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Date seed characterisation, substrate extraction and process modelling for the production of polyhydroxybutyrate by *Cupriavidus necator*

R.G. Yousuf [a,b], J.B. Winterburn [a,*]

[a] School of Chemical Engineering and Analytical Science, The Mill, The University of Manchester, Manchester M13 9PL, UK
[b] Chemical Engineering Department, University of Baghdad, Iraq

HIGHLIGHTS

- The feasibility of using waste date seeds for PHB production is demonstrated and modelled.
- A yield of PHB on substrate consumed of 0.46 was achieved at a PHB accumulation of 73%.
- Reduction of nitrogen and oxygen availability increase PHB accumulation.

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ABSTRACT

Poly-3-hydroxybutrate (PHB) is a biodegradable polymer synthesised via bacterial fermentation as a means of storing carbon and energy under unbalanced growth conditions. The production cost of petroleum-based plastics is currently lower than that for biopolymers, and the carbon source is the most significant contributor to biopolymer production cost.

A feasibility study to assess the suitability of using a date seed derived media as an alternative for PHB production under various stress conditions was investigated. 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 supplementation. 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.

1. Introduction

Plastics are high molecular weight compounds which consist of a vast number of simpler molecules called monomers, commonly synthesised from the petroleum-derived material.

Most conventional plastics are non-biodegradable and accumulate as solid waste at the end of use. Plastics also contribute to global warming through increasing CO_2 levels in the air due to emissions produced during their manufacture and disposal, via incineration or landfill. For example about 1.5 tons of CO_2 are emitted per ton of polyethylene terephthalate (PET) bottles recycled (Gironi and Piemonte, 2011).

The first stage to overcome these issues is to find alternative polymers with a high degree of biodegradability which can be produced via the application of modern biotechnology using waste streams from agriculture and food production as raw materials. One such microbially produced biopolymer of particular interest is Poly-3-hydroxybutrate (PHB), which is accumulated as intracellular inclusion bodies by many microorganisms. Intracellular PHB serves as an energy store for the microorganism, being produced when the surrounding environment comprises of unbalanced conditions which limit growth; such as nitrogen limitation in the presence of an excess of carbon (López et al., 1995).

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 used as direct replacements for commonly used synthetic polymers. PHB has attracted attention as a biodegradable, biocompatible and thus promising drop-in replacement for petroleum-based plastics in different industrial applications such as packaging, paper coatings, moulded materials, adhesives and performance additives (Batcha et al., 2014).

* Corresponding author.
E-mail address: james.winterburn@manchester.ac.uk (J.B. Winterburn).

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Various microorganisms are capable of producing PHB such as photosynthetic bacteria, *archaebacteria*, as well as gram negative and positive bacteria. *Cupriavidus necator* is a gram-negative bacterium and 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).

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., 2014).

Date palm, also known as *Phoenix dactylifera* L., 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 (Sanaa and Shanab, 2014). 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 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 date seed waste 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, 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 (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., 2004).

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 purpose of this research 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). As a part of this study kinetic parameters for microbial growth and PHB synthesis in batch fermentation by *C. necator* were determined.

2. Materials and methods

2.1. Date seeds preparation

Date seeds were purchased from a local Iraqi supermarket, washed to remove any remaining date flesh and dried overnight at 60 °C. The date seeds were then milled in a heavy-duty grinder (UMA PHARMA) and the fine powder preserved at −20 °C for subsequent use.

2.2. Determination of ash, dry matter and moisture content

The ash content and dry matter were calculated, according to AOAC, (1990), while moisture content was measured using the Nennich method (Nennich and Chase, 2007).

2.3. Determination of crude fibre and protein

The Borchani method was used to determine the crude fibre content of the date seeds (Borchani et al., 2010) and the protein content was calculated using the Wang method (Wang et al., 2004).

2.4. Quantification of fructose

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. The absorbance of fructose standards and samples was measured at 490 nm using a spectrophotometer, UV-mini1240 (Shimadzu, USA). The amount of fructose was calculated as a percentage of the sample on a dry basis.

2.5. Determination of pH

To determine the pH date seed powder was suspended in distilled water, then the mixture was stirred for one hour, centrifuged at 10,000 rpm for 5 min and finally the pH was measured using a standard pH probe (Mettler Toledo, USA) (Salakkam, 2012).

2.6. Determination of reducing sugar

Total reducing sugar concentration was measured using the DNS method described by Gusakov et al. (2011). A standard curve of five points (0.2–1 mg/ml) was made using maltose solution.

2.7. Determination of total carbohydrate

Carbohydrate content was measured by using the Anthrone method proposed by Zhao et al. (2011). A five-point calibration curve was generated using varying volumes, 0.2–1 ml, of 10 g/l glucose solution, with supernatant from the hydrolysed date seed media being analysed. All standards and samples were transferred to measuring tubes, and the total volume topped up to 1 ml using