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Biorefining palm oil agricultural refinery waste for added value rhamnolipid production via fermentation

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ABSTRACT

Rhamnolipids (RL) production by *Pseudomonas aeruginosa* PAO1 is a potentially attractive route to add value to palm oil refinery agricultural by-products; palm fatty acid distillate (PFAD) and fatty acid methyl ester (FAME). The results showed maximum RL concentration of 3.4 and 2.5 g L^{-1} when using 10 g L^{-1} PFAD and FAME respectively; while using 20 g L^{-1} PFAD and FAME, the RL concentrations achieved were 3.2 and 3.1 g L^{-1}, respectively. The predominant congener produced was identified as dirhamnolipid, Rha-Rha-C10-C10. The RL produced reduced surface tension to 29–32 mN m^{-1} with a CMC value of 19 mg L^{-1}. A high emulsion index with kerosene, 40% and sunflower oil, 46% were measured. This work demonstrates the potential for the utilisation of palm oil refinery agricultural by-products, PFAD and FAME, as low cost and renewable substrates for RL production in integrated palm oil biorefinery systems.

1. Introduction

Biosurfactants are extracellular microbial surfactants that are amphipathic and have high emulsifying activity and surface activity (Ji et al., 2016). Biosurfactants have several advantages compared to synthetic surfactants, such as biodegradability, low toxicity, stability and effectiveness at extreme pH, salinity and temperature and their potential to be produced from renewable substrates (George and Jayachandran, 2013). These features give biosurfactants potential for use as green alternatives to chemical surfactants that can be applied in environmental remediation, as antimicrobial agents, as emulsifiers and stabilisers in the food industry, in enhanced oil recovery processes and medical applications (Elshikh et al., 2016; Moya Ramírez et al., 2015).

Chemical structure plays a major role in biosurfactant classification into glycolipids, lipopeptides, phospholipids, fatty acids and polymeric compounds (Lourith and Kanlayavattanakul, 2009). Rhamnolipids are one of the low molecular mass glycolipid type biosurfactants, being produced mainly from *Pseudomonas aeruginosa* strains that are being intensively studied by researchers (Irorere et al., 2017). There are two main types of rhamnolipids with one (monorhamnolipid) and two (dirhamnolipid) rhamnose sugar moieties, attached to one or two β-hydroxy fatty acid chains (Gudiña et al., 2016). To date, several companies have been producing rhamnolipids in relatively large quantities such as Rhamnolipid Incorporated (http://rhamnolipid.com/), Rhamnolipid Holdings (http://www.rhamnolipidholdings.com/) and AGAE Technologies (http://www.agaetech.com/), thus showing rhamnolipids have enormous potential for commercialisation into various products and application areas.

There are several factors that affect rhamnolipid production which include the carbon source, nitrogen source and environmental factors. *Pseudomonas aeruginosa* is capable of using water immiscible free fatty acid containing substrates such as sunflower oil, olive oil and soybean oil to produce rhamnolipid. Glucose, glycerol, ethanol and mannitol are commonly used hydrophilic carbon sources, with results showing that hydrophobic substrates give better rhamnolipid production compared to hydrophilic substrates (Nitschke et al., 2011). Nitrogen sources also play an important role in rhamnolipid production. It has been reported that when the nitrogen became limiting there was overproduction of rhamnolipid and cell growth become stationary (Reis et al., 2011). Furthermore, environmental factors such as pH, temperature, agitation speed, strain type and age, oxygen availability and culture media affect rhamnolipid production and cell growth (Banat, 1997).

Despite intensive research efforts into the development of large scale biosurfactant production processes, there still exists the main challenge of high production costs that limit the wider use of biosurfactants (Dobler et al., 2016). The high cost of rhamnolipids

Abbreviations: RL, Rhamnolipids; PFAD, Palm fatty acid distillate; FAME, Fatty acid methyl ester; CMC, Critical micelle concentration; EI, Emulsion index; CPO, Crude palm oil; PPGas, Protease Peptone Glucose Ammonium Salt Media

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production mainly arises from the expensive substrate and downstream processes, making the use of rhamnolipids costly, especially for low added value applications such as environmental remediation (Banat et al., 2014). One of the ways to reduce the production cost is the utilisation of some industrial/agro-industrial waste or by-product which could provide a low cost fermentation substrate and an opportunity to generate added value, as well as making use of wastes that can potentially have a harmful impact on the environment (Moya Ramírez et al., 2015).

The Malaysian palm oil industry is one of the most important contributors to the Malaysian economy with substantial export earnings of Malaysian Ringgit (RM) 60×10^9 and high employment rate. Globally, 34% of palm oil production is obtained from Malaysia, where in 2015 the planted area was 5.64×10^6 hectares (MPOB, 2016). Palm oil industry made Malaysia the second largest producer in the world with crude palm oil (CPO) production in 2016 of 17.3×10^6 tonnes (MPOB, 2017). The refining process of CPO produces a by-product called palm fatty acid distillate (PFAD) that is low value, renewable and abundant waste substrate. It is estimated that 6.2×10^5 tonnes of PFAD are produced annually, which accounts for 3.6% of the total CPO processed. Recently, PFAD has been used as a feedstock for the oleochemical industry, animal feed and, as a source of vitamin E in the cosmetics industry and has also been extensively studied for the production of biodiesel (Abdul Kapor et al., 2017; Hosseini et al., 2015).

PFAD is a by-product of palm oil refining, produced in agricultural palm oil refinery mills. At room temperature, PFAD is a yellow solid, which turns to a dark brown liquid at temperatures above the melting point of ≈40 °C and FAME (fatty acid methyl ester) is the biodiesel that can be derived from PFAD via the esterification process (Chabukswar et al., 2013). The esterification of PFAD to produce FAME, an existing valorisation route, significantly affects its physical properties, changing from solid to liquid at room temperature. PFAD and FAME are mainly composed of fatty acids such as oleic acid, pentadecanoic acid, tridecylic acid, palmitic acid, stearic acid, palmitic acid and others with carbon chain lengths from C_{11} to C_{18} (Nazren Radzuan et al., 2016). There is much interest in biodiesel (FAME) produced from PFAD because of its comparable properties to the diesel fuel produced from petroleum and the increasing demand for biofuels by both developing and developed countries (Yadav et al., 2010). The high production cost of biodiesel, however, is a big challenge that inhibits its industrial growth (Lokman et al., 2014).

There are many studies in which free fatty acids derived from waste or low-value by-products, such as olive mill waste, waste frying oil, glycerol and soap stock, have been used for rhamnolipid production (Lovaglio et al., 2015). De Faria et al. (2011) used raw glycerol produced from biodiesel production as a sole carbon sources for rhamnolipid production which yield 1.36 g L^{-1} meanwhile Moya Ramírez et al. (2015) used olive mill waste produce much lower rhamnolipid production of 0.56 g L^{-1}. George and Jayachandran, (2013) present a study that produce 1.97 g L^{-1} of rhamnolipid from used coconut oil meanwhile soap stock from refining vegetable oil process was used as substrate for rhamnolipid production by Benincasa et al. (2004) producing 15.8 g L^{-1}. All the studies mentioned used different types of *P. aeruginosa* strain thus it shows its ability to used fatty acid as main substrate for rhamnolipid production, and the production from water-immiscible substrate such as fatty acid has better production than water –soluble substrate like glucose and glycerol (Banat, 1997).

In this present research, the high fatty acid content of PFAD and FAME have been investigated as sole carbon sources for rhamnolipid (RL) production using *Pseudomonas aeruginosa* PA01 in a minimal culture medium. Cell growth, RL production, RL yields and RL characteristics were determined and compared to other reports of value added biosurfactant production routes using agricultural wastes. The novelty of this study is the production of significantly increased amounts of RL when using minimal media, compared to our previous study, using PFAD as the sole carbon source, demonstrating the technical feasibility of producing meaningful amounts of RL from palm oil refinery mill by-product. In a broader biorefining strategy, it is advantageous to have alternative valorisation routes for PFAD and FAME which can be utilised based on the prevailing market conditions. One option is to use the FAME as a substrate for RL production, thus providing an alternative valorisation route that could be employed as part of a wider palm oil biorefinery industry. This investigation expands our knowledge and understanding of the usage of palm oil refinery agricultural waste for RL production and its potential to be transformed into a valuable product that can be used in various applications.

2. Materials and methods

2.1. Esterification of PFAD

Palm fatty acid distillate (PFAD) was converted into fatty acid methyl ester (FAME) by esterification. The FAME produced was subsequently used as a substrate to produce rhamnolipids by fermentation. PFAD was obtained from Sime Darby-Jamolina and The Cucurbit Company Sdn. Bhd., Malaysia and dried in a drying oven at 70 °C for one day. The esterification was then carried out using a 10:1 ratio of methanol to PFAD, with the addition of sulfuric acid (2.5% weight of PFAD) as a catalyst. The reaction was then carried out at 100 °C for 1 h, using a reflux condenser. The product was then cooled, transferred to a separating funnel and washed with hot distilled water until the bottom water layer becomes evident and the pH reached 7.

2.2. Microorganism

Pseudomonas aeruginosa PAO1 was supplied by the School of Biomedical Sciences, Ulster University from their culture collection. The strain was stored at −80 °C as master stock and working culture was preserved at 4 °C on nutrient agar plates.

2.3. Media and culture condition

To prepare cultures *P. aeruginosa* PAO1 was first spread onto a nutrient agar petri dish and incubated at 37 °C for 24 h. There were two types culture medium used in this fermentation. The first culture medium used for seed culture was the Protease Peptone Glucose Ammonium Salt (PPGas) medium which consisted of 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 10 g L^{-1} peptone, 19 g L^{-1} Tris-HCl, 1.5 g L^{-1} KCl, 1 g L^{-1} NH_4Cl and 1% glucose. The second culture medium used was minimal medium (MM) which consisted of 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 1.0 g L^{-1} KCl, 0.3 g L^{-1} K_2HPO_4, 1.0 g L^{-1} $NaNO_3$. Trace elements for MM were 2 g L^{-1} $C_6H_5Na_3O_7{\cdot}2H_2O$, 0.28 g L^{-1} $FeCl_3{\cdot}6H_2O$, 1.4 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 1.2 g L^{-1} $CoCl_2{\cdot}6H_2O$, 1.2 g L^{-1} $CuSO_4{\cdot}5H_2O$ and 0.8 g L^{-1} $MnSO_4.H_2O$.

Fermentation experiments were conducted in 5 L shake flasks in triplicate, using 1L of MM supplemented with the desired amount of glucose, PFAD or FAME and were carried out at 37 °C over three days. Inocula were prepared in two stages; in stage 1 50 mL of PPGas medium containing 1% glucose in a 250 mL flask was inoculated with one loop of bacteria into and grown at 37 °C for 24 h. In stage 2, 40 mL of stage 1 culture were transferred to 400 mL PPGas media with 1% of glucose in a 2 L flask and grown for 24 h. For the preparation of the final inoculum, 100 mL of stage 2 culture was centrifuged for 10 min and the cell pellet resuspended in 100 mL of sterile distilled water. This was then used to inoculate the 1 L MM in 5 L shake flask. The initial concentration of the fermentation was maintain at 0.2 g L^{-1}.

2.4. Growth measurement

Cell growth was quantified by measuring optical density (OD) from which dry cell weight (DCW) was calculated using a linear correlation $DCW = 0.4639(OD) + 0.0276$ with $R^2 = 0.87$ for glucose and

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