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Production of Surfactin from *Bacillus subtilis* ATCC 21332 by Using Treated Palm Oil Mill Effluent (POME) as Fermentation Media

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Abstract. Surfactin, a lipopeptidic biosurfactant from *Bacillus subtilis* can only be produced under appropriate fermentation conditions and one of the factor being considered is their nutrient source. Conventionally, production of surfactin had been practised by utilizing commercial laboratory media in either both small and large scale fermentation. Alternative media options are being sought from agro-based wastes in order to minimize the production cost due to its relatively abundant and inexpensive raw materials. Palm oil mill effluent (POME), an agricultural waste from palm oil industry has been reviewed as a promising candidate that could potentially to be exploited. This study investigated on the feasibility of POME as fermentation media in surfactin production by using prominent surfactin producer of *B. subtilis* American Type Culture Collection (ATCC) 21332. Nutrient analysis showed POME consisted of significant amount of fermentable sugars, nitrogenous compounds, and essential elements that could support the bacterial growth and surfactin production. Fermentation study evaluated that POME media at various concentrations (10, 30, and 50%) were capable to produce surfactin with different yields. The highest surfactin amount was achieved by using 50 % (v/v) of POME compared to other concentrations studied.

Keywords: Surfactin, lipopeptides, *Bacillus subtilis* ATCC 21332, palm oil mill effluent (POME), fermentation

1. Introduction

Biosurfactant or microbial derived surface-active agent had gained attention in the past few decades because of their biodegradability, low toxicity, ecological acceptability, and ability to be produced from renewable and cheaper substrates [1]. Among of biosurfactants existed, lipopeptides are of great interest because of their high surface activities and therapeutic potential [2]. Surfactin is one of the most excellent biosurfactant so far known belongs to the lipopeptide family excreted by *B. subtilis* spp. [3]. The rising interest on this macrolide lipopeptide is due to its exceptional amphiphilic character, which is responsible for its efficient surface-active properties as it reduces the surface tension of water from 72 to 27 mN/m at a concentration as low as 0.005% [4]. Furthermore, it possesses remarkable membrane-active properties, resulting in a number of biological properties such as antibacterial, antiviral, antimycoplasms, and hemolytic ability [5].

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The unique feature of surfactin has caught a worldwide attention and many research attempts are being studied for their production process. Surfactin production was known by fermentation of substrates mainly from carbon-rich substrates of sugars, vegetable oil or starch as their carbon source. However, agricultural wastes have been considered as alternative option in replacing of those commercial carbon sources due to its low-in-cost and relatively abundant.

Malaysia is essentially an agricultural country in which palm oil industry is growing rapidly, thus making the country is the world's leading producer and exporter of palm oil replacing Nigeria as the chief producer since 1971 [6]. However, wet process of palm oil milling consumes a large amount of water. About 5 to 7.5 tonnes of water required to produce 1 tonne of crude palm oil and thus accumulates a by-product waste which is known as the palm oil mill effluent (POME) [7].

POME, a collodial suspension containing 95-96% water, 0.6-0.7% oil and 4-5% total solids including 2-4% suspended solids that are mainly consisted of debris from palm fruit mesocarp generated from three main sources of sterilizer condensate, seperator sludge and hydrocyclone wastewater [8]. In the year 2004, about 40 million tonnes of POME was generated from 372 mills in Malaysia [9]. Direct POME dischargal might causes environmental hazard as it has been reported high in biochemical oxygen demand (BOD) (25 000 mg/L), chemical oxygen demand (COD) (53 630 mg/L), oil and grease (8370 mg/L), total solids (43 635 mg/L) as well as suspended solids (19 020 mg/L) [10]. Despite of its hazardous attributes, POME is bountiful with numerous nutritional values of carbohydrates, nitrogenous compounds, lipids and minerals in which favour for further biotechnological uses.

Exploitation of this agricultural waste has been studied to a great extent for various fermentation bioproducts of organic acids [11], enzymes [12], acetone-butanol-ethanol (ABE) [13], biohydrogen [14], penicillin antibiotic [15] as well as plant fertilizer [16]. Therefore, encouraging results from these findings might have to suggest the great potential of POME that could be implemented in surfactin production.

The aim of the study is to investigate the ability of POME as media substrates for surfactin production by using *Bacillus subtilis* ATCC 21332. Since POME compositional analyses showed high in essential nutrients, exploitation of this potential substrates was suggested to be studied extensively. Bright future from this study may contribute for Malaysian development in surfactin production and also to alleviate the environmental issues regarding on POME's massive accumulation.

2. Materials and Methods

2.1. Sample Pretreatment

Raw POME was collected from Seri Ulu Langat Palm Oil Mill Sdn Bhd, Dengkil, Kajang, Selangor around the year of 2011 and stored at 4^0C. Sample pretreatment included of centrifugation, filtration and autoclaving steps. Raw POME was centrifuged at 10 000 rpm for 10 min to obtain three layers formation. The second lower part of layer was filtrated through Whatman No. 1 and GF/C filter papers to obtain the clarified filtrates. The POME filtrates was collected and pH value was adjusted to 7.0 ± 0.1 before subjecting to autoclaving at 121^0C for 20 min.

2.2. Characterization of POME

POME filtrates was analyzed for their chemical properties in term of nutritional content and pH value. Total carbohydrates and reducing sugars content were analyzed by referring to Phenol-Sulfuric Acid [17] and Somogyi-Nelson [18] methods respectively. Total organic nitrogen was determined by using Kjedahl method and minerals content of Mg, Ca, Na, Cu and Fe were quantified by using atomic absorption spectroscopy (AAS).

2.3. Medium Preparation

Preparation of different POME concentrations was performed by dilution of POME filtrates into several concentrations of 10, 30, 50, and 70% (v/v) in 100 mL volume. Each media diluent was deposited into 250 mL conical flask. The nutrients supplemented into each media were NH_4NO_3 (0.05 M), KH_2PO_4 (0.03 M), Na_2HPO_4 (0.04 M), $MgSO_4$ (8.0×10^{-4} M), $CaCl_2$ (7.0×10^{-6} M), $FeSO_4$ (4.0×10^{-6} M), and Na_2 EDTA (4.0

x10^{-6} M). A solution of the added nutrients without POME was served as negative control. Each media prepared was adjusted its pH value to 7.0 ± 0.1 with sterilized 1 N NaOH.

2.4. Microorganism and Inoculum Preparation

Bacillus subtilis strain 21332 derived from American Type Culture Collection (ATCC) (Rockville, Maryland, USA) was maintained on nutrient agar (NA) at 4^0C. Preparation of inoculum suspension was performed by inoculating 2 loopfuls of bacterial isolates into 100 mL of glucose-nutrient broth (40 g/L glucose and 13 g/L NB) and incubated at 30 ^{0}C, 150 rpm for 24 h. Only cultures with cells concentration around $10^8 – 10^9$ cell/mL were used as inoculum.

2.5. Fermentation

Fermentation of POME substrate was carried out by inoculating with 2 % (v/v) of inoculum suspension and incubated at 30 ℃, 150 rpm for 120 h in shaking incubator. A volume of 2 mL of samples were withdrawn every 24 h interval times (0, 24, 48, 72, 96, and 120 h) for determination of bacterial growth and surfactin concentration.

2.6. Determination of Bacterial Growth

Bacterial cells concentration was determined by total plate count method and recorded as log CFU/mL. Each fermentation broth at defined time-intervals was serially diluted and spread plated for colony counting.

2.7. Determination of Surfactin Concentration

In determination of surfactin concentration, culture sample at time intervals were withdrawn and centrifuge at 8 000 rpm for 10 minutes, subsequently filtered through 0.22 µm Nylon membrane filter. The supernatant samples were analyzed using a method described by Isa et al., [19] with slight modification by Wei and Chu [20]. The optimal conditions of detection were achieved by using an HPLC (Agilent Technologies, 1200 Series,USA) equipped with C-18 column (Agilent Zorbax Eclipse C18 , 250 mm × 4.6 mm, 5µm), and detected at 205 nm with a Variable Wavelength Detector (VWD). The system was operated at a flow rate of 1.5 mL/min with solvent system of 3.8 mM trifluoroacetic acid (TFA) in 80 % acetonitrile (ACN) under isocratic mode. Surfactin from *Bacillus subtilis* (Sigma Chemicals Co., St. Louis, Missouri, USA) was served as reference standard for calibration curve.

3. Results and Discussion

3.1. Chemical Characteristics of POME

The amount of nutritional contents varies according to the location of palm oil plantation and milling factories [21]. Due to that reason, analysis on POME sample obtained is suggested. Generally, chemical analyses on POME filtrates sample showed a significant amount of primary nutrients of carbohydrates, nitrogenous compounds, and essential elements. Results were tabulated as in Table 1. Carbohydrates content in POME was investigated from water-soluble centrifugal fraction and it consists of glucose, reducing sugars, and pectin. However, the amount of total soluble carbohydrates was found at low concentration of 0.390 g/100mL (equivalent to 3.9 g/L) [22]. The amount of total carbohydrates content measured at 17.38 g/L in this study was found higher than Ho et al. [22].

Total Kjedahl nitrogen was measured at 0.016 ± 0.03 % indicates the low nitrogen content in POME. Previous literature also mentioned the nitrogenous content in POME was reported significantly in low amount [23]. Nitrogen content in POME originally from organic nitrogen form (protein) and converted gradually to ammonia compound with molecular weight of 17-35 g/mol by time [23]. The protein fraction is tightly associated to insoluble part of POME as that perhaps account for the low digestibility as being found by Devendra [24].

Mineral nutrients are inorganic elements that have essential and specific functions in oil palm plant metabolism [25]. Due to that reason, some of minerals content from palm fruits might have been retained in POME during the milling and extraction process. In this analysis, Na was found in high concentration of 1014.00 ± 76.00 mg/L, followed by Ca, Mg, Fe, and Cu at 93.00 ± 3.00 mg/L, 29.62 ± 0.03 mg/L, 19.25 ± 0.85 mg/L, and 0.05 ± 0.03 mg/L respectively.

Table 1: Compositions of POME Filtrates Utilized as Fermentation Substrate

POME component	Concentration/value
pH	4.0 – 4.2
Total carbohydrates	17.38 ±0.45 g/L
Reducing sugars	6.79 ±0.31 g/L
Total Kjedahl nitrogen	0.016 ±0.03 %
Mg	29.62 ±0.03 mg/L
Ca	93.00 ±3.00 mg/L
Na	1014.00 ±76.00 mg/L
Cu	0.05 ±0.03 mg/L
Fe	19.25 ±0.85 mg/L

3.2. Bacterial Growth and Surfactin Production

Bacterial growth curve of 10, 30, 50 and 70 % POME was shown as in Fig. 1. Study of bacterial growth revealed that 10, 30, and 50 % POME were capable to support bacterial growth. In general, all media showed that bacterial growth reached early stationary phase during 24 h incubation time. However, no bacterial growth was observed when 70% POME media was utilized. Among of all media studied, 10% POME showed the fastest bacterial growth with doubling time (t_d) of 5.10 h, followed by 30% POME (t_d = 5.32 h), and 50% POME (t_d = 5.95 h). Bacterial population grew faster in decreases of POME concentration. However, 10% POME showed a rapid decline at 48 h, while bacterial growth for 30% POME showed a gradual decline at the same time. A slow growth rate was observed for 50% POME with a continuous escalates of bacterial growth until 96 h.

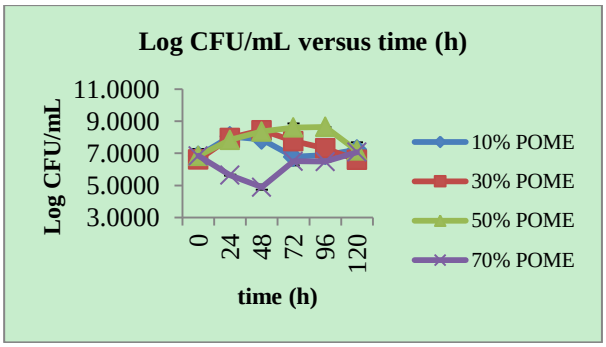

Fig. 1: The patterns of growth curve of *B. subtilis* ATCC 21332 in various concentrations of POME media

Surfactin production from *B. subtilis* ATCC 21332 on different POME concentrations was presented as in Fig. 2. All concentrations of POME studied showed a positive surfactin production with exception to 70 % POME media. Maximal surfactin concentration (30 – 35 mg/L) was quantified by 50 % POME. POME composition of 10 % and 30 % showed a comparable result in surfactin production. However, no production of surfactin was observed for 70 % POME since growth was not occurred.

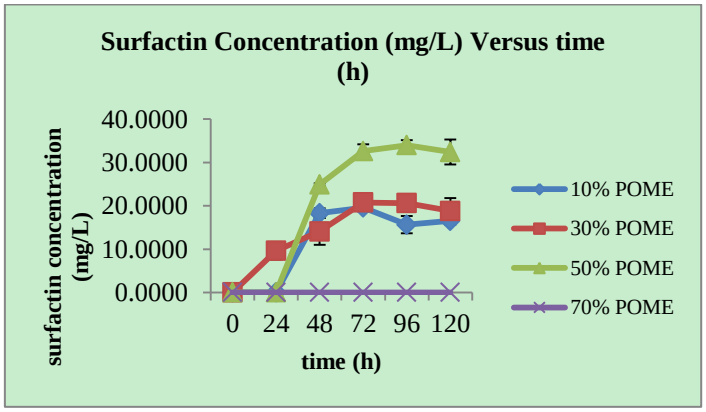

Fig. 2: Production of surfactin (mg/L) in 10, 30, 50 and 70% POME at time-intervals of incubation period

Surfactin is synthesized during the stationary phase when nutrients in the culture media are limited [5]. Previous studies reported that most of biosurfactant production is growth associated [26], [27]. However, another fermentation study on *Pseudomonas aeroginosa* reported that high yield of rhamnolipid biosurfactant can only be produced under growth-limiting conditions [28]. In this study, a parallel relationship between the bacterial growth and surfactin production was observed for all concentrations of POME media. Therefore, this could explain the production kinetics of surfactin in 10, 30, and 50 % POME media. The onset of surfactin production was initiated at 24-48 h incubation time in which the period of stationary phase was achieved.

Based from the results, it can be hypothesized that optimal balance of nutrient content plays major role in determining surfactin production. Carbon and nitrogen were confirmed as the important key factors that affecting mostly of all kind of biosurfactants production [29]-[31]. According to Fonseca et al. [32], the best results was obtained for medium containing crystal sugar (10 g/L) and NH_4NO_3 (4.0 and 1.3 g/L) under 250 rpm agitation rate corresponding to C/N ratio of 3 and 9. Previous literature usually adopts a C/N ratio around three and agitation of 150 rpm for maximal surfactin production. Davis et al., [33] obtained the highest quantity of surfactin cultivating *B. subtilis* ATCC 21332 in medium of glucose (10.0 g/L) and ammonium nitrate (4.0 g/L), which corresponds to a C/N ratio of 11. Ghribi and Ellouze-Chaabouni [34], reported that a C/N ratio of 7 would enhances the biosurfactant production of *B. subtilis* SPBI by using glucose and ammonium chloride media as the carbon and nitrogen sources respectively. Fonseca et al., [32] conducted an optimization study of carbon/nitrogen (C/N) ratio on biosurfactant yield reported that low C/N ratio in fermentation media with higher rates of agitation cause higher percentage in surface tension reduction. Our finding discovered the highest amount of surfactin concentration was quantified in 50% POME medium in which possesses the C/N ratio of 2.170. This value was found correlates to most of previous reports as C/N ratio around 3 as the ideal condition to be applied in surfactin production.

4. Conclusions

POME comprises of valuable organic substances and inorganic minerals. Although it is high in carbohydrates and sugar content, the nitrogenous content was quantified significantly in low amount. Thus, in order to reuse it as a functional fermentation media, raw POME must be pretreated through centrifugation, filtration and nutrient enhancement steps. This preliminary study on the capability of POME as alternative media revealed its bright potential as a promising fermentation medium for more economics and environmental friendly production.

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