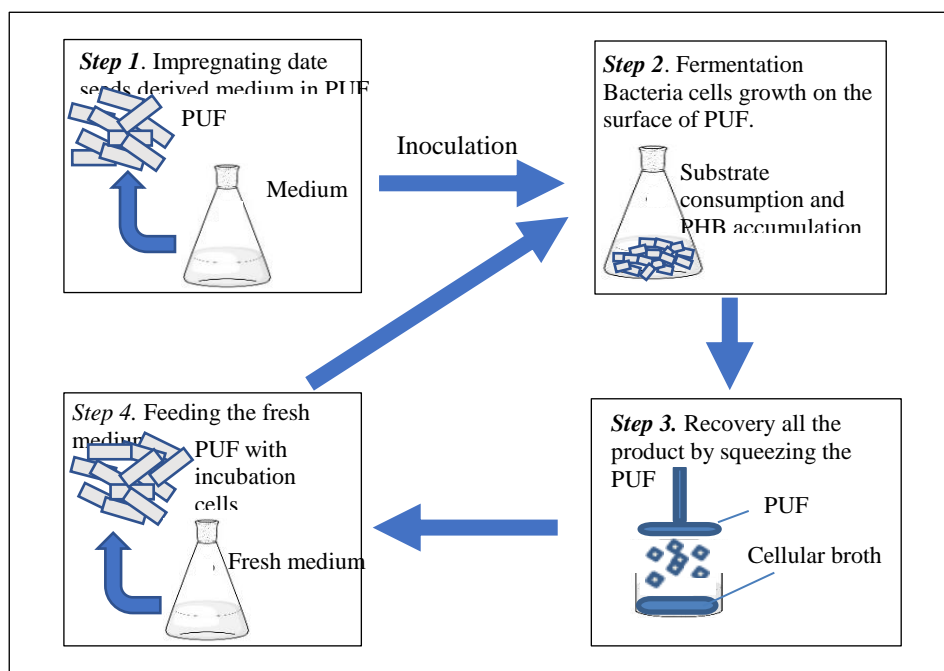


Figure 4.7. Schematic diagram of repeated batch *C. necator* fermentation by solid state fermentation using PUF.

4.3.6 Polymers identification and characterisation

4.3.6.1 Transmission Electron Microscopy (TEM)

An appropriate amount of diluted sample was mixed with a fixative solution [a mixture of formaldehyde (4%) and glutaraldehyde (2.5%) in 0.1M cacodylate buffer]. Then, fixed with a tannic acid solution (1%), osmium tetroxide (1%) and uranyl acetate (1%), and finally dehydrated in alcohol. The sample was settled in epoxy resin and cut to be monitored under TEM.

4.3.6.2 Scanning Electron Microscopy (SEM)

The SEM was conducted to examine the effectiveness of washing steps on the biomass recovery from PUF during fermentation time. After fermentation and each washing step, the one piece of PUF was subjected to SEM (JEOL JSM 5600LV, 115, Japan) analysis. The PUF samples were cut into thin pieces and lyophilised. Images were taken at different acceleration voltages magnifications (max. of 20 kV).

4.3.6.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

The polymer sample (1 mg/ml) was dissolved in spectrochem grad deuteriochloroform (CDCl_3). The ^1H NMR spectra of the sample were obtained at 400 MHz by using the Bruker model advance 400 ^1H NMR spectrometer (Sindhu et al., 2011).

4.3.6.4 Gel Permeation Chromatography (GPC)

The sample solution was prepared by adding 20 mg of PHB sample to 10 ml of CHCl_3 . The solution was filtered using 0.2 μm polyamide membrane filter. The analysis conditions were a flow-rate of 1.0 ml/min by using PLgel guard plus two mixed bed-B columns at 30 °C and 1 ml/min flow rate and the chromatography was analysed using Cirrus GPC (Version 3.2) software.

4.3.6.5 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is a method used to identify the sample composition. FTIR spectra with attenuated total reflectance were obtained at room temperature on a FTIR-8400S spectrometer. The scanned wavenumber range was 4000-600 cm^{-1} . All spectra were recorded at a resolution of 4 cm^{-1} , and 64 scans were averaged for each sample.

4.3.6.6 Thermal analysis using Differential Scanning Calorimeter (DSC)

DSC is a technique of measuring the thermodynamic properties, melting temperature and fusion heat of the sample and reference cell which are contentiously monitoring at the same temperature. This machine is useful in the crystallization measurement, and it is commonly used in polymer studies. The glass transition, melting temperature and crystallinity of PHB obtained from fermentation using date seeds oil as a substrate supplemented with mineral media were determined by DSC (PERKIN ELMER, Inc., USA) equipped with Intracooler 2P in Department of Textile Engineering, Chemistry and Science. Samples of 5 mg were placed in aluminium pans and loaded into the sampler and two scan cycles of heating and cooling were conducted within the temperature range from -25 to 200°C with a scanning rate of 10°C/min were tested. The information of enthalpy of fusion (ΔH), melting temperature (T_m) and crystallinity temperature (T_c) was determined from the second cycle. The crystallinity of PHB samples was calculated as shown in equation 11:

$$x_c = \frac{\Delta H}{\Delta H_0} \times 100 \quad (11)$$

Where: x_c degree of crystallinity, ΔH and ΔH_0 is crystallization enthalpy of PHB sample and ΔH corresponding with 100% crystalline PHB which assumed to be 146 J/g. (Barham et al., 1984).

4.3.6.7 Biodegradability

Biodegradation experiments were conducted in multi-purpose compost medium (which prepared by mixing 50 gm of compost with 100 ml of tap water). The weighed

samples were prepared by placing each sample in a non-degradable mesh bag then burying them 5 cm below the surface. The containers were kept at 30°C, and the medium was irrigated evenly with 5 ml of water once a week. Degraded samples were removed every week for up to 20 weeks, then washed with water with gentle agitation, and then dried at 60 °C. After drying and in order to measure accurately, the specimens were left for 24 h in the same lab environmental conditions (where the initial samples weights were measured) to reach equilibrium point with ambient humidity, after that, the samples were re-weighed. The weight loss percentage was calculated using equation 12:

$$W_{loss} (\%) = \frac{W_0 - W_1}{W_0} \times 100 \quad (12)$$

Where W_0 is the weight dried sample before biodegradation, while W_1 represents biodegraded sample weight.

4.4 Theory and calculation

4.4.1 Statistical analysis

All data required statistical analysis were carried out in triplicates while experiments were repeated twice. T-test (p-value) was used to investigate the significant difference between the results. Significance and related information for the data sets analysed are given in the caption of each relevant figure.

4.4.2 Productivity calculations

4.4.2.1 Growth kinetics

During the growth phase, cell growth population over the time is straight proportional to biomass amount present in times constant, in the case of specific growth rate constant. Hence, it can be expressed as in equation 13:

$$\frac{dx}{dt} = \mu x \quad (13)$$

X : biomass, t : time, μ : specific growth rate.

The specific growth rate (μ) was estimated by plotting $\ln(X/X_0)$ against time during the exponential growth phase; the slope is defined as μ . X_0 and X represent the biomass concentration at the beginning and end of the exponential phase at t_0 and t , respectively.

4.4.2.2 The specific growth rate (μ)

The specific growth rate (μ) was estimated by plotting $\ln(X/X_0)$ against time during the exponential growth phase; the slope is defined as μ . X_0 and X represent the biomass concentration at the beginning and end of the exponential phase at (t_0 and t), respectively.

4.4.2.3 Yield of product / substrate and biopolymer content

The yield of product and substrate on biomass /substrate were calculated using the equation 14 while the PHB content (amount of PHB in dry pellet), amount of PHB in dry mass, was determined using equation 15 and 16:

$$y_{p/x} = \frac{\text{Product formed (g/l)}}{\text{max. biomass concentration (g/l)}} \quad (14)$$

$$y_{x/s} = \frac{\text{biomass formed (g/l)}}{\text{substrate consumed (g/l)}} \quad (15)$$

$$\% \text{ PHB} = \frac{\text{Max. PHB concentration (g/l)}}{\text{max. biomass concentration (g/l)}} \quad (16)$$

4.4.3 Mathematical modelling of fructose extraction from date seed powder

In the food industry, various processes depend upon internal diffusions, such as leaching in a liquid-solid operation in which solutes can transfer from the solid to liquid phase, controlled by internal diffusion of solute (Nickerson, 1968).

The extraction is a function of the rate at which compounds are dissolved and the equilibrium concentration with the liquid. The mass transfer stage involves the transfer of the solute from the solid matrix into the solvent's bulk.

In order to understand date seed processing the diffusivity of the solute is evaluated; assuming that the rate limiting step is the diffusion of the solute from the solid into the liquid. The latter can be applied when negligible external resistance to mass transfer is assumed; therefore, the extraction rate increases with a large concentration gradient (Vega-Mercado et al., 2001), equation 17:

$$\frac{\partial c}{\partial t} = \nabla(-D \cdot \nabla C) \quad (17)$$

where C is the solute concentration, t is extraction time and D is the diffusion coefficient. Considering the date seed particles to be spherical in shape and the concentration difference

$$C^* = \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left(-\frac{\pi^2 n^2 D}{r^2}\right) \quad (19)$$

of the sugar will be relevant in the radial direction only Fick's second law can be rewritten as in equation (18):

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 y}{\partial r^2} \quad (18)$$

As the extraction of substances from food materials is controlled by internal diffusion, the solutions to Fick's second law were used to determine the diffusivity coefficient, assuming the latter is constant with concentration. C^* is a dimensionless concentration defined as the ratio of extract concentration at time t to the initial concentration (C_0) while C_{eq} is the equilibrium concentration [$C^* = (C - C_{eq}) / (C_0 - C_{eq})$].

The boundary conditions used were:

$$C^* = 0, t \geq 0 \text{ and } r \pm R; C^* = 1, t = 0 \text{ and } 0 < r < R.$$

Giving solutions in the form:

Equation 19 above is used here to describe the increasing fructose concentration that is extracted from date seed powder during leaching using hot water.

In the food extraction process, the external resistance is assumed negligible and the diffusion based on one-dimension transport (R jaguerre, 1985). Therefore, the solution of the first term of the series can be used with little error When rearranging equation (19) the logarithmic expression can be written as below:

$$\ln C^* = \ln\left(\frac{6}{\pi^2}\right) - \frac{\pi^2 D}{r^2} t \quad (20)$$

And in order to obtain a straight line, where the slope represents the diffusivity of fructose extracted at different temperatures, equation 20.

$$D = D_0 \exp(-E/RT) \quad (21)$$

where D_0 is an exponential factor, E is the activation energy, R is the universal gas constant (8.314 J K⁻¹ mol⁻¹), and T (K) is the absolute temperature of leaching (Pedreschi et al., 2009).

4.4.4 Mathematical modelling of cell growth and PHB accumulation

Bacterial growth can be expressed as a function of the bacterial concentration in the culture medium at a given time. When the growth limiting substrate is present at low concentrations, the cell growth rate is proportionally low; therefore, the specific growth rate (μ) is expressed as a function of substrate concentration and the biomass (x) increases with time regards to the availability of substrate. In order to better describe the cell growth kinetics, the Monod equation and the logistic equation were introduced (Khan et al., 2013):

$$\frac{\partial x}{\partial t} = \mu \cdot x \quad (22)$$

$$\frac{\partial x}{\partial t} = \mu \left(1 - \frac{x}{x_m}\right)x \quad (23)$$

Where X_m is the population size at stationary phase. From integrating equation (22), with $x = x_0$ at $t = 0$ as initial conditions:

$$x_t = x_0 e^{\mu t} \left[1 - \frac{x_0}{x} (1 - e^{\mu t})\right] \quad (24)$$

Equation (24), known as the logistic equation, gives a sigmoidal variation of biomass which empirically represents two phases of cell growth, the exponential and stationary phases, during which most PHB is produced.

The biomass concentration depends on the initial and maximum attainable cell concentrations with respect to time, which varies with fermentation conditions and the microorganism used. The basic kinetic model for growth and non- growth associated PHB production under nitrogen limitation was proposed by Leudeking-Piret (Pazouki et al., 2008), where the product formation rate depends upon both the growth rate and biomass concentration in a linear fashion, equation 25:

$$\frac{\partial p}{\partial t} = \alpha \frac{\partial x}{\partial t} + \beta x \quad (25)$$

where α and β are the associated and non-associated growth constants respectively. In order to evaluate the constants (α and β) we divide equation 25 by x and assume $k = \frac{1}{x} \frac{\partial x}{\partial t}$, yielding:

$$\frac{1}{x} \frac{\partial p}{\partial t} = \alpha k + \beta \quad (26)$$

When the experimental cell growth data are plotted as $\frac{1}{x} \frac{\partial x}{\partial t}$ against k the result is a straight line where α is equal to the slope while the β is equal to the intercept. P and x represent the PHB polymer and dry cell weight concentration. The biomass concentration with respect to time depends upon the initial and final biomass concentrations.

Theoretically, this model is based on three assumptions: (a) $y_{x/s}$ is constant (b) death-rate of the cell is negligible, and (c) also, the lag phase period is negligible. In a batch culture, the combined of Monod and Luedeking–Piret model can be used to describe quite simply, with three set of mass balance equations, the cell mass (equation 21), product concentration (equation 25) and the limiting substrate (equation 25) with respect to time (t):

$$\frac{ds}{dt} = -q_s \cdot X \quad (27)$$

q_s , the rate specific substrate consumption, is related to μ according to:

$$q_s = \frac{\mu}{y_{x/s}} \quad (28)$$

where $y_{x/s}$ known as substrate yield coefficient which is defined as:

$$y_{x/s} = -\frac{dx}{ds} \quad (29)$$

Also, $y_{x/s}$ can be utilised to develop a relation between biomass and substrate concentrations, by assuming it has a constant value:

$$s = \frac{1}{y_{x/s}} \cdot (x_m - X) \quad (30)$$

Where x_m is the cell biomass concentration in the stationary phase, by substituting equation (30) in equation (21), the cell balance equation becomes:

$$\frac{dx}{dt} = \frac{\mu m \cdot \left[\frac{1}{y_{x/s}} \cdot (x_m - X) \right]}{Ks + \left[\frac{1}{y_{x/s}} \cdot (x_m - X) \right]} \cdot X \quad (31)$$

By re-arranging the above equations, the cell balance equation becomes:

$$\frac{dx}{dt} = \frac{\mu m \cdot (x_m - X)}{Ks y_{x/s} + (x_m - X)} \cdot X \quad (32)$$

By integrating equation 30 using $X = X_0$ at $t = 0$ as the initial condition, the resulting solution is;

$$\left[\frac{x_m + k_s \cdot y_{x/s}}{x_m} \right] \ln \left(\frac{X}{x_0} \right) - \left[\frac{k_s \cdot y_{x/s}}{x_m} \right] \cdot \ln \frac{(x_m - X)}{(x_m - x_0)} = \mu_m \cdot t \quad (33)$$

Equation (33) is an analytical relation, but non-explicit for X , which given all growth-related parameters (μ_m , k_s and $y_{x/s}$, are known. X_0 and S_0 are given, and X_m is calculated from equation (30) with the conditions $X = X_0$ and $S = S_0$. Equation 33 can be used to evaluate the

growth parameters by linear regression on batch culture data. Partially integrating to solve for product formation, from equation 27;

$$P-P_0 = \alpha \cdot (X-X_0) + \beta \cdot \int_0^t x \cdot dt \quad (34)$$

The difficulty in the integration of equation 34 is the last term since the solution for X is not explicit., therefore, by integrating by parts:

$$\int_0^t x \cdot dt = X \cdot t - \int_{x_0}^x t \cdot dx \quad (35)$$

In order to obtain an analytical solution to equation 35, it was temporarily assumed that t is the dependent variable. Integrating with respect to X between X and X_0 , the following solution was obtained;

$$\begin{aligned} \int_{x_0}^x t \cdot dx = \frac{1}{\mu_m} \left[\left\{ x \cdot \ln \frac{x}{x_0} - (x - x_0) \right\} + \frac{K_s \cdot y_{x/s}}{x_m} \cdot \left\{ (x_m - x) \cdot \ln \left(\frac{x_m - x}{x_m - x_0} \right) + \right. \right. \\ \left. \left. X \cdot \ln \left(\frac{x}{x_0} \right) \right\} \right] \end{aligned} \quad (36)$$

Then, by substituting equation (35) in (33), the result is;

$$\left[\int_0^t x \cdot dt = x \cdot t - \frac{1}{\mu_m} \left[x \cdot \ln \frac{x}{x_0} - (x - x_0) + \frac{k_s \cdot y_{x/s}}{x_m} \cdot \left\{ (x_m - x) \cdot \ln \left(\frac{x_m - x}{x_m - x_0} \right) + x \ln \left(\frac{x}{x_0} \right) \right\} \right] \right] \quad (37)$$

$$\left[\int_0^t x \cdot dt = \frac{1}{\mu_m} \left[(x - x_0) - k_s \cdot y_{x/s} \cdot \ln \left(\frac{x_m - x}{x_m - x_0} \right) \right] \right] \quad (38)$$

Then, equation (38) can be utilised to develop an explicit solution for evaluating the concentration of the product with respect to concentration of biomass during batch fermentation and controlled by a combined Monod and Leudeking-Piret kinetic model, equation 39;

$$P-P_0 = \alpha \cdot (X-X_0) + \left\{ \alpha + \frac{\beta}{\mu_m} \right\} \cdot (x - x_0) - \frac{k_s \cdot y_{x/s} \cdot \beta}{\mu_m} \cdot \ln \left(\frac{x_m - x}{x_m - x_0} \right) \quad (39)$$

The substrate utilisation kinetics are shown in equation (39), which assumes that the substrate is converted to cell biomass and product only, neglecting the substrate consumed for maintenance:

$$\frac{\partial s}{\partial t} = -\frac{1}{y_{x/s}} \left(\frac{\partial x}{\partial t} \right) - \frac{1}{y_{p/s}} \left(\frac{\partial p}{\partial t} \right) \quad (40)$$

From equation 29 and equation (40) it follows,

$$-\frac{\partial s}{\partial t} = \left(\frac{1}{y_{x/s}} + \frac{\alpha}{y_{p/s}} \right) \left(\frac{\partial x}{\partial t} \right) + \frac{\beta}{y_{p/s}} x \quad (41)$$

By assuming:

$\lambda = \frac{1}{y_{x/s}} + \frac{\alpha}{y_{p/s}}$ and $\eta = \frac{\beta}{y_{p/s}}$, and by solving eq. 29 using the same steps in the product, the

final equation becomes;

$$s_t = s_0 - \lambda \left[\frac{x_0 \exp(\mu t)}{1 - \left(\frac{x_0}{x_m}\right)(1 - \exp(\mu t))} - x_0 \right] - \eta \left(\frac{x_m}{\mu}\right) \ln \left[1 - \frac{x_0}{x_m} (1 - \exp(\mu t)) \right] \quad (42)$$

From the equation above it can be observed that the substrate concentration may continue to decrease due to product synthesis even after cell mass reaches the stationary phase (x_m).

4.4.5 Kinetic parameters of date seeds oil extraction process

The solvent extraction process is a diffusion of a solvent into solid cells of the oily raw material to obtain a solution of the oil dissolving in the solvent, and different types of solvents can be used for extraction (L. A. Johnson, 1983). The oil extraction solvent method follows the leaching and diffusion theory. Since there is no reaction between the oil and the solvents, the mass transfer kinetic model was used to represent the experimental data and to assume that the mass transfer is controlled the rate of oil extraction from the solid (date) to the solvent. Mass transfer rate can be written as:

$$\frac{dx}{dt} = k.A(CA_i - CA) \quad (43)$$

Where dx/dt is the mass transfer rate of the date seeds oil (g/s); CA and CA_i , concentration of date seeds oil in the solvents and at equilibrium at time t (g/m³), respectively; k is mass transfer coefficient (m/s) and A is surface area for mass transfer (m²).

Because the extraction was conducted at a constant volume was through the experiment, equation 43 can be written as:

$$\frac{dx}{dt} = \frac{K.A}{V} (CA_i - CA) \quad (44)$$

Rearrange:

$$\frac{dx}{dt} = k.a (x_i - x) \quad (45)$$

Where, a and k , are volumetric mass transfer coefficients. After integration equation 43 and applying condition: where at the beginning of the extraction process, the mass of date seeds oil is zero in liquid, then the equation 44 can be written as equation 46:

$$x = x_i (1 - \exp(-k.a.t)) \quad (46)$$

Rearranging the equation 46 in terms of yield per mass of date seeds solid, the kinetic model will be:

$$Y = Y_i (1 - \exp(-k.a.t)) \quad (47)$$

Where, Y and Y_i is a yield of date seeds oil; (k and a) are volumetric mass transfer coefficient.

Non-linear least squares fit method was used to fit the data in order to determination the mass transfer value (a and k) numerically (Meziane et al., 2006).

CHAPTER

FIVE

5 Production of PHB using feedstock derived from waste date seed hydrolysate

5.1 Introduction

In this chapter, an investigation into the potential of a novel feedstock derived from waste date seed for PHB production by *C. necator* is presented. This begins with the characterisation of the waste date seed (Section 5.2), then the preparation of the waste date seed media, using various solvents and methods, is discussed in Section 5.3. This is followed, in Section 5.4, by a study of microbial growth using waste date seed derived media, with and without supplementation of some essential nutrients, to analyse the behaviour of *C. necator*. Various types and concentration of nitrogen were added to the media to give the understanding of whether nitrogen could increase the ability of the *C. necator* to grow under such conditions. Therefore, a series of experiments designed to investigate this, were carried out and are discussed in Sections 5.4, 5.5 and 5.6. Furthermore, it is interesting to study the effect of working volume (oxygen transfer coefficient) on bacterial growth and PHB accumulation, as shown Section 5.8. Also, a model of the system was developed, and the kinetic growth parameters were determined and fitted to the experimental data, Section 5.10. Finally, since the scale up of PHB production is extremely important if a viable waste to biopolymer bioprocess is to be developed the cell growth and PHB production by *C. necator* in a 1.7 L fermenter were also studied, with results presented in Section 5.11. Following on from this results chapter, in Chapter 6 a similar study into PHB production, using the oil derived from waste date seed is presented. The results of both chapter 5 and 6 were taken as a basis for the study into PHB production using co-substrate, presented in Chapter 7.

5.2 Characterisation of waste date seed

The water-soluble fraction produced by the hydrolysis of lignocellulosic materials, which contains fermentable carbohydrates, can be utilised for several biological purposes including biopolymer and alcohol production. Many factors affect the production of biopolymers with the main issues being the difficulty in synthesising biopolymers from inexpensive raw materials at high yields via fermentation processes. Agricultural waste and by-products are renewable sources of inexpensive carbon and nutrients which have no competing food worth. Because date seeds have a high nutrient content there is an opportunity to use them as a source of substrate for PHB production via fermentation.

Characterisation of waste date seed was carried out to on a dry basis determine the total reducing sugar, protein, ash content, moisture, oil content, total carbohydrate, and fructose concentrations. The results of the various analyses are shown in Table 5.1.

Table 5.1. Partial characterisation of the date seed powder used in this study.

<i>Characteristic</i>	<i>Value</i>
Ash content (% , db)	1.76
Moisture (% , db)	1.8
Dry matter content (% , db)	98
Protein (% , db)	4.8
Crude fibre (w/w % db)	18.3
Total sugar (w/w % db)	19
Total reducing sugar (mg/g db)	330
Total carbohydrate (w/w % db)	70
Oil content (% , db)	9.3
pH	5.6

db: dry basis

Table 5.1 shows that sugar is one of the major component categories in the date seeds, including 18% (db) fructose. The date seeds powder contains a low amount of oil content, 9.3 %, while according to the literature, about 10-12 % of date seeds weight is oil (Borchani et al., 2010). Furthermore, results showed that the protein content of date seeds (4.8 %) which is not high compared with other waste material such as rapeseed and wheat bran. A normal value of ash content in date seeds is between 1% and 4% and the variation among samples looks to be rather little (Arun et al., 2014). In this study, the ash content of the date seeds was found to be 1.76%.

The fructose source in date seeds could be belonged to break down the sucrose molecules into fructose and glucose or may be because presence of fructans, non-reducing, quite soluble in hot water which considered a source of fructose in most seeds plants. fructans found in stem tissues and storage organs of plant from a wide range of various families and their seeds (MacLEOD and McCorquodale, 1958).

By using simple chemical treatment, which is achieved under mild conditions, all the nutrients required for bacterium growth can be extracted. Although the constituents of date seeds vary depending on several factors such as, cultivar, growing location (climate) and season of cultivation (Hu and Duvnjak, 2004), the characteristics of the seeds presented that they were whole within the ranges reported in the literature (Besbes et al., 2004b). The results for protein, oil content and moisture were lower than those reported by Basuny, 2011; El-Shurafa et al., 1982) but higher than (Saad et al., 1984; Nehdi et al., 2010) and these

differences, as mentioned previously, are most likely due to natural variation between different date seed types.

The result of the date seed characterisation shows that the seeds could be used as a rich media for bacterium growth and accumulation PHB as all essential nutrients for cell growth are present, along with a fructose content sufficient for use as a substrate for PHB production.

Based on the characterisation results, date seeds are rich in the carbohydrate, therefore it was expected to find various sugars in water-soluble fraction after hydrolysis of date seeds which could be utilised for further biological purposes. Therefore, date seed derived media was analysed, and the soluble sugar and ethanol concentrations measured after hydrolysis for 20 min, at solid to liquid ratios of 1:12 and 1:8 w: v at 121°C and the results are presented in Table 5.2.

Table 5.2. Soluble sugar and ethanol concentrations in waste date seed derived media prepared using the steam-treatment method.

<i>Concentration (g/l)</i>	<i>Value (s)</i>
<i>1:12 (w: v)</i>	
Fructose	10.8 ± 0.5
Sucrose	9.2 ± 0.3
Glucose	12.5 ± 0.1
Acetic acid	3.7 ± 0.3
Ethanol	4.2 ± 0.2
<i>1:8 (w: v)</i>	
Fructose	8.4 ± 0.4
Sucrose	10.2 ± 0.3
Glucose	14.8 ± 0.3
Acetic acid	5.5 ± 0.5
Ethanol	6.2 ± 0.2

Each value represents the mean ± SE.

From the results, it can be seen that at a 1:8 w: v ratio, the highest sugar content was glucose 14.8 g/l of water soluble fraction followed by sucrose at 10.2 g/l, with ethanol and acetic acid also present at concentrations of 5.45 g/l and 6.15 g/l, respectively. While at 1:12 w: v ratio, it was observed that all the concentration values are lower. During steam treatment of the lignocellulosic material, organic acids formation, mainly acetic acid, from

hemicellulose materials leads to acid-hydrolysis of cell wall components and a considerable amount of sugar degradation is expected, phenolics and organic acids are also produced. Finally, it can be concluded that the hydrolysate produced from steam-treated date seeds material being rich in soluble carbohydrates, therefore, the high nutrients content in date seeds leads them to be a candidate raw material for many biological purposes including PHB production.

5.3 Preparation of date seeds hydrolysate media

5.3.1 Effect of boiling time and temperature on fructose concentration

In order to obtain a high concentration of fructose in the media which can later be used for PHB production, various extraction conditions including higher temperatures and longer extraction times were tested. The first method was hydrolysis of date seed powder using water as a solvent at different temperatures and time. At the beginning, there is no fructose concentration was detected when a date seeds powder ($d \geq 1\text{mm}$) was mixed with water as a solvent at ratios of 5:1, 10:1, and 15:1 (v/w) and extraction temperatures of 25 °C and 50 °C for 30 min, 3, and 10 h. When the extraction temperature increased to 90°C, fructose concentration being detected as shown in Figure 5.1. Figure 5.1 shows the fructose concentration against extraction time, using water and ground date seeds ($d \geq 1\text{mm}$).

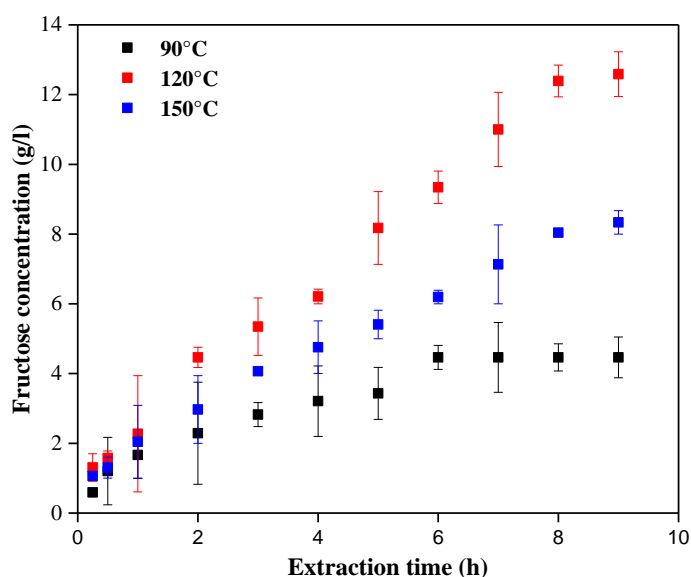


Figure 5.1. Kinetic study for fructose extraction from date seeds A: effect of temperature and time on fructose concentration using three different temperature (■ 90°C, ● 120°C, ▲ 150°C).

Data are shown for three different temperatures, and average values of fructose concentrations from triplicate repeats are plotted. The fructose concentration tends to increase with both time and temperatures of 90°C and 120°C, after which increasing the temperature to 150°C decreased the fructose concentration obtained. The maximum fructose concentration obtained was 12.95 g/l at 120°C and 10 h of extraction time. Increasing the temperature from 120°C to 150°C decreased the fructose concentration from 12.95 to 9.35 g/L after 10 h of extraction. Figure 5.2 shows the fitting of the mass transfer model to fructose extraction data at different temperatures (90°C, 120°C and 150°C).

At high temperatures, dehydration reactions of hexoses (fructose) and Maillard reactions may occur when amino acids (nitrogen) and reducing sugars are found in the same media, therefore; fructose is probably consumed by these reactions as it is extracted by sucrose hydrolysis (Cazor et al., 2006). Furthermore, the low amount obtained due to the rapid denaturation of soluble proteins of date seeds under high temperature, which then entrapped the soluble sugars hence reduced their extractions (Kim and Hwang, 2003). This may explain the lower fructose yield at 150°C. Moreover, the fructose concentration increased from 0.65 to 4.6 g/l when the extraction time was increased from 1 to 10 h at 120 °C.

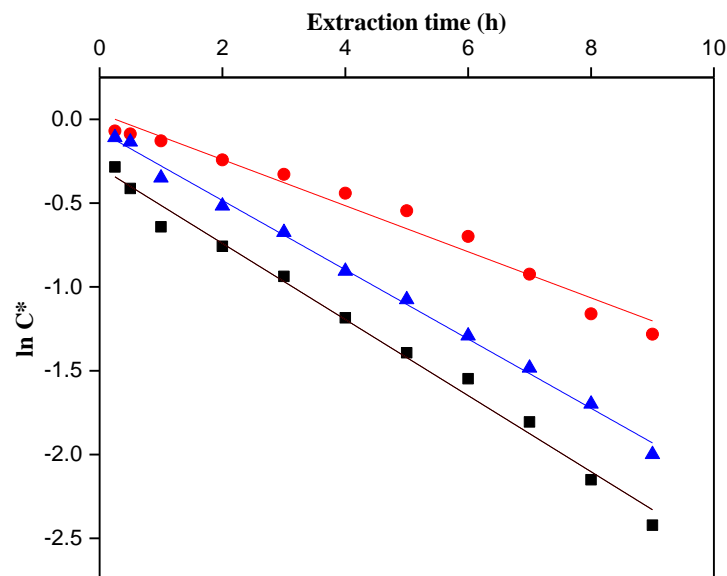


Figure 5.2. Kinetic study for fructose extraction from date seeds data of fructose concentration fitted on mass transfer model.

The Arrhenius relationship for the diffusivity coefficient for fructose leaching from date seeds powder at deferent temperatures, 90°C, 120°C and 150°C is plotted in Figure 5.3.

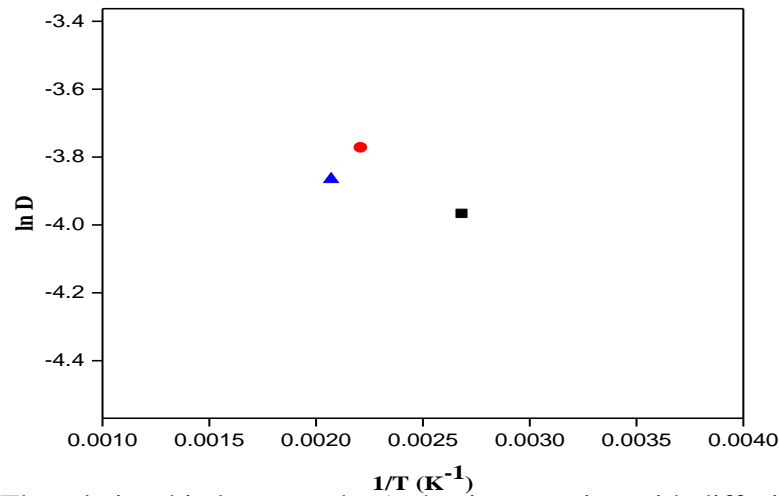


Figure 5.3. The relationship between the Arrhenius equation with diffusivity coefficient for fructose leaching from date seeds powder at deferent temperature.

The t-test for fructose concentration values was carried out to examine the effect of on fructose concentration, there was no significant difference in fructose concentration values between 120 and 150°C ($p = 0.35$) while there is a significant difference between 100°C to 150°C ($p = 0.01$) and 90°C to 120°C ($p = 0.004$) values, see Table 5.3.

Table 5.3. Diffusivity values and t-test of mass transfer model of fructose extraction from date seed powder at various temperatures.

<i>Parameter</i>	<i>Temperature</i>			<i>t-test (p-value)</i>		
	90°C	120°C	150°C	90°C and 120°C	100°C and 150°C	120°C and 150°C
Diffusivity coefficient (D m ² /s)	1.3×10^{-11}	3.1×10^{-11}	2.5×10^{-11}	0.004	0.01	0.35

These results agree with the results reported by Mohan et al., (Mohan et al., 2015) and Rubio et al., (Rubio et al., 1998) which state that in hydrolysis processes the concentration of fructose increased with increasing the temperature until a specific maximum temperature, after which any further increase in temperature resulted in decreasing fructose yield.

5.3.2 Fermentable sugars obtained using dilute-acid and alkali hydrolysis methods

Acid hydrolysis is an established process which has the potential to enable the production of greater amounts of soluble sugars in a shorter time, in comparison with less harsh hydrolysis methods. The fructose concentrations in water soluble fraction obtained from hydrolysis date seeds under different conditions were the target of this study because of the wild strain of *C.necator*, which is used in our research, can metabolise just fructose (Fukui et al., 2014a).

Two types of acids, HCl, H₂SO₄, and one base, NaOH, were tested in order to study the effect on sugar yields, mainly fructose, under various concentrations and temperatures and results of this study are presented in Table 5.4. By using HPLC analysis, liquids type that loading through the preparation of media led to a corresponding variation in the fructose extracted from the seeds. The autohydrolysis treatment process is confirmed to be effective for fructose recovery, where (Chandrasekaran and Bahkali, 2013) reported that the maximum concentrations of fructose was 16 g/l. While this study the maximum concentration obtained was 9.66 g/l, 8.96 g/l when H₂SO₄ and HCl, respectively were used after 2.5 h.

In general, the concentration of fructose in the aqueous extracts was almost 14% higher compared to the acid hydrolysis, results shown in previous section, making the choice of the simpler hydrolysis, using only water, the preferable option in this case.

Table 5.4. Fructose concentration (g/l) in date seeds waste under various extraction conditions and extractant.

<i>Temperature</i>	<i>100 °C</i>			<i>100 °C</i>		<i>115 °C</i>		<i>130 °C</i>	
	(5:1v/w)			(10:1v/w)		(10:1v/w)		(10:1v/w)	
solvent-to-sample ratio									
Extraction time	H ₂ SO ₄	HCl	NaOH	H ₂ SO ₄	HCl	H ₂ SO ₄	HCl	H ₂ SO ₄	HCl
15 min	2.65	1.56	0.88	2.95	2.06	6.25	3.65	1.35	1.09
30 min	4.58	2.36	charred	5.04	3.19	8.36	4.26	3.51	1.99
1.5 h	7.15	3.58	charred	7.46	3.98	9.43	5.52	5.34	2.95
2.5 h	8.67	5.27	charred	8.96	5.89	9.86	8.96	6.69	3.89
3 h	8.15	4.96	charred	8.35	5.23	9.45	8.36	6.55	3.15

The effect of various types solvents, H₂SO₄, HCl and NaOH, on date seeds after hydrolysed using (5:1v/w) ratio and 100°C are shown in Figure 5.4.



Figure 5.4. Effect of various types of solvents, H₂SO₄, HCl and NaOH, on date seeds after hydrolysis using (5:1v/w) ratio and 100 °C.

There is an increase in the fructose concentration extracted from waste date seed with the increasing extraction time and temperature in the range studied. From Table 5.4, it can be seen that the fructose concentration increased from 2.65 to 8.05 g/l when using H₂SO₄ hydrolysis at 100 °C. Followed by extraction with HCl, the concentration was 1.56 g/l at 30 min and then increased to 4.96 g/l at 3h, regardless of liquid-to- solid sample ratio (15:1v/w).

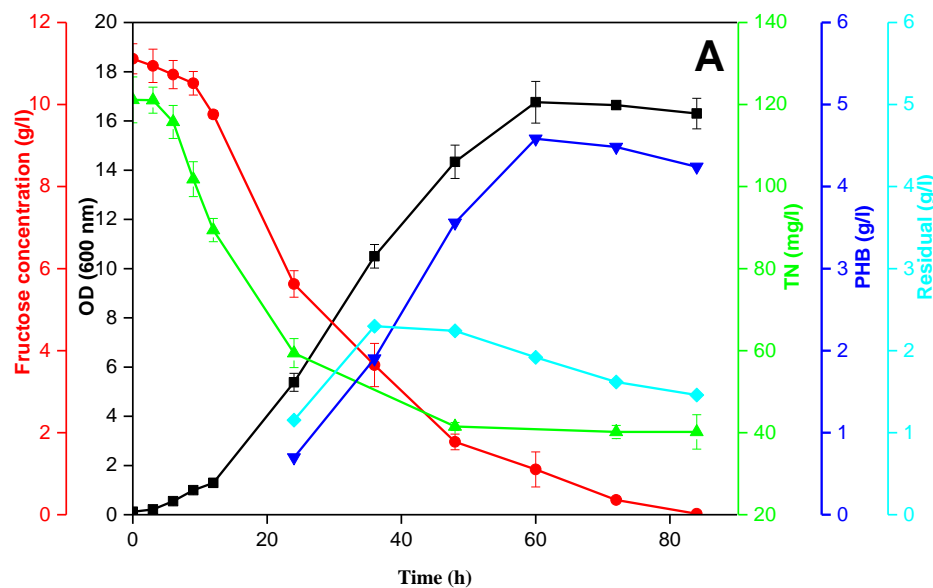
When extraction was conducted at 100°C, using NaOH the date seeds were completely charred after 30 min and gave a maximum concentration of fructose of 0.88 g/l. Extraction with H₂SO₄ at 115°C resulted in a higher fructose concentration than extractions at 130°C, under the same conditions, whilst the extraction performed with a liquid -to-solid sample ratio of 10:1 and at 115°C for 2.5 h rise to 9.86 g/l of fructose, also which was significantly higher than results obtained at 100 °C and 130 °C ($P < 0.05$).

Kim and Hwang, (Kim and Hwang, 2003) found significant differences among various ratios of liquid-to-solid sample and concluded that the optimal ratio was 5:1 to effectively extract soluble sugars from the defatted meal of soybean. The effect of dilution could impair the amount of soluble sugars when using the excessive amount of solvent or by decreasing the extractability using inadequate quantity of liquid type. Nevertheless, the two

ratios of liquid-to-solid sample ratio gave similar results in this study. Liquid types, temperature, liquid-to-solid sample ratio and extraction time may exert various effects on the sugars extraction from date seeds. The results showed that time ($P < 0.05$), temperature and liquid significantly impacted the sugar extraction from date seeds. Under these relatively moderate conditions, it indicates less impact in the hexoses formation and this is mainly because of monosaccharides decomposing into less desirable materials during the hydrolysis process (Journal et al., 2012). These materials, include a product of hexose dehydration, hydroxymethyl furfural (HMF) and furfural along with acetic acid, take place during initial hemicelluloses decomposition, as a result of acetyl groups hydrolysis which linked to the sugar, and later inhibit fermentation, leading to a decrease in bioproduct yield (Romero et al., 2007). The production of these inhibitory compounds increases when hydrolysis occurs at higher acid concentrations and temperatures (Hamelinck et al., 2005). In conclusion, in this study the media derived using these methods did not use because, ultimately the fructose concentration obtained was less, as well as influence these materials on environment and their economic cost.

5.3.3 Effect of extraction method: boiling and steam treatment on PHB production

Two techniques were used to hydrolyse the date seeds: boiling with reflux system and steam-treatment by autoclave. In order to study the effect of each technique on bacterial cell growth and PHB production, shake flask experiments were carried out and all experiments were applied in duplicate (at least). The average values of biomass, PHB concentration, TN and fructose concentration were measured, and the results are shown in Figure 5.5.



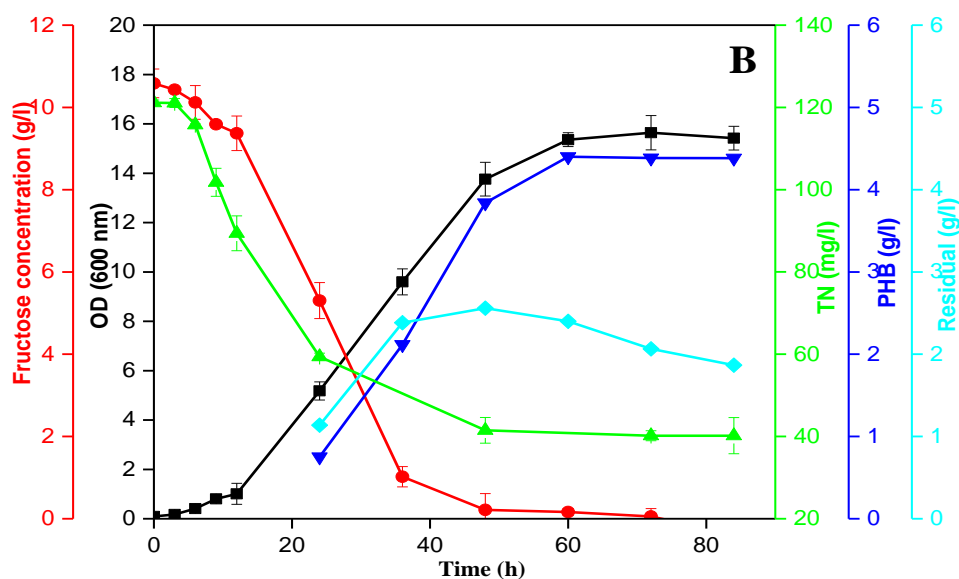


Figure 5.5. *C. necator* growth in media derived from date seeds contains about 11 g/l fructose as initial concentration. The incubation conditions were 30°C, 200 rpm for 84 h. A: experiment 1 (Boiling method), B: experiment 2 (autoclave). ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass.

The results indicate that the *C. necator* bacterium is able to grow in the date seed derived media and produce PHB for both techniques, as well as, there was no significant difference between both techniques for all parameters including OD_{600nm}, total biomass and PHB concentration. The first experiments were carried out using 100 ml working volume with media hydrolysed by a boiling method while the second set of experiments was conducted using 100 ml of autoclaved date seed media (as described in Section 4.3.2.10).

From Figure 5.5 it can be observed that in general the lag phase was finished at 12 h, and then a steady increase in OD can be seen until 60 h, where the phase begins, with PHB accumulation occurring throughout. Although some PHB production during the early growth phase was observed, most PHB was produced during the latter stages of the fermentations, which PHB started to accumulate significantly after 48 h, while biomass growth gradually slowed. These experiments revealed that maximum OD_{600nm} of 6.3 g/l and PHB concentration of 4.6 g/l was achieved after 60 h for experiment B; following experiment A which gave a OD_{600nm} of 6.1 g/l and PHB concentration 4.58 g/l. The maximum specific growth rate was 0.13 h⁻¹ for both experiment A, and B. The total nitrogen (TN) concentration was also studied, and it was observed that 66% of TN was consumed in the first 48 h, after which the TN remained constant at a concentration of 40 mg/l. In both experiments, A and

B, the initial concentration of fructose was 11 ± 0.6 g/l and the fructose concentration was consumed after 48 h of fermentation. The PHB content was 75% (w/w) for experiment A and 73% (w/w) for experiment B. The overall yield $y_{p/s}$; $y_{x/s}$ and PHB productivity were calculated and the values were 0.66 and 0.45, respectively for experimental A while were 0.68 and 0.46 for experiment B. Overall results indicate that there was no significant difference between the media prepared using different methods (boiling and autoclave), both mediums gave the all essential nutrient for bacteria growth. And it was shown that no material toxic to the microbial cell is produced during steam treatment of date seeds.

Samples were taken at the different stage of the fermentation course in order to observe the PHB granules accumulation inside the *C. necator* cells and the results were shown in Figure 5.6. Figure 5.6 A, 36 h of fermentation time, shows there was a lot but small granules of PHB and some cell debris scattered in the medium, also, it can be seen some bubbles around the cell may somehow damage the cell, causing cell disruption. Furthermore, at 48 h the culture was in the exponential phase and the TN concentration had decreased to less than half the start concentration, more and bigger PHB granules were observed. A releasing PHB granules into the culture and dead cell disrupting was also observed.

Beyond 72 h, (Figure 5.6 C), the proportion volumes of cell that occupied by granules of PHB were not much various. By 48 and 72 h, around 5% of both dead cells and PHB-free cells can be observed, while about 95% of cell containing PHB were also spotted. Figure 5.6 D presents the zoom into PHB granules.

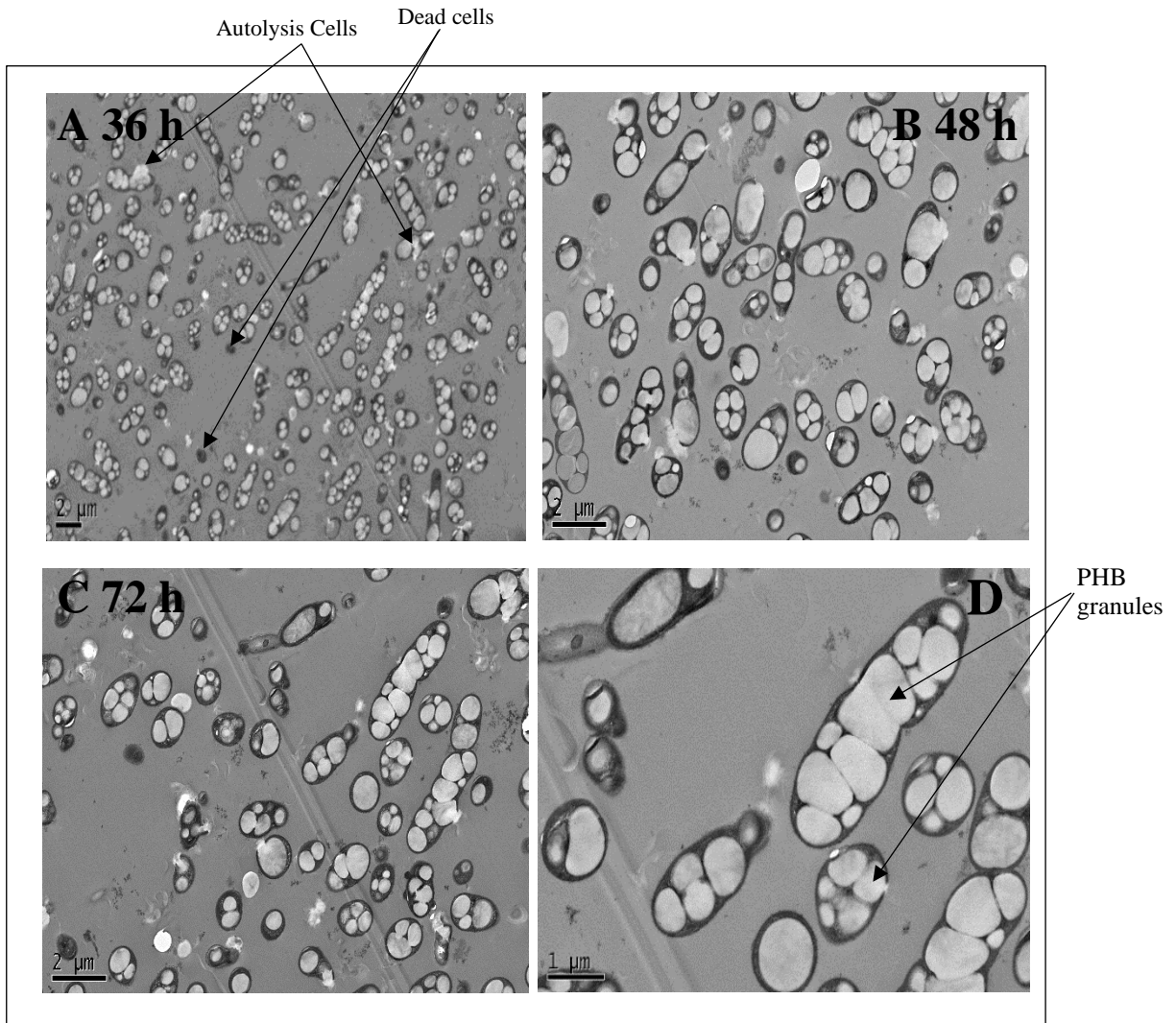


Figure 5.6. TEM images of *C. necator* producing PHB as intracellular granules at different stages of fermentation using date seed hydrolysate media. The granules of PHB are the white/grey fractions inside the biomass cells.

Note that although *C. necator* bacteria have flagella, it was difficult to notice them by this method due to the embedded the cells could be cut in any angle and direction. To notice the bacteria flagella arrangement, the biomass cells should be stained negatively and monitored under electron microscopy (EM) (Makkar and Casipa, 1987). However, in this study the existence of *C. necator* flagella was not of importance compared to the existence of PHB.

5.4 Effect of supplementation of nutrients on *C. necator* growth

Since the previous experiments demonstrate that *C. necator* bacterium is able to grow and accumulate PHB, an attempt for increasing the cell growth and PHB production was studied in this section. Different experiments were conducted by supplementing the date seeds derived media each time with various essential nutrients in order to examine the effect of these nutrients on both cell growth and PHB accumulation. Figure 5.7 presents the growth curves of *C. necator* over a 96 h cultivation period for the date seeds derived media tested with and without supplementation.

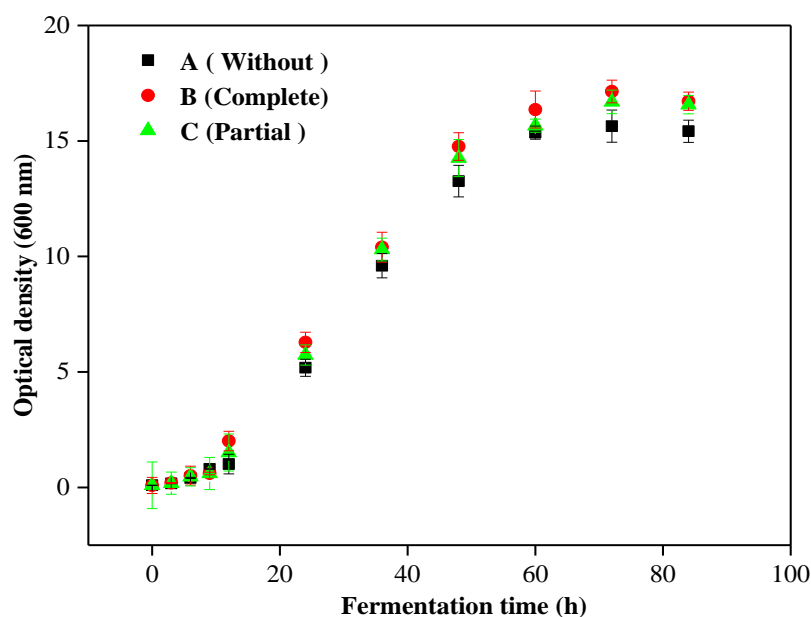


Figure 5.7. Optical density trend obtained from the growth of *C. necator* for 96 h and using date seeds derived media under three different conditions: (A) the media without supplement, (B) when the media supplemented with all nutrient that essential for microbial growth and (C) when the media supplemented with partial nutrient.

Similar biomass growth trends were noticed for all types of media (A, B and C) and all the curves with final OD_{600nm} and total biomass being almost the same ($P > 0.05$). All media types showed exponential growth for the first 60 h, then the stationary phase started. The corresponding OD_{600nm} for media A and C were 15.22 and 16.32, respectively, while media B gave the highest OD_{600nm} of 16.82. The results for OD_{600nm}, total biomass, PHB productivity and yield coefficient ($Y_{p/x}$) during the fermentation period are shown in Table 5.5.

Table 5.5. Summary of the results obtained during *C. necator* growth in date seed derived media supplemented with three different nutrients.

<i>Media type</i>	<i>Total biomass (g/l)</i>	<i>PHB concentration (g/l)</i>	<i>PHB Content (%)</i>	<i>$Y_{p/x}$</i>	<i>μ_{max} (h^{-1})</i>	<i>Productivity (g/l.h)</i>
A	6.3	4.6	73	0.46	0.13	0.11
B	6.43	4.9	75	0.48	0.136	0.13
C	6.59	4.8	73	0.46	0.133	0.13

The corresponding total biomass, for media A and B were 6.3 ± 0.05 g/l and 6.43 ± 0.13 g/l, respectively, while media C gave the highest total biomass, of 6.59 ± 0.25 g/l. The analysis results revealed that there was no significant difference between all types of media for all parameters including total biomass, PHB concentration and content. Hence the date seed media can be utilised as an inexpensive rich media for bacteria growth and PHB production without supplementation, as all required nutrients for biomass growth are present, along with a fructose content high enough for use as a substrate for PHB accumulation.

5.5 Effect of nitrogen types and concentration in PHB production

Nitrogen is essential for organism growth and nitrogen limitation is generally the constraint required to trigger PHB accumulation, whereas high nitrogen concentrations lead to greater biomass growth with little to no PHB formation (Albuquerque et al., 2007). In normal conditions, microorganism synthesises their cell materials, such as proteins, and grow, but under nutrient restricting conditions bacteria could shift their protein production to PHB accumulation for survival. Or in other words, after complete consumption of nitrogen, growth is no longer possible so the microorganism channels its metabolic energy into the production of energy reserve materials, such as PHB (Bajaj and Abbass, 2011). Therefore, nutrient environmental/control stress is required to prompt PHB production (Elsayed et al., 2013).

The aim of this section is to understand and select a nitrogen source and concentration to supplement with the intention of increasing cell number and hence PHB production, because the date seeds have a low nitrogen content, e.g. not much protein. Therefore, in this section organic, inorganic and complex nitrogen sources, urea, ammonium sulphate and yeast extract, at various concentrations were tested to study the effect of adding

nitrogen to the media derived from date seeds on *C. necator* growth and PHB production. As shown in Figure 5.8, the general trend of bacterial cell growth for were similar for all nitrogen types, showing exponential growth for the first 60 h and then a subsequent stationary phase.

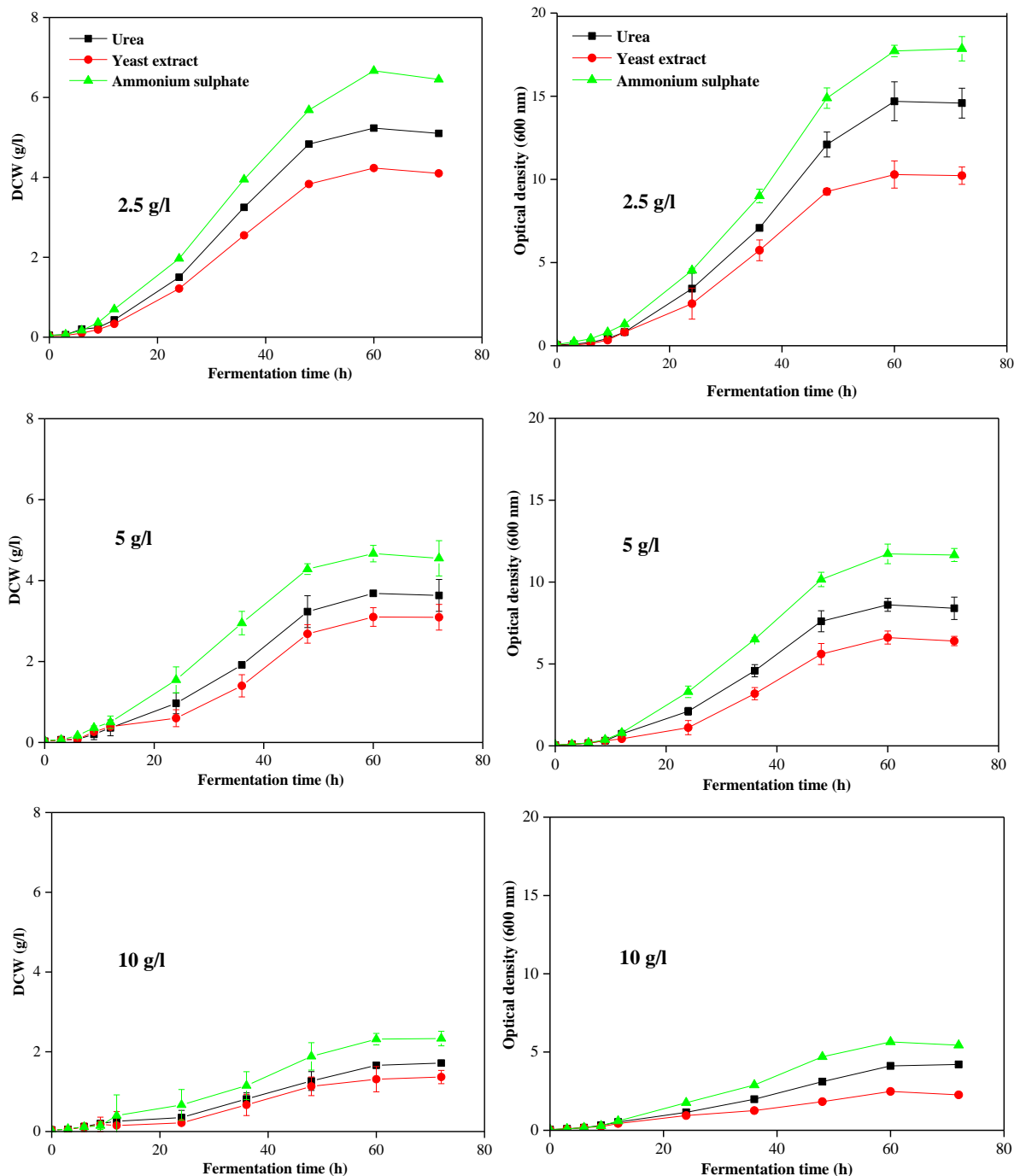


Figure 5.8. Growth profiles of *C. necator* over 72 h of cultivation period in the date seeds derived media supplement with various types and concentration of nitrogen. The incubation conditions were 30°C, 200 rpm.

Among the various nitrogen sources examined, ammonium sulphate supported the greatest total biomass accumulation, urea supported the moderate growth of the bacterium, whereas, yeast extract was comparably ineffectual as a source of nitrogen. Both OD_{600nm} and biomass were higher when other nitrogen sources were replaced by ammonium sulphate; this could be due to the inability of *C. necator* to metabolise urea and yeast extract efficiently compared with ammonium sulphate. In general, both OD_{600nm} and total biomass reached the maximum at a nitrogen source concentration of 2.5 g/l for all types and the values were 17.1, 14.5 and 10.2 for ammonium sulphate, urea and yeast extract, respectively while the maximum total biomass values were 7.2, 5.1 and 4.3 g/l. Figure 5.9 presents the PHB content accumulated under various types of nitrogen at 2.5 g/l concentration.

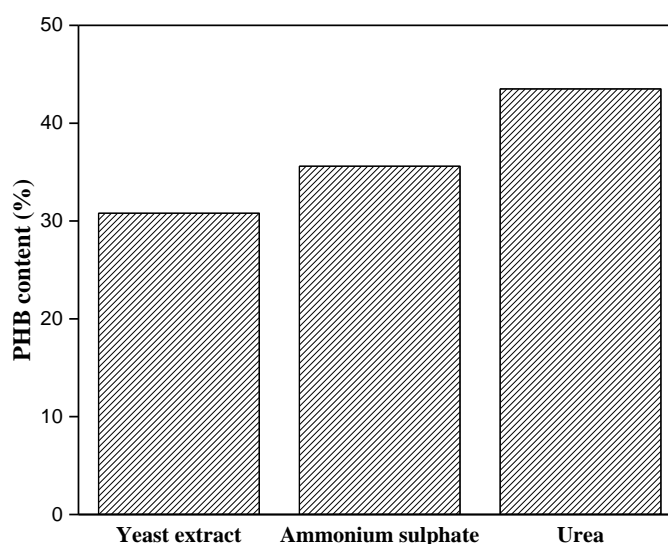


Figure 5.9. Percentage of PHB content for 2.5 g/l of different types of nitrogen source, yeast extract, ammonium sulphate and urea, when the *C. necator* growth in media derived from waste date seed for 72 h.

From Figure 5.9 it can be seen that urea supported greater PHB accumulation compared with other types. The highest PHB was accumulated with urea (43.5%) followed by ammonium sulphate (35.6%), while yeast extract (30.2 %) supported least PHB accumulation. Results from this study indicate that at lower concentrations of all sources types tested, both cell growth and PHB content raised significantly (Figure 5.9). In contrast, at high concentrations, the biomass accumulation was quite low. Normally, it was reported that PHB accumulation in photosynthetic bacteria is associated with nitrogen availability in media (Khatipov, 1998). Urea was optimal nitrogen source for PHB production compared to $(\text{NH}_4)_2\text{SO}_4$ as well as yeast extract in many microorganisms including *C. necator* (Grothe et al., 1999), (Koutinas et al., 2007). Figure 5.10 presents the variation of specific growth

rate with nitrogen types and concentration and the results were indicated the maximum specific growth rate was 0.21 h^{-1} for ammonium sulphate followed by urea and then yeast extract of $(0.16, 0.13 \text{ h}^{-1})$ for the same concentration, 2.5 g/l , while the specific growth rate value was the lowest when the nitrogen concentrations were increased for all types.

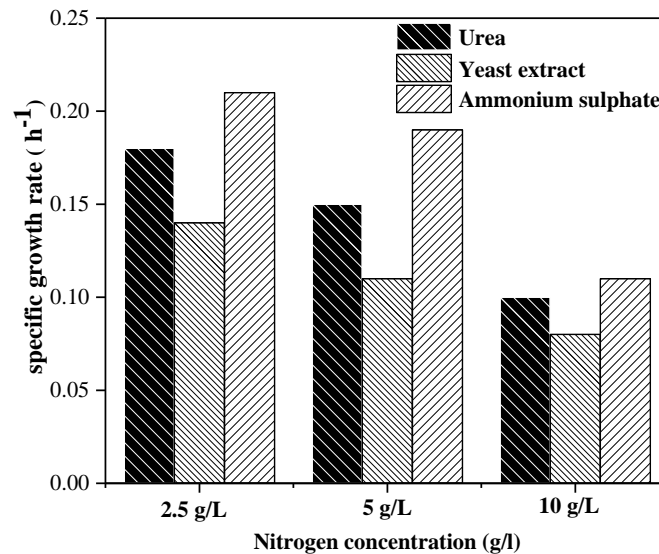


Figure 5.10. Variation of specific growth rate (μ) as function of the initial concentration of nitrogen for three types of nitrogen supplement into media derived from date seeds contains around 11 g/l fructose as initial concentration. The incubation conditions were 30°C and 200 rpm .

The effect of various nitrogen sources such as ammonium sulphate, yeast extract, and urea on the growth and PHB production by *C. necator* was studied by Sreya Kumbhakar et al., (Sreya Kumbhakar et al., 2012) and studies showed that both ammonium sulphate and yeast extract gave the raised biomass growth while PHB accumulation was originated highest for ammonium sulphate in comparison with yeast extract. This result agreed with (Sangkharak and Prasertsan (Sangkharak and Prasertsan, 2008) which reported that PHB accumulation was found to be growth associated and urea supported maximum PHB production (0.93 g/l). Belal, 2013, (Belal, 2013) studied the impact of various concentrations of the best nitrogen source (ammonium sulphate) on PHB accumulation by *C. necator* and *Pseudomonas stutzeri E114*. PHB yields accumulated were between 3.6 and 4.1 g/l PHB yield, respectively. It was found that 1 g/l of ammonium sulphate supported highest PHB yield (0.377 g/100 ml) in comparison to other concentration. According to our results can be concluded that inorganic nitrogen sources such as different salts of ammonia resulted in the accumulation of the higher amount of PHB than organic nitrogen and complex sources. Our results agree with the results reported by (Alkando and Ibrahim, 2011).

5.6 PHB Production using two different fermentation strategies

As demonstrated the previous section, urea with 2.5 g/l concentration was the best nitrogen source for PHB accumulation, in this section fermentation experiments were carried out in shake flasks supplemented with 2.5 g/l urea to study the influence of nitrogen supplementation on cell growth and PHB accumulation. The results obtained are represented in Figure 5.11.

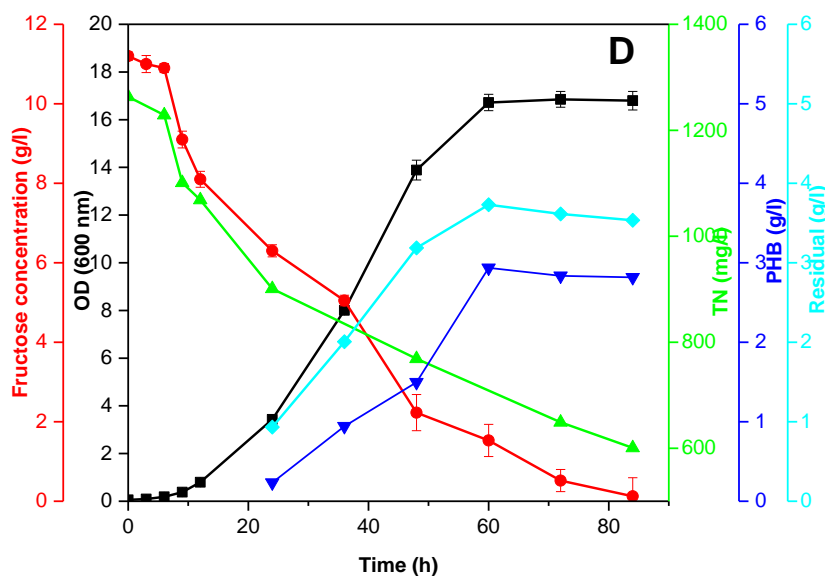


Figure 5.11. The *C. necator* time course growth in media derived from date seeds contains around 11 g/l fructose as initial concentration and supplemented with 2.5 g/l urea. Incubation conditions were 30°C, 200 rpm. ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass.

From Figure 5.11, it can be seen that PHB started to accumulate significantly after 48 h, while cell biomass gradually slowed down. A maximum PHB concentration and total biomass of 2.9 and 6.7 g/l, respectively, were reached at 60 h of fermentation while PHB content was 43.3%. The specific growth rate was 0.21 h^{-1} for the excess nitrogen experiment over the same fermentation time. The amount of nitrogen present in the media has a significant influence on bacterial growth and PHB accumulation, TN concentration decreased with time, from 130 mg/l to reach 60 mg/l after 24 h and reaching a constant value of 35 mg/l at 60 h to the end of the fermentation period. Based on the results of this section, two different strategies of nitrogen feeding: two-stage batch culture and fed-batch culture were conducted trying to control nitrogen concentration in the media to enhanced PHB accumulation. Three-time points for the induction of PHB accumulation in bacteria cells through introduction of nitrogen restriction, either by changing to a nitrogen limited culture or by feeding media of nitrogen limited at regular intervals, were studied. The results of this study are presented in the next sections:

5.6.1 PHB production using two-stage fermentation

The two-stage cultivation process was first used by (Tanaka and Ishizaki, 1994) which achieved promising results. Based on the results in the previous section, the first stage of the fermentation was terminated, after 60 h, and then the second stage of fermentation, was started. Three-time points, 57 h, 60 h and 63 h, were tested to try to increase PHB accumulation through the addition of date seed derived media. Figure 5.8 presents the *C. necator* time profile growing in a media derived from date seeds, with the second batch fermentation starting at three different points. From the results, it can be seen that all parameters values indicated that *C. necator* grew exponentially during the first 60 h of incubation with highest OD_{600nm} and PHB concentration being 24.3 and 7.29 g/l, respectively. As illustrated in Figure 5.13, PHB content value boosted as the second stage of cultivation proceeded being 77.8 % at 60 h of induction. For the second stage and according to fructose consumption analysis during the process, the initial concentrations of fructose were 11.1 g/l, 10.8 g/l and 11.3 g/l at 57 h, 60 h and 63 h, respectively, and all the fructose was consumed after almost 50 h of fermentation for all induction times. The yield of PHB ($Y_{p/s}$) was 0.26 at the end of first stage of incubation, while for the second stage this value increased to 0.39, 0.53 and 0.43 g/g for 57, 60 and 63 h induction times. During the second stage of fermentation, TN concentration decreased with time, from 756.36 mg/l to reach 531.96 mg/l after 36 h and reaching a constant value of 450 mg/l at 50 h to the end of the fermentation period for 60 h of induction time. While for 63 h of induction time, TN concentration was reduced from 748.61 mg/l to 412.6 mg/l at 50 h and remained constant until the end of fermentation. At 57 h, TN concentration was 733.31 mg/l to reach a constant value 396.36 at 50 h.

The concentration of PHB at introducing time of 60 h was significantly higher than those for 57 h and 63 h, away from giving the highest concentration of PHB at the end of the second stage. When nitrogen limitation was introduced at 60 h and 63 h, PHB productivity and yield were significantly higher, in spite there was no significant variation between all introduced time 60 h and 63 h. Relative to all parameters (PHB concentration, PHB content, PHB productivity and $Y_{p/s}$), 60 h induction resulted in the higher performance compared to 57 h and 63 h. Over the 60 h induction process, rising time of fermentation beyond 63 h did not have a significant impact on all parameters. However, there were significant differences in both PHB and total biomass concentration between all samples taken at 36 h and 50 h of the second stage.

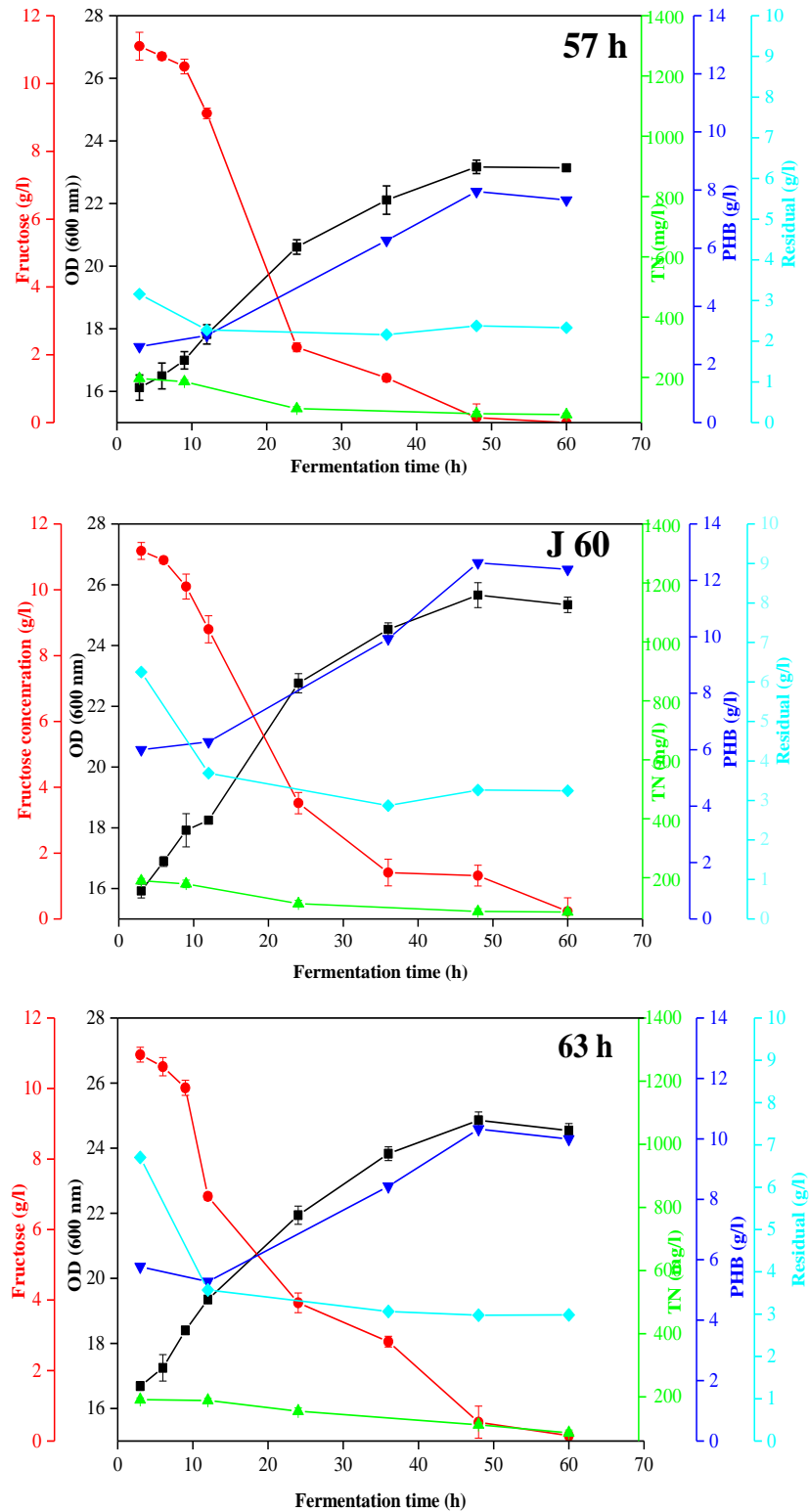


Figure 5.12. *C. necator* time profile growth in date seeds derived media, during second stage of two-stage nitrogen limited batch fermentation at three different time of transferring.

● Fructose, ● TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass.

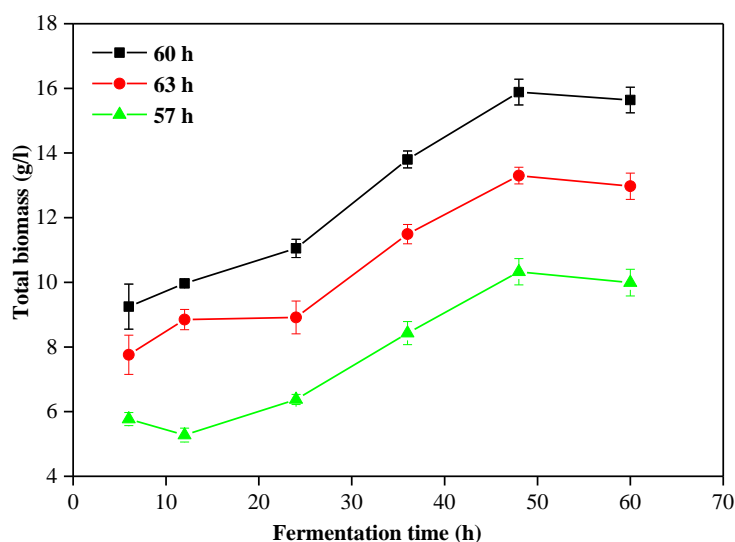


Figure 5.13. Dry cell weight (biomass) trends during second stage of two-stage nitrogen limited batch fermentation at three different time of transferring using *C. necator* growth in date seeds derived media. ■ 60 h, ● 63 h, ▲ 57 h.

The results of the fermentation experiment of two-stage nitrogen limited media indicate that total biomass increased for all induction times until 50 h then stationary phase was started, giving the highest value of total biomass of 8.6 g/l when the inducing applied at 60 h of fermentation time of the first stage (Figure 5.13). While the values of time 57 h and 63 h were 5.34 g/l and 7.75 g/l, respectively. Also from the figure it can be noticed that the residual biomass was not changed indicating there was not very much growth occurring as the total biomass increase is due to polymer accumulation.

According to OD_{600nm} and total biomass values at the end of first stage fermentation for three different induction times (57 h, 60 h and 63 h), samples at 60h had significantly higher for both OD_{600nm} and total biomass. While for second stage fermentation, all parameters (total biomass, OD_{600nm} , PHB concentration, PHB content, $Y_{p/x}$, and PHB productivity), various sampling points after the introduction of nitrogen limitation for three different induction times as shown in Table 5.6, designated that induction time to have a significant influence on PHB accumulation. During the second stage, each parameter measured was analysed, there was no significant difference between the OD_{600nm} and total biomass for 60 and 63h runs, but they were both higher than 57 h. The productivity of the process was 0.04 g/l.h at the end of the first stage of growth (72 h) while increased to being 0.092, 0.13 and 0.081 g/l.h at 57, 60 and 63 h, with corresponding concentration of PHB in fermentation broth was 6.98 g/l, 7.21 g/l and 6.13 g/l. respectively, at the end of the second fermentation period.

Therefore, the process in which nitrogen limitation was reached at 60 h and the second stage of fermentation proceeded for a further 60 h was identified as a means of increasing biopolymer yield. Table 5.6 shows the values of PHB yield coefficient ($Y_{p/x}$) and PHB productivity over the first and second stage period of fermentation and the value of $Y_{p/x}$ was 0.42 at 60 h of inducing time which was comparable to the results reported by (Wang et al., 2013).

Table 5.6. PHB accumulation by *C. necator* during first and second stage of two stage of fermentation.

Time	First stage			Transferring time	Second stage		
	Yield		Productivity		Yield		Productivity
	$Y_{x/s}$	$Y_{p/s}$	(g/l.h)		$Y_{x/s}$	$Y_{p/s}$	(g/l.h)
72 h	0.23	0.26	0.04	57 h	0.28	0.35	0.092
				60 h	0.32	0.42	0.14
				63 h	0.29	0.39	0.08

5.6.2 B-PHB production using fed-batch culture

Similar parameters those studied in two-stage batch fermentation were determined for fed-batch fermentation. Figure 5.14, presents the time profile of *C. necator* growth in media derived from waste date seed supported with nitrogen using feeding batch technique in order to study the effect of feeding date seed media on cell growth and PHB accumulation, after nitrogen limitation was reached. from the Figure 5.14, PHB concentration values exhibit a similar trend compared with two-stage cultivation process, giving the final PHB content was 59 %, 69 % and 49.9% for 57 h, 60 h and 63 h, respectively. While the corresponding concentrations of PHB were 8.1 g/l, 10.2 g/l and 12.43 g/l at 57 h, 60h and 63 h, respectively. All fructose was consumed during 57 h, 60h and 63 h run while the corresponding final value of PHB yield coefficients ($Y_{p/s}$) during this process were 0.35, 0.42 and 0.39 at 57 h, 60 h and 63 h, respectively, Table 5.7. The total nitrogen concentration consumption was 197.63 mg/l for 57 h, 202.09 mg/l for 60 h, and 192.27 mg/l for 63 h. $Y_{p/x}$ increased over the fermentation process with the maximum values being 0.42 for 60 h induction, 0.39 for 57 h and 0.35 for 63 h. Productivity values of the process ranged from 0.11 to 0.12, 0.17 and 0.15 g/l.h, respectively. At 60 h, the productivity value induction was the highest at 0.17 g/l.h.

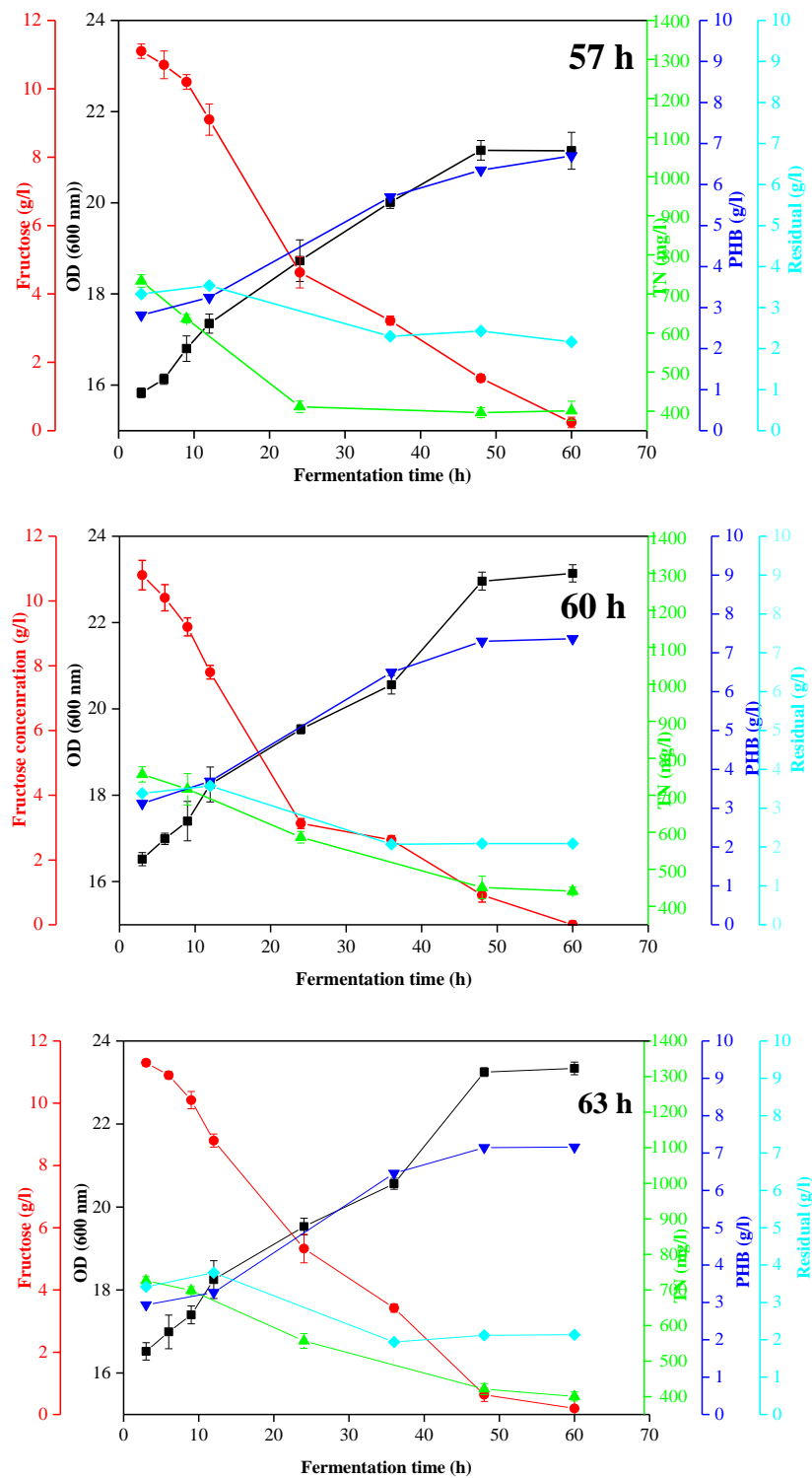


Figure 5.14. Variation of cell growth, PHB accumulation and fructose consumption during the fed-batch fermentation using *C. necator* in media derived from date seeds contains around 11 g/l fructose as initial concentration, ● Fructose, ▲ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass.

Figure 5.15 shows the total biomass trend up to the times when feeding was started to introduce limitation nitrogen in the hydrolysate media, and it can be seen that the total biomass followed a growing trend for all time points after starting feeding. For 60 h, the highest total biomass obtained was 15.63 g/l at 60 h after first feeding during fed-batch fermentation, while that for 57 h and 63 h final total biomass was 9.99 g/l and 12.79 g/l, respectively.

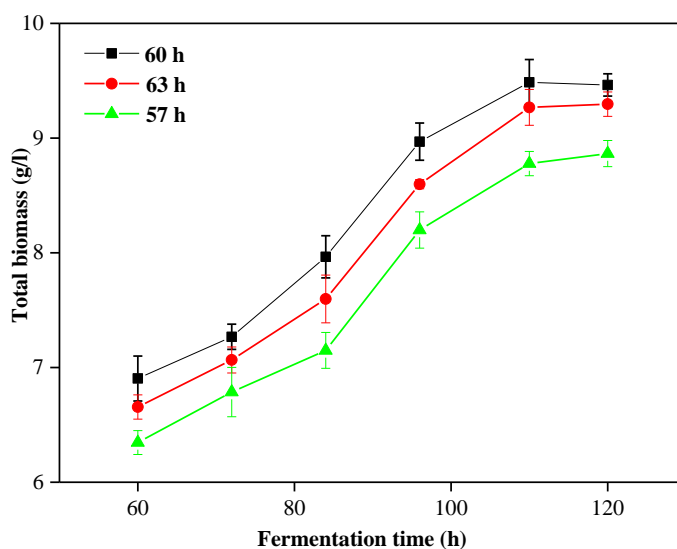


Figure 5.15. Total biomass weight after initiating feeding during fed-batch fermentation, second stage, at three different time using *C. necator* growth in date seeds derived media.

■ 60 h, ● 63 h, ▲ 57 h.

Considering all time points and parameters, significant variation ($P \leq 0.05$) among the three various points of time for introducing nitrogen limitation concentration by started feeding of nitrogen limited medium were investigated. For 60 h, the final values of PHB concentration and total biomass samples obtained at 50 h were significantly higher ($P \leq 0.05$) than those obtained from 57 and 63 h. In spite of PHB content of samples induced by starting nitrogen limitation at 60 h was the highest, it did not differ significantly ($P > 0.05$) between 57 and 63 h samples. However, the values of 57 h and 60 h were significantly different ($P \leq 0.05$) from values obtained from 63 h initiation. Values from the 60 h experiments had significantly higher ($P \leq 0.05$) final value of PHB productivity and yield at the end of fermentation time when a comparison was performed among the three experiments (57 h, 60 h and 63h initiation).

Statistical analysis to investigate the influence of sampling time (fermentation progression) over the 60h run indicated there was no significant difference ($P > 0.05$) in PHB content, productivity, $Y_{p/x}$, and total biomass at 50 and 60 h. Compared to 50 h, however, there was a significant increase ($P \leq 0.05$) in the concentration of PHB when fermentation process was achieved up to 50h. For 57 h samples, 60 h gave significantly higher ($P \leq 0.05$) PHB concentration and total biomass but there was no significant variation ($P > 0.05$) in PHB yield, productivity and content. Also, for 63 h samples, there were no significant differences ($P > 0.05$) among 36 h, 50 h and 60 h for all parameters estimated. Hence, it can be concluded that 60 h time inoculation was the appropriate time for starting nitrogen limitation by feeding media under nitrogen limited in a fed-batch fermentation process and the 60 h fermentation time initiation, was an optimal accumulation period.

Table 5.7. PHB accumulation by *C. necator* during first and second stage of fed-batch culture fermentation

<i>Before feeding</i>				<i>After feeding</i>			
<i>Time</i>	<i>Yield</i>		<i>Productivity (g/l.h)</i>	<i>Feeding time</i>	<i>Yield</i>		<i>Productivity (g/l.h)</i>
	$Y_{x/s}$	$Y_{p/s}$			$Y_{x/s}$	$Y_{p/s}$	
72 h	0.63	0.42	0.11	57 h	0.53	0.35	0.12
				60 h	0.59	0.42	0.17
				63 h	0.56	0.39	0.15

5.7 Comparison of the PHB Production using two fermentation modes: two-stage batch and fed-batch under optimised conditions

According to statistical analysis which was conducted to compare different parameters belong to PHB accumulation from optimised conditions of both fed-batch and two-stage fermentation (T-test (p-value), Microsoft Excel® 2016). The two-stage values were significantly lower ($P \leq 0.05$) than fed-batch process. Furthermore, biomass production because the extended exponential phase is a major advantage why fed-batch fermentation has been widely used thus far (Tsuneo Yamane et al., 1996b). For the productivity, concentration, content, and $Y_{p/x}$ of PHB there was no significant difference between both fed-batch and two-stage fermentation process ($P > 0.05$). Apart from being easier to do compared to fed-batch fermentation, a two-stage batch process requires the same amount of sugar as indicated by total sugar amount used during the fermentation.

In conclusion, in this study two-stage batch fermentation with introduction of nitrogen limitation at 60 h with a 60 h post induction nitrogen restricted phase was noticed as the optimal fermentation system for PHB accumulation by *C. necator* using date seeds derived media. The final value of PHB concentration was higher (6.72 g/l vs. 2.97 g/l) than that obtained by (Khanna and Srivastava, 2006) using shake flasks (500 ml) with time set at 95 h using optimised media without nitrogen limitation. The yield coefficient ($Y_{p/x}$) of PHB though slightly less was comparable (0.14 vs. 0.56) to the one from studied published by (Azhar et al., 2009) who reported a work under conditions of nitrogen limited with the first step lasting 24 h. This study is a step ahead in the direction of decreasing process time while increasing or maintaining PHB yield coefficient and is in line with the main goal of this project. It is expected that results obtained from this work can be utilised to use low cost fructose-based substrates including date seeds wastes as media for PHB production to further reduce the expense of PHB accumulation. From the previous results it can be inferred that the amount of nitrogen present in the media has a significant influence on bacterial growth and PHB accumulation.

5.8 Effect of oxygen concentration on cell growth and PHB accumulation

Generally, when the microorganisms are grown aerobically and normally under non-restricted oxygen conditions, a biopolymer with higher molecular weight is accumulated (Jung et al., 2013). But the incremental viscosity of broth creates a layer on the surface of cells which acts as a diffusion barrier, transfer of oxygen to the cell becomes more and more difficult. The concentration of dissolved oxygen becomes limiting in fermentation processes of high demand of oxygen (fast growing microbial, high biomass, and biopolymer production) or when a high resistance to the mass transfer is presented by the rheological properties of broth such as production of xanthan gum (Santos et al., 2000) and (Lo et al., 2001). Hence oxygen supply can be the controlling stage in bioprocesses industry and scale-up of biosynthesis systems aerobically (Garcia-choa and Gomez, 2009), (Seviour, 1996), (Jung et al., 2013). Therefore, it was worthwhile to investigate the effect of oxygen concentration on cell growth and biopolymer accumulation in the date seed derived culture using four various aeration conditions (different medium-to-flask volumes, 1:1, 1:2, 1:5 and 1:10). The results of these experiments are shown in Figure 5.16.

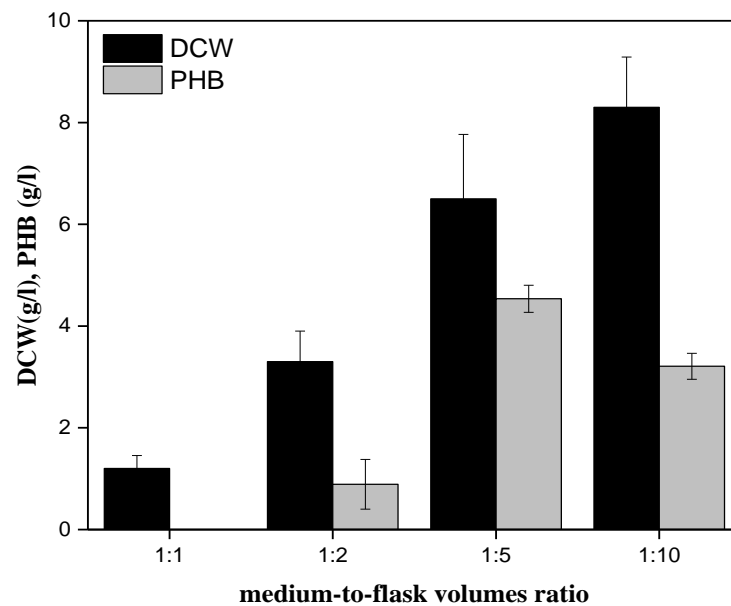


Figure 5.16. Cell growth and PHB accumulation by *C. necator* grown with shaking in flasks in date hydrolysate media without any supplementation under the four culture conditions results shown are mean values.

The results indicate that the maximum growth 8.3g/l was obtained at working volume 1:10, then reduced gradually by incrementing the working volume reaching 6.5 g/l at 1:5. whilst PHB was not accumulated at working volume 1:1 and started it producing at a working volume of 1:2 (3.3 g/l and PHB content 27%). As expected, cell biomass increased with reducing the ratio of the medium: flask volume, whilst reducing this ratio to value enhanced the PHB production reaching its maximum value at 1:5. However, PHB production was found to be repressed at low working volume: flask volume ratios, perhaps due to the negative effect of high oxygen concentration on PHB accumulation. These results revealed that although, adequate concentration of oxygen was required for increasing cell mass, PHB accumulation was negatively affected by excess oxygen availability. Small variations in oxygen concentration availability could lead to significant differences in the metabolite distribution of *C.necator*. These changes, that reflect the metabolic modifications which take place due to develop cell growth in this optional aerobic. Generally, moderate agitation rate led to the highest amounts of PHB accumulation, that is useful in production processes due to decrease aeration associated problems and related costs. Oxygen transfer rate is the most important parameter implied in the design and operation of aeration and agitation of bioreactors and in scale-up (Ccug, 2014) and (Goh Mei Fong, 2008).

5.9 Overview of PHB production under improved conditions

In order to compare between all the growth conditions that investigated previously, three different experiments: experiment 1 (Figure 5.5), experiment 2 (N and O₂ limitation), C: experiment 3 (N limitation), D: experiment 4 (O₂ limitation), as described in section 44.2.3.2, were compared to detect the best conditions for PHB production using date seed derived media. All shake flask experiments were achieved in duplicate (at least) and the average values of cell biomass, PHB accumulation, total nitrogen and fructose concentration were measured. The results of experiment B was described previously in section 5.3.3 while experiment C was carried to study the effect of working volume and hence oxygen availability on bacterial growth and PHB production. The influence of oxygen in *C. necator* growth was determined in experiments at flask scale and the results are shown in Figure 5.17. By maintaining a constant flask volume, different culture volumes were tested to obtain different oxygen mass-transfer coefficients.

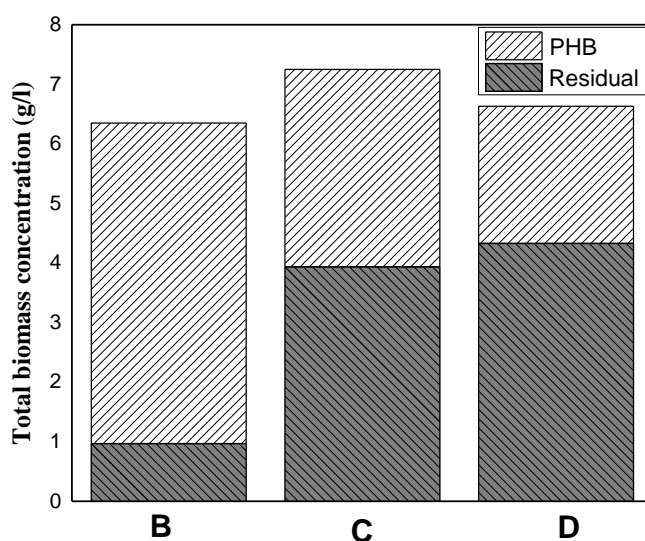


Figure 5.17. Growth of *C. necator* in media derived from date seeds with 11 g/l initial fructose concentration. The incubation conditions were 30°C, 200 rpm for 84 h. A: experiment 1 (Boiling method), B: experiment 2 (N and O₂ limitation), C: experiment 3 (N limitation), D: experiment 4 (O₂ limitation) for date seed derived media.

Figure 5.18 shows the total biomass distributed across PHB and residual biomass concentration in experiments B, C and D. The results indicate that experiment B gave the highest PHB value of 73% w/w when both O₂ and N were limiting, while a lower value of 43.3% w/w was obtained when nitrogen was in excess, experiment D.

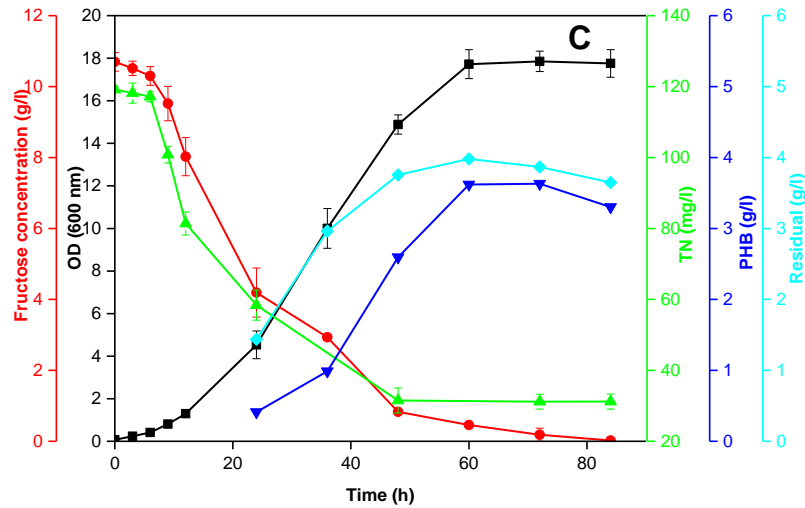


Figure 5.18. Dry matter concentration distributed across PHB and residual biomass concentration in three different experiments; B: experiment 2 (N and O₂ limitation), C: experiment 3 (N limitation), D: experiment 4 (O₂ limitation) for the media derived from date seeds.

A lower volume of media, higher transfer area to volume ratio, will allow a better oxygen transfer. Using equation 1, k_{La} was calculated as 94 min⁻¹ for 50 ml culture volumes, while 54 min⁻¹ for 100 ml, which approximately doubles when the volume is decreased by half. From the results, it can be seen that the higher the k_{La} value gave a higher the cell biomass in the culture broth as shown in Figure 5.19.

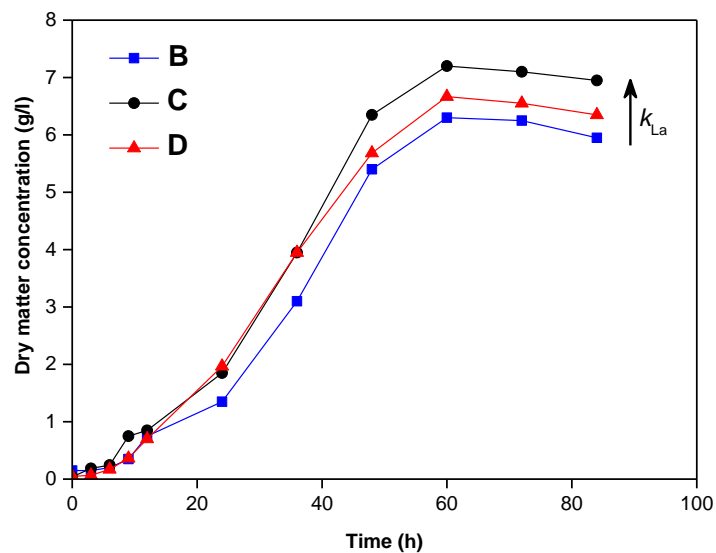


Figure 5.19. Effect of increasing of k_{La} on dry matter concentrations in three different experiments; B: experiment 2 (N and O₂ limitation), C: experiment 3 (N limitation), D: experiment 4 (O₂ limitation) for date seed derived media.

Furthermore, experiment D was carried out using two shake flask experiments to study the influence of N-limitation at the lowest k_{La} (higher volume), to simulate low oxygen supply with and without nitrogen limitation. The comparison of the experimental results is shown in Figures 5.19 and Table 5.8. Nitrogen, 2.5 g/l urea, was supplemented in order to avoid nitrogen limitation and N were limiting, while a lower value of 43.3% w/w was obtained when nitrogen was in excess, experiment D, the results presented in section 5.6.

The specific growth rate in the nitrogen limitation experiment and with low oxygen supply batch culture was 0.17 h^{-1} (experiment B) whilst it was 0.21 h^{-1} for the excess nitrogen experiment over the same fermentation time (experiment D). The amount of nitrogen present in the media has a significant influence on bacterial growth and PHB accumulation, TN concentration decreased with time, from 130 mg/l to reach 60 mg/l after 24 h and reaching a constant value of 35 mg/l at 60 h to the end of the fermentation period. The results were compared to the relevant literature detailing PHB production using various agriculture wastes, as summarised in Table 5.8.

According to the comparison of the results of this study to the literature, as shown in Table 5.8, in general, cell growth and PHB synthesis, as measured by the appropriate yields, were in the expected range, or slightly higher than the literature Figures reported. The range of $y_{p/s}$ values achieved using waste date seed derived media, 0.26 – 0.46 g/g (depending on fermentation conditions), is well in excess of the yields achieved by (Baei et al., 2011) using sugarcane molasses and compares favourably to that reported by (Steinwandter, 2014a) using a chicory root based media. The total percentage PHB accumulation as a proportion of dry matter achieved from the date seed derived media is also comparatively higher those reported in the aforementioned studies. This means that the hydrolysed date seed media produced not only provides the necessary conditions for the propagation of *C. necator* but also supports significant PHB synthesis, with a high PHB content in the range of 73-75% of total dry matter being reached.

Table 5.8. Overview of PHB synthesis by *C. necator* with different systems and carbon sources.

<i>Reference</i>	<i>Carbon source (g)</i>	<i>Biomass concentration (g/l)</i>	<i>PHB concentration (g/l)</i>	<i>Productivity (g_{PHB}/l h)</i>	<i>PHB content (%wt/wt)</i>	$y_{x/s}$	$y_{p/s}$	<i>Fermentation type</i>
(Baei et al., 2011)	Fructose	11	5.8	0.37	53	0.5	0.27	Shake flasks
(Aramvash et al., 2015)	Fructose	4.67	2.16	-----	46.2	----	----	Shake flasks
(Mulchandani et al., 1989)	Fructose	6.2	3.78	-----	59	----	0.25	Batch fermenter
(Khanna and Srivastava, 2005b)	Fructose	3.5	1.4	-----	40	----	----	Batch bioreactor
(Seo et al., 1998)	Fructose	30	8	0.32	26.7		0.21	Shake flasks
(Steinwandter, 2014b)	Chicory root hydrolysed media	3.8	1.7	0.009	43	----	0.38	Batch fermentation
(Chaijamrus and Uduy, 2008)	Sugarcane molasses	8.9	1.3	0.17	14.6	0.55	0.06	Shake flasks
This work	A	6.1	4.58	0.12	75	0.66	0.45	Shake flasks
	B	6.3	4.6	0.11	73	0.68	0.46	
	C	7.2	3.9	0.088	54	0.58	0.35	
	D	6.7	2.9	0.04	43.3	0.23	0.26	

5.10 Modelling and simulation of biomass growth and PHB accumulation in batch fermentation

The kinetics of *C. necator* growth, product formation and substrate consumption were described mathematically in order to understand the coupling between the associated rates. In general, batch microbial growth is described by two phases exponential growth, where μ is constant, and a stationary phase (when $dx/dt = 0$). The Monod, logistic, Leudeking-Piret models and substrate utilisation model were used to simulate the profiles of cell growth, PHB accumulation and substrate consumption during the complete course of fermentation to provide adequate representation of *C. necator* growth and PHB synthesis. The differential equations (as described in section 4.4.4) were solved using R language computer programming and Microsoft-Excel, and the response of the models was compared to the experimental data. Plots of actual experimental data and simulated results from the mathematical model are shown in Figure 5.20 and all calculated parameter values are given in Table 5.9. The model parameters were first estimated by solving equations (28), (32) and (40) and then these values were applied to predict the dynamics of fermentation for the growth rate of biomass, product formation and substrate utilisation. The models have been successfully used to simulate the profiles of cell biomass, PHB product formation, and the consumption of substrate concentration during the fermentation period and Figure 5.20 shows that there is a good agreement between the simulation results and the experimental data. The simulation results using the logistic model compare favourably with the experimental data and the model is capable of accurately describing cell biomass, PHB accumulation and substrate utilisation. The theoretical predictions were found to be a good agreement with all the experimental results and they show that the logistic model for biomass growth and Leudeking-Piret model for intracellular polymer accumulation are close to reality. From the results, it can be seen that the accumulation rate is linearly proportional to the cell biomass growth rate, meaning that both growth associated and non-growth associated PHB accumulation occurred (see α and β values in Table 5.9). Thus, the proposed model is beneficial in describing the batch cell growth and PHB accumulation in *C. necator* under conditions of nitrogen depletion.

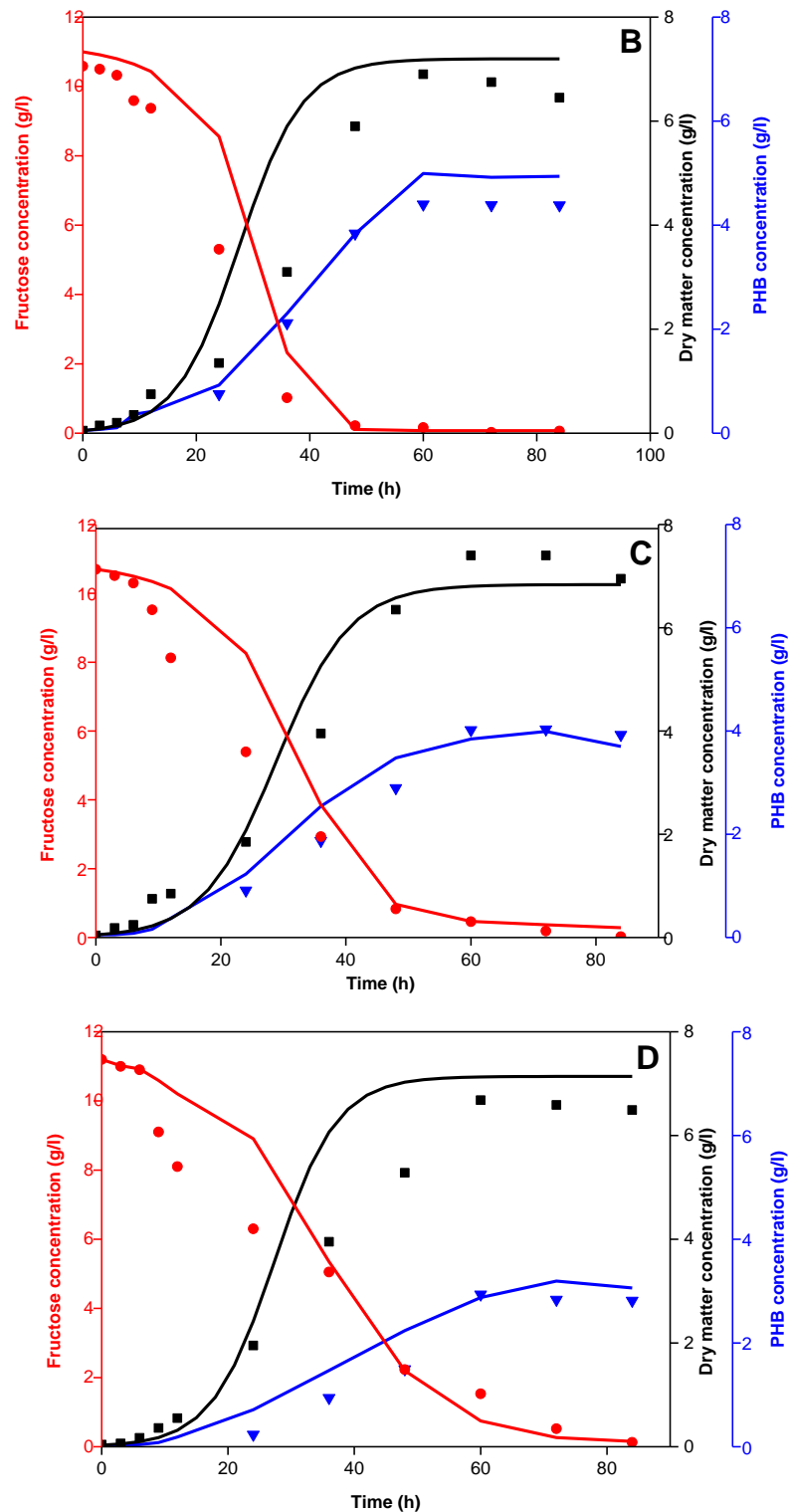


Figure 5.20. Comparison of time course profiles for experimental (symbols) and predicted data (lines), showing biomass, PHB accumulation and substrate utilisation of *C. necator* in batch fermentation and using date seed derived media under various conditions. B: experiment 2 (N and O₂ limitation), C: experiment 3 (N limitation), D: experiment 4 (O₂ limitation).

Table 5.9. Comparison of model parameters and the value of correlation coefficient for PHB production in media derived from date seeds at in various conditions using *C.necator*.

<i>Reference</i>	<i>Fermentation type</i>	<i>Carbon source (g/l)</i>	μ_m (h^{-1})	<i>K</i> (g/l)	<i>a</i> (g/g)	β (g/g h)	R^2
(Karbasi et al., 2011)	Fed-batch	Fructose	0.303	22.83	0.48	0.034	-----
(Khanna and Srivastava, 2006)	Batch	Fructose	0.21	-----	0.58	0.0016	0.994
This work	Shake flask	A	0.13	3.24	0.54	0.038	0.995
		B	0.13	3.19	0.52	0.035	0.993
		C	0.17	5.63	0.4	0.001	0.997
		D	0.2	7.14	0.1	0.0005	0.997

5.11 Scale up the PHB production from shake flask to fermenter

Scale up is the process which small scale production (flasks) is converted to a large-scale production (bioreactor). In other words, scale up is to carry out an experiment in bulk, after the best conditions have been determined using screening experiments. Both definitions point out to a process in which the data from an experimental scale operation is used in a larger scale unit for larger production (Seletzky, 2007). The first parameter was investigated in this section is OD_{600nm}, which is considered to be the most important factor. The effect of DO level in the bioreactor was studied by increasing the concentration from 20 to 50% and the results are shown in Table 5.10. It was observed that total biomass was increased when dissolved oxygen level increased while PHB concentration was reduced with the increase in DO level.

Table 5.10. Effect of DO (%) level on accumulation of PHB using 1.7 L batch bioreactor contain 1L date seed derived medium. The values were estimated after 48 h of fermentation.

<i>DO %</i>	<i>Maximum total biomass (g/l)</i>	<i>Maximum PHB (g/l)</i>	<i>Residual (g/l)</i>
20	9.55	6.98	2.57
30	11.87	9.48	2.39
40	12.52	5.32	7.2
50	12.81	4.37	8.44

The highest total biomass (12.81 g/l) and PHB concentration (9.48g/l) were achieved at 50 and 30% DO level, respectively. Based on the results, it was found that DO concentration in the fermentation media enhanced the cell growth; however, PHB production was found to be increased towards limitation level of oxygen.

This result indicated that appropriate oxygen level is required for cell development, and depletion level of oxygen was favourable for PHB production. PHB production was almost twice that at lower DO concentrations compared to the higher ones. This might be because of the fact that insufficient provision of oxygen to the bacteria cell may reduce oxidation of NADH and lead to PHB accumulation (Ariffin, 2012), (Anderson and Dawes, 1990) and (Choong-Kyung Kang, Satoshi Kusaka, 1995). Generally, from the results, it can be concluded that both oxygen and nitrogen limitation does not enhance cell biomass but markedly promote the PHB production and are essential in getting the maximum PHB level.

The time profile of *C. necator* growth and PHB accumulation using batch fermentation in date seeds hydrolysate media in 1.7 L bioreactor with aeration supplied at 30% DO level and without supplementation are shown in Figure 5.21. This medium was tried to demonstrate the performance of *C. necator* in date seeds derived media after improved their ability to grow and produced a meaningful amount of PHB in shake flasks scale.

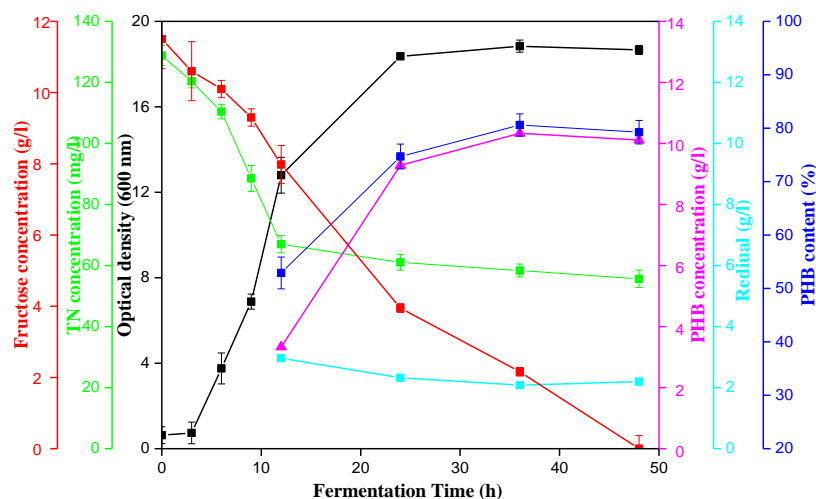


Figure 5.21. Time profile for cell growth, fructose, total nitrogen utilization and PHB accumulation by *C. necator* using date seeds derived medium in 1.7 L bioreactor at 30% DO level.

The plot of fermentation time against OD_{600nm} revealed that the cell biomass grew fast after inoculation and growth reached the exponential phase after 6 h of fermentation and total biomass increased steadily for about 24 h. Although total biomass continued to increase until 50 h, cell concentrations referred to residual biomass stopped increasing after 12 h and tended to decrease after that. The reason that some nutrients, possibly nitrogen, might be limited, impacting cell proliferation, maybe leading to cell autolysis (Yanagida et al., 2011).

Total nitrogen concentration was determined during the fermentation, which starting with 136.86 mg/l to reach a constant value after 12 h of fermentation at a value of 72.56 mg/l and almost still at this value until the end of the fermentation time. However, as can be seen in the Figure 5.21, PHB accumulation was observed at 12 h. This implies that the concentration of nitrogen decreased to the level that stimulates PHB synthesis in the cells. So, the increase in total biomass after 12 h was due to intracellular PHB accumulation. The highest total biomass obtained from this defined medium was 12.37 g/l at 36 h, but this decreased to 10.8 g/l by the end of the experiment. From the results shown in Figure 5.21, it is obvious that the increment in biomass was due to PHB production. The PHB concentration increased sharply between 12 h and 36 h reaching 9.9 g/l when the biomass concentration reached the highest, resulting in about 80% (w/w) PHB content. The reduction in PHB content to about 77% at the end of the fermentation period might be due to the release of PHB into the medium because of cell autolysis.

The bacterial growth is mainly associated with the consumption rate of nitrogen. For the first 12 h, lower fructose consumption by *C. necator* was noticed. The consumption of fructose within the time range was about 5.24 g/l which is almost half of the total fructose concentration in the culture broth. On the other hand, the TN concentration was found to be reduced drastically from the initial value and reached a constant amount after 12 h of the fermentation period. This result indicates that initially, mainly nitrogen consumption attributed the bacterial growth, in addition to that, other organic compounds including amino acids and minerals, that were characterised previously in date seeds derived media, could be utilised as supplementary growth substrates by the microbes (Ahmad et al., 2012). Then, the biomass growth was contributed by the expansion of the cells because of PHB production inside the cells.

It can be seen that the PHB production was about doubled that is, 40% to 80%, from 12 h to 36 h of fermentation period. From fructose consumption and PHB production profiles, it can be noticed that the detectable depletion of fructose in the media from 35 h onwards can be associated with PHB production. These results agree with the findings of other studies that reported PHB production is favoured by presenting an excess of carbon source and the limited supply of other essential minerals such as dissolved oxygen and nitrogen (Beaulieu et al., 1995) and (Koller et al., 2009).

The $Y_{x/s}$ and $Y_{p/s}$ were 0.71 and 0.43, respectively while the maximum productivity of PHB was 0.15 g/l.h. A similar value of PHB content with some improvement in biomass was achieved in this study compared to the shake flask experiments under optimal conditions due to various conditions which prevail in the shake flasks and bioreactor; some of these conditions include temperature agitation and aeration. The increase in total biomass was mainly contributed by the cell expansion due to PHB accumulation inside the cells. The results agree with the findings of other studies that reported PHB production is favoured by presenting an excess of carbon source while limited supply of dissolved oxygen and nitrogen (L. Madison and Huisman, 1999), and (D Kalaiyezhini and Ramachandran, 2015).

It is interesting to observe that *C. necator* completely utilised the fructose in the date seed derived media. Compared to the previous studies in shake flasks, batch studies in 1.7 L bioreactor using renewable sugars (fructose) from date seeds waste showed superior results in *C. necator*, possibly due to the controlled conditions that improve the performance of the fermentation. It can be concluded that the bioreactor fermentation reduces the time of fermentation compared with the shaken flask scale while PHB content was higher when *C. necator* was grown in shake flasks compared to experiment conducted in the fermenter. Furthermore, the hypothesis was that the reduced availability of oxygen in shake flasks experiments relative to the fermenter could be the reason for the variation in PHB production. Therefore, reduce the DO concentration in the fermenter to 30%, total biomass raised slightly then levelled off, but no rise in PHB production was noticed.

5.12 Summary

PHB Production from date seed hydrolysate media as an inexpensive, nutrient-rich media, with a kinetic model describing the process was presented in this chapter. The first step started with the characterisation of date seeds to have an idea whether they contain most of the essential nutrient that required for the microbial growth. Subsequently, study of the growth of PHB-accumulation bacterium, *C. necator* H16, in media derived from as nutrient-rich media containing fructose as a sole carbon source.

It was found that results include fructose extraction from date seeds and a mass transfer model to describe the process, demonstrating that the high nutrient content of date seeds makes them a promising raw material for microbial growth and that a meaningful amount of PHB can be produced without nutrient supplementation and a significant agreement between the literature and experimental findings in terms of the range of PHB content using waste materials as a media.

Two different approaches have been used to extract the nutrients; boiling with a reflux system and steam-treatment by autoclave and the maximum concentration of fructose obtained was 11 ± 0.5 g/l. The results show that the date seeds hydrolysate media has viable properties for PHB accumulation through bacterial fermentation without any supplementation and the maximum total biomass and PHB concentrations were 6.3 g/l and 4.6 g/l respectively, with a PHB content of 73%, when an initial fructose concentration of 10.8 g/l was used.

In order to investigate the effect of nitrogen on the bacterial growth and PHB production, various types and concentration of nitrogen were supplemented to the date seeds derived media. A dramatic improvement in total biomass 7.75 g/l, μ_{\max} (from 0.09 h^{-1} to 0.21 h^{-1}) and PHB concentration 5.34 g/l were achieved and when the media supplied with 2.5 g/l urea, hence urea encouraged PHB concentration compared with $(\text{NH}_4)_2\text{SO}_4$ and yeast extract under the same conditions. Three points of time for the induction of PHB accumulation in bacterium cells over the introduction of nitrogen limitation, either by feeding nitrogen limited media at selected intervals or by switching to a nitrogen limited media were tested. These results were used to determine the optimum conditions for PHB production utilising date seeds derived media with and without supplementation and different fermentation technique, hence may be to scale-up the process in the future.

Different experiments were carried out on the influence of oxygen concentration (oxygen transfer coefficient) on cell growth and PHB production by *C. necator* resulted in maximum PHB content and concentration 69% and 4.53 g/l, respectively, at working volume of 1:5.

As culture volume was increased the oxygen availability decreased and hence a decrease in the biomass concentration obtained was observed. As such the highest culture volume corresponds to the experiment with the most oxygen limitation. By applying these conditions with the addition of nitrogen limitation, it was found that PHB accumulation was significantly increased. Both logistic and *Leudeking-Piret* models have been successfully used to simulate the profiles of cell biomass, PHB product formation, respectively, and results show that there is a good agreement between the simulation results and the experimental data.

CHAPTER

SIX

6 Production of PHB using waste date seed oil extract

6.1 Introduction

Plant oils are essential agricultural products that are obtained from different crops, such as rapeseed, soybean, and oil palm. Depending on the plant species, the fatty acids types and their distribution that present in oil will be varied. Traditionally, plant oils have been utilised not only in the food industry, but also they can be processed into other chemical products such as polymers (Harding et al., 2007), surfactants (Vasileva-Tonkova et al., 2006), fine chemicals and fuels (Kalscheuer et al., 2006). Through the TAGs cycle, bacteria secrete lipases to catalyse the release of fatty acids from the oil and then convey into the cell to catabolised via the β -oxidation cycle (Riedel et al., 2014).

In this chapter, an investigation into the technical feasibility of PHB production using date seed oil as a sole carbon source is presented. The first step was extraction date seed oil investigating the various type of conditions including solvent types (Section 6.2.1), particle size (Section 6.2.2), temperature (Section 6.2.3) and extraction time (Section 6.2.4). Analysis of date seed oil was investigated in Section 6.2.5. Effect of different oil types (date seed, vegetable, rapeseeds, and sunflower) and concentration on cell growth and PHB production was presented in Section 6.2.7 while Section 6.2.8 discusses the effect of oils concentration on bacteria growth and PHB accumulation. *C. necator* growth and PHB production in shake flasks with date seed oil are discussed in Section 6.2.9.

Although, date seed oil can be utilised as feedstock of carbon for *C. necator* cultivation using a basic method of cultivation, it was difficult to monitor oil consumption during the experiment due to the hydrophobic properties of the date seed oil which forms a layer on the top of the aqueous medium, leading to heterogeneous condition at the start of the cultivation. *C. necator*, at some point of fermentation time, will create homogeneous conditions during emulsification of the date seeds oil because of excretion of an external lipase (Lu et al., 2013). However, until the emulsion of oil is made, no estimation of oil consumption is possible. Furthermore, the time needed for date seed oil to be emulsified served to expand the lag phase of the culture, because the oil is not significantly bioavailable for bacterial cell growth now. Therefore, Arabic Gum (GA) was used as the emulsifying agent to improve cell growth and hence the PHB production. GA a natural glycoprotein material produced by the acacia tree and is known as a suitable agent for emulsifying oil cultivations with *C. necator* and is already utilised in the industry of food as thickener,

emulsifier and stabiliser (Goodrum et al., 2000) and (Qi et al., 1991). According to the literature, GA cannot be used as either nitrogen or carbon source during fermentations with *C. necator* and it does not significantly affect cell growth or PHB production (Budde et al., 2010).

Various approaches were discussed as followed: the evaluation of potential using GA as carbon and nitrogen source are also presented in Section 6.4.1, while Section 6.4.2 contains the profile of *C. necator* growth of in minimal medium with GA at various concentrations. The solvents used for quantification of date seed oil which allowed oil consumption to be observed is discussed in Section 6.4.3. The effect of using emulsified date seed oil on bacterial growth and PHB accumulation was first studied in Section 6.4.4 at shake flask scale while scale up of the process to (1.7 L) bioreactor scale is discussed in Section 6.4.5. Section 6.4.6 presents the effect of using fed batch bioreactor on cell growth and PHB production using emulsified date seed oil as a carbon source. Comparison between shake flasks and bioreactor scale is discussed in Section 6.4.7. The summary of this section, is presented in Section 6.5.

6.2 Results and discussion of oil extraction and microbial growth

6.2.1 Date seed oil extraction

The extraction of date seed oil was investigated, and recovery maximised by testing various types of organic solvents, particle size, temperatures and extraction time, to give the understanding of the baseline oil yield achievable and the fatty acid content. It is, however, critical to the environmental performance of the bioprocess that future scale up is based on the use of green solvents, in line with industry trends towards reduction in organic and chlorinated solvent use.

6.2.1.1 Effect of solvent

Date seed oil was extracted using three different solvents, hexane, methanol/chloroform mixture (MCM) and petroleum ether (PE), in order to study the effect of the solvent on the oil yield. For each solvent, an extraction time of 2 h and a particle size of ≤ 1 mm were selected, and the percentage of oil yield was calculated, Figure 6.1. The results indicate that a maximum oil yield of 9.3 % was obtained using MCM, followed by hexane, 5.38 %, and PE, 2.96 % at 120°C, 160°C and 180°C respectively, depending on the boiling point of each solvent. The oil yield obtained using MCM was 1.8% and 3.2% greater than the yields for hexane and PE, respectively. Sayyar et al., (Sayyar et al., 2009) report

that the jatropha seed oil extraction yield using hexane was 1.3% more than for PE under the same conditions, in agreement with the yields observed in this study.

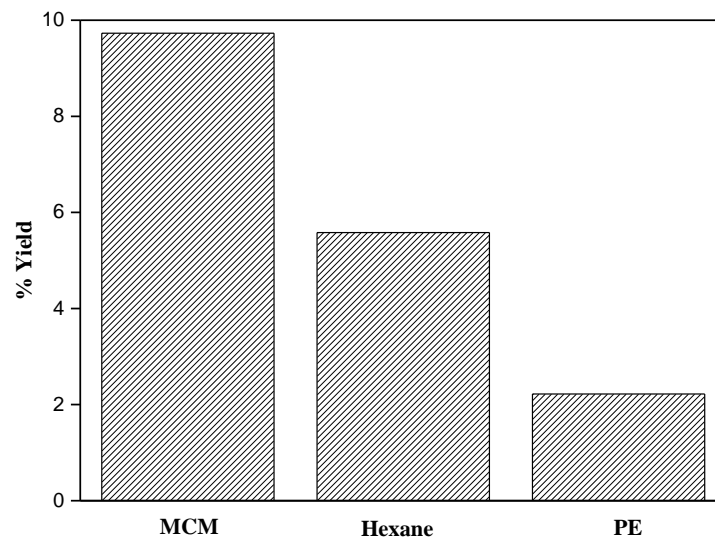


Figure 6.1. Kinetic study of date seed oil extraction effect solvent types on oil yield using particle size ≤ 1 mm.

6.2.1.2 Effect of particle size

The effect of two different particle sizes, ≤ 1 mm and 2-4 mm, on the oil extraction rate using different solvents is illustrated in Figure 6.2. The oil yields for all types of solvents are lower, by nearly half, for a particle size of 2-4 mm as compared to a particle size of ≤ 1 mm, indicating that the extraction rate from fine date seed particles is higher compared to that from coarser particles. The lower extraction rate for the larger particle size was expected, due to the lower total surface area. The solvent has the ability to penetrate into the core of the seed to extract the oil and this is restricted in the case of the larger particles (Sayyar et al., 2009). Sulaiman et al., (Sulaiman et al., 2013) found that the rate of oil extraction from rambutan kernel seeds increases with decreasing particle size during oil extraction. The same result was obtained by Ebewele et al., (Ebewele et al., 2010) when they extracted oil from peanuts, who found that when the particle size was reduced from 3.35 – 4.75 to 0.86 – 1.19 mm, the total oil yield was increased from 36% to 82%. The results confirm the effect of the particle size on extraction processes for by-product materials, namely that the grinding step enhances the recovery of oil by increasing the surface area in contact with the liquid fraction.

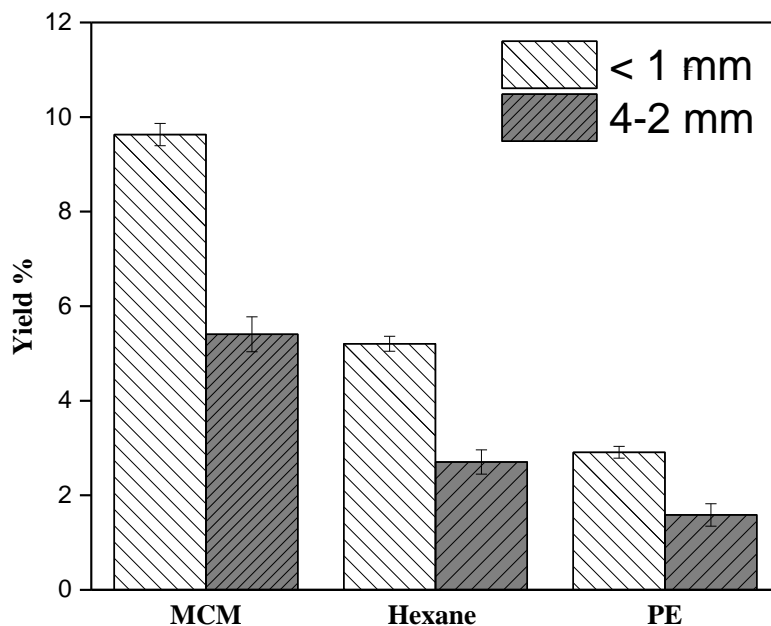


Figure 6.2. Kinetic study of date seed oil extraction shows effect of date seed particle size (≤ 1 and 2-4 mm) on the oil yield using three different types of solvents.

6.2.1.3 Effect of temperature on oil yield

Generally, solvent extraction is conducted at a temperature close the solvent boiling point, which decreases the viscosity of oil and develops its solubility in the solvent, ensuring the process efficiency (Gandhi et al., 2003). In this study, oil extraction was carried out across a range of temperatures from 80°C to 180°C, depending on the solvent used and its boiling point. The impact of varying temperature on the date seed oil yield is presented in Figure 6.3. It was observed that the date seed oil yield increased with increasing temperature and oil yields of 9.3%, 5.38% and 2.69% were obtained at 180°C, 160°C and 120°C and using an MCM, hexane and PE respectively.

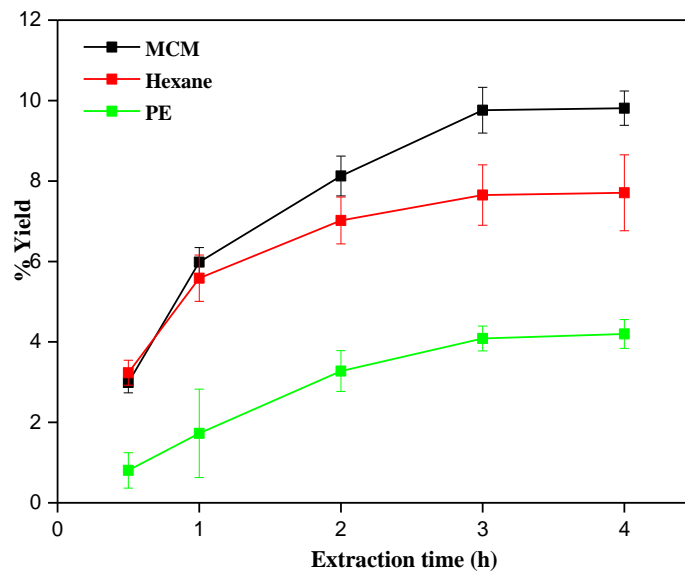


Figure 6.3. Kinetic study of date seed oil extraction which presents the effect of temperature on oil yield using particle size ≤ 1 mm, using three different types of solvents.

According to the results, it can be confirmed that the mass transfer coefficient of the oil extraction increases by increased the temperature. The same behaviour has been reported for sunflower seed oil extraction processes (Topallar and Geçgel, 2000), (Perez et al., 2011) and Jatropha (Sayyar et al., 2009) and (Amin et al., 2010) using n-hexane as a solvent.

6.2.1.4 Effect of extraction time

Extraction of oil from the date seeds of ≤ 1 mm particle size was carried out at 100°C, 140°C and 160°C with MCM, hexane and PE. The extraction data are plotted in Figure 6.4, as total oil recovery against time. Figure 6.4 shows that the oil yield increases with time for all the solvents tested and that the rate of extraction is high during the first three hours. The maximum quantities of oil recovered were 9.3%, 5.4% and 3.5% with the MCM, hexane and PE respectively, when the extraction process lasted for four hours. The high initial extraction rate was due to the mass transfer driving force being large when the oil concentration in the fresh solvent was low. The extraction rate subsequently decreased with increasing oil concentration in the solvent, due to the reduction in mass transfer driving force (Treybal, 1968).

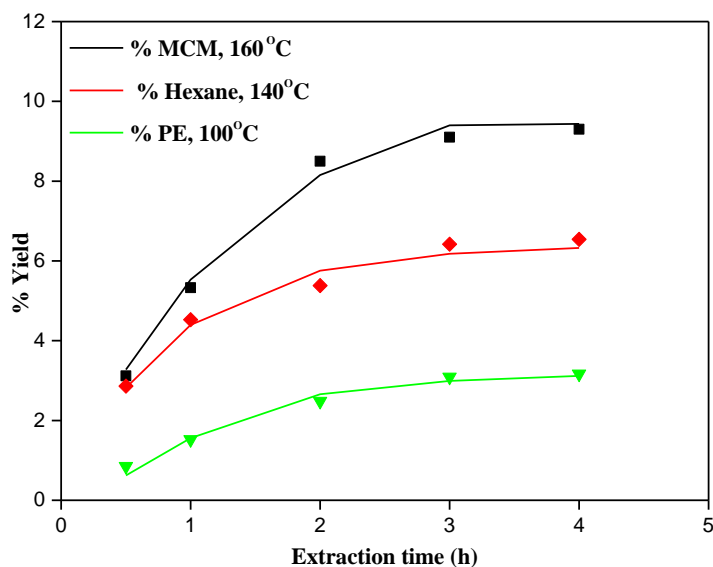


Figure 6.4. Kinetic study of date seed oil extraction shows the effect of extraction time, on oil yield using particle size ≤ 1 mm, using three different types of solvents.

In general, the mass transfer of the solute from the solid to the liquid phase accelerates when there is a large concentration gradient between the solute and the solvent, as the extraction progresses the oil diffusion rate decreases as the concentration of the oil in the solvent increased. Finally, the extraction process reached an equilibrium point when the highest amount of oil was obtained and remained constant without significant change, these results agree with (Treybal, 1968) and (Sayyar et al., 2009). The extraction process reached an equilibrium point when the highest amount of oil was obtained and remained constant without significant change and these results agree with Liauw et al., (Liauw et al., 2008) and Sulaiman et al., (Sulaiman et al., 2013). In Figure 6.5 a non-linear least square fit method was used to fit the oil yield data for different solvents, to determine the mass transfer coefficient, Table 6.1. From the results, it can be seen that the oil yield is lower compared to oily crops, because of the lower oil content of date seeds. However, as part of a wider waste valorisation strategy it is important to maximise added value by extracting all fermentable carbon sources from the waste date seed (Yousuf & Winterburn, 2016). There are also opportunities to use edible date seed oil in pharmaceutical and cosmetic products (Besbes et al., 2004a) and (Al-shahib and Marshall, 2003).

Table 6.1. Parameters fitted and comparison of mass transfer coefficient for oil extraction from different materials using soxhlet extraction.

<i>Raw material</i>	<i>Solvent types</i>	<i>T (°C)</i>	<i>Yield (%)</i>	<i>K.a (s⁻¹)</i>	<i>Reference</i>
Coconut waste	Hexane	80	21.9	0.386×10^{-3}	(Sulaiman et al., 2013)
Jatropha seeds	Acidic Hexane	60	21.24	0.13×10^{-3}	(Yu et al., 2002)
Sunflower seed	n-Hexane	60	25.42	0.107×10^{-3}	(Topallar and Geçgel, 2000)
Neem	n-Hexane	50	44.29	7.29×10^{-3}	(Liau et al., 2008)
Date seeds	MCM	160	9.3	0.78×10^{-3}	This work
	Hexane	140	5.3	0.59×10^{-3}	This work
	PE	100	3.4	0.46×10^{-3}	This work

6.2.1.5 Date seed oil analysis

The fatty acid composition of date seed oil was analysed using GC-MS with the free fatty acid composition shown in Figure 6.6. The results show that oleic and lauric acid are present in the highest amounts among unsaturated and saturated fatty acid, respectively for samples extracted with three different solvents, hexane, MCM and PE. According to Nehdi et al., (Nehdi et al., 2010) the date seed cultivars in United Arab Emirates can be classified as oleic-linoleic or oleic-lauric oil depending on the two major fatty acids present. Iranian date seeds cultivars yield oleic-lauric oil, whilst linoleic acid was present in the lowest quantity among the five major fatty acids. The third most abundant fatty acid in the study by Nehdi et al., was linoleic acid, while Biglar et al., (Biglar et al., 2012) reported myristic acid as a third major fatty acid. The analysis presented here is in broad agreement with studies of other varieties of date palm, such as Akbari et al., (Akbari et al., 2012) as well as the results similar to the results reported by Nehdi et al., (Nehdi et al., 2010) which they studied Deglet Nour seed oil. While our results slightly different from Besbes et al., (Besbes et al., 2004b) results where they obtained the oleic acids the highest fatty acid followed by linoleic, lauric and then palmitic acid for Allig seed oil. Generally, the major fatty acid composition in date seed oil is different across date varieties and climate conditions.

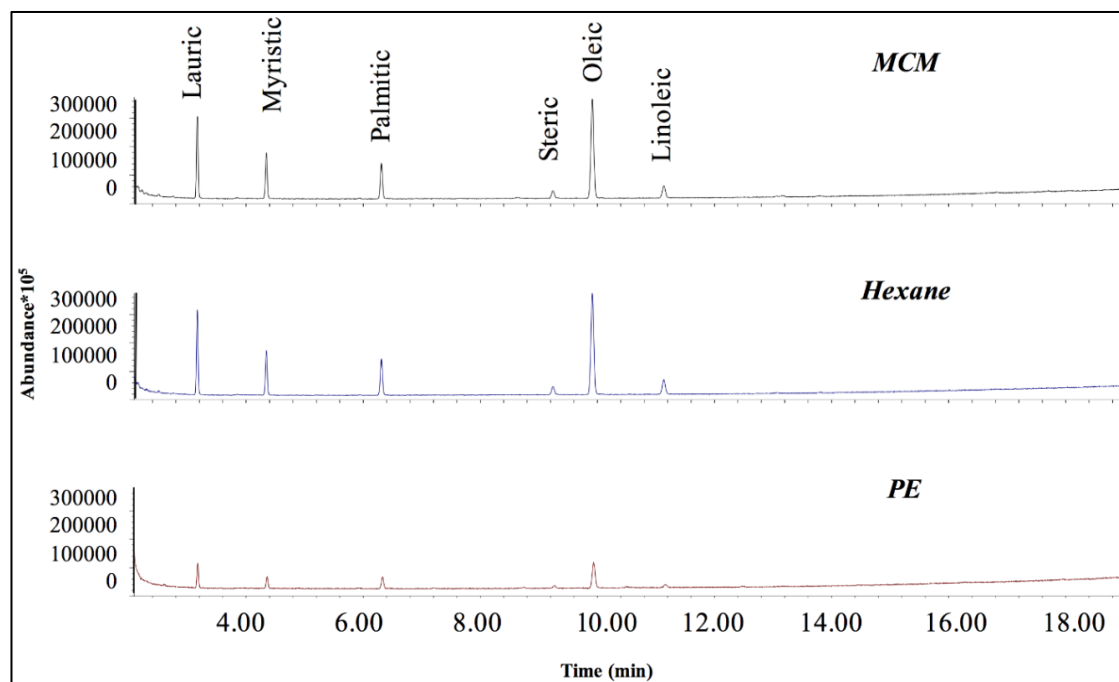


Figure 6.5. The fatty acid composition of date seed oil extracted using three different solvents, MCM, hexane and PE.

6.2.2 PHB synthesised in non-emulsified date seed oil

6.2.2.1 Effect of different oil types and concentration on cell growth and PHB production

In order to study the effect of various types of oil (date seed oil, vegetable, rapeseeds, and sunflower oil at concentration of 15 g/l) on *C. necator* growth and PHB accumulation, three shake flask experiments were conducted. The cultivation conditions were performed at 30 °C and 200 rpm under aerobic conditions. OD_{600nm} and PHB accumulation were measured at least in duplicate and the average value are presented in Figure 6.7 and 6.8. From the figures, it indicates that *C. necator* can metabolise date seed oil and accumulate PHB as other types of oil.

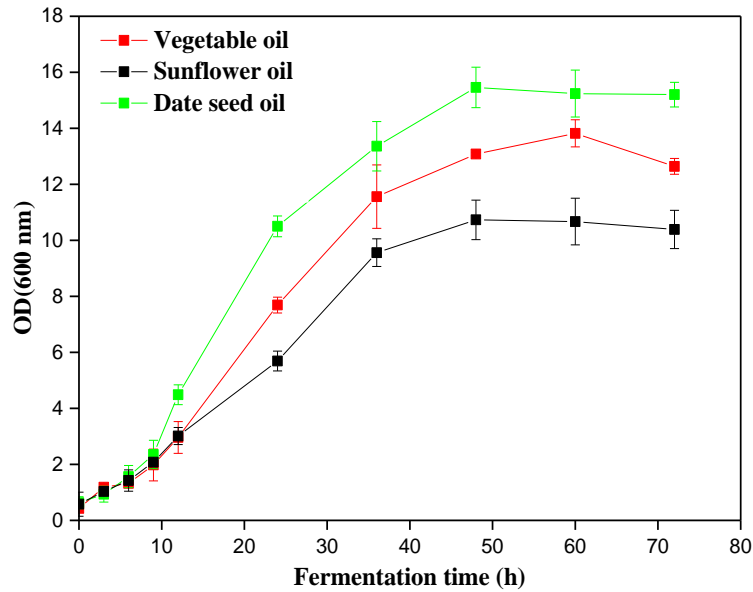


Figure 6.6. Optical density trends for *C. necator* growth in mineral media contained 15 g/l of different types of oil; date seed, vegetable and sunflower, under fermentation concentration at 30 °C and 200 rpm under aerobic conditions.

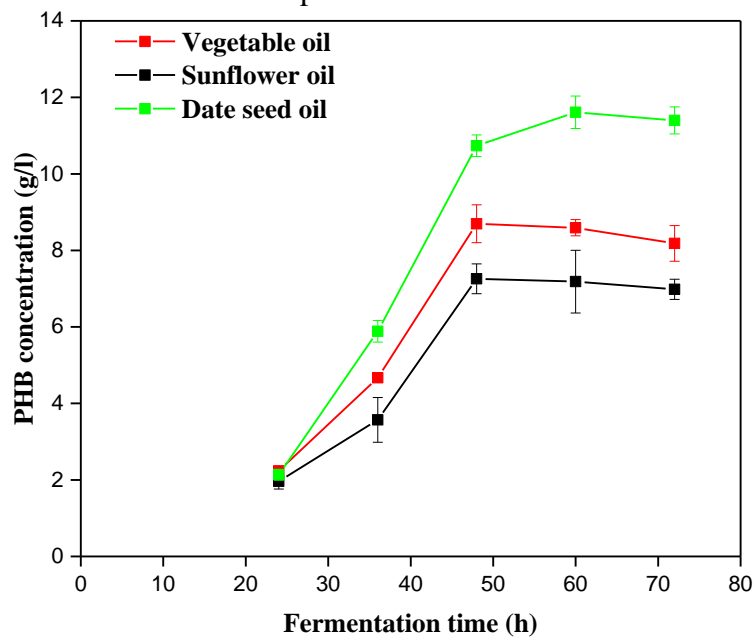


Figure 6.7. PHB concentration trends for *C. necator* growth in mineral media contained 15 g/l of different types of oil; date seed, vegetable (rapeseed) and sunflower.

The results from small scale fermentations with 15 g/l oils in mineral media, as described in section 4.2.3.7, are shown in Figure 6.7. Among these tests in this study, date seed oil not only supported the cell growth but also enhance PHB synthesis. However, sunflower oil less supporting for PHB synthesis, as shown in Figure 6.8. The growth trend was similar for date seed, vegetable (rapeseeds) and sunflower oil, and after 72 h the OD

600nm for the date seed oil fermentation had risen to 15.3 while the other two fermentations only had 10.3 and 9.8.

The PHB content for all three fermentation conditions have an optimum value around 48 h and these levels are reducing after this time, due to utilisation of PHB as a substrate in the cell metabolism. Analysing the data for the PHB content, t-test ($P < 0.05$) shows that date seed oil performs significantly better than vegetable and sunflower oil from 12 h onwards. Fermentations of *C. necator* with date seed oil obtained a significantly higher PHB content than with vegetable and sunflower oil. While a maximum concentration of 11.4 g/l can be achieved with date seed oil, only 6.9 g/l was synthesised.

6.2.2.2 Effect of oils concentration on bacteria growth and PHB accumulation

After demonstrated, in the previous section, that *C. necator* are able to grow and accumulate PHB using date seed oil as a carbon source, in this section different concentration, 5 to 25 g/l, of each types oil were tried in order to determine the optimum oil concentration to gain a high concentration of PHB. Figure 6.9 presents the specific growth rate, μ at different oil concentrations were used to evaluate the cell growth. Each experiment was applied in duplicated (at least) and the average values were presented.

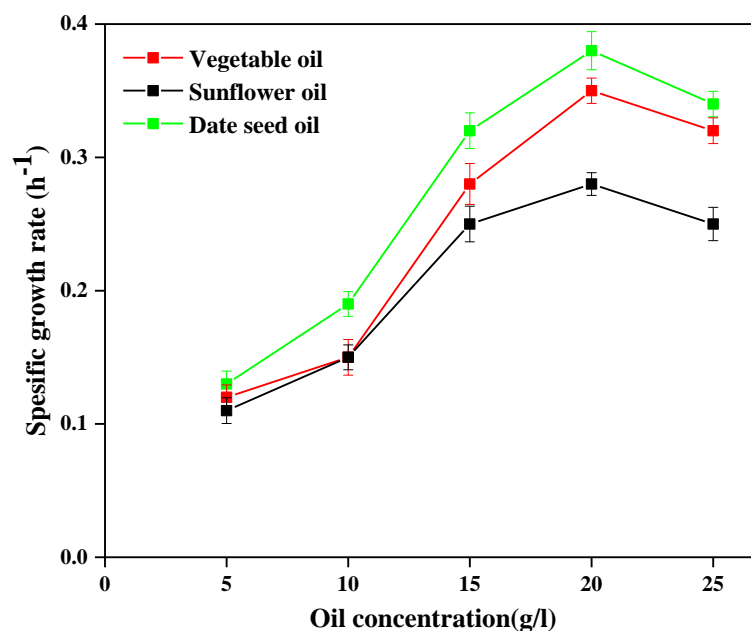


Figure 6.8. Variation of specific growth rate as the function of the initial of three various oils, date seed, rapeseed and sunflower oil concentration.

The figures indicate that cell growth increased with increase the oil concentration for all concentrations, until reached the certain concentration the OD_{600nm} started decreased. The specific growth rate, μ , was calculated for each concentration and type and the trend was the same. The results show that the cells grew fastest during the first day of cultivation and gave a maximum value for μ of 0.38 h⁻¹ when 20 g/l initial date seed oil concentration was used for all types, as shown in Figure 6.11. While the maximum μ for vegetable and sunflower oil were 0.35 h⁻¹ and 0.28 h⁻¹ respectively. The μ of the bacteria cell was increased by increasing the oil concentration up to 20 g/l and then a slight decrease was observed at 25 g/l.

6.2.2.3 *C. necator* growth and PHB production in shake flasks with date seed oil

In order to demonstrate the technical feasibility of using date seed oil as a substrate for PHB production a series of small-scale shake flask batch fermentations using *C. necator* in media containing 20 g/l of date seed oil as a carbon source were conducted. Experiments were carried out in triplicate and the average values of DCW, PHB concentration, content and total nitrogen were shown in Figure 6.10. It was observed that *C. necator* is able to grow on non-emulsified date seed oil and produce PHB, on the other hand, the oil gradually emulsified as *C. necator* grew, though the mechanism through which this emulsification happens is unknown. Many bacteria are known to secrete surfactants (E. Rosenberg á E. Z. Ron, 1999), however there is no evidence that *C. necator* produces these types of emulsifiers. Through the breakdown of triacylglycerol (TAGs), such as free fatty acids (FFAs), mono-glycerol or mono-ethylene glycol (MAGs) and diacylglycerol (DAGs) it is possible that polar lipids released and can themselves emulsify the date seed oil. Furthermore, some studies report that the putative lipase, encoded by gene H16-A1322 (Gene ID: 4249488), is crucial for the robust growth of *C. necator* on non-emulsified media (Brigham et al., 2013).

As seen in Figure 6.10, the initial cell growth (optical density) was similar for all oil types tested. At 48 h, the OD_{600nm} for the date seed oil fermentation reached 18.8, while OD in the other two fermentations were 15.8 and 12.9, for vegetable and sunflower oil, respectively. The total cell dry weight for date seed, vegetable and sunflower oils were 14.35 g/l, 12.1 g/l and 10.2 g/l, respectively at 48 h (Table 6.2). The PHB content for all three fermentation experiments reached a maximum value at 48 h. A slight decrease in PHB levels was noticed after this time, most likely due to PHB utilisation as a carbon source. The PHB production curves were found to be distinctly different for all oil types. The most PHB was produced in the fermentation with date seed oil, 11.8 g/l, achieved after 48 hrs, while only 9.1 g/l and 7.4 g/l were synthesised after 48 hrs using vegetable and sunflower oil as the

carbon source. Correspondingly flasks fed date seed oil as PHB content of 82% was reached after 48 hours, while the PHB content was 75% and 72% for vegetable and sunflower oil.

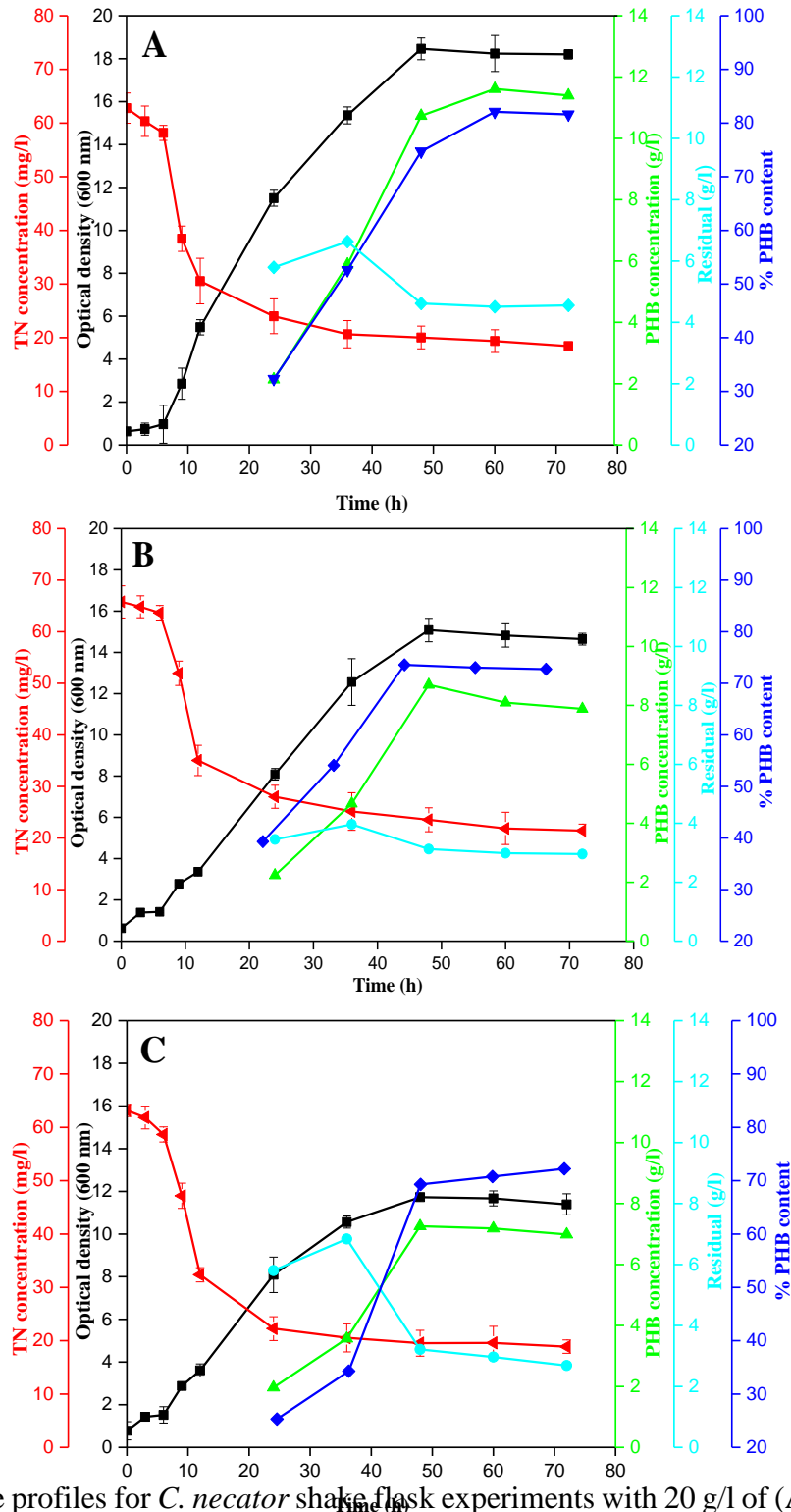


Figure 6.9. Time profiles for *C. necator* shake flask experiments with 20 g/l of (A) date seed oil, (B) vegetable oil, (C) sunflower oil and incubation conditions were 30°C, 200 rpm.

The higher PHB accumulation in experiments with date seed oil may be ascribed to the fatty acid composition of date seed oil, as described in section 4.3.3.6, which may mean that the fatty acids in date seed oil making are more accessible and simpler for the microorganism to metabolise (Brigham et al., 2013). In this study, the PHB production was low in comparison to *C. necator* batch fermentations with using different oils, performed by (Rehm et al., 1998) and (Younas et al., 2015). They achieved PHB concentrations between 6 to 11 g/l in 25 h using mineral medium and the conditions described by (Yu et al., 2002). The medium used contained both extracted nutrient components and salts, which may explain the increased PHB production. The amount of nitrogen available in the media plays an important role in the trade-off between cell growth and PHB synthesis (Dobroth et al., 2011). For all three substrates used, date seed, vegetable and sunflower oil, the initial total nitrogen concentration was around 65 mg/l and about 70% of the total amount was consumed to reach the steady state at 48 h of 23 ± 0.8 mg/l for both vegetable and sunflower oil, while being slightly lower for the date seed media of 16 mg/l. The PHB accumulation mechanism has been described as a de novo (production of complex molecules from simple ones) route in that fatty acids will be transformed into acetyl-CoA by oxidation cycles (Amin et al., 2010).

According to the fatty acid analysis for both vegetable and sunflower oil, it was observed that unsaturated fatty acids are present in both, much more so than in date seed oil. Therefore; it is possible that saturated fatty acids are more readily taken up and converted to acetyl-CoA than unsaturated fatty acids. Generally, cell growth and PHB accumulation were in the expected range and the PHB content for date seed oil is greater than that achieved by Khan et al., (Khan et al., 2013) using *Jatropha* oil and also compares favourably to those reported by Kamilah et al., (Kamilah et al., 2013) using waste cooking oil. Furthermore, the percentage of PHB accumulation achieved from the date seed oil is comparatively higher than those reported in the aforementioned works. Thus, date seeds oil could be used to accumulate a significant amount of PHB, with a high content of 82% of total dry cell weight being reached.

From the results of this chapter, it can be noticed that the TN concentration, in general, less than the nitrogen that used in the experiments in chapter five, date seed derived media, and this could be the hydrolysis media has much more nitrogen forms that not accessible to the microorganism while in the oil experiments just one type of nitrogen was used, ammonium sulphate, which is considered the best nitrogen source for the microorganism growth.

Table 6.2. Overview of PHB production by *C. necator* in shake flasks batch fermentation includes oil types, key results for polymer (PHB) accumulation and reference.

<i>Reference</i>	<i>Carbon source type</i>	<i>Maximum biomass concentration (g/l)</i>	<i>PHB concentration (g/l)</i>	<i>PHB content (% wt/wt)</i>	<i>Fermentation type</i>
(Khan et al., 2013)	Jatropha oil	11.6	8.6	74	Shake flask
(Park and Kim, 2011)	Soybean oil	15	13	86	Shake flask
(Kamilah et al., 2013)	Waste cooking oil	15	10.7	72	Shake flask
This work	Date seeds oil	14.35	11.8	82	Shake flask
	Vegetable oil	12.1	9.1	75	
	Sunflower oil	10.2	7.4	72	

6.2.3 PHB synthesised using emulsified date seed oil

6.2.3.1 Results and discussion of PHB synthesised in emulsified date seed oil

The goal of this study is to investigate the effect of using emulsified date seed oil, emulsified by GA, on *C. necator* growth and subsequently PHB accumulation. This was achieved by study various approaches as follows:

6.2.3.1.1 Evaluation of potential Arabic Gum(GA)

A- GA as alternative carbon source

The first step studied in this section was the potential of using GA as a carbon source for cell growth, therefore, different fermentation experiments were conducted using mineral media, as described in section 4.4.2.3, containing various GA concentrations as an alternate carbon source. The results of this study were presented in Figure 6.11, which shows *C. necator* growth in mineral media contained GA agent with various concentrations; 0.1, 0.3 and 0.5, as a carbon source. From Figure 6.11, it can be noticed that *C. necator* did not grow in the mineral media at any concentration of GA and consequently bacteria cannot use GA as a carbon source for the cell growth.

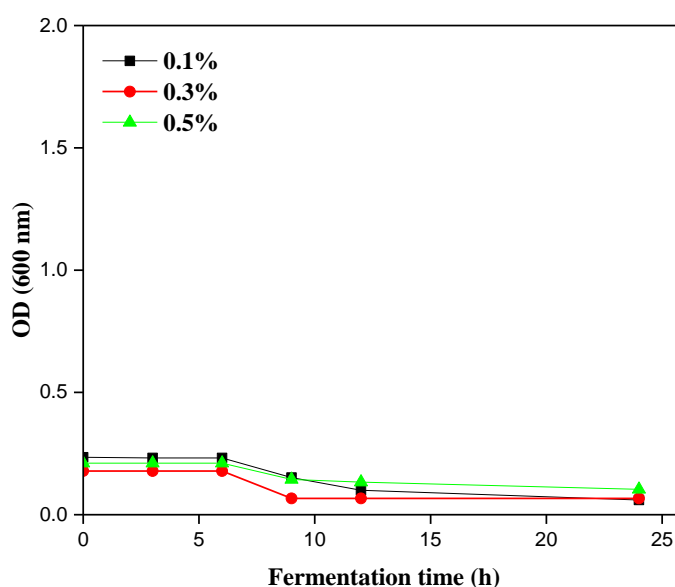


Figure 6.10. *C. necator* growth on mineral media contained GA agent as an alternate carbon source under various concentrations; 0.1, 0.3 and 0.5.

From Figure 6.11, it can be observed that the GA cultures showed that OD_{600nm} values did not increase at any time points and for any concentration and the values were decreased after about 9 h of fermentation time, indicating that no significant growth was taking place, hence GA cannot use as a carbon source for the cell growth.

This result agrees with the results reported by Budde et al., (Budde et al., 2011) and Volkering et al., (Volkering et al., 1998) when investigated various types of emulsifiers agents including GA. They found that the bacteria could not utilise the emulsifiers, SDS; Triton; Tween and GA, as an alternate carbon sources for *C.necator* growth using plant oil emulsified in mineral media.

B- GA as nitrogen source

Since GA is a glycoprotein, it has the potential to utilise as a nitrogen source for *C. necator* growth. This was investigated using medium cultures with 20 g/l date seed oil and 0.1% concentration of GA as the sole potential nitrogen source, after remove the $(\text{NH}_4)_2\text{SO}_4$ from the original culture. Along with controls that contained normal salts with 20 g/l date seed oil and without GA. Triplicate cultures were inoculated to an initial OD_{600nm} of 0.2. After 24 h of incubation, the mean value of OD_{600nm} of the control culture was 0.356, while the mean OD_{600nm} of the GA cultures was decreased to reach 0.096, as shown in Figure 6.12. The figure was presented the *C. necator* growth on mineral media contained GA agent as a nitrogen source and 20 g/l date seed oil as a carbon sources.

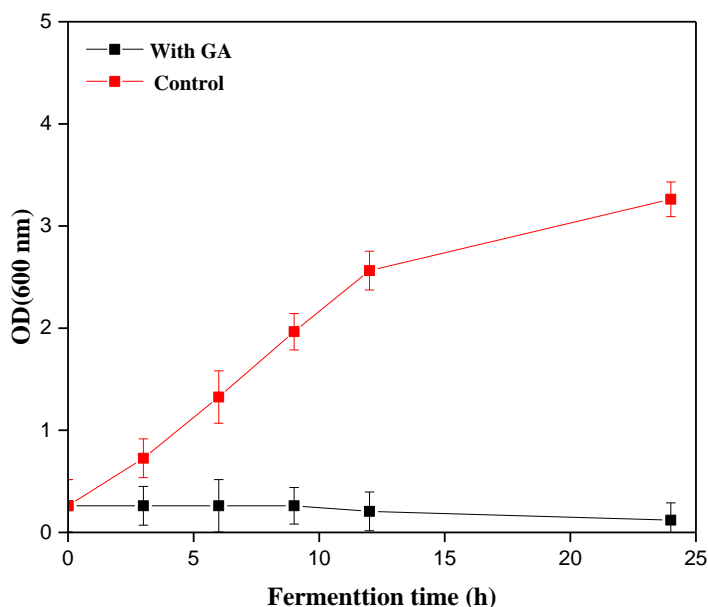


Figure 6.11. *C. necator* growth on mineral media contained GA agent as a nitrogen source and 20 g/l date seed oil as a carbon sources.

From the Figure 6.12, the raise in OD_{600nm} of the control can be attributed to the changes in both cell growth and cell morphology caused by PHB storage. The GA cultures exhibited a constant value in OD_{600nm} and after around 10 h the value was started to decrease, while the fact that there was small difference between the GA cultures and the controls should be observed if the bacteria used GA.

Therefore, results indicate that GA does not serve as an alternative nitrogen source for *C. necator* growth and these results agree with the results reported by (Riedel, 2016) who found that GA agents was not a nitrogen source for *C. necator* growth using plant oil as a carbon source. Based on the results obtained from this section which agreed with the results reported in the literature, for examples the study reported by Budde et al.,(Budde et al., 2011), it is clear that GA was the best emulsifying agent to utilise in oil cultures.

6.2.3.1.2 Growth of *C. necator* in minimal medium with date seed oil and GA

In order to gain insight into the influence of GA on *C. necator* cell growth and PHB accumulation, various concentrations of GA were studied to investigate these concentration may influence measurements. *C. necator* grew in minimal medium containing 20 g/l of date seed oil in the presence of different concentrations of GA: 0.1%, 0.3%, 0.5% and 0.7% and the results are plotted in Figure 6.13. From Figure 6.13, it can be noticed that the high concentration of GA is significantly influence cell growth which increased by decreasing the GA concentration and it did not grow when the GA was at a very high concentration.

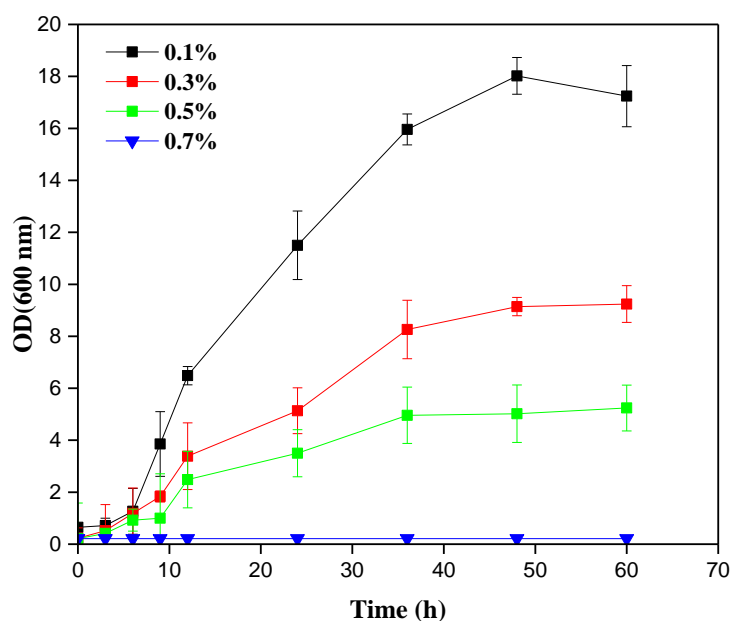


Figure 6.12. *C. necator* growth in minimal medium containing 20 g/l of date seed oil and presence different concentration on GA, 0.1%, 0.3%, 0.5% and 0.7%, under growth conditions, 30

Presence of GA does significantly effect OD_{600nm} value, where was 18.2 at 0.1% GA concentration and it was 5.2 when the GA concentration increased to 0.5% while high concentration (0.7%) of GA prevents the cells of *C. necator* to grow and this affect increased by increasing the GA concentration, hence no mass of precipitated material can be measured over the cultivation time.

At the main time, the effect of GA on PHB accumulation was investigated and the results presented in Figure 6.14. From Figure 6.14 presents the PHB accumulation inside *C. necator* using minimal medium containing 20 g/l of date seed oil and presence various concentration on GA, 0.1%, 0.3%, 0.5% and 0.7%.

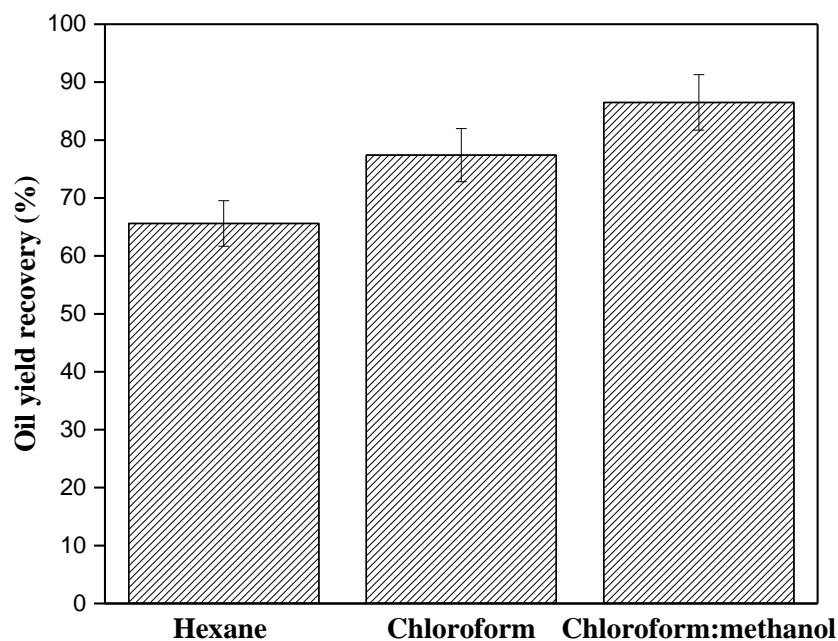


Figure 6.13. PHB accumulation inside *C. necator* in minimal medium containing 20 g/l of date seed oil and presence different concentration on GA, 0.1%, 0.3%, 0.5% and 0.7%.

From the results, it can be seen that PHB concentration curve has similar trend that obtained from OD_{600nm}, which decreased by increasing the GA concentration. However, as cells grown in media with GA made 10% less PHB granules than cells not exposed to GA. The value of PHB content was 75.5% at 0.1% but it was reduced to 53% by increased the concentration to 0.5%. In spite of this reduction, *C. necator* grown in the presence of GA medium still accumulated significant amount of PHB, while there were no PHB granules accumulated when the concentration raised 0.7% and this may be belong to high concentration of GA worked as an inhibitor for the cell growth.

6.2.3.1.3 Date seed oil quantification

The common method that was found in the literature for measuring the concentration of oil in bacterial growth culture that relied on hexane as a solvent and this method was not quantitative. Therefore, quantification of date seed oil from culture samples using hexane, chloroform, and a mixture of chloroform: methanol (2:1) were tested. chloroform: methanol mixtures have long been applied to extract oils from biological samples.

In order to accurately measure the total amount of oil present in a culture, an extraction method must also retrieve these polar lipids. The results of effect of solvent types on amount recovery of date seed oil from *C. necator* culture are shown in Figure 6.15.

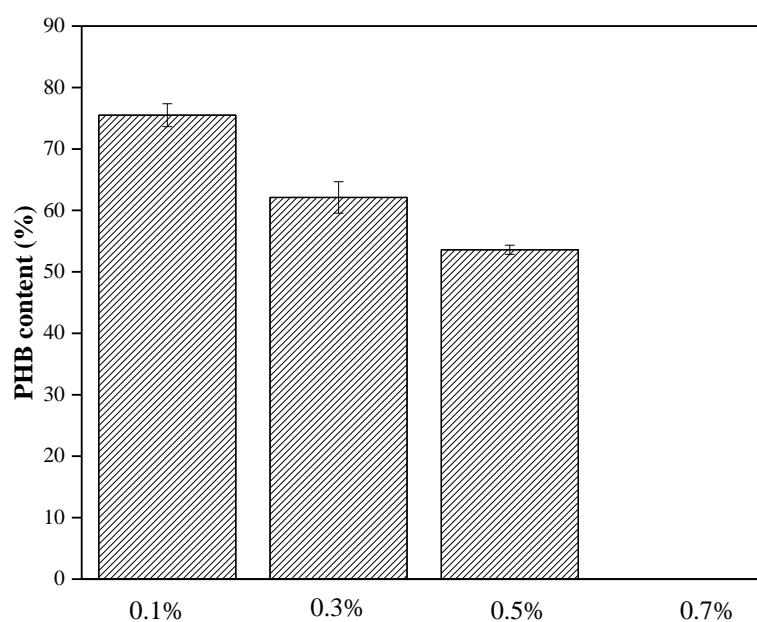


Figure 6.14. Effect of solvent types on amount recovery of date seed oil from *C. necator* culture.

The results obtained from Figure 6.14 indicates that chloroform: methanol mixture recovered greater than 86% of the oils from each sample, following by chloroform and then hexane which recovered about 77% and 68% of oils, respectively. Therefore, chloroform: methanol mixture was used for measuring oil concentration in all the following experimental samples, as it yielded better results than the other solvents. Furthermore, control standards were also prepared contained 0.3% of GA but without oil. The mass retrieved from all these samples was insignificant, indicating that GA is not recovered by the solvent utilised in this study. On the other hand, when cell pellets are existing in a sample, some of the oils in the media are associated with the cells, therefore, estimate how much oil was associated with the cell pellets by washing them with hexane after the washing step with water was also studied.

MCM cannot be applied for this step because the chloroform can extract PHB granules from biomass. The results from these experiments, which were repeated four times, indicated that if the hexane wash step is included in the oil recovery method, the maximum observed oil concentration increases by just about 3% given slightly influence this has on the measurement. Hence, this step does not need to be added in the standard procedure, but could be included if higher accuracy is required.

These results agreed with Iverson et al., and (Iverson et al., 2001) which reported that hexane cannot be applied to quantitatively recover oleic acid and/or palm oil, and it can be used if sodium hydroxide (NaOH) was added to the sample to break the emulsions. Even with NaOH treatment, however, oleic acid could not be retrieved efficiently. In summary an oil recovery method was developed to enable the monitoring of the consumption of oil by the cells.

6.2.3.1.4 Emulsified date seed oil fermentations

Investigation the effect of using emulsified date seed oil on bacterial growth and PHB accumulation was first studied at the shaken flask scale to earn more information on the production scaling up production. The agitation speed was kept constant at 200 rpm during the duration of the fermentation and the fermentations was continued until the optical density value of the culture began to reduce. At this time, the maximal PHB production was expected to have been achieved. The cultivation period was extended for further hours after death phase was reached in order to have better understanding on the activities of the *C. necator* towards the accumulated biopolymer (PHB). Fermentation experiments were carried out in medium containing 20 g/l date seed oil emulsified with 0.1% GA and cell growth and oil utilisation were measured and the results of these experiments are presented in Figure 6.16.

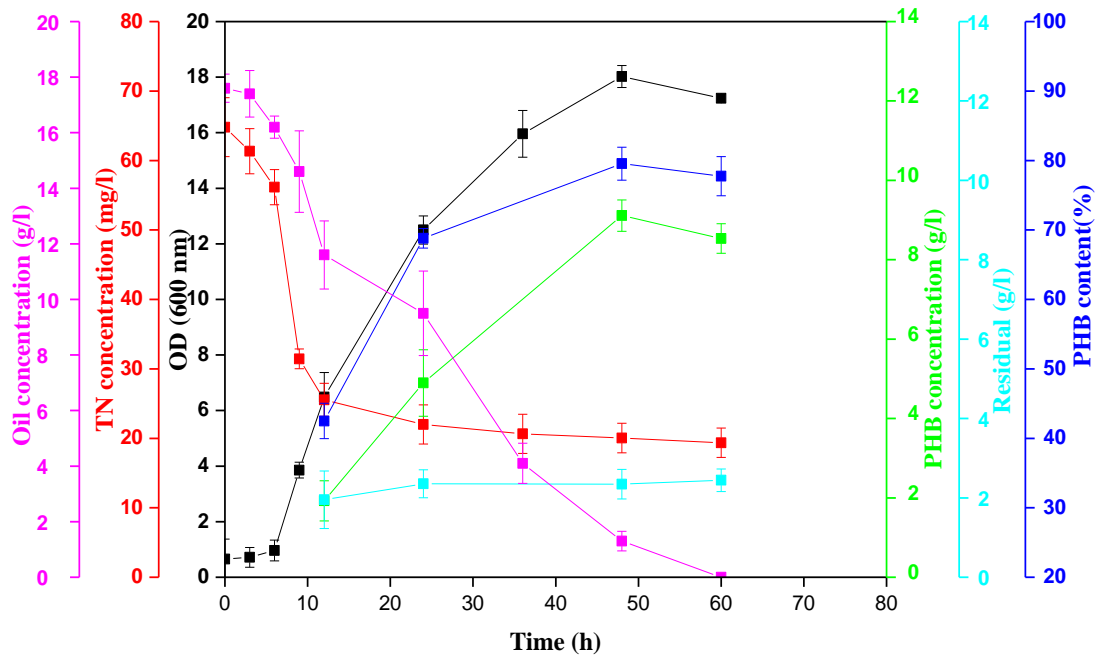


Figure 6.15. *C. necator* growth in a media contained 20 g/l as a sole carbon source date seed oil emulsified with 0.1% GA. The incubation conditions were 30°C and 200 rpm for 48 h. ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass

Figure 6.16 shows *C. necator* growth in a media containing 20 g/l date seed oil emulsified with 0.1% GA. The incubation conditions were 30°C and 200 rpm for 48 h. The results demonstrate that the new method using emulsified date seed oil reduced the lag phase of the culture, and that accurate values of oil consumption early in the were able to be determined experiments and it was observed considerable increase in OD_{600nm} by the 6 h time point. From Figure 6.16 it can be noticed that after 24 h of cultivation time, the value of total nitrogen in the medium was reached a constant value at 29 mg/l, and the value of residual biomass reached a maximum. By 50 h, 79.5% of total biomass consisted of PHB. Triplicate repeats of fermentation experiments were carried out, with the small error bars indicating that this method permits for reproducible growth of *C. necator*. The initial measure oil concentration in the medium was 17.2 g/l, which is less than the 20 g/l added to each flask. This contradiction is attributed to oil recovery percentage the by the solvent.

There was a significant consumption of oil by the cells which initially observed between the 9 and 12 h time points and continued throughout the experiment, although the consumption rate reduced over time and measurement of oil utilisation allows for product yields calculation. It can be found that during the entire fermentation course, PHB was accumulated at a yield of 0.61 g_{PHB}/g_{oil}. These values are the same other yields value from plant oils reviewed in the literature by (Kahar et al., 2004b).

Plant oils have been proposed to be more effective carbon sources for commercial PHB accumulation than sugars (Akiyama et al., 2003b). Wild strain of *C. necator* is able to grow on non-emulsified date seed oil, these media exhibit changeable lag phase, and representative samples cannot be taken early time in the experiments. It was decided to use GA as the emulsifying agent for the cultures of date seed oil, because it produce stable emulsions and did not affect *C. necator* growth significantly. From the above fermentation experiments it has been found two minor problems with using GA: first, some of the materials settled down when autoclaved, and second, the existence of GA led to a slight reduce in PHB accumulation. Neither of these problems had a major effect on the results of *C. necator* growth and PHB accumulation experiments. *C. necator* fermentations with emulsified date seed oil demonstrated the efficiency of this method. Cultures had short lag time and were highly reproducible. The *C. necator* cells produced high levels of biopolymer, with PHB content of 79.5% of DCW reached after 50 h.

6.2.3.1.5 Fed-batch fermentation

Fed-batch fermentation system is one of the best techniques of obtaining a high cell density with the highest potential amount of PHB (Akaraonye et al., 2010b). Studies were carried out to monitor the influence of intermittent addition of oil to the bioreactor trying to develop the PHB productivity. Date seed oil was intermittently added to the culture after a few hours of start fermentation, when a reduce in dissolve oxygen (DO) from the initial set up (100%) was observed to have reduced to 30% (as described in section 4.2.4.1.5). *C. necator* behaviour in large scale was monitored as to how that could affect PHB accumulation. A bioreactor (1.7 L) was used for further experiments and the results presented in Figure 6.17.

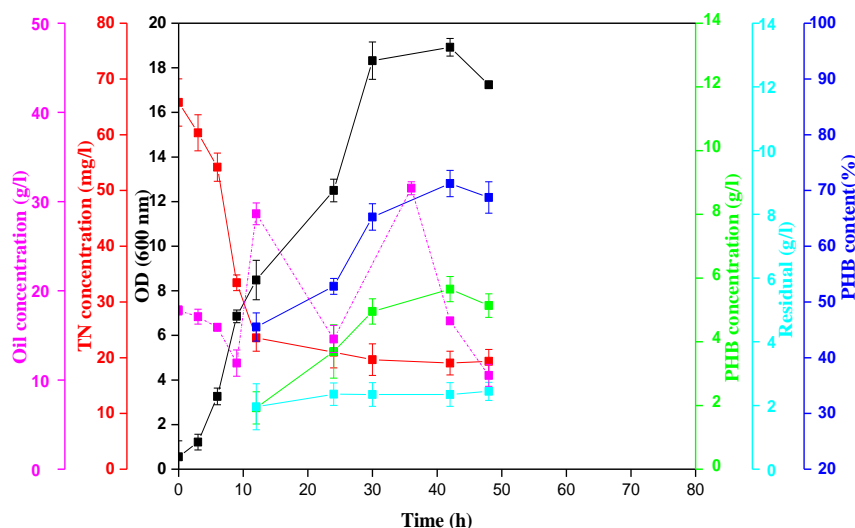


Figure 6.16. *C. necator* growth in a media contained 20 g/l as a sole carbon source date seed oil emulsified with 0.1% GA and using 1.7 l bioreactor.

Figure 6.17 presents the profile of the parameters monitored during *C. necator* growth in a media contained 20 g/l as a sole carbon source date seed oil emulsified with 0.1% GA and using 1.7 L bioreactor. It can be observed that the fermentation time is less compared with the shake flask experiment, in which increase in cell growth continued until 30 h, exponential growth, followed by stationary growth that lasted until 42 h of the cultivation time.

From the analysis conducted, it was observed that the cell growth phase started after 3 h of fermentation time and continued until 30 h. The bacteria showed a stationary cell growth phase after 30 h and this lasted until 42 h of the total fermentation time. Further extension of the cultivation time beyond 48 h led to reduction in the growth of the bacteria.

The highest OD_{600nm} achieved at the beginning of intermittent addition of oil (at 12 h of fermentation) was 8.45 g/l which was reached to 18.32 g/l at 30 h while the highest OD_{600nm} obtained at the end stationary phase was 19.1 g/l. The PHB content at the start of intermittent addition of oil was 45.2 %. This value was found to have developed, at end of raised cell growth and stationary phase, to 65.2 % and 81.2 % respectively. The PHB concentration obtained at the onset of intermittent addition of oil was 1.35 g/l.

This value was observed to increase significantly to 11.36 g/l at the end of stationary phase. Further extended in the fermentation time led to a reduce in the PHB accumulated over the stationary phase. At the initial of intermittent addition of oil, 1.95 g/l of residual was quantified which increased to 4.59 g/l at the end of exponential phase and then was

observed to have reduced to 3.34 g/l during the stationary phase but increased significantly during the death phase.

Also from the Figure 6.17, it can be seen that the accumulation of PHB increased rapidly after the depletion of TN while residual tended to reduce due to cell lysis. For this reason, the raise in DCW was considered because of PHB accumulation. The concentration of TN was also evaluated, and it was noticed that 70% of TN was consumed in the first 12 h of fermentation, after that the TN stayed constant at a concentration of 25 mg/l. On the other hand, the initial concentration of oil measured was 17.6 ± 0.8 g/l after 3 h and was consumed to 10.2 g/l after 48 h of fermentation.

6.2.3.1.6 Comparison between shake flask and bioreactor scale

Based on the statistical analysis which was carried out to compare various parameters belong to PHB production using emulsified date seed oil as a carbon source in shaken flask and bioreactor scale (T-test (p-value), Microsoft Excel® 2016). Overall parameters values was compared over the *C. necator* growth in salts media medium containing date seed oil as the main carbon source showed that more DCW was accumulated by the organism in bioreactor than in shaken flask. The results were presented in Figure 6.18 which shows the comparison of DCW, PHB concentration and content in shaken flask and 1.7L bioreactor.

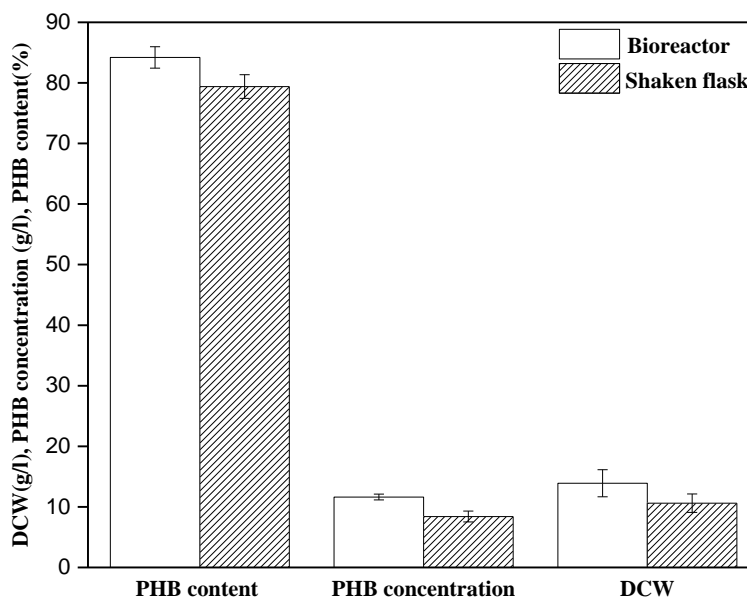


Figure 6.17. Comparison of DCW; PHB concentration and content accumulated by *C. necator* using salts media and date seed oil as the main carbon source in shaken flask (at 60 h fermentation) and bioreactor levels (at 48 h).

From the figure, it can be seen that the bioreactor process parameters were significantly higher ($P \leq 0.05$) than shaken flask. Decrease in the values of total, PHB concentration and content were found in the shaken flask study as well as a shorter cultivation time of 48 h for obtaining maximum PHB concentration was achieved with the bioreactor as opposed to 60 h observed in shaken flask investigations.

The final value of PHB content was higher (84.1% vs. 73.9%) than that obtained by Schmidt et al., (Schmidt et al., 2016) using shake flasks with time set at 72 h using 20 g/l emulsified soybean oil nitrogen limitation while Budde et al., (Budde et al., 2011) obtained the same value when applied emulsified plant oil as a carbon source. Also Spp Magar et al., (Spp Magar et al., 2015) obtained similar results when they used emulsified cotton seed oil as a carbon source for growth of *C. necator* and PHB production. Spp Magar et al., found that the emulsifying agent led to slight reduced in PHB accumulation. While our results disagree with the results published by Kahar et al., (Kahar et al., 2004b) who reported that the PHB yield increased about 7.9% when the media supplemented with minie mulsified vegetable oil. This study is a step ahead in the direction of decreasing process time and increasing PHB yield and is in line with the main goal of this study.

6.3 Summary

As mentioned previously, the cost of the raw material (substrates) is one of the main reasons for the high cost of PHB synthesis and one promising class of carbon sources for industrial PHA production are plant oils, because of it highly carbon content in these materials. In general, oil derived from waste and by-product materials is lower in price when compared to pure substrates, such as edible vegetable oils, that can be used for microbial growth and PHB accumulation. Therefore, in this chapter both a kinetic study of oil extraction from date seeds under various conditions and the potential of using recovered oil as alternative carbon source were investigated. The results demonstrate that the mass transfer model is beneficial in describing the oil extraction from date waste and *C. necator* is capable of synthesising PHB, utilising date seed oil as a sole carbon source and accumulating a high with a high content of PHB concentration. Hence, in this chapter, the feasibility of waste date seed valorisation via PHB production utilising extracted date seed oil is demonstrated. The most PHB was accumulated in the fermentation with date seed oil, 11.8 g/l, obtained after 48 h, while only 9.1 g/l and 7.4 g/l were produced for the same time using vegetable and sunflower oil, respectively. The PHB content of 82% was reached after 48 h, while the PHB content was 75% and 72% for vegetable and sunflower oil.

Although *C. necator* accumulates high levels of PHB and can utilise oil effectively, it was difficult to quantify the oil consumption in a quantitative and reproducible way due to the heterogeneity of the two phases of the medium. *C. necator* secretes a lipase to emulsify the hydrophobic oils in the aqueous culture medium and the natural emulsification leads to an increased culture lag phase, where it is difficult to take the homogenous sample from the medium until the oil has been emulsified. To overcome this obstacle, a new method was improved in which date seed oil was emulsified in growth media using GA as the emulsifying agent after demonstrated that GA did not affect *C. necator* growth and cannot be utilised as a nutrient source (carbon or nitrogen) by the bacteria. *C. necator* was grown in the emulsified oil media and PHB accumulation was measured over time. Therefore, a part of this study was presented a date seed oil emulsification method using GA as an emulsification agent, in which allowed reducing the lag phase in culture, since the date seed oil was bioavailable beginning from the inoculation of the culture. Through the pre-emulsification of the oil, it has potential to take homogeneous samples during the entire duration of the fermentation. The emulsified date seed oil media can be applied to both shake flasks and fermenter.

Various experiments were carried out using emulsified date seed oil, monitoring cell growth and PHB production by *C. necator*, resulting in maximum PHB content and concentration 79.5 % and 9.53 g/l, respectively, after 48 h of fermentation time and this value is less than the value obtained from using non-emulsified oil. With the improved chloroform: methanol method extraction recovered greater than 86% of the oils from each sample, following by chloroform and then hexane which recovered about 77% and 68% of oils, respectively. Hence, it was possible to quantify the date seed oil that was consumed during the cultivation time and to create an oil profile. Also Based on the results, it can be concluded that the shaken flasks parameters, total biomass and PHB content and concentration were significantly higher than bioreactor process, 5.56 and 71.3% of total biomass was obtained when the fed batch bioreactor was applied.

CHAPTER

SEVEN

PART A

7 PHB production using mixed substrate and by ACSSEF

7.1 Introduction of PHB production using mixed-substrate

When bacteria grow in cultures containing minimal media supplemented with two types of carbon source (mixed-substrate media), they exhibit two different types of behaviour dependent on the type and concentration of the carbon sources in the culture. When substrates are present in high concentrations, sequential and diauxic growth is observed and the substrate which provides the highest rate of growth is used preferentially from the mixture. Whilst in other cases, when the substrate concentrations are low, simultaneous utilisation of the different compounds present in the culture seems to be the general response (Monod, 1942).

Sequential and diauxic utilization are commonly ascribed to catabolite repression by the catabolite activator protein regulatory system (cAMP-Crp). This activates transcription through binding to particular sites on the DNA which in turn interacts with RNA Polymerase. (Müller-Hill, 1996), (Deutscher et al., 2006) (Narang and Pilyugin, 2007) and (Busby and Ebright, 1999), although specific studies have presented the cAMP-Crp regulation system to be either not sufficient for diauxic growth (Okada et al., 1981) or not necessary (Inada et al., 1996). Another possible theory was presented based on that the adoption of the second substrate is not blocked but rather that it's use is inhibited. Although this results in sequential use of the two substrates, it does not lead to diauxic growth. Diauxic growth is not the unique response noticed when mixed substrates utilisation is investigated, several substrates don't interfere with using of each other (this combination possible even stimulate the growth), in which condition the two compounds are utilised simultaneously.

The first part of this chapter is to investigate of the effect of utilising mixed-substrates, oil and fructose derived from waste date seed, on PHB accumulation using batch and fed-batch fermentation. Batch cultivation was applied at three fructose to oil ratios; 1:1,1:3 and 3:1 and the results are presented in Section 7.2.1. The results of fed-batch cultivation, from feeding date seed oil at 48h of fermentation time, are shown in Section 7.2.2.

7.2 Results and discussion

All the fermentation experiments were carried out using shake flask scale at least in duplicate for each condition. All parameters including OD_{600nm}, PHB concentration and content, TN and DCW were evaluated and averaged values presented in the figures, as follows:

7.2.1 Mixed-substrate for PHB production using batch cultivation technique

To study and discuss *C. necator* behaviour on mixed substrates derived from waste date seed (hydrolysate media contains fructose and emulsified date seed oil using 0.1% GA as described in chapter 6), fermentation experiments were conducted as described in section 4.2.4.1.7. The results of these experiments are presented in Figure 7.1 which shows the time course profile of fermentation in a media containing mixed substrate, date seed oil and hydrolysate media containing fructose as a carbon source for cell growth. From the figure it was observed that *C. necator* follows diauxic growth and the fructose is the preferred substrate over the oil.

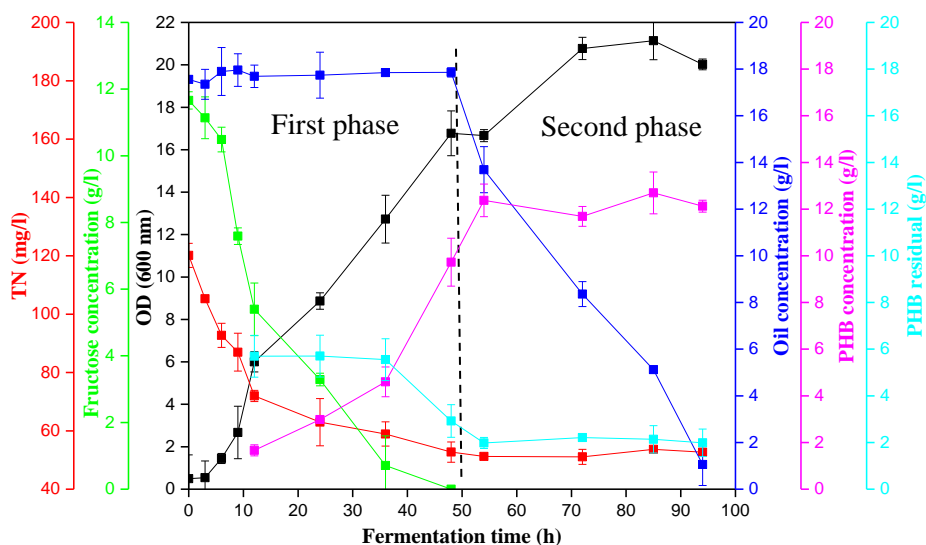


Figure 7.1. Time course profile of *C. necator* fermentation in a media containing mixed substrate, emulsified date seed oil without mineral media and hydrolysate media containing fructose as a carbon source, under fermentation conditions 30°C and 200 rpm. ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass, ■ PHB concentration.

In the first phase of growth, only fructose was used for the cell growth, which was completely consumed after 48 h of fermentation to give OD_{600nm} and total biomass of 16.77 and 12.65 g/l, respectively, with a PHB content of 60.9 %. Total nitrogen concentration dropped from 120.33 mg/l to reach 52.4 mg/l after 48 h and continued with this value to the end of fermentation time. After exhaustion of fructose production of oil-metabolising enzymes occurred, which allowed a slower (second) growth phase on oil. There was a lag phase to start the second exponential phase for the second substrate, oil. The maximum total biomass and PHB accumulation were 14.38 g/l and 85.9 % respectively.

The initial oil concentration measured in the mineral media was 17.5 g/l, which is less than the 20 g/l added to each flask. This discrepancy is attributed to the fact that some date seed oil collected on the walls of the flasks and was thus removed from the media. Consumption rate of oil continued throughout the experiment, although the rate reduced over time. Significant consumption of date seed oil by the bacteria cells was first monitored after 50 h of fermentation time. The oil concentration reduced from 17.89 g/l to be 0.98 g/l after 40 h of the starting of the second phase of fermentation. It was found that over the course of the entire fermentation, PHB was formed at a yield of 0.61 g_{PHB}/g_{oil}. These value is similar to other yields from oil produce from reported in the literature (Kahar et al., 2004c).

7.2.1.1 Mixed-substrate media at ratio (1:1 fructose to oil)

In this experiment hydrolysate date seed media contained fructose and mineral media contained emulsified date seed oil at a ratio of 1:1 were used as a mixed substrate for *C. necator* growth and PHB production. The details of the experimental procedure followed are described in 4.4.1 while the results of the study are shown in Figure 7.2. The figure shows that *C. necator* can metabolise both fructose and oil following a diauxic growth.

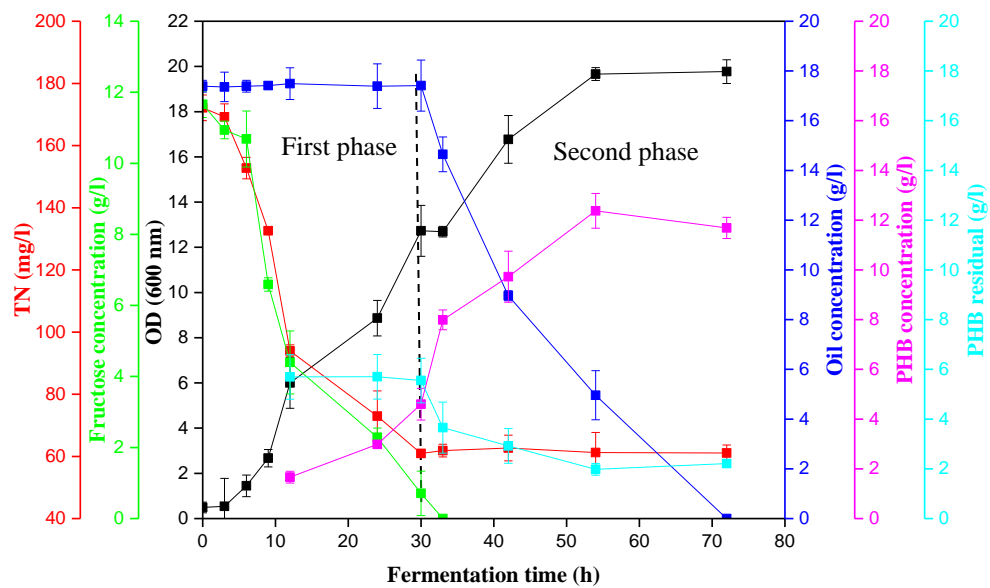


Figure 7.2. Diauxic growth of *C. necator* on a mixture of date seed hydrolysate media (fructose) and mineral media contained 20 g/l of emulsified date seed oil at ratio (1:1 fructose to oil), under fermentation conditions 30°C and 200 rpm. ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass, ■ PHB concentration.

The behaviour of *C. necator* in this experiment was the same as the previous results shown in Figure 7.1. From Figure 7.2, the first growth phase lasted for 30 h and then the second phase was noticed after about 3 h of lag phase, which was an indication of the depletion of the first substrate composition in the culture and the switch to growth utilising the second, oil, substrate. The cell growth was then found to have increased until 50 h, after which the cell growth slowed down, and the stationary phase was entered.

The maximum total biomass achieved by the end of first stage of fermentation was 11.86 g/l while the further extension of the fermentation time into the stationary growth phase led to further biomass accumulation, reaching 15.22 g/l by the end of fermentation period (72 h). The maximum PHB concentration before the consumption of the second substrate was 45.3 % of DCW. This value was found to have increased to 84.1 % DCW at the end of the fermentation period. At the end of fermentation 72 h, the concentration of PHB was found to increase to 12.8 g/l. The residual cell mass was found to decrease steadily through the fermentation with 3.6 g/l reached, before the initiation of feeding, which decrease to 2.06 g/l at the end of stationary phase and the reduction of residual biomass could be caused by cells lysis.

7.2.1.2 Mixed-substrate media at ratio (1:3 fructose to oil)

In this experiment, another substrate media ratio, hydrolysate date seed media (fructose) and mineral media (emulsified date seed oil) at a ratio of 1:3 was investigated to study the effect on *C. necator* behaviour, and hence PHB accumulation. All the parameters, OD_{600nm}, DCW, TN and PHB concentration were determined and the results are presented in Figure 7.3. From this study it was indicated that *C. necator* followed the similar behaviour, Diauxic growth and metabolise fructose firstly, as in the previous ratio.

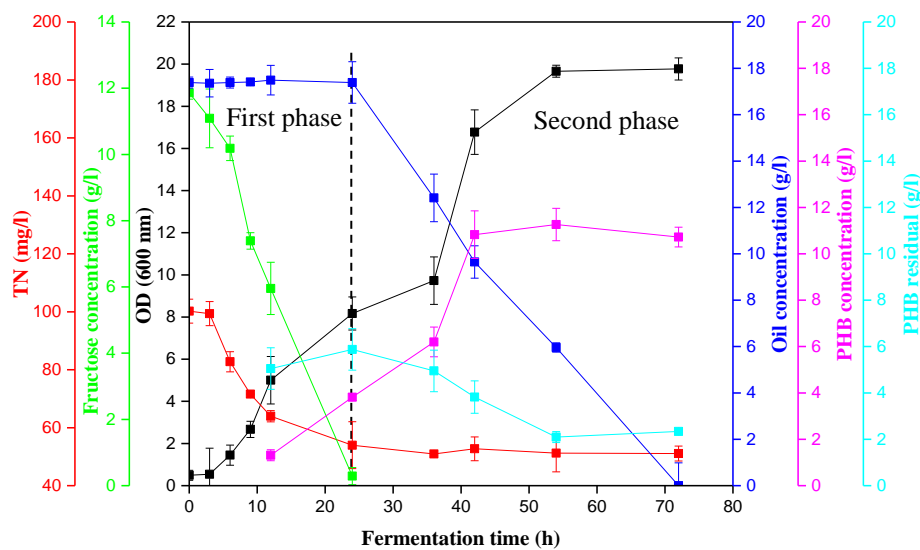


Figure 7.3. Diauxic growth of *C. necator* on a mixture of date seed hydrolysate media (fructose) and mineral media contained 20 g/l of emulsified date seed oil at ratio (1:3 fructose to oil), under fermentation conditions 30°C and 200 rpm. ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass, ■ PHB concentration.

From Figure 7.3, it can be noticed that the lag phase was finished at 6 h, and then a sharp increase in OD_{600nm} can be seen until 24 h, where the second phase begins after 3h of lag time, with PHB accumulation starting throughout. *C. necator* metabolised the fructose as a first substrate, giving a maximum OD_{600nm} of 12.27 after 36 h of fermentation, while the maximum PHB concentration was 5.94 g/l. Subsequently, these values were raised to be 19.6 and 10.96 g/l for OD_{600nm} and PHB concentration respectively by the end of fermentation time (72 h). In spite of some PHB production during the early growth phase being observed, most PHB was produced during the latter stages of the fermentation, PHB started to accumulate significantly after 24 h, whilst residual biomass growth gradually slowed. After that the oil concentration decreased during the second growth phase and was consumed completely after 72 h of cultivation.

This experiment revealed that maximum total biomass of 13.46 g/l and PHB content of 81.5% was obtained after 60 h of cultivation with maximum specific growth rate 0.14 h^{-1} . TN concentration was also investigated, and it was observed that 67% of TN amount was consumed in the first 36 h, after which the TN remained constant at a concentration of 61 mg/l. The initial fructose concentration was 11.6 g/l consumed completely after 24 h of fermentation to give PHB content of 49.3% (w/w).

7.2.1.3 Mixed-substrate media at ratio (3:1 fructose to oil)

In this section, hydrolysate date seed media (fructose) and mineral media (emulsified date seed oil) at a ratio of 3:1 was used to investigate the effect of this ratio on *C. necator* behaviour and hence PHB accumulation. All the parameters, $\text{OD}_{600\text{nm}}$, DCW, TN and PHB concentration were determined and all the results of this section are presented in Figure 7.4. From the figure, it was obvious that *C. necator* metabolise fructose completely after 48 h, after that, they started to metabolise oil to allow the second growth phase to begin.

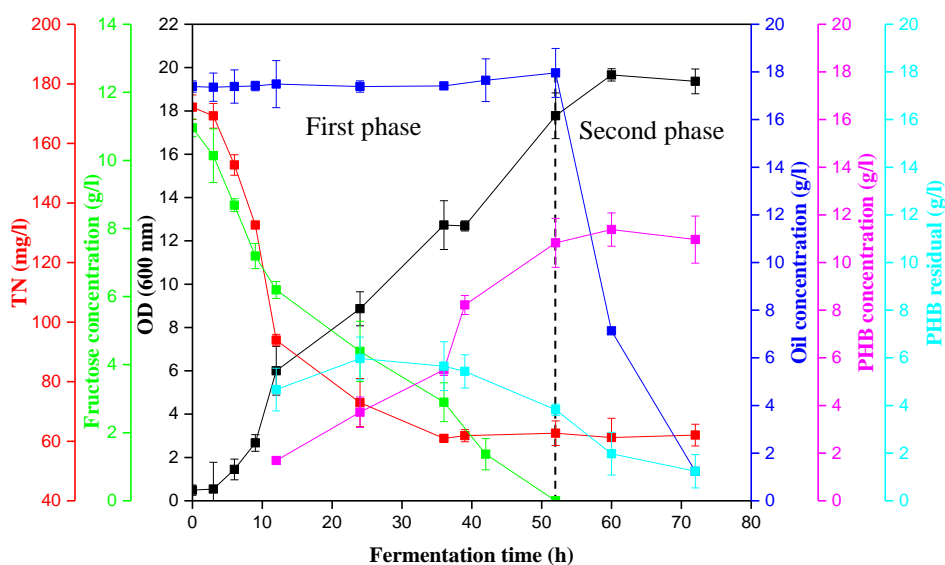


Figure 7.4. Diauxic growth of *C. necator* on a mixed-substrate of date seed hydrolysate media (fructose) and mineral media contained 20 g/l of emulsified date seed oil at ratio (3:1 fructose to oil), under fermentation conditions 30°C and 200 rpm. ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass, ■ PHB concentration.

It is obvious that *C. necator* metabolised the fructose for 52 h of the fermentation time to give the highest total biomass of 14.65 g/l whilst the PHB concentration was 11.26 g/l. The second growth phase started after 52 h of fermentation to reach the stationary phase after 24 h and the both DCW and PHB increased to 11.15 g/l and 10.85 g/l respectively.

Even with this ratio (3:1 of fructose to date seed oil) *C. necator* followed similar behaviour as for the previous ratios, the difference being the length of first and second growth phases. From the results obtained in this section it can be concluded that emulsified oil is the preferred substrate over fructose in *C. necator* which utilised them sequentially to follow diauxic growth. In general, various bacteria do not give the similar response to the combination of the mixed substrates. A combination that follows diauxic growth in one microorganism may not work in other (Chong et al., 2017). Furthermore, a material that is the preferred substrate for one microorganism properly a secondary substrate for another. There is a relation between the ability of the material to act as a preferred carbon source (substrate) and the rate of growth which it will sustain. Although not invariably, in many cases, the substrate existence and the growth allowing a higher rate of growth prevents the using of a second (poorer) substrate in batch culture (Harder and Dijkhuizen, 1982).

7.2.2 Mixed-substrate for PHB production using fed-batch cultivation

7.2.2.1 Effect of feeding pure oil after 48 h of fermentation time

Fed-batch fermentation of *C. necator* was tried to further investigation of the influence of feeding another substrate on PHB accumulation. All parameters including OD_{600nm}, PHB concentration and carbon source consumption were monitored and all average parameters are presented in Figure 7.5. In this study an exponential fed-batch fermentation was proposed to attain high PHB concentrations at the end of the fermentation. From the figure, it was observed that feeding oil after 48 h of fermentation, *C. necator* did not metabolise the oil and the fermentation finished after 80 h.

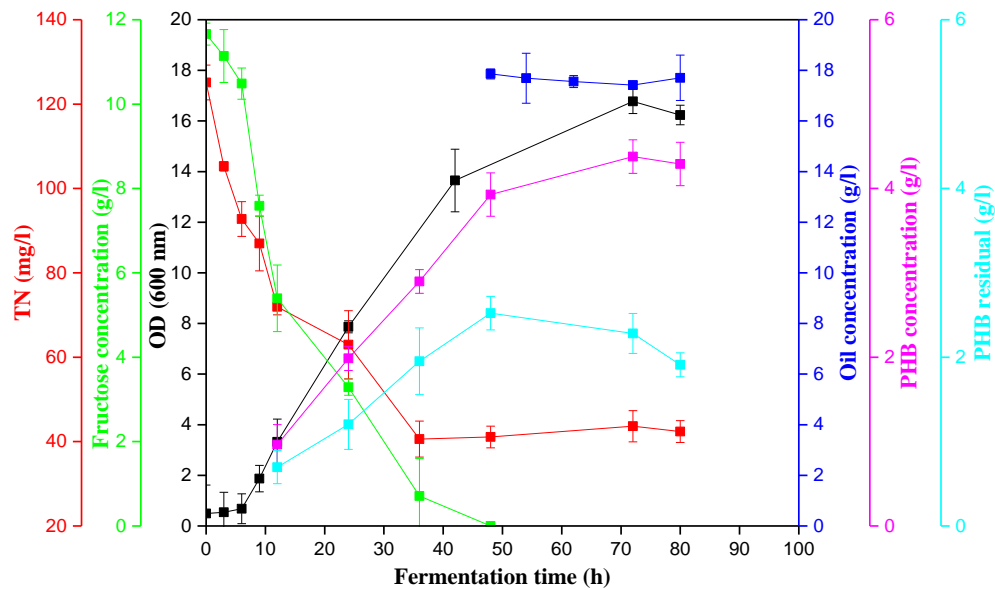


Figure 7.5. Time profile of *C. necator* growth in a media hydrolysate from waste date seed then feeding oil after 48 h of fermentation time, under fermentation conditions 30°C and 200 rpm. ● Fructose, ◆ TN, ▲ PHB, ■ OD (600 nm), ▼ Residual biomass, ■ PHB concentration.

As shown in Figure 7.5, these cultivations started with a batch period, until maximum μ (0.17 h^{-1}) was reached (48 h of fermentation). After that time, the second substrate, date seed oil, was fed to the culture. The bacterial growth is mainly associated with the consumption rate of nitrogen. The amount of nitrogen present in the media has a considerable influence on bacterial growth and PHB production, TN concentration reduced with time, from 125 mg/l to reach 45 mg/l after 36 h and reaching a constant value to the end of the fermentation period. The highest total biomass obtained was 14.36 g/l at 36 h but this decreased to 13.6 g/l by the end of the experiment. From the results, it is obvious that the increase in biomass was due to PHB accumulation. The PHB concentration increased sharply between 9 h and 48 h reaching 12.37 g/l when the biomass concentration reached its highest value, resulting in about 85% (w/w) PHB content. The reduction in PHB content to about 81% at the end of the fermentation period might be due to the PHB releasing into the culture because of cell autolysis.

For the first 6 h, lower fructose consumption by *C. necator* was observed. The consumption of fructose within the time range was about 6.24 g/l which is almost half of the total fructose concentration in the culture and to consumed completely at 48 h. On the other hand, the TN concentration was found to be reduced drastically from the initial value and reached a constant value after 12 h of the fermentation period.

PART B

7.3 PHB production using ACSSF system

7.3.1 Introduction

In spite of the potential of solid state fermentation (SSF) systems, many physical aspects related to the availability of nutrients and heterogeneity of the medium are serious constraints. To overcome these issues, it is convenient to use inert supports which have well-characterised properties as well as being biologically inert. In this chapter, results from SSF experiments using inert carrier support material (known as adsorbed-carrier solid state fermentation, ACSSF) are presented. The ACSSF process consists of biologically inactive, porous and heterogeneous material called an inert support, in which inoculum and culture media are absorbed. Cell growth occurs under controlled conditions of aeration within appropriate reactors preserved at a constant temperature. In this study, many aspects are discussed; *C. necator* growth using an ACSSF system is presented in section 7.3.1. A scanning electron microscopic study was applied to investigate the cell growth on polyurethane foam (PUF) inner pores and surface, and how a washing step affects the biomass recovery is discussed in section 7.3.2.2. Many factors affected the ACSSF system such as incubation time, particle size, initial moisture, PUF depth and effect of container size are presented in sections 7.3.2.3, 7.3.2.4, 7.3.2.5, 7.3.2.6 and 7.3.2.7 respectively, while the effect of repeat batch ACSSF is shown in section 7.3.2.8. The summary of this chapter is presented in Section 7.4.

7.3.2 Results and discussion of ACSSF

In this part of the study, *C. necator* growth and the factors affecting the ACSSF kinetics system include the carrier particle shape, size, initial moisture, incubation time, depth and container size were investigated, as follows.

7.3.2.1 *C. necator* growth on PUF

In order to investigate *C. necator* growth using an inert support (PUF) impregnated in a medium derived from waste date seed, different fermentation experiments using PUF were conducted. The results of these experiments are presented in Figures 7.6 (A-D) which shows *C. necator* growth in a medium derived from waste date seed using ACSSF fermentation system, and scanning electron micrographs of the PUF after four days of fermentation.

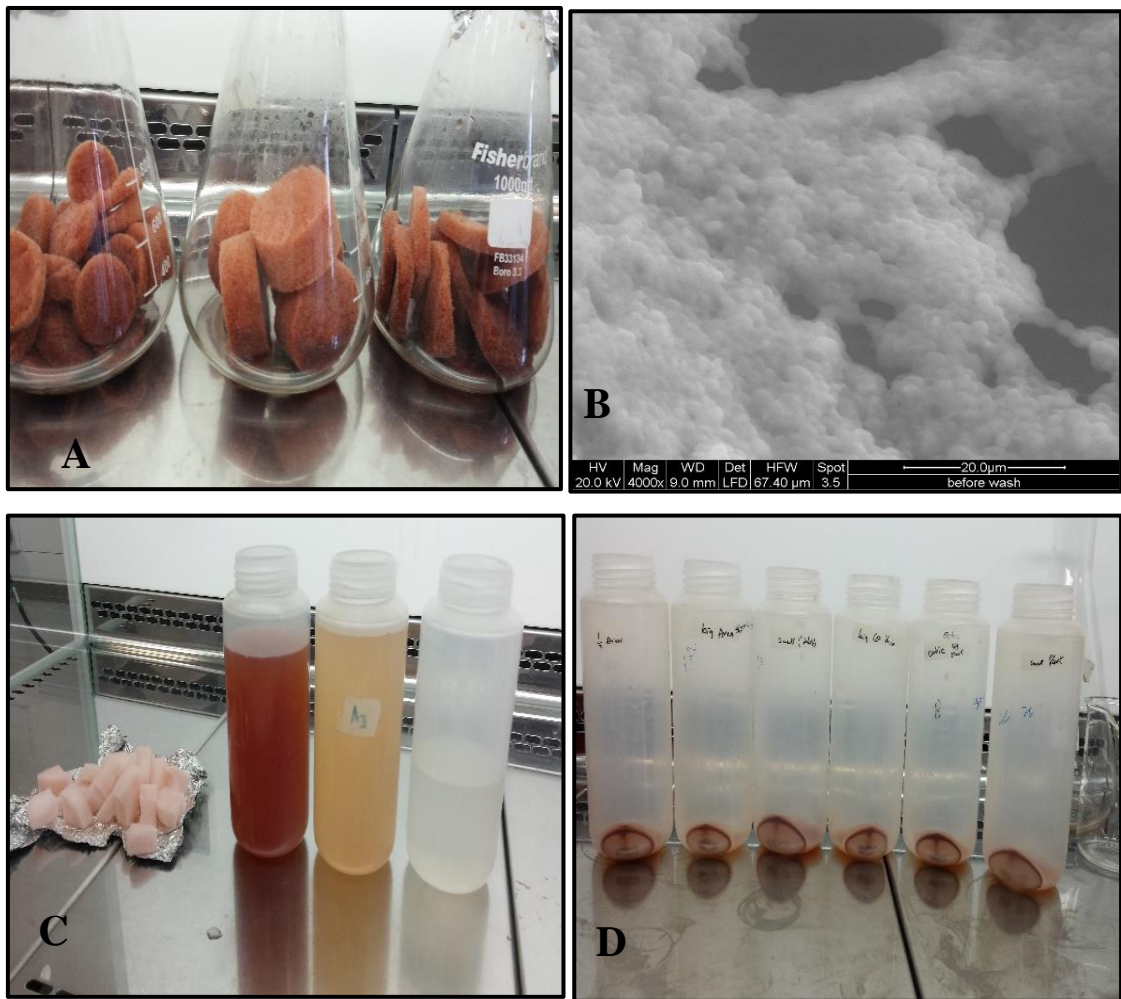


Figure 7.6. *C. necator* growth in a medium derived from waste date seed using SSF fermentation system, applying PUF as an inert support; (a) *C. necator* immobilisation and growth on PUF impregnated with a media derived from waste date seed (b) scanning electron micrographs showing *C. necator* growth on PUF after 4 days of fermentation (c) reveals the biomass retrieved by repeated washing using water, (d) biomass recovered from PUF after four washes.

From Figure 7.6 it can be noticed that immobilised *C. necator* has the ability to grow on both surfaces and inside the various shapes of PUF, with a maximum biomass concentration of 0.05 ± 0.01 g/g PUF. Figure 7.6 B. SEM image was taken to demonstrate the capability of *C. necator* growing in PUF, while Figure 7.6 C, presents the effectiveness of washing times on biomass recovery from the PUF after four days of fermentation. Recovered biomass from PUF after four times of washing and centrifuged at 7000 rpm and 15 min, as shown in 7.6 D.

7.3.2.2 Scanning electron microscopic (SEM) study

Due to the high internal surface area PUF allows for a large number of cells to be adsorbed and immobilised in the supporting structure. Furthermore, PUF provides a continuous, homogenous aerobic condition until the end of incubation time. However, the recovery of bacterial biomass from the medium after fermentation is a quite difficult task.

Since PHB granules are produced as intracellular inclusion bodies within the microorganism cytoplasm, it is essential to be able to extract the bacterial cells from the PUF efficiently and easily. After fermentation, SEM images were taken in order to check the effectiveness of biomass retrieval from PUF, images are shown in Figure 7.7. From figure 7.7 it can be observed the extremely good growth of *C. necator* on the PUF and all most there is no biomass cells left after four wash cycles.

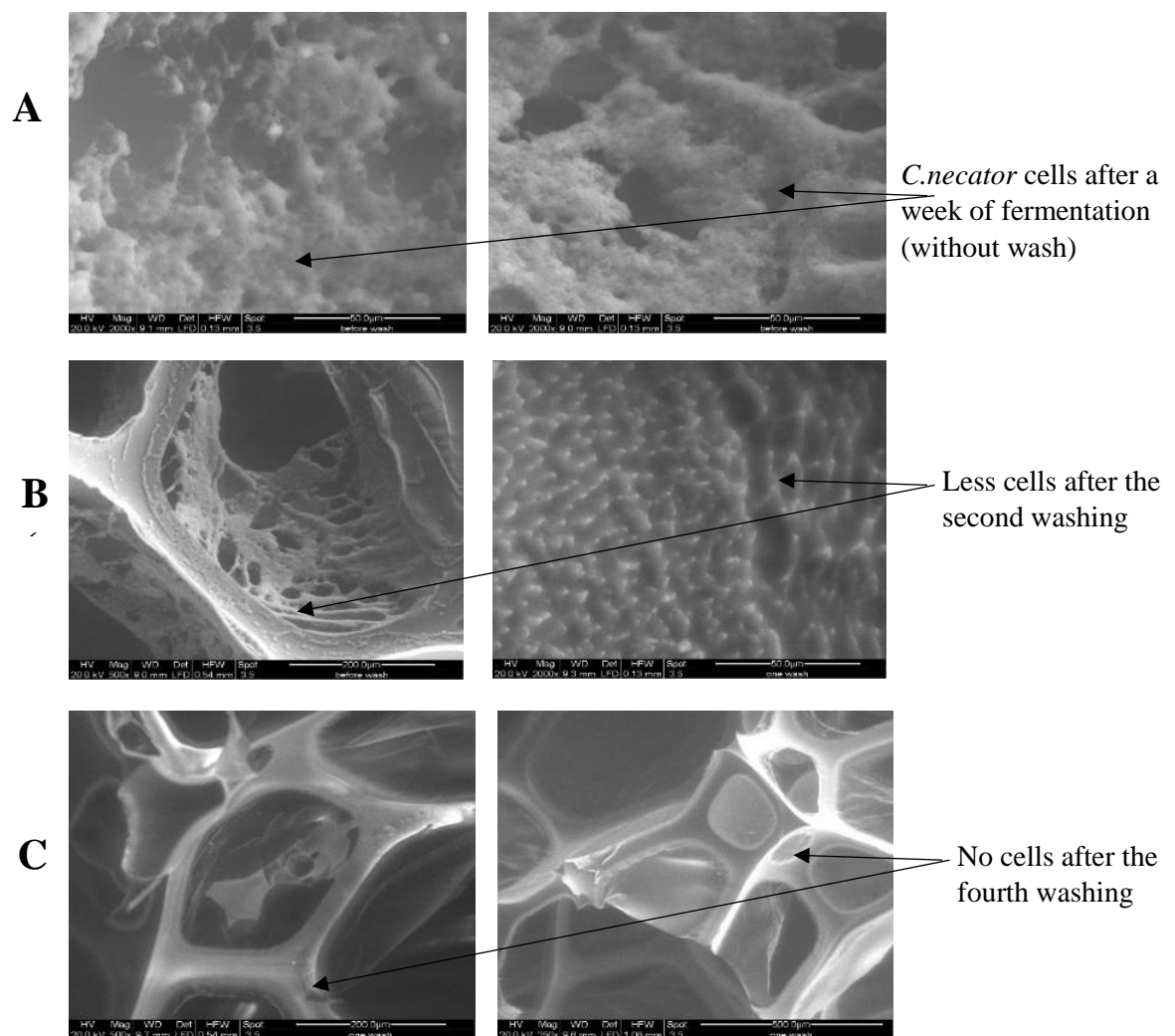


Figure 7.7. Scanning electron micrographs images taken for *C. necator* after growth in a media derived from waste date seed containing 11.8 g/l fructose concentration (A) PUF after four days of fermentation (without washing) (B) second washing, and (C) after fourth washing.

After each washing step using distilled water, PUF samples were observed and SEM images were taken. The SEM images revealed that most of the cell biomass was retrieved by repeated washing. The images of SEM revealed that the fourth wash almost completely retrieved the cell biomass. Although this method is effective on a lab scale, it is not efficient on the industrial scale because it requires large quantities of water as well as difficulty controlling the system.

7.3.2.3 Effect of incubation time on cell growth and PHB accumulation

In the case of *C. necator* it is imperative to estimate the optimum incubation period because *C. necator* can accumulate PHB and then subsequently consume it as nutrient depletion increases with prolonged fermentation time (Benoit et al., 1990) and (Nam and Ryu, 1985). In order to investigate the effect of incubation time on cell biomass growth and PHB accumulation, fermentation experiments were conducted at a range of incubation times, one week, and the effect of incubation time on PHB production and biomass is presented in Figure 7.8.

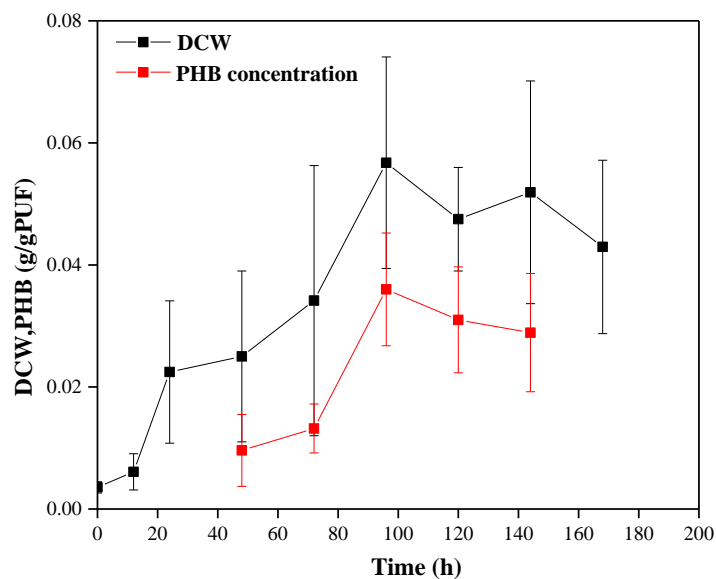


Figure 7.8. Effect of incubation time on cell growth and PHB production by *C. necator* using waste date seed derived media under incubation conditions, 30 °C and 170 h.

From the results in figure 7.8, it was evident that biomass and PHB concentration were quite low 0.022 ± 0.021 g/g PUF, for the first 24 h and then a drastic rise could be seen to give the maximum value after 96 h 0.056 ± 0.013 g/g PUF and then decreased. PHB accumulation followed the similar trend which produced 0.006 ± 0.011 g/g PUF after 24 h and then increased to PHB 0.036 ± 0.025 g/g PUF after four days, 96 h; the results indicate

that the long fermentation time would lead to excessive consumption of PHB content. Considering the actual accumulation, the incubation time was controlled in almost four days (96 h). A decrease in biomass and PHB after four days can be explained as a result of PHB utilisation by the *C. necator* for further growth. From the results it is obvious that the error associated with these measurements is large, due to difficulties in sampling related to the heterogeneity present in SSF system. Therefore, in the next experiments for this study, the flask contained the samples be fermented for one week and the biomass will recover as a whole by the end of the fermentation period.

7.3.2.4 Screening of PUF particle size

Both particle size and the specific surface area of the carrier are the most important factors affecting both the microbial activity and the oxygen transfer rate in SSF system. Therefore, selecting an appropriate particle size will meet the oxygen transfer rate and the growth of microorganisms (Nampoothiri and Pandey, 1996). In this study, 2 g of PUF at a various size, A, B, C and D immobilised with date seeds derived media that used for *C. necator* growth of and PHB production. The maximal biomass and PHB obtained were presented in Figure 7.9. From the figure, it can be observed that both biomass and PHB concentration increased by increasing the size, surface area, of the carrier until the particular point and then both were decreased.

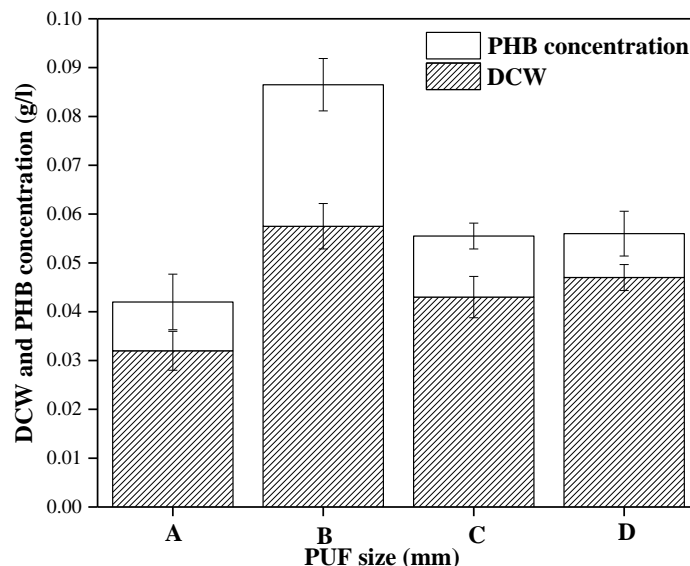


Figure 7.9. Effect of PUF on cell growth of *C. necator* and PHB production in a medium derived from waste date seed. (A: 5×5×5 mm, B: 10×10×5 mm, C: 15×15×5 mm, D: 20×20×5 mm).

In general, the results show that when the PUF size was increased from size (A) to size (C), both DCW and PHB concentration were increased, while when the size increased to size D, there was no significant difference compared with size C. The highest PHB concentration 0.032 ± 0.002 g/g PUF, 52%, and biomass 0.061 ± 0.003 g/g PUF when size B was used, while they were 0.008 ± 0.001 g/g PUF, 17.7%, and 0.045 ± 0.002 g/g PUF, respectively at size A. When PUF was at size C, the maximal biomass was obtained 0.011 ± 0.002 g/g PUF, 29.7%, while PHB was 0.037 ± 0.001 g/g PUF. Finally, size D gave a PHB concentration 0.013 ± 0.001 g/g PUF, 27.4% and biomass 0.038 ± 0.002 g/g PUF.

The effect of particle size is based on the fact that the larger the particle size, the lower the specific surface area, which limits the adsorption and growth of microorganisms. Smaller particle size will obtain less porosity, but the specific surface area will be increased (Hu et al., 2011). From the results, it can be concluded that with larger particles, the surface area available for bacteria growth is less, while the interparticle space and hence the porosity increase, but with smaller particles of the substrate the situation is reversed. These two opposing factors perhaps interact and consequently determine the activity and growth of microorganisms.

The oxygen transfer role into a void space impacts the microorganism growth, and a compromise must always be made regarding the substrates composition with the particle sizes for optimum mass transfer and activity effects. These results agree with Oriol et al., (Oriol et al., 1988).

7.3.2.5 Effect the PUF depth on bacterial growth and PHB production

The PUF depth is the most important factors which impact mass and heat transfer in solid state fermentation system. Four various depths of PUF PHB were investigated for cell biomass growth and PHB accumulation, and the results showed in Figure 7.10. The results show that the biomass concentration and PHB content increased with increasing the PUF depth up to a maximum at 15 mm and then subsequently decreased when the depth was further increased to 20 mm.

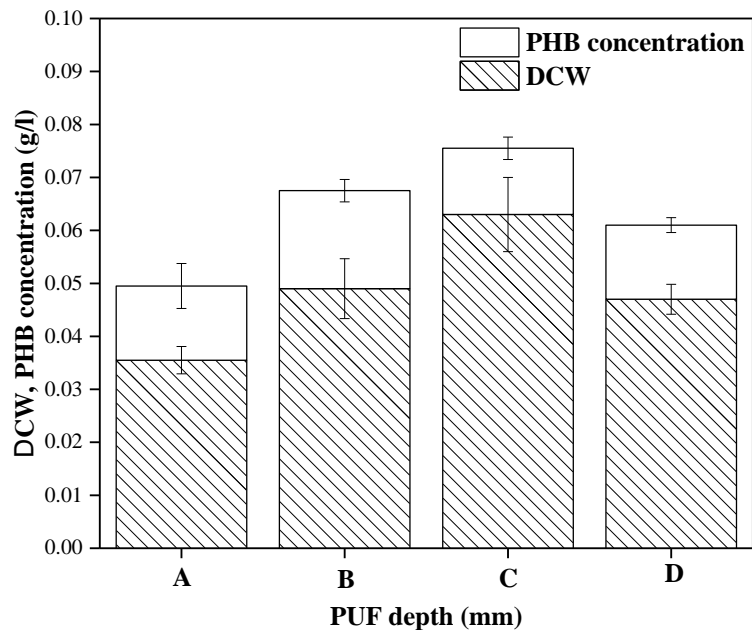


Figure 7.10. Effect the depth of PUF on *C. necator* growth and PHB production after four days of fermentation time and using date seed derived media. A: 5×5×5 mm, B: 5×5×10 mm, C: 5×5×15 mm, D: 5×25×20 mm.

The highest total biomass yield and PHB concentration were 0.063 ± 0.011 g/g_{PUF} and 0.0165 ± 0.01 g/g_{PUF} respectively, at a depth of 15 mm, PHB content of 25 %, A dramatic improvement in total biomass and PHB concentration were achieved when the depth of inert carrier, PUF, was increased, due to improving oxygen transfer rate and hence benefiting the growth of microorganisms and PHB production while for further increase in the depths, both growth and PHB production were decreased. Increasing depth after the specific level was quite high negatively impacted the respiration of the bacteria and this is could be due to increasing the volume compared to surface area of PUF which causing decreased in oxygen transfer.

7.3.2.6 Effect of initial moisture content on the production of PHB

Moisture is a critical factor in ACSSF processes as bacteria need a water activity for growth. The moisture in SSF process exists in the form of absorbed or complex within the solid matrix, which is considered to be advantageous for the growth due to the possible effective oxygen transfer process (Prabhakar et al., 2005). If the amount of the water is insufficient and does not provide for good diffusion of gases and solutes, the metabolism of the cell can be slowed or stop, due to a lack of substrate or through accumulation of inhibitory metabolites in or close to the cell (Gervais and Molin, 2003). In this section, different fermentation experiments were carried out applying various ratios of broth amounts to PUF; the ratio of solid/liquid was in the range 1:5–1:35. The efficiency ratio of solid/liquid on the biomass production and PHB accumulation was studied. The result is shown in Figure 7.11. Generally, from the results, both biomass and PHB concentration were increased by increasing the initial moisture until reaching a maximum after which the biomass decreased.

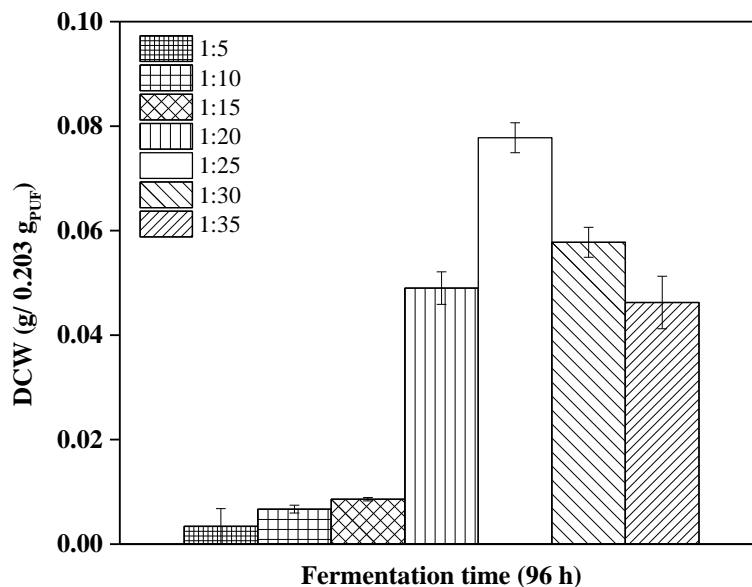


Figure 7.11. Effect of solid–liquid ratio on *C. necator* growth and PHB production after four days of fermentation time and using date seed derived media using adsorbed carrier solid-state fermentation.

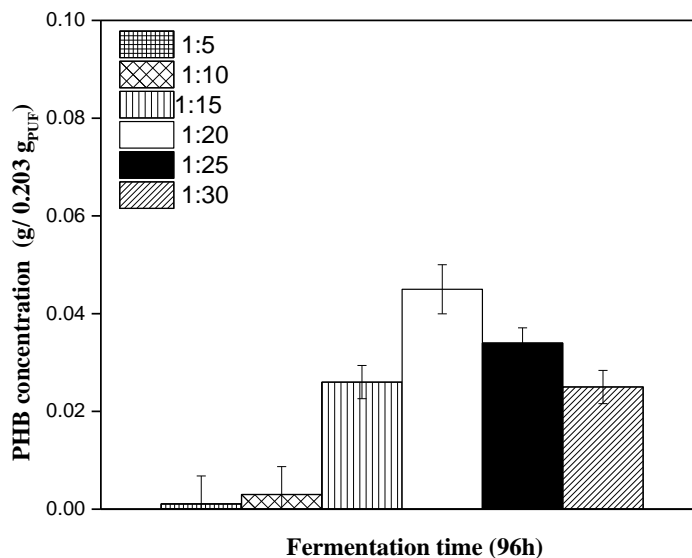


Figure 7.12. Effect of solid–liquid ratio on *C. necator* growth and PHB production after four days of fermentation time and using date seed derived media using adsorbed carrier solid-state fermentation.

From Figures, 7.11 and 7.12, it can be noticed that when the solid/liquid ratio was 1:25 the bacterial produced the highest biomass and PHB concentration of 0.077 ± 0.002 g/g PUF and 0.045 ± 0.001 g/g PUF, respectively, due to the broth being absorbed uniformly on the PUF surface, and there was no considerable gradient distribution of fermentation broth. With the decrease in the ratio of solid/liquid, the content decreased; this was because there was also little media, and hence nutrients, to satisfy bacterial growth.

For further increasing in solid/liquid ratio, 1:35, free media appeared due to the restricted water-absorbing capacity, and both biomass and PHB concentration were decreased to 0.046 ± 0.003 g/g PUF and 0.025 ± 0.001 g/g PUF, respectively. The film thickness of the liquid on the PUF surface increased, while the porosity reduced; the oxygen transfer rate was limited, hence inhibited the microorganism's growth. This could be due to the lower amount of biomass present in 1 ml inoculum, which might be insufficient to utilize the nutrients present in the production medium. Further increase in inoculum size leads to decrease in the biomass accumulation. When the moisture level higher than optimum, the porosity decreases because promotes the development of stickiness, reduces both gas volume and diffusion, which results in reduced oxygen transfer rate (Ramesh and Lonsane, 1990).

7.3.2.7 Effect of container size on *C. necator* growth and PHB accumulation

As the effect of initial moisture was studied, it was necessary to investigate the effect of container size on cell growth and PHB production (effect of oxygen supply on cell growth and PHB accumulation). Fermentation experiments were carried out using $(2 \pm 0.002 \text{ g})$ of PUF which inoculated with 25 ml of culture at three different flask sizes (250 ml, 500 ml and 1L), and the results were presented in Figure 7.13. The results show that *C. necator* growth was increased when the flask size increased, whilst conversely the PHB concentration decreased by increasing the container size.

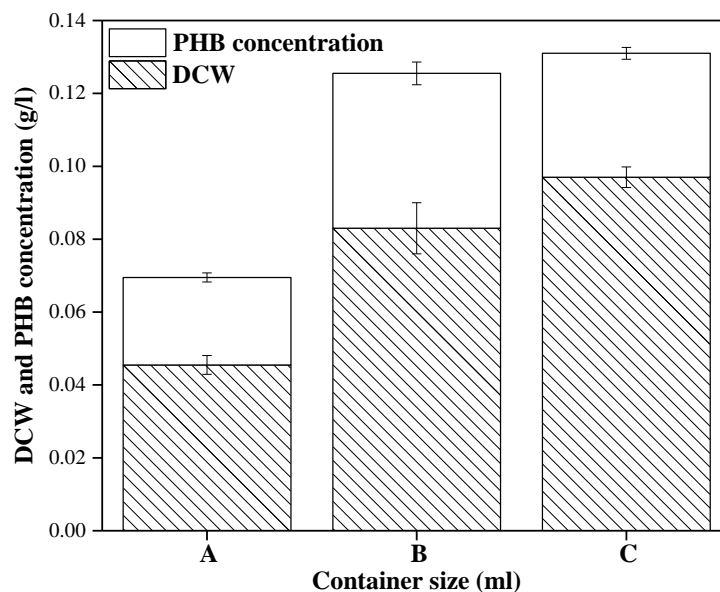


Figure 7.13. The effect of container size, contained PUF as a carrier support, on *C. necator* growth and PHB production in a media derived from waste date seed.

The results in Figure 7.13 show that the maximum biomass was achieved $0.097 \pm 0.001 \text{ g/g PUF}$ at size C (1 L), with a PHB concentration of $0.034 \pm 0.0012 \text{ g/g PUF}$. The highest PHB concentration of $0.045 \pm 0.002 \text{ g/g PUF}$ was obtained at size B (500 ml), from a biomass concentration of $0.081 \pm 0.001 \text{ g/g PUF}$. Oxygen plays an essential role in the biosynthetic system within cells. Some bacteria, such as *C. necator* could produce large numbers of PHB under oxygen restriction conditions. Limitation of accessible oxygen inhibits growth and PHB production by individual organisms in a specific way (Annur et al., 2008).

PHB production was suppressed at higher oxygen transfer rate. (Barron, 1955) suggested that the harmful influences of oxygen rate on biochemical materials were because of non-specific oxidation of enzymes. Similarly, an effect of aeration on bacterial growth and PHB production was reported for various bacteria (Stockdale et al., 1968), (Senior et al., 1972) and (Pal et al., 1999).

7.3.2.8 Repeated batch bioreactor

The effect of repeated batch bioreactor on cell growth and PHB production was studied using date seeds derived media, and the results were shown in Figure 7.14. A 20 ml of media was fed to the culture broth, as described in Section 4.2.4.3.7 after squeezing the same volume (20 ml) of the medium after 24 h of fermentation time then repeated every 24 h. Biomass production and PHB accumulation were analysed every 12 h during the fermentation period. Both total biomass and PHB concentration were continued increasing until the end of fermentation time.

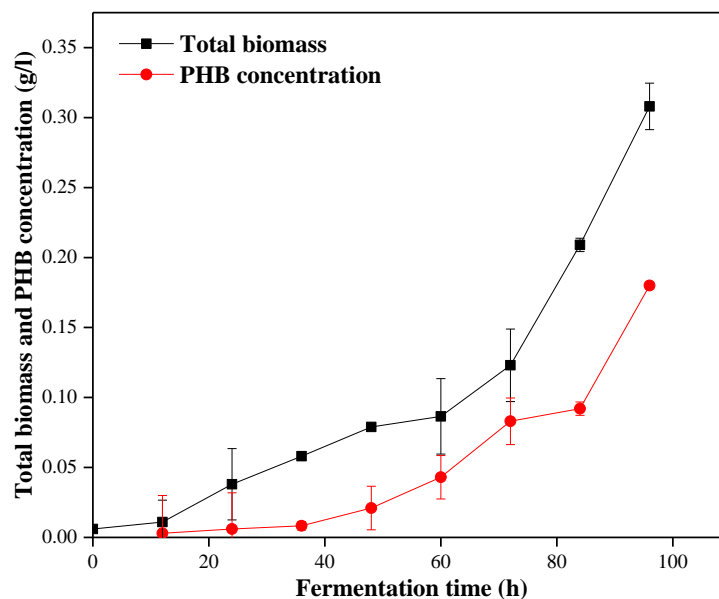


Figure 7.14. Batch fermentation kinetics of *C. necator* growth at repeated batch cultivation using solid-state fermentation using PUF as a carrier support.

The total biomass concentration was 0.011 ± 0.001 g/g_{PUF} g/l with PHB concentration was 0.003 ± 0.002 g/g_{PUF} at 12 h of fermentation time then were increased to 0.308 ± 0.001 g/g_{PUF} and 0.18 ± 0.001 g/g_{PUF}, respectively at the end of fermentation, after 96 h, which represent the highest biomass and PHB achieved. These results indicate that the repeated batch PHB production could be achieved by maintaining PHB concentration at a high value and retrieving a part of the biomass periodically. In this method, PHB concentration is about 3.9 times higher than that of normal SSF batch cultivation

7.4 Summary

In general, this chapter was divided into two parts; the first part, part A, about the investigation the effect of the mixed substrates, fructose and oil derived from waste date seed, on the *C. necator* growth and PHB accumulation. Whilst the second part, part B, a study of PHB production by *C. necator* under ACSSF process using PUF as inert support.

In part A, different fermentation techniques have been applied: batch cultivation using various ratio of substrate (1:1, 1:3 and 3:1 fructose to oil) and fed-batch: feeding oil after 48 h of fermentation time. The results from this study indicate that *C. necator* can metabolise both substrates sequentially, diauxic growth, taking fructose as a preferable substrate over oil for all conditions while they followed normal growth curve, single substrate when the second substrate (oil) fed to the culture after 48 h of fermentation. When a pure oil was added into a date seed derived media containing fructose at 10.8 g/l concentration, *C. necator* consumed the all fructose firstly, after 48 h of fermentation time, to give the highest OD_{600nm} and PHB concentration and total biomass were 16.77 and 12.65 g/l, respectively, with PHB content of 60.9%. A dramatic improvement in total biomass were 14.38 and PHB content 85.9 % were achieved when the second fermentation finished, and all oil was used by the end of fermentation time. Although all the ratios gave the same trend for both biomass and PHB concentration, the ratio of (1:1 fructose to oil) produced the highest biomass and PHB concentration of 15.22 g/l and 12.36 g/l (with PHB content 84.1 %), respectively. Followed by the ratio (3:1 fructose to oil) which gave biomass 14.65 g/l with PHB 11.26 g/l. While biomass and PHB concentration were 13.46 g/l and 9.16 g/l respectively, when the ratio (3:1 fructose to oil) was used. *C. necator* did not follow the diauxic growth when the oil was fed to the date seed derived media contained fructose as first carbon source to produce the highest biomass and PHB concentration of 14.36 g/l and 12.32 g/l, with 85% w/w PHB content, after 48 h of fermentation.

Although submerged fermentation system (liquid fermentation) has been the focus of most researches in this area of the study, in the last decade solid state fermentation has paid the attention of many researchers because of advantages including low-cost waste materials availability as solid substrates for the microbial growth as well as easier downstream processing. Consequently, in this study, the possibility of PHB production in a solid culture and compared the result with defined liquid culture under similar conditions was investigated. Waste date seed was chosen as culture media for microbial growth and -PHB formation.

Results showed a maximum PHB content in the range 70-80% in liquid fermentation within 48-60 h while the maximum PHB content of 5.98 g/l was achieved in ACSSF within one week of fermentation time. This confirms that submerged fermentation processes are preferable for this system. The major inherent issue associated with PHB accumulation in SSF systems is the biomass retrieval from bacterial cells. This restriction can, however, be overcome by applying PUF as an inert support was presented in Part (A). The results reveal the potential of SSF strategy utilising PUF as an inert carrier for the PHB production and was a successful alternative to traditional SSF method with ease. The results showed that the cell biomass recovered from PUF after fermented using date seed derived media was appreciable.

Different images for the fermentable PUF were taken using SEM machine and the results indicated that almost all cell biomass revealed from PUF after four washes with water. The next step was investigated various factors that influencing the cell biomass and PHB accumulation including fermentation time, surface area and the PUF depth. The obtained results showed that both biomass and PHB concentration increased by increasing the surface area and the depth to give the highest values of 0.011 ± 0.002 g/g PUF, 29.7% while PHB was 0.037 ± 0.001 g/g PUF, respectively by increasing the surface area. Other conditions were investigated in an attempt to increase the *C. necator* growth and consequently PHB accumulation, therefore, a repeated batch bioreactor system was conducted, based on the assumption that repeated batch bioreactor would help *C. necator* to grow more. The results showed that the *C. necator* grew 3.9 times higher than that of one stage batch fermentation through only 4 cycles of repeated fermentation giving the maximum cell biomass and PHB yield of 0.308 ± 0.001 g/g PUF and 0.18 ± 0.001 g/g PUF, respectively. Based on the obtained results, it can be concluded that ACSSF can be utilised as an alternative strategy for PHB production. However, both further standardisation and the economics of the process are required to be assessed for further scale-up of the process.

CHAPTER

EIGHT

8 Characterisation of extracted PHB

8.1 Introduction

PHB is a thermoplastic, completely biodegradable polyester which has the high degree of crystallinity and a melting temperature in the range 165-170°C. PHB is piezoelectric, has optical activity and good membrane barrier properties. It has mechanical and physical properties similar to those of isotactic polypropylene polymer (IPP), being brittle and stiff, with poor effective flexibility and resistance. The degree of brittleness depends on the crystallinity, micro-structure and glass transition temperature. When stored at room temperature PHB becomes more brittle over time (Valappil S. P et al., 2007). In order to fully, positively identify and analyse the products accumulated by *C. necator* after fermentation of substrates derived from waste date seed (fructose, oil, mixed-substrate and ACSSF system), various characterisation techniques were applied and the results are presented in this chapter. This chapter contains different analytical techniques were used to determine the monomeric compositions of PHB; Fourier Transform Infrared (FTIR) spectroscopy as well as Nuclear Magnetic Resonance (NMR) were used to observe the structure of polymer chains. NMR is an analytical chemistry technique which can be utilised to explore content, purity and molecular structure of polymer samples; analysis results are shown in Section 8.2.2.1. FTIR spectroscopy is also a routine technique which can be applied to investigate the molecular structure of polymers, with results obtained from this technique being discussed in Section 8.2.2.2. PHB is thermal unstable at temperatures above its melting point, which causes significant difficulty in processing by applying traditional methods. PHB crystallinity was determined using Differential Scanning Calorimetry (DSC) by quantifying the heat associated with fusion state, and this heat is presented as percent crystallinity (X_c), this technique and the results are reviewed in Section 8.2.2.3. Gel permeation chromatography (GPC) is applied to determine the molecular weight of the polymer samples and results obtained are presented in Section 8.2.2.4. Biodegradability was the last PHB property investigated in this study and the results are described in Section 8.2.2.5. Finally, a summary of the findings presented in this chapter is given in Section 8.2.

8.2 PHB characterisation results and discussion

8.2.1 Extraction of biopolymer

Solvent extraction is the common method used to recover PHB from cell biomass and is applied routinely in the laboratory because it is quick and simple. This method has advantages over other methods of PHB extraction, such as digestion method, mechanical disruption, and alkaline solution treatment (B. Kunasundari, 2011), in terms of efficiency, due to the ability to separate any endotoxins which would otherwise cause polymer degradation. Consequently, it is possible to extract higher purity PHB with greater molecular weights (Jacquel et al., 2008).

Extraction using chloroform, as described in Chapter 4 section 4.3.3.3, was applied to recover PHB from *C. necator* biomass, with the maximum yield achieved using this method was 95% (w/w). Figure 8.1 presents the PHB extracted from *C. necator* inclusion bodies.

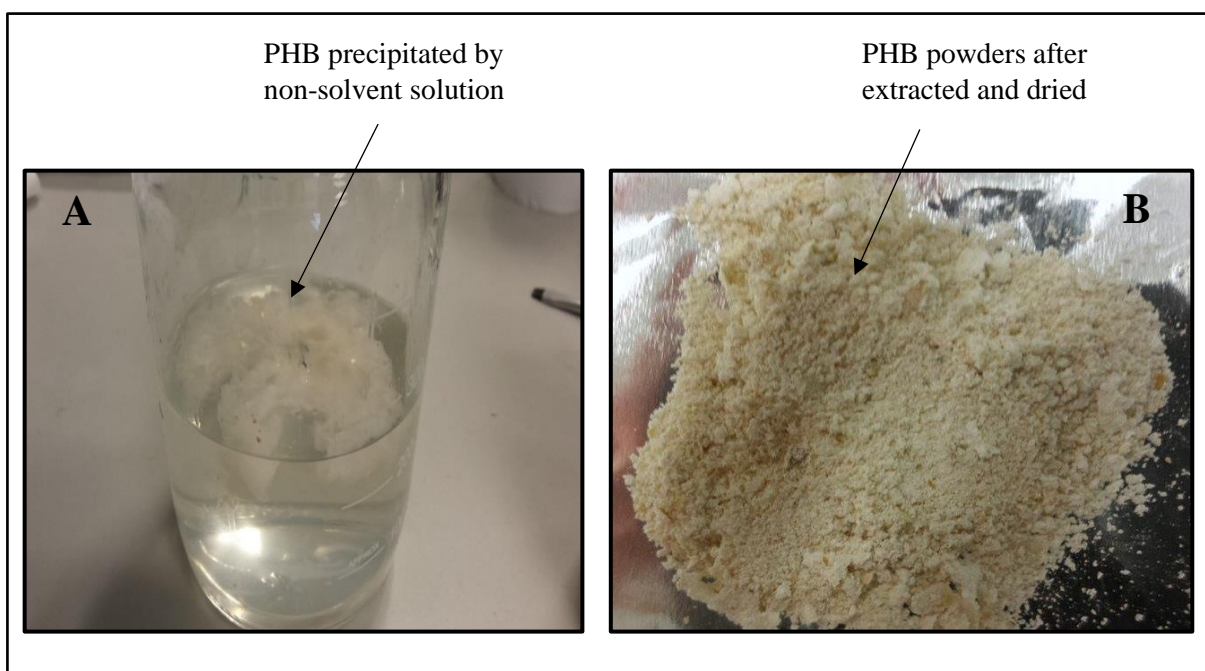


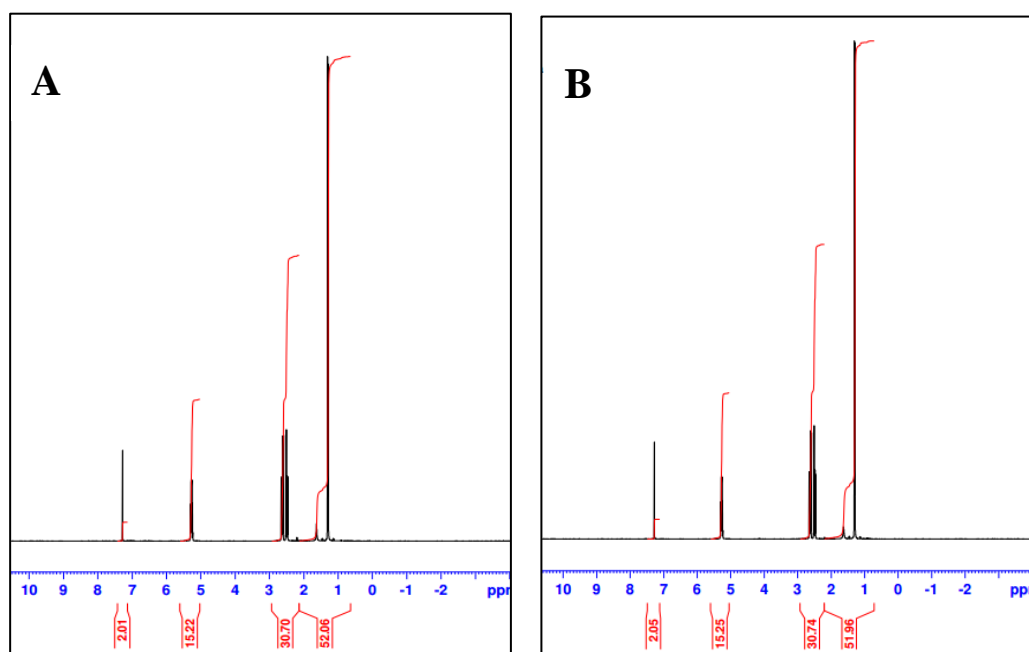
Figure 8.1. (A) PHB precipitated using non-solvent solution (methanol and distillate water), (B) the PHB after extraction from *C. necator* inclusion bodies after drying for 2 days at 60 °C.

This result was agreed with the result that reported by Fei et al., (Fei et al., 2016) which obtained the maximum yield of PHB of 94% when extracting from *C. necator* using chloroform under 130°C and 30 min without any pre-treatment.

8.2.2 Characterization of the extracted biopolymer

8.2.2.1 ^1H NMR analysis

In order to determine the structural composition of the PHB synthesised from date seed derived media, as the structure presented in Chapter 2 section 2.5, ^1H NMR method was applied. Figure 8.2, presents ^1H NMR peaks for PHB sample accumulated by *C. necator* grown on various types of carbon source derived from waste date seed and fermentation system. All the results show there is no difference between the samples produced from the different waste date seed derived substrates and fermentation strategies. ^1H NMR spectra of PHB synthesised by *C. necator* were obtained with major peaks because of resonance absorption representing the methyl (CH_3), methylene (CH_2) and methine (CH) groups, in 3-hydroxybutyrate (3-HB).



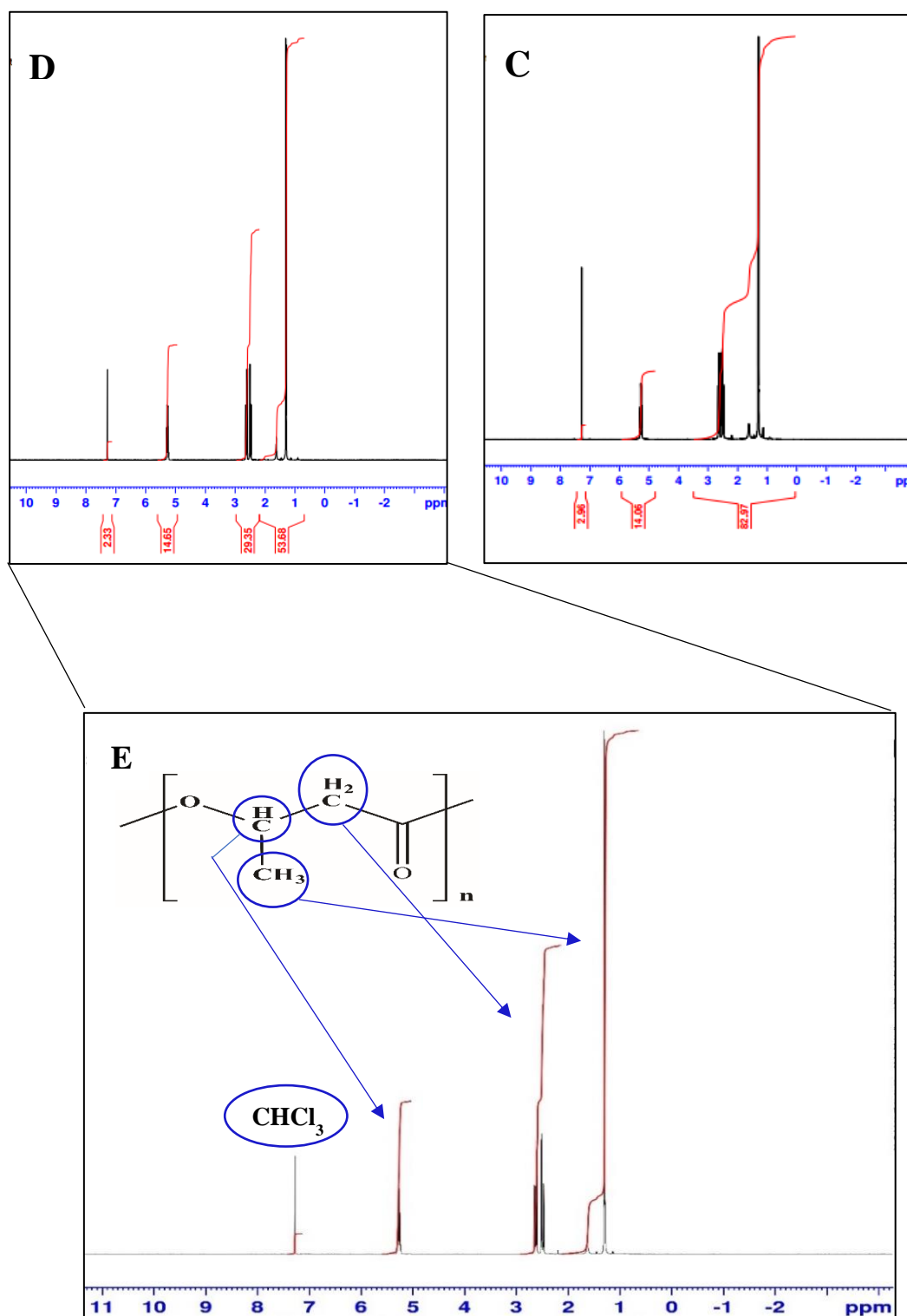


Figure 8.2. ^1H NMR of PHB accumulated by *C. necator* grown on (A) date seed hydrosate media using fructose as a carbon source, (B) date seed oil as a carbon source, (C) mixed-substrate and (d) PHB produced using ACSSF system while (E) zoom out to present the chemical shift signals of the HNMR spectrum and proton positions.

The ^1H NMR spectra show chemical shifts at 1.2, 2.4, 2.7 and 5.3 indicating the presence of a proton in position 3, 2 and 1 respectively, which represent the CH_3 , CH_2 and CH groups, while chemical shifts at 7.3 belong to chloroform, the solvent used in sample preparation. The molecular composition of the PHB as indicated by the chemical shifts that generate a backbone structure ($\text{CH}_2\text{-CH}$) attached to a CH_3 group (Jan et al., 1996) and (Zhu et al., 2010). The results of the present study match results reported by (Pal and Paul 2002) which found that the spectra of CH_3 at 1.2 ppm, CH_2 at 2.6 ppm and 5.3 ppm for CH group. Saruul *et al.*, (Saruul et al., 2002) identified that the peak values are at 1.2, 2.5 and 5.2 ppm for CH_3 , CH_2 , CH groups, respectively. As well as (Li et al., 2016) reported that the chemical shift signals of HNMR spectrum of extracted polymer were (1.26-1.28), (2.45-2.62) and (5.23-5.27) for CH_3 , CH_2 and CH respectively. Based on the literature, (Chaijamrus and Udupuay, 2008) found the chemical shifts range of the commercial PHB were (1.24-1.3) for CH_3 , (2.48-2.59) for CH_2 and (5.16-5.35) or CH . The results of this study exactly matched with the peaks of standard PHB, as identified in the literature, confirming the identity of the 3-HB monomer in the biopolymer.

8.2.2.2 Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was carried out in order to know the functional groups that present PHB samples extracted from *C. necator* when different carbon sources were used, date seed hydrolysate media, date seed oil and ACSSF system. FTIR spectra were recorded at a wavelength range from 400 to 4000 cm^{-1} . Figure 8.3 shows the FTIR spectra of PHB extracted from *C. necator* (A) using different carbon sources derived from waste date seed, fructose and oil, and (B) using ACSSF system. The results showed that the functional groups for PHB sample were identified as C-O, C-H and C=O. The methane group (C-H) showed a strong band in the range of 3047- 2914 cm^{-1} while the carbonyl group (C=O) occurred a strong band in the range of 1731-1673 cm^{-1} . The medium-strong of (CH_3) bond happened at 2359 cm^{-1} and 1342 cm^{-1} and a medium- weak (CH_2) stretching bond appear at 1430-1300 cm^{-1} . Also, medium-weak (C-C) stretching bond at 1949 cm^{-1} was obtained. An aliphatic asymmetrical (O-H) bond occurred at 3442 cm^{-1} .

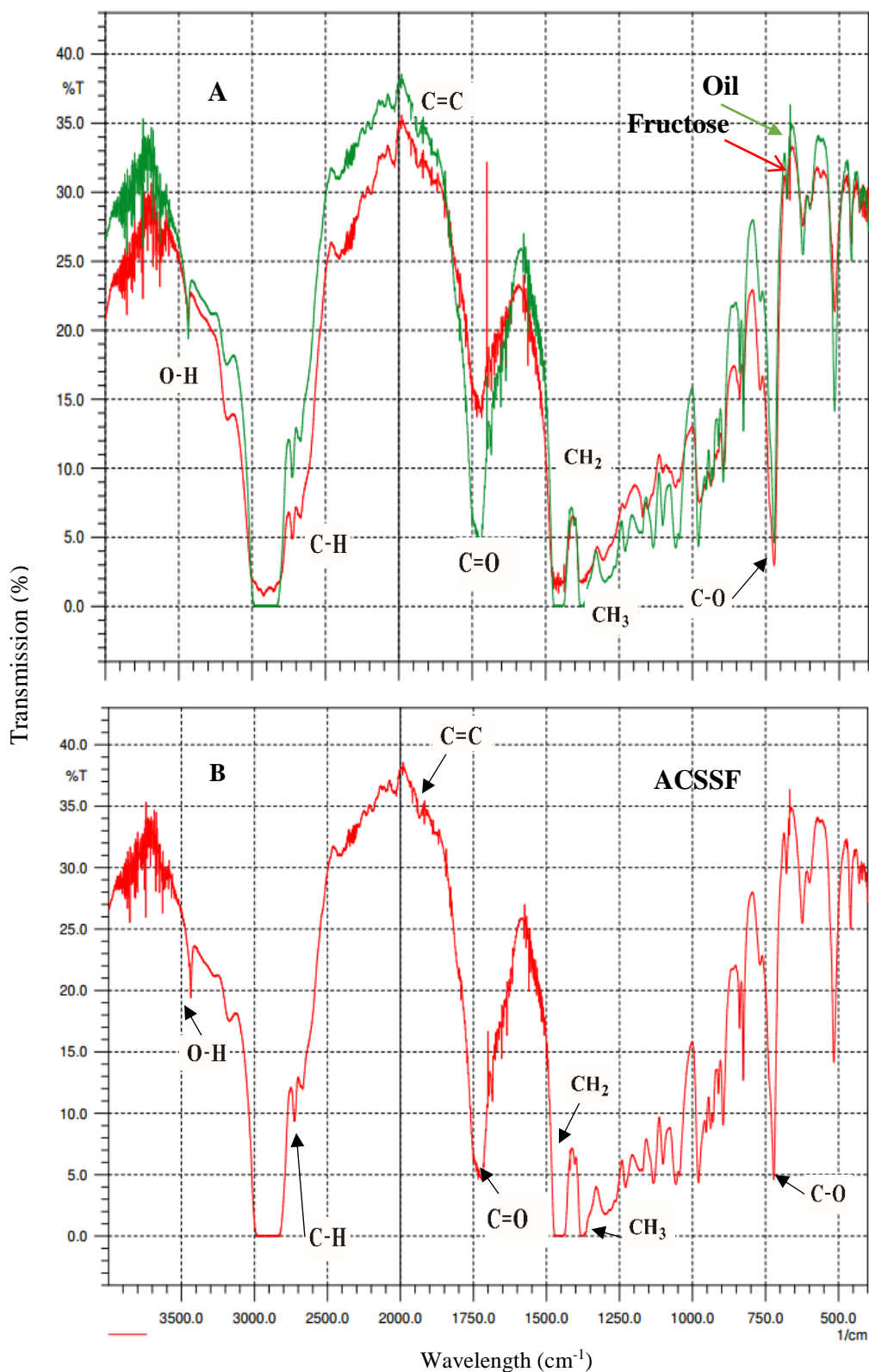


Figure 8.3. FTIR spectra of PHB extracted from *C. necator* (A) using different carbon sources derived from waste date seed, fructose (red) and oil (green), (B) using ACSSF system.

The spectra for PHB samples were also compared to the FTIR spectrum of standard PHB, as reported in the literature, and found to be composed of identical peaks positioning, meaning that all extracted biopolymers (produced in this work) are PHB. There were no significant differences noticed amongst the FTIR spectra for all PHB extracted samples. However, peaks intensity observed for PHB produced by date seed hydrolysed media were greater than the other PHB samples. The results obtained by this work are exactly agreed with the other researchers (Santhanam and Sasidharan, 2010) and (Shah, 2012). (Kumar and Prabakaran, 2006) reported that the spectrum results obtained from analysing PHB sample extracted from *C. necator* were recorded in the range of 100-4000 cm^{-1} . The FTIR spectra confirm that all PHB samples from date seed hydrolysed media, date seeds oil and ACSSF system are chemically identical. Furthermore, the results reported by Misra et al., (Misra et al., 2000) which found that the spectra of FTIR of the intact cells at positive absorption at 1724 cm^{-1} can be utilised as a device to screen the PHB synthesising microorganisms. Shamala et al., (Shamala et al., 2003) recovered PHB from a *Bacillus spp.* and subjected to FT-IR spectra. There were many intense absorption spectra typical to PHB, C=O and C-O groups respectively.

8.2.2.3 Thermal analysis using Differential Scanning Calorimetry (DSC)

All PHB samples were analysed using DSC to study the thermal properties including melting temperature and degree of crystallinity. The PHB tested was a crude product obtained via a single chloroform solvent extraction. The PHB extraction step gives purity in the range 80-90%, with little degradation (Manangan and Shawaphun, 2010) and (A. Aramvash et al., 2015).

DSC curves of PHB samples were obtained from two scan cycles of heating and cooling, conducted across a temperature range of -25 to 200°C at a scanning rate of $10^{\circ}\text{C}/\text{min}$, as presented in Figure 8.3. During the first heating cycle, the melting peak for the PHB samples was in the range of 171 - 173°C for and there was no detection of a glass transition for all the samples. The fact that no glass transition was observed is either due to T_g being much lower than the $\approx 2^{\circ}\text{C}$ expected or that at the resolution and scanning rate used the small change in heat flow associated with T_g was not picked up. In terms of material behaviour polymers with a lower T_g are generally tougher and have a higher degree of crystallinity, as T_g relates to the segmental mobility of the polymer chains. It could be that the lack of an observed T_g means that the PHB produced is in a rubbery state in the temperature range explored.

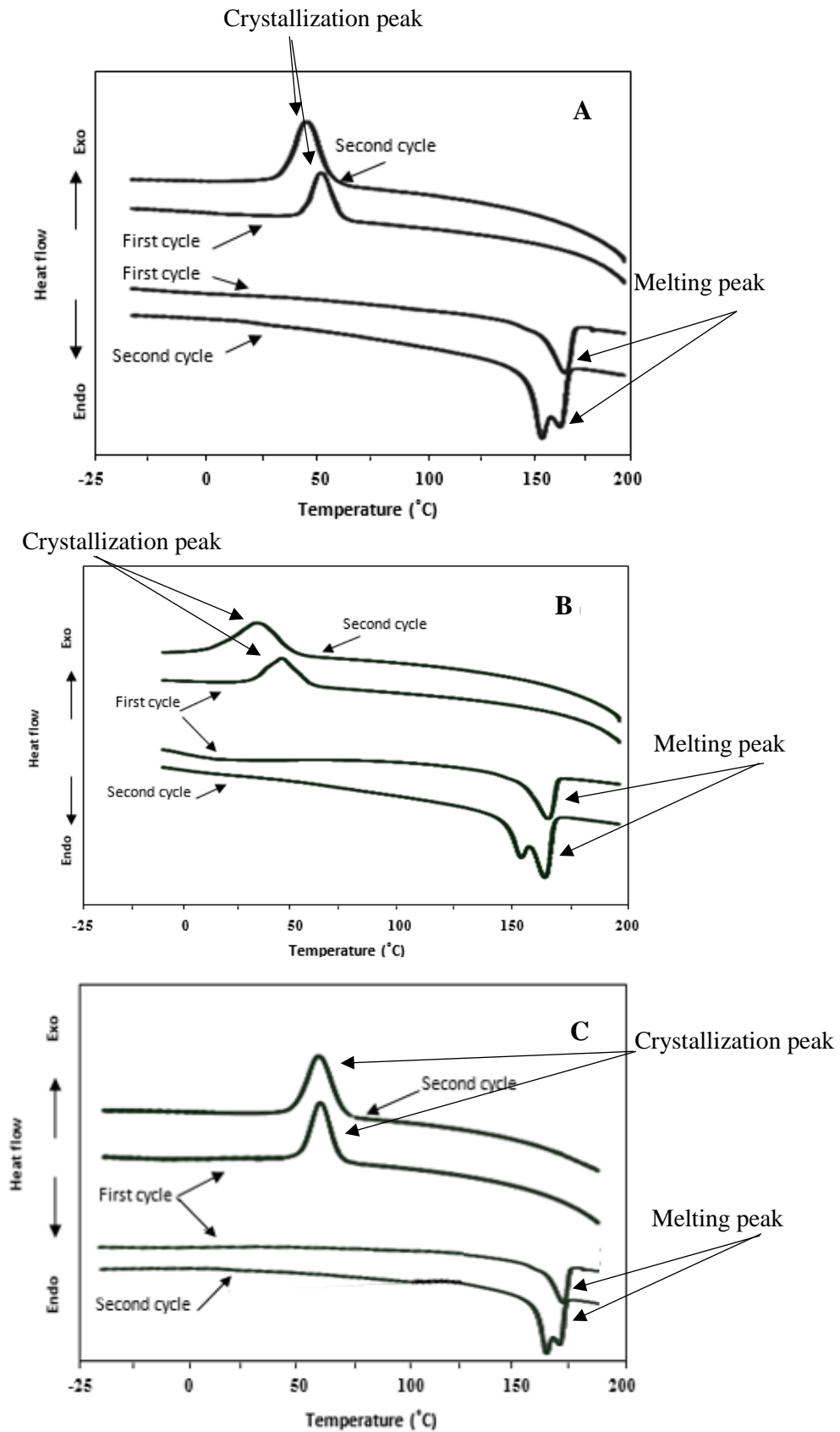


Figure 8.4. DSC curves for PHB sample obtained from (A) date seeds oil, (B) date seed hydrolysate media and (C) ACSSF system. Two scan cycles of heating and cooling were conducted within the temperature range from -25 to 200°C and scanning rate of $10^{\circ}\text{C}/\text{min}$.

During the cooling process, a crystallisation peak appeared at the range of 95-98°C. During the second heating run the PHB samples show a double melting peak, corresponding to the melting of “reformed” and re-crystallised PHB. Furthermore, there is a significant decrease in melting point compared with that of the first heating run, likely due to chain scission degradation of PHB. The peak representing the PHB melting point in the second scan appeared at 163-165°C, the corresponding crystallisation temperature was 88-90°C and the degree of crystallinity was 65-68 %.

These results agree with Rohini et al., (Rohini et al., 2006) who reported that the melting temperature of PHB pure is around 160-180°C whilst the crystallization temperature and degree of crystallinity are 90-120°C and 50-70%, respectively. The results for PHB produced from date seed oil in this study agree with those reported for standard PHB. Labuzek and Radecka, (Labuzek and Radecka, 2001), reported that PHB melting temperature ranged from 173.84 °C to 176.33 °C which was a slightly higher than compared with the value obtained in this study. While Pal and Paul (Pal and Paul, 2002) and the melting point of PHB was 171.34 °C which was comparable to the results showed in the present study. Consequently, characterisation of PHB produced from *C. necator* using substrate derived from the waste date seed confirmed not only the product purity but also presented the expected the plastic qualities.

8.2.2.4 Gel permeation chromatography (GPC)

Average molecular weight (M_w) and a number average molecular weight (M_n) of PHB can differ from 0.2 to more than 6×10^6 Da, with this variation based on concentration and type of substrate, availability of nutrients and growth conditions such as temperature and pH, the extraction method, as well as the microorganism type, controls the biopolymer molecular weight (Kusaka et al., 1997), (G. Q. Chen, 2010), (Doi, 1995) and (Lee, 2000). Thus, the ability to characterise M_w and M_n is critical in evaluating if the biodegradable polymers produced will have similar or superior properties to their petroleum-based counterparts (Myshkina et al., 2008). The molecular weight distribution determines the biopolymers end-use properties as it influences the supra macromolecular and macromolecular structures. It is known that the PHA molecular weight that synthesised biologically is much higher when compared with that chemically achieved (Higuchi-Takeuchi et al., 2016).

The PHB mechanical properties deteriorate when the weight average molecular weight is less than 0.4×10^5 Da, but for thermoplastic applications weight, average molecular weight value should be more than 0.6×10^6 Da.

Molecular weight analysis of the PHB isolated from *C. necator* using date seed hydrolysed media, date seeds oil, mixed-substrate and ACSSF system were carried out to determine the M_w , M_n , Refractive Index (RI) and Polydispersity which can calculate as shown in equation (48) and the GPC chromatograms results for all samples are listed in Table 8.1:

$$\text{Polydispersity} = \frac{\text{Average molecular weight (Mw)},}{\text{Number average molecular weight (Mn)}} \quad (48)$$

Table 8.1. Molar mass averages and polydispersity index of PHB samples produced from *C. necator* using various types of carbon sources including date seed hydrolysed media, date seed oil, mixed-substrate and another type of fermentation such as ACSSF system.

Sample	M_n (Daltons)	M_w (Daltons)	PD (M_w / M_n)	RI (ml)
PHB-Date seed oil	244	2.157×10^6	8.8	17.2
PHB-Date seed hydrolysed media	466	3.166×10^6	6.7	17.1
PHB-mixed-substrate	223	4.277×10^6	3.9	15.8
PHB-ACSSF system	191	0.975×10^6	5.1	17.3

From table 8.1, it can be seen that The M_w of the PHB produced from date seed hydrolysate media, date seed oil and mixed-substrate were in the expected range, while PHB obtained from using ACSSF system was less than other PHB samples. From the results, it is shown that PHB accumulated using mixed-substrate carbon source showed the highest M_w of 4.277×10^6 . Taidi *et al.*, (Taidi et al., 1994) have been reported that raise in M_w value of PHB can be facilitated by addition source of the substrate in the bacterial culture.

The weight average molecular weight and polydispersity of PHB produced were in the range 4.277×10^6 to 0.975×10^6 Da and 8.813 to 5.104, respectively. As there exist a large variety of PHA structures the weight average molecular weight can vary from 10×10^4 to 10×10^6 (G. Q. Chen, 2010). This variation is a result of the microorganism type, growth conditions and PHB extraction method. The polydispersity of PHA samples is usually in the range 1.2 – 6.

The higher value here indicates an increased degree of the variation in the PHB sample, likely due to the solvent extraction procedure used for recovery of the intracellular PHB.

The RI was also compared with all samples having similar RI values, except PHB obtained by the mixed-substrate source which was slightly lower. The significant difference in the value of RI between samples could be of concern if the PHB samples are prepared to be identical, due to the considerable differences in both RI and M_w can cause large impacts on the end-use of polymers properties. Variations in M_w and M_n of PHB can impact the biodegradability and thermo-plasticity of the polymers thus the differences in the M_w in PHB samples may not only impact the end-use properties but also the environmental effect of a product made with PHB. Generally, still the M_w of PHB samples at approximately 10^6 has a wide range of application in the medical field and so on (Guadalupe N.V., et al., 2013). According to the literature, it can be observed that the value of PHB M_w may be affected by the mechanism of PHB synthesis (synthetase) to some extent. When the concentration of nutrients was kept at a certain amount, both activity and concentration of PHB synthetase can achieve at an appropriate quantity for the high M_w of PHB synthesised in the strains.

On the other hand, it is important to know that the PHB content in the bacterial cells did not affect the M_w value of PHB significantly. The reduction in M_w and the raise of the PD value could be because of the depletion the availability of carbon source in the culture during long fermentation times (Xin et al., 2011). The carbon source depletion results in the bacteria using PHB granules as energy reserves and all microorganisms that produced PHB contain intracellular depolymerase for the PHB degradation. The degradation process happens along the polymer chain in a random way and could result in the significant variation of polymers chain length. The high rate of PHB producing resulted in the use of the available carbon source, the rest carbon source in culture was not enough to sustain the PHB production, therefore; microorganisms might have begun to use the PHB granules as energy reserves hence depolymerisation of PHB chains would give increase to a diversity of polymer chain lengths causing the decrease in M_w and increase in PD (Peña et al., 2014)

8.2.2.5 Biodegradability

The most attractive feature of PHB is its degradation in the natural environment. Biodegradability is known as the capability of the material to be broken down, particularly into innocuous products, via the action of the living organisms, such as microorganisms. In the natural environment, this job is taken care of by fungi and bacteria. They earn precursors

for the component of the cells and energy for their energy-requiring biological systems from this breakdown. Hence biodegradation can be considered as nothing more than a catabolism process. Biopolymer properties are related to their biodegradability and both the physical and chemical properties of biopolymer influence the biodegradation mechanism (Boskhomdzhieva et al., 2010). Biodegradation of biopolymers was achieved using various processes but with enzymes and microorganisms shows to be the most efficient process. When polymers are utilised as substrates for microorganisms, estimation of their biodegradability must not only be associated with their chemical structure, but also on their physical properties such as temperature, glass transition and crystallinity. The relationship between degrading microorganisms and biodegradability for different types of biodegradable polymers has been reported (Muthukumar and Veerappapillai, 2015).

In this work, biodegradation experiments were conducted in multi-purpose compost medium. The weighed samples were prepared by placing each sample in a non-degradable mesh bag then burying them 5 cm below the surface as described in Chapter 4 section 4.10.5. Biodegradation of PHB sample that extracted from *C. necator* after fermentation of substrates, fructose and oil, derived from waste date seed and ACSSF system by microorganisms was tested and an induction time for the biodegradation process can be observed. The results of this method are presented in Figure 8.5.

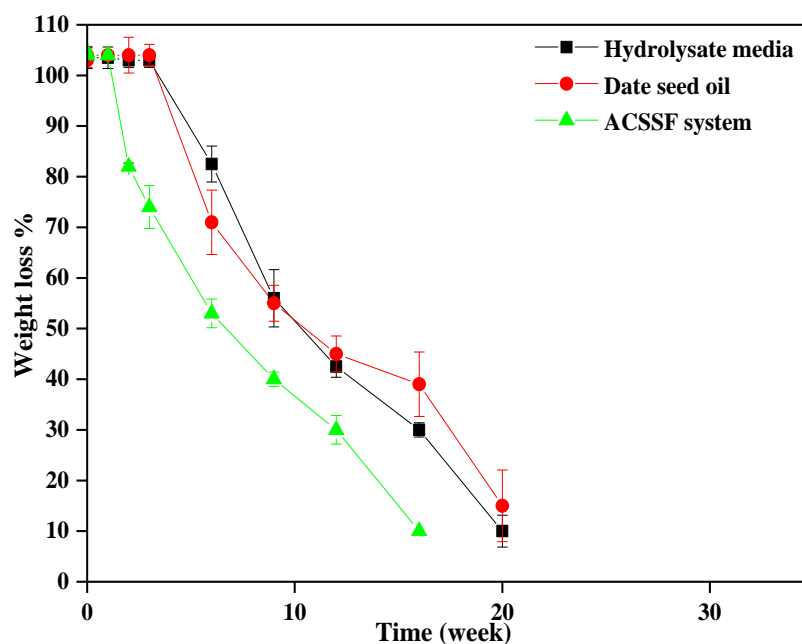


Figure 8.5. Represent the degradation weight-loss percentages (%) of PHB samples, which buried in compost medium as a function of time.

Figure 8.5 shows the degradation weight-loss percentages of PHB samples, which buried in compost medium as a function of time and calculated by Eq. (4.10). Since PHB is not soluble in water, its weight value in water shows an increment over the two weeks because of the water absorption before reach a constant value.

All PHB samples showed an increase in weight up to 102% and 102%,104% respectively, at the beginning of the compost burial test and this may occur due to water absorption by the samples.

PHB-ACSSF samples begin to degrade after three weeks, the weight change was a rapid decrease to become 60 % after about nine weeks and more decrease was observed to be completely degraded after fifteen weeks. While the other samples start degrading after about five to six weeks and significant weight loss was observed after twenty weeks biodegradation time. The results agree with the results reported by Zhang (Zhang, 2010) which found that the weight for both PHB and Biomer P226 was increased up to 101% and 102%, respectively. At starting of the burial in soil and as the time is increased to 10 weeks, the weight values reduce rapidly due to the biodegradation process. As well as Numata et al., (Numata et al., 2008) reported that the maximum rate of PHB degradation obtained at a moisture level of 55% and 60 °C was up to 85% within 7 weeks. Bonartsev et al., (Bonartsev et al., 2007) found that that the biodegradation rate of PHB depends on M_w of PHB, which the rate increase by decreasing the M_w , when tested different M_w (300, 450,1000 and 1500 kDa). PHB M_w of 1000 kDa completely degraded after 3 months while 1500 kDa degreed after 6 months.

8.3 Summary

In this chapter, the molecular structure and thermal properties of the PHB produced from *C. necator* during fermentation using substrates derived from waste date seed, with submerged and solid state fermentation, were determined with results being broadly comparable to those of a PHB standard. The extracted PHB was characterised by ¹HNMR and FTIR in order to investigate the chemical structure, and the spectra results for all PHB samples were found to be identical in terms of peak positioning. In addition, DSC was used to study the thermal properties of PHB, where the present study demonstrated that the biodegradable PHB produced all samples, is highly crystalline, 65-78 %, with a melting temperature range of 171-176 °C.

GPC was used to determine the molecular mass of all recovered PHB samples and to comparing the obtained results with other studies using various types of bacteria and substrates, where the M_w obtained were 2.157×10^6 , 3.166×10^6 , 4.277×10^6 and 0.975×10^6 Da for samples recovered using date seed hydrolysate media, date seed oil, mixed substrate and ACSSF system respectively, to enable their usage in various applications.

Furthermore, the biodegradability for all PHB samples produced from date seed hydrolysate media, date seed oil and ACSSF system showed an increase in weight up to 102% and 102%, 104% respectively, at beginning of the compost burial test. This was due to water absorption by the samples and significant weight loss was observed for PHB- ACSSF samples with *C. necator* after three weeks biodegradation time to lose about 90% after fifteen weeks while the other samples starting degraded after five weeks to complete after twenty weeks. Increasing the molecular weight (M_w) of the biopolymer reduced its degradability, PHB with higher M_w ($M_n > 4000$) was degraded slowly more than that with low M_n (Lim et al., 2005) and (Tokiwa et al., 2009b).

From the results presented in this chapter, it can be concluded that the thermal characteristics of the PHB produced were not impacted by the carbon source type, no significant variation was observed in the properties of the polymer obtained from date seed hydrolysate media, date seed oil and ACSSF system.

CHAPTER

NINE

9 Conclusion and future work

9.1 Conclusion

The production of polyhydroxybutyrate (PHB), a biodegradable polymer that can be accumulated intracellularly by a wide range of microorganisms, could be a potential solution for the environmental issues caused by the enormous utilisation of non-biodegradable petroleum based plastics. Selecting an appropriate carbon source and an adequate strain of bacteria might lead to a successful process for the economical and efficient competitive PHB accumulation. The findings of this research project, described throughout the thesis, are focused on a series of media preparation/extraction, fermentation and biopolymer characterisation experiments that were conducted to appraise the feasibility of using of waste date seed to synthesise PHB. The results achieved from the various research studies demonstrate the technical feasibility of using waste date seed as a renewable raw material for the production of value-add biopolymer.

The novelty of this work lies in the development of a bioprocess in which waste date seed is utilised as the feedstock for biopolymer production. This is the first time that the carbohydrate and oil fractions of waste date seeds have been used as the carbon sources for PHB production. The findings of this project lead to the wider outlook that the general feedstock derived from the date palm, by-product or seeds, have potential to be used to produce a broad range of bio-products based on the microorganisms applied. The current work has enabled the development of a waste date seed based bioprocess for the production of PHB. Both the carbohydrate and oil fractions of the waste can be used as substrates for fermentation and PHB production by *C. necator* with PHB production typically in the range of 4-12 g/l at an accumulation of 73-85% being achievable. Maximum dry cell weight and PHB concentrations were 6.3 g/l and 4.6 g/l respectively, giving a PHB content of 73%, when an initial fructose concentration of 10.8 g/l was used. While a maximum dry cell weight of 14.35 g/l was obtained with a PHB content of 82%, giving a PHB concentration of 11.77 g/l when the date seed oil was used as a substrate.

The initial analysis of waste date seed indicates that they could be utilised as a media for bacterium growth, *C. necator* H16, and PHB production as all crucial nutrients for growth are present, along with a fructose content sufficient for utilisation as a substrate for PHB accumulation, as presented in **Chapter 5**.

Also reported in **Chapter 5**, the results including fructose extraction from date seeds and a mass transfer model to describe the extraction process, demonstrating that the high nutrient content of date seeds makes them a promising raw material for microbial growth and that a meaningful amount of PHB can be produced without nutrient supplementation.

Furthermore, both a kinetic study of oil extraction from date seeds under different conditions and the potential of using recovered oil as alternative carbon source were studied. The results demonstrate that the mass transfer model is beneficial in describing the date waste oil extraction and *C. necator* is capable of synthesising PHB, utilising non-emulsified date seed oil as a sole carbon source and accumulating a high amount of PHB being demonstrated, as shown in **Chapter 6**. Although bacterium *C. necator* accumulates high levels of PHB and can utilize oil effectively, it was difficult to quantify the oil consumption due to the heterogeneity of the two phases of the medium. To overcome this hurdle, waste date seed oil was emulsified using GA as the emulsifying agent after demonstrated that GA did not affect *C. necator* growth and cannot be utilised either carbon or nitrogen source. However, as cells grown in media with GA made 10% less PHB granules, 73% of total biomass reached, than cells not exposed to GA. This method is a useful lab experimental scale; it is unlikely to have industrial applications because the GA cost.

Moreover, the results show that the *C. necator* can metabolise both substrates (fructose and oil) sequentially, diauxic utilisation growth, taking fructose as a preferable substrate over oil for all conditions tested. Whilst they followed normal growth curve, as a single substrate, when the second substrate (oil) fed to the culture after 48 h of fermentation as presented in **Chapter 7**. The results of this chapter show that date seed hydrolysate media adsorbed on PUF is suitable for the growth of *C. necator* and this system is a potential alternative fermentation for traditional SSF for PHB production with ease. However, the selection of washing number, foam size, depth of foam and initial moisture ratio for the solid-state fermentation is the most factors that necessary to study. The results proved that the ACSSF system is to be a successful alternative fermentation for traditional SSF for PHB production with ease.

The results confirmed that all the samples produced from previous **Chapters, 5, 6 and 7**, are PHB after characterised by HNMR, FTIR as well as DSC, as presented in **Chapter 8**. The average molecular weight of PHB produced from SMF and SSF were in the range 4.257×10^6 to 0.975×10^6 Da and this variation based on concentration and type of substrate, availability of nutrients and growth conditions such as temperature and pH, extraction method as well as the microorganism type controls the biopolymer molecular weights.

Variations in M_w of PHB can impact the biodegradability and thermo-plasticity of the polymers thus the differences in the M_w in PHB samples may not only impact the end-use properties but also the environmental effect of a product made with PHB. Generally, still the M_w of PHB samples at approximately 10^6 has a wide range of application in the medical field and so on. The spectra for PHB samples were also compared to the FTIR spectrum of PHB standard and was found to composed of identical peaks positioning; this means that all extracted biopolymers (produced in this work) are PHB. There were no significant differences noticed amongst the FTIR spectra for all PHB extracted samples. It can be concluded that the PHB structures of the different types of polymer were not impacted by the variation of carbon source type, no significant variation was observed in the polymers properties obtained from date seed hydrolysate media, date seed oil and ACSSF system even for the same substrate, various bacteria can accumulate PHAs with a different composition. This result is expected because normally the wild strain of *C. necator* accumulates mono-polymer, PHB, unless incorporating another organic acid as precursors.

Overall, the feasibility of producing PHB from waste date seed has been demonstrated, with the results obtained in this thesis demonstrating that high added-value PHB can be efficiently biosynthesised from waste date seed. Linked with optimised fermentation processes the use of renewable substrates, such as waste date seed, can be applied to potentially decrease the PHB production cost and hence enhance PHB usage in different applications, however, further investigation for industrial applications is needed. The novel and significant results obtained in this study invite a wider outlook that the general feedstock derived from waste date seed has the potential to be utilised to produce a wide range of bioproducts depending on the microorganisms utilised. Further improvement of this process to develop nutrient production efficiency, yields of product and subsequent process integration into the biopolymer production process could well be a significant contribution to the improvement of a sustainable biopolymer industry.

The possibility of synthesis PHB from a media derived from waste date seed has been demonstrated. The process development and study of economic feasibility is therefore the next stage towards industrialised production of PHB. The economic feasibility of using date seeds as feedstocks for PHB production was evaluated by comparing the direct value of the raw material, date seed, to the value of the PHB produced for a basis of 100,000 kg PHB produced by the process.

According to the literature it can be seen that the values of date seed powder (10-50 \$/ton) is quite low, and the process need extraction of soluble sugar or oil from the seeds, (energy source) and this relatively cheaper comparing with the cost of the raw materials. From the other point of view, converting the waste to PHB might be more economic, particularly if the residual biomass after the fermentation and PHB extraction can be used as a protein rich feed. Choi and Lee (1997) showed a breakdown of the cost associated with PHB production from different systems. This presented that the raw materials accounted for 40% of the total cost of production therefore, using low cost feedstock such as waste date seed could help to reduce the PHB production cost considerably.

A flow sheet for PHB production (appendix A), with estimated mass balance for a production of 100,000 kg PHB per annum is proposed. The flow sheet consists of both upstream processes as well as economic downstream processes chosen from the literature (Posada *et al.* 2011). The mass balance for upstream processing was calculated based on results shown in Chapter 5, whereas the results obtained from the date seed derived media (without supplementing) experiment was used for calculation of the mass balance for PHB fermentation.

On a basis of 100,000 kg PHB, converting the waste date seed to PHB can yield about 21,500 \$ (according to the PHB global market) while nothing can obtain if the waste is sold or thrown a way. Also, from the producer of PHB point of view, using low cost raw materials, substrate, could decrease the PHB production cost, which in turn enables a decrease in the PHB price in the market.

9.2 Recommendations for future work

Although this project has provided many important findings and promising results and hence advanced our understanding of the biosynthesis of PHB using waste date seed, many potential perspectives could be required for the development of the process and subsequently, opportunities for potential industrial applications, are as follows:

➤ Since PHB production is mainly dependent upon the concentration of cells, development of cell proliferation can be contributed to increasing the PHB production instead of growth. Fed-batch and continuous fermentation can be applied using a feeding solution containing both carbon source and nitrogen to sustain the bacterium cell growth. An excess amount of nitrogen in the culture media prevents the bacteria cell from accumulating PHB granules, by encouraging the metabolism towards cell proliferation.

It has also been reported, in many publications, that the C/N ratio affects PHB accumulation in *C. necator* (Amirul *et al.* 2008). By controlling the ratio of the feeding solution, it can be possible to develop PHB accumulation. Xu (2007) studied the influence of various feeding solutions on cell growth and PHB production and found that, for the same glucose concentration, feeding solutions containing 1,480 mg/l FAN can yield 63% higher residual than feeding a solution containing 300 mg/l. As a result, Xu (2007) produces 25% more PHB biomass.

➤ To increase the range of applications that polymers can be used for, there is a need to be capable to tailor the material properties. Therefore, the next step towards development the applicability of biodegradable biopolymers is to investigate the synthesis of co-polymers, by feeding various precursors, depending on the metabolic pathways available or strain engineering, to produce specific PHA. These types of polymers have different thermal and mechanical properties which can be tailored. For instance, the incorporation of (3HV) monomers to produce (PHBHV) lowers the crystallinity and melting point of PHB, hence, increasing toughness and reducing stiffness. This makes it easier to acquire and process, favourable properties for commercial applications (Poirier *et al.*, 1995). On the other hand, the principles of metabolic engineering can be applied to fully understand the biosynthetic pathways for PHB production, not only to enhance the PHB production from bacteria, but also to expand diverse sugars present in waste date seed utilisation ability and to synthesise novel PHA copolymers with potentially better properties.

➤ Concerning to the economic and design evaluation of PHB production processes, future investigations will focus on a deeper economical evaluation of the PHB producing processes. Though not only cost competitiveness should be taken in account, but also the competition of raw materials fermentation in the food markets, thus PHA production must not represent a threat to global food security, which may be in the case of wheat utilisation, for example.

➤ The statistical method, including response surface, analysis can be applied to optimise the production of PHB using waste date seed to achieve higher yield. As well as optimisation using various modes of fermentation such as fed-batch and continuous would also be interesting. On the other hand, future investigations should focus on solid-state fermentation design and solid bed temperature control to increase bacterial growth, to get high hydrolysis performance.

➤ In general, the thermal and mechanical properties of PHB are poor. Hence its processing is a difficult task. Therefore, polymer blending is one of the most successful methods to improve the processing and the quality of the products. Various materials could be utilised as a polymer blending including thermoplastic starch, polyethene glycol (PEG) and lignin (Khanna and Srivastava, 2008). The study will, therefore evaluate the chemical-physico properties of the blends, investigate appropriate processing conditions for the blend preparation, asses the thermal- mechanical properties of the blends, as well as find the potential environmental degradation mechanisms. Furthermore, in the processing of PHB-blend could be monitoring the molecular structure of the PHB-blend, evaluation a kinetic study for the PHB degradation reaction.

➤ It would be beneficial to study adding of chemicals to the bacterial fermentation media. The addition or removal of particular chemicals, such as trace magnesium, phosphorus and sodium, induces the organism into a stress condition, in which they may synthesise greater amounts of PHB. It has been reviewed that strains of resistant *C. necator* can synthesise greater PHB yield with the addition of polymyxin to the fermentation broth. As well as the emphasis was put on modelling PHB production by pure culture and co-substrate of *C. necator* on substrate extracted from waste date seed. A model must set up, calibrated, and validated applying experimental data and is ready for use in view of process optimization. Such as, studying various possible feeding strategies can lead to enhanced PHB productivity.

Whatever the subsequent area of the research, the essential thing to be considered is to establish a comprehensive model of economic analysis and environmental impact to produce PHB utilising renewable biomass sources. A balance between product yield, quality and operating cost is required to be made in order to make this bioconversion functionally feasible and economically at scale.

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