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Palm oil mill effluent as a low-cost substrate for bioflocculant production by *Bacillus marisflavi* NA8

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Abstract

Background: Palm oil mill effluent (POME) generated from the palm oil milling process contains high organic matter from which the molecule of interest for bioconversion such as fermentable sugar can be derived. This study examined the enzymatic hydrolysis of complex celluloses and polysaccharides present in POME with the aim to release the simple sugars as low-cost fermentable feedstock convertible into bioflocculant.

Results: The maximum concentration of fermentable sugar produced after 24 h of hydrolysis was 30.5 g/L. The POME hydrolysate served as a carbon source for *Bacillus marisflavi* NA8 to produce bioflocculant. The optimum conditions obtained were an initial pH of 7.0 and a temperature of 37 °C with an inoculum size of 5 % (v/v), yielding 6.4 g/L or 32 kg/t bioflocculant from POME.

Conclusion: This finding indicated that POME could be utilized as a low-cost substrate to improve the feasibility of commercial production of bioflocculant.

Keywords: Palm oil mill effluent, Enzymatic hydrolysis, Sugar, Bioflocculant, *Bacillus marisflavi* NA8

Background

Malaysia is one of the world's largest producers and exporters of palm oil and its products. The palm oil demand grows as it is cheap and has high oxidative stability (Gobi and Vadivelu 2013). The oil palm-planted area has reached 5.39 million ha in 2014 (MPOB 2014). In parallel, with higher palm oil production, the amount of its by-products from the palm oil milling process has also increased. Currently, the fresh fruit bunches (FFB) are processed by about 440 palm oil mills. Empty fruit bunches (EFB), mesocarp fibres, palm kernel shell, palm kernel cake and palm oil mill effluent (POME) are the wastes generated from palm oil extraction process. Among these wastes, POME makes up the largest portion. It is estimated that about 0.67 tonnes of POME is generated from 1 tonne of FFB processed. Hence in 2014, about 67.28 million tonnes of POME was generated from 100.42 million tonnes of FFB in Malaysia.

The bioconversion of POME into higher-value products has been recognized as an attractive alternative for waste management strategy. In bioconversion, the rich organic residue in POME is used as a substrate for specific microorganisms to grow and consume while concurrently producing biomass and the targeted products, e.g. bioenergy and biochemicals. One of such products, i.e. bioflocculants, has recently attracted huge scientific and biotechnological consideration. Bioflocculants have been widely used in various industrial processes, including water treatment (Buthelezi et al. 2009; Li et al. 2009), wastewater flocculation and settling (Gong et al. 2008; Li et al. 2013), wastewater colour removal (Solis et al. 2012; Zhang et al. 2009), sludge dewatering (More et al. 2010; Yang et al. 2012), heavy metal removal (Chien et al. 2013; Mikutta et al. 2012), toxic organic compound removal (Zhang et al. 2011), landfill leachate treatment (Renou et al. 2008) and soil remediation and reclamation (More et al. 2014).

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Bioflocculants from microbial source have drawn an increasing interest because they are biologically active, biodegradable, nonpolluting and harmless to the environment, hence can be a substitute for conventional chemical flocculants. Since the substrate in use for bioflocculant production is a critical cost factor, cheap and renewable feedstock should be explored as alternatives to commonly used substrates such as glucose, fructose, sucrose and L-glutamate. Utilization of waste materials such as wastewater could largely reduce the production cost, improve the feasibility of commercial production of bioflocculant and ultimately minimize the disposal/pretreatment cost. Various wastewaters from organic sources, e.g. soybean juice (Huang et al. 2001), fishmeal (Zhou et al. 2003), dairy (Wang et al. 2007), brewery (Zhang et al. 2007), starch (Li et al. 2008), potato starch (Pu et al. 2014; Yan and Yun 2013), livestock sludge (Peng et al. 2014), rice stover hydrolysate (Guo et al. 2015), formaldehyde (Zhao et al. 2016) and methanol wastewater (Cao et al. 2015), have been used as substrates for bioflocculant production in an attempt to lower the production cost.

The fact that POME contains substantial concentration of carbohydrates, proteins, lipids, nutrients and minerals favourable for microbial growth makes it a potential fermentation substrate. However, exploration of POME as a substrate for bioflocculant production is still lacking. So far, only a few studies were reported on isolating bioflocculant-producing microorganisms from POME, i.e. *Staphylococcus cohnii* spp. (Wong et al. 2012), *Chryseomonas luteola* (Syafalni et al. 2012), *Bacillus marisflavi*, *Bacillus toyonensis* and *Stenotrophomonas daejeonensis* (Nurul Adela et al. 2015a). The potential of POME as a carbon source was only recently reported by Aljuboori and co-workers (2014). However, this study reported on the usage of raw POME as a substrate. In POME, there are two forms of carbohydrates, i.e. insoluble and soluble carbohydrates. The amount of total soluble carbohydrates is very low compared to that of the insoluble ones having high molecular weight such as cellulose, hemicellulose and starch (Ho and Tan 1983). Therefore, it is necessary to hydrolyse the complex carbohydrates in POME prior to fermentation to aid or speed up the microbial process. In this study, raw POME was firstly enzymatically hydrolysed to release the fermentable sugars. The hydrolysed POME was then used as a carbon source to examine its viability for bioflocculant production by *B. marisflavi*. The optimal conditions in POME hydrolysis and the resulting bioflocculant production by *B. marisflavi* were also examined.

Methods

Raw material

Fresh POME sample was collected from a palm oil mill located at Labu, Negeri Sembilan, Malaysia. The sample was stored at 4 °C prior to analysis and further treatment.

Bioflocculant-producing microorganism

The strain used for fermentation of POME in this study was *B. marisflavi* NA8. It was isolated from the aerobically treated POME (Nurul Adela et al. 2015a).

Enzyme

The enzyme used was obtained from Universiti Kebangsaan Malaysia (UKM) namely UKM-enzyme Formulation-3 (F3). F3 is a mixture of lignocellulases, i.e. endoglucanase produced by submerged fermentation of a genetically modified strain of *Pichia pastoris*. F3 also contains a mixture of CTec and xylanase with a total enzyme activity of 1493 U/mL. One unit of enzyme activity is defined as the quantity of enzyme to produce reducing sugars equivalent to 1 μmol of glucose per minute from oil palm EFB as a substrate under standard assay conditions at pH 5.0 and 50 °C.

Enzymatic hydrolysis of POME

The enzymatic hydrolysis was performed on raw POME (100 mL) in a 250-mL flask with a screw cap. The reaction mixture consisted of 1 % (v/v) enzyme loading (1 mL enzyme per 100 mL of POME). The sample was then incubated at 50 °C, 150 rpm for 72 h in a rotary incubator shaker (Innova-40, New Brunswick Scientific, USA). Sample aliquots were withdrawn at 6-, 24-, 48- and 72-h intervals and analysed for the released sugars.

The parameters affecting the hydrolysis of POME, i.e. incubation time (6, 24, 48 and 72 h), substrate loading (COD of 10,000, 20,000, 40,000, 60,000 and 80,000 ppm), enzyme loading [0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 % (v/v)] and addition of different non-ionic surfactants [Polysorbate 20 ($C_{58}H_{114}O_{26}$), Polysorbate 80 ($C_{64}H_{124}O_{26}$) and Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$)] at different concentrations [0.05, 0.1, 0.2, 0.5 and 1.0 % (v/v)], were examined and optimized. All experiments were performed in triplicate.

Medium preparation

The conventional growth medium (GM) for bacterial growth and bioflocculant production was prepared according to Zhang et al. (2007) consisting of glucose (20 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), $(NH_4)_2SO_4$ (0.2 g/L), K_2HPO_4 (5 g/L), KH_2PO_4 (2 g/L), urea (0.5 g/L) and yeast extract (0.5 g/L). The pH of the medium was adjusted to 7.0. The medium was sterilized by autoclaving at 121 °C

for 15 min. The POME medium was prepared similarly by replacing glucose with 20 % (v/v) of POME hydrolysate. The POME hydrolysate was prepared via enzymatic hydrolysis (as described in 2.4) for 24 h.

Bioflocculant production by *Bacillus marisflavi* NA8

One loopful of the pure single colony from the seed culture was inoculated into 10 mL of nutrient broth (NB) in a 50-mL Falcon tube overnight to promote the microbial growth. The production of bioflocculant by *B. marisflavi* NA8 was performed in a 250-mL flask with a screw cap containing 100 mL of POME medium and GM (as control). The flask was inoculated with 10 % (v/v) of the overnight seed culture and incubated at 37 °C with agitation at 150 rpm. Samples were withdrawn at 12-, 24-, 48-, 72- and 96-h intervals and monitored for cell growth and flocculating activity. The culture broth was used to determine the bacterial flocculating activities.

The effect of initial pH on POME fermentation was studied by varying the pH at 1 unit interval from 3.0 to 10.0. The POME was adjusted to the desired pH using 5 M H_2SO_4 or NaOH prior to inoculation. The cultures were incubated at 37 °C with an agitation rate of 150 rpm for 24 h. The effect of temperature on bioflocculant production was studied by varying the temperature as 30, 34, 37 and 40 °C. The POME sample was incubated at the optimum pH obtained. Likewise, inoculum volumes of 1, 2, 5, 10 and 20 % (v/v) in proportion to the fermentation volume were used for bioflocculant production. Other conditions were kept constant. The effect of nitrogen source on POME fermentation was studied using seven different nitrogen sources, i.e. yeast extract (YE), meat extract (ME), peptone, ammonium chloride (NH_4Cl), ammonium sulphate $(NH4)_2SO_4$, glutamic acid and urea at 1 % (w/v), respectively.

Bioflocculant extraction

The fermentation broth was centrifuged at 4000 rpm for 15 min to remove bacterial cells. To the obtained supernatant, two volumes of cold ethanol were added, stirred till white cotton-like flocs were formed and then left to stand overnight at 4 °C. The resulting precipitate was collected by centrifugation at 4000 rpm for 15 min, rinsed and redissolved in distilled water. The precipitate was dried to obtain crude bioflocculant and used for analysis.

Analytical procedures

Reducing sugar analysis

The reducing sugar concentrations were analysed using a dinitrosalicylic acid (DNS) assay (Miller 1959). The reducing sugar concentration was calculated using a glucose standard curve having a linear regression of $y = 0.707x + 0.099$ with $R^2 = 0.997$.

Measurement of flocculating activity

Flocculating activity was determined according to Kurane et al. (1994) with slight modifications. Five grams of kaolin clay was suspended in 1 L of distilled water to make a suspension concentration of 5 g/L. One hundred mL of kaolin suspension, 3 mL of 1 % (w/v) $CaCl_2$ and 3 mL of culture supernatant were added into a 500-mL flask. The mixture was agitated vigorously for 60 s, then poured into a 100-mL measuring cylinder and allowed to settle for 5 min at room temperature. The optical density (OD) of the clarifying supernatant was measured at 550 nm with a UV spectrophotometer (Genesys, Thermo Scientific, USA), and the resulting flocculating activity was determined as follows:

$$\text{Flocculating activity (\%)} = [(A - B/A)] \times 100\ \%,$$

where A and B represent the OD of the control and sample measured at 550 nm, respectively.

Biomass concentration

Bacterial growth was estimated by measuring the OD of the fermentation broth at 600 nm with a UV–Visible spectrophotometer and then converted it to cell dry weight (CDW) (g/L) as biomass concentration. The culture broth was centrifuged at 4000 rpm for 10 min to separate the cells. The supernatant was removed and the cells were dried at 80 °C to a constant weight as a measurement of cell growth. The standard curve was prepared based on OD 600 versus CDW. The prepared standard curve had a linear regression of $y = 0.5x + 0.0555$ with $R^2 = 0.9986$.

Functional group analysis

The crude bioflocculant was analysed using a Fourier transform infrared (FTIR) spectrometer (Perkin-Elmer System, USA). The dried bioflocculant powder was used for spectral measurement in the frequency range of 4000–650 cm^{-1}.

Results and discussion

Enzymatic hydrolysis of POME

The characteristics of raw POME and POME hydrolysate are shown in Table 1. POME hydrolysate showed higher BOD and total organic carbon (TOC) than raw POME indicating that they were degraded by the enzyme into reducing sugars. It is proved that endoglucanase was able to hydrolyse POME as it had ability to break and digest carbohydrates present in POME. The time course study of POME hydrolysis was firstly conducted to find its optimum reaction time in producing reducing sugars. The test was carried out at a fixed volume of enzyme loading (v/v of substrate). The reducing sugar profile produced by the enzymes is shown in Fig. 1a. It showed that the

Table 1 Characteristics of raw and enzymatically hydrolysed palm oil mill effluent (POME) used in the experiment

Parameters	Unit	Raw POME	Enzymatic POME hydrolysate
Biochemical oxygen demand (BOD)	mg/L	16,900 ± 5671	59,483 ± 1200
Chemical oxygen demand (COD)	mg/L	82,685 ± 7112	72,727 ± 6389
Total solid (TS)	mg/L	73,806 ± 3227	78,956 ± 4337
Total volatile solid (TVS)	mg/L	61,680 ± 1351	67,611 ± 3852
Solid suspended (SS)	mg/L	46,254 ± 591	27,067 ± 1006
Oil & grease (O&G)	mg/L	8540 ± 169	9033 ± 368
Total nitrogen (TN)	mg/L	1218 ± 86	1530 ± 36
Volatile fatty acid (VFA)	mg/L	1130 ± 77	1287 ± 120
Total organic carbon (TOC)	mg/L	7500 ± 2474	23,250 ± 6010
pH		4.34	4.47
Temperature	°C	80 – 90	50
Moisture content	%	95 – 96	95.8 – 99.1

highest production of sugar was obtained at 24 h with enzyme addition as compared to the control at 48 h under the same conditions. The addition of 1 % (v/v) enzyme was able to produce 27.13 ± 2.8 g/L from 100 % POME (undiluted), compared to the control, 12.45 ± 1.4 g/L at 24 h. The POME was completely hydrolysed within 24 h and gradually deceased thereafter. Thus, the subsequent optimization experiments at different substrate, enzyme and surfactant loadings were conducted only within 24 h.

Effect of substrate loading, enzyme loading and surfactant addition

Substrate loading may influence POME hydrolysis depending on the availability of reaction sites for enzyme to adsorb onto. At the fixed 1 % (v/v) enzyme loading for 24 h, enzymatic POME hydrolysis of an increase in substrate loading gave higher yield of the reducing sugar (Fig. 1b), i.e. 0.443 ± 0.04 g/g COD. However, a decline in sugar yield occurred when POME COD was >60,000 ppm. As higher substrate loading is always preferable to maximize the enzyme reactivity, POME in this study was not diluted prior to hydrolysis process. Figure 1c shows that the highest sugar was obtainable at 2.5 % (v/v) enzyme loading, i.e. 34.36 ± 1.35 g/L. However, the sugar production at 1.0 % (v/v) was relatively high (32.47 ± 2.14 g/L). Since lower enzyme loading is always preferable to reduce processing cost, the enzyme concentration of 1.0 % (v/v) which gave comparable sugar production was used.

The presence of surface-active agent may increase the enzymatic reactivity in POME hydrolysis. Surfactants may also stabilize the enzymes, effectively preventing enzyme denaturation during hydrolysis (Kristensen et al. 2007). In previous study, non-ionic surfactants were found effective in enhancing the enzymatic conversion of cellulose into soluble sugars (Nurul Adela et al. 2015b). However, as shown in Fig. 1d, after 24 h of incubation of POME with enzyme along with surfactant, only Triton X-100 (0.5 %, v/v) showed a slight increase in sugar production compared to the control. Other surfactants (Tween 20 and Tween 80) did not show any improvement at all concentrations tested. Thus, it was unnecessary to add surfactants in enhancing the hydrolysis of POME.

Time course production of bioflocculant

In bioconversion of POME into bioflocculants, the factors affecting the flocculating activity in culture broth, i.e. production time, initial pH, temperature and nitrogen source, were investigated. A previously isolated *B. marisflavi* NA8 from aerobically treated POME (Nurul Adela et al. 2015a) was used to assess the fermentability and efficiency in POME hydrolysis. In this time course study, utilization of 100 % POME was challenging since the colour of POME could result in high absorbance reading thus contributing to negative flocculating activity. The fermentation by *B. marisflavi* NA8 was therefore verified using 20 % (v/v) POME hydrolysate for 96 h to examine the capability of the bacteria to produce bioflocculant. As shown in Fig. 2, the bacterial cell quickly adapted to the POME medium at the beginning of fermentation and its growth increased exponentially at the early stage of fermentation. The bioflocculant production commenced at early stage and continuously increased and reached a maximum activity of 80 % within 24 h in POME medium as well as conventional GM. The cell growth in POME was higher than that in GM showing the suitability of POME for culture grown. The highest flocculating activity was peaked at the early stationary phase, i.e. 24 h, indicating that biosynthesis was responsible for the production of bioflocculant. Interestingly, this had resulted in short culture period for bioflocculant secretion by *B. marisflavi* NA8. This finding was similar with that by *B. mojavensis* showing the highest flocculating activity

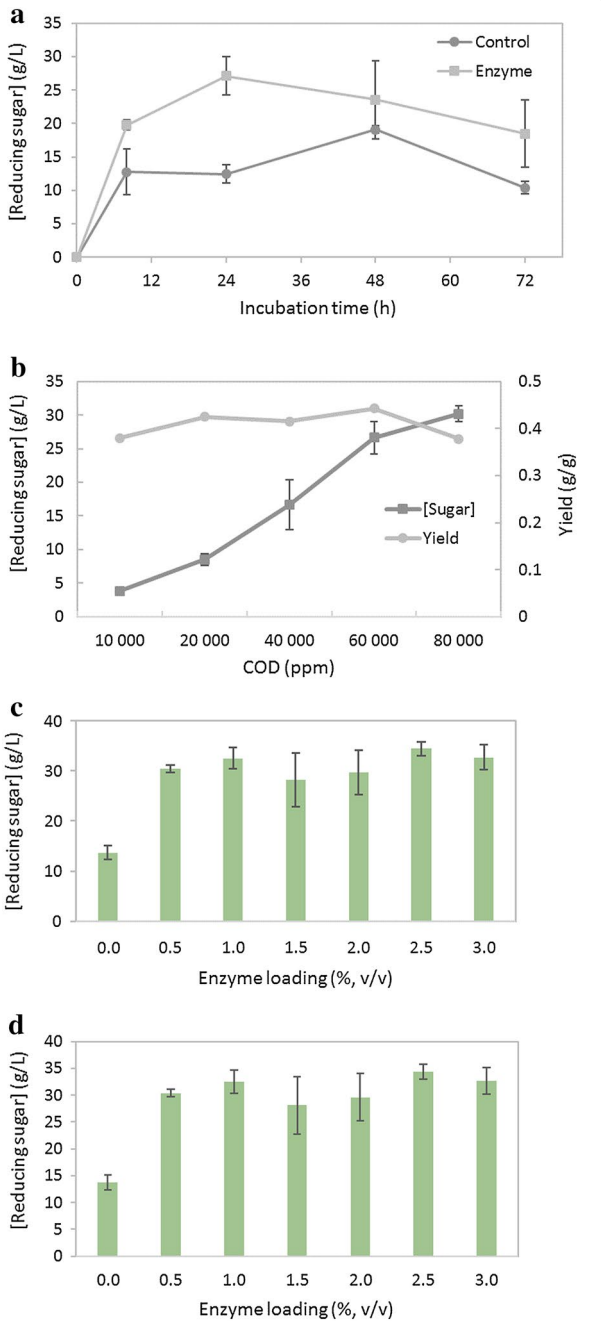

Fig. 1 Optimization of enzymatic hydrolysis of POME: **a** time course of reducing sugar released during enzymatic hydrolysis of 100 % POME using 1 % (v/v) enzyme loading for 72 h; **b** effect on substrate loading; **c** effect on enzyme loading; and **d** effect on surfactant addition. *Error bars* indicate standard deviation ($n = 3$)

within the same period (Elkady et al. 2011). Therefore, this period was used to harvest bioflocculant in the subsequent experiments.

After 24 h, the cell growth had stagnated as the cells entered the stationary phase causing a decrease in flocculating activity. In many cases, this represented the maximum flocculating activity in the so-called late logarithmic phase and early stationary phase, before it began to decrease further due to deflocculation of enzyme activity and cell lysis (Aljuboori et al. 2013; Salehizadeh and Yan 2014). The flocculating activity decreased slowly at 48 h, and by 72 h, *B. marisflavi* reached its death phase.

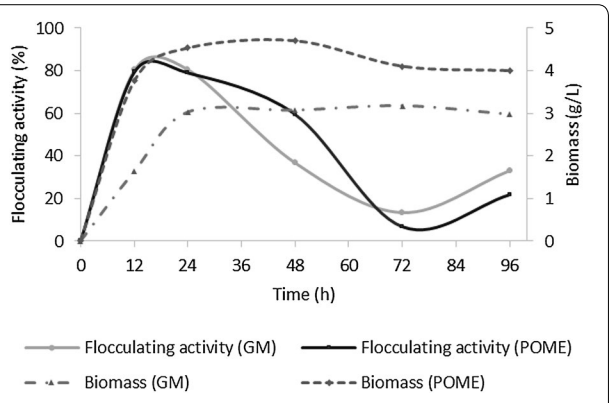

Fig. 2 Time course of bioflocculant production by *Bacillus marisflavi* NA8 in conventional growth medium (GM) and palm oil mill effluent (POME)

Effect of initial pH, temperature, inoculum size and nitrogen source

The culture conditions, i.e. initial pH, temperature, inoculum size and nitrogen source, on bioflocculant production in POME culture medium were investigated. The initial pH of the culture can affect the electric charge of the cells and the oxidation–reduction potential, nutrient assimilation and enzymatic reaction of bioflocculant producers (Salehizadeh and Shojaosadati 2001; Xia et al. 2008). Initial pH for bioflocculant production differs from one microorganism to another. Figure 3a shows the significant influence of the initial pH on cell growth and bioflocculant production by *B. marisflavi*. The highest flocculating activity was achieved at 24 h of fermentation with a neutral pH 7.0. This result was in agreement with other findings which also reported that bioflocculant producers from genus of *Bacillus* performed the best at neutral pH, e.g. *B. mojavensis* (Elkady et al. 2011), *B. clausii* (Adebayo-Tayo and Adebami 2014), *B. licheniformis* (Li et al. 2009), *B. subtilis, B. fusiformis* and *B. flexus* (You et al. 2008). As a result, an initial pH of 7.0 was used for the subsequent experiments.

Temperature is one of the most important parameters influencing bioflocculant production. The culture temperature associates well with the metabolism of microorganisms in use (Giri et al. 2015; Salehizadeh and Shojaosadati 2001). Figure 3b shows that fermentation at

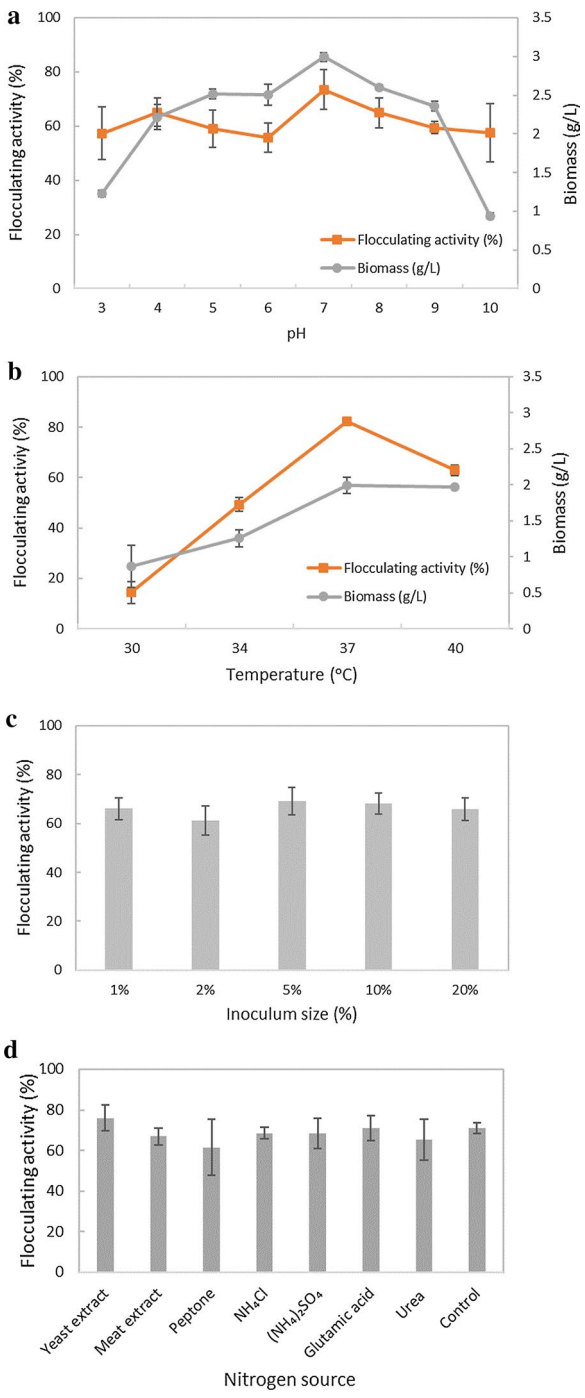

Fig. 3 Effect of culture conditions on bioflocculant production from palm oil mill effluent by *Bacillus marisflavi* NA8: **a** initial pH, **b** temperature, **c** inoculum size and **d** nitrogen source. Error bars indicate standard deviation ($n = 3$)

37 °C yielded the highest flocculating activity reaching 80 % with 2.9 g/L of cell biomass. Fermentation at either a lower or above the optimum temperature decreased the bioflocculant production by *B. marisflavi* NA8. In particular, lower temperature was the most unsuitable and least efficient. Other microorganisms which favour higher temperature that perform well at 37 °C are *B. clausii* (Adebayo-Tayo and Adebami 2014), *B. licheniformis*, (Li et al. 2009), *Agrobacterium* sp. (Li et al. 2010) and *Halomonas* sp. (Sam et al. 2011).

In the present study, the use of 5 % (v/v) of *B. marisflavi* NA8 in POME medium resulted in the highest flocculating activity. Figure 3c shows that higher inoculum concentrations (10 and 20 % v/v) did not correspondingly increase the bioflocculant production. Rather, a slight decrease was observed. Small inoculum size prolongs the lag phase, while a large inoculum causes excessive overlapping of niches of the strain, thus restraining bioflocculant production (Salehizadeh and Shojaosadati 2001). As shown in Fig. 3d, *B. marisflavi* NA8 could efficiently produce bioflocculant using POME hydrolysate without having to add nitrogen. Yeast extract could provide a positive attribute for the process than the control and other supplementation. On the other hand, addition of meat extract, peptone and urea slightly reduced the bioflocculant production as evidenced by a lower flocculating activity. This indicated that POME by itself was enriched with proteins sufficient for microbial growth. The suitability of *B. marisflavi* NA8 to grow in POME without additional nitrogen source could largely reduce the production cost of bioflocculant.

Functional group analysis of crude bioflocculant

The FTIR spectrum obtained showed some functional groups of POME-derived bioflocculant produced by *B. marisflavi* NA8 (Fig. 4). The trend in the FTIR spectrum of the bioflocculant was similar to most of the bioflocculants produced in the literature by different microorganisms. It showed a broad stretching frequency at 3275 cm^{-1}, which was a common characteristic of hydroxyl and amino groups (Li et al. 2009). A weak C–H asymmetric stretching vibration band was observed at 2920 cm^{-1}. Stretching mode observed at 1628 cm^{-1} indicated the presence of C = O in an amide group and the frequency at 1409 cm^{-1} was the result of COO^{-} symmetrical vibration (Zhang et al. 2013). The vibration band between 1000 and 1100 cm^{-1} suggested the presence of saccharide derivatives. These evidently showed the presence of common functional groups favourable for flocculation, i.e. hydroxyl, carboxyl and amino groups. These functional groups could serve as binding sites for divalent cations as well as suspended particles, in fact they were the preferred groups for most adsorption processes to take place causing floc formation (Comte et al. 2006; Wang et al. 2011).

Previously, POME with TOC concentration (12,710 mg/L) would produce 2.73 g/L purified

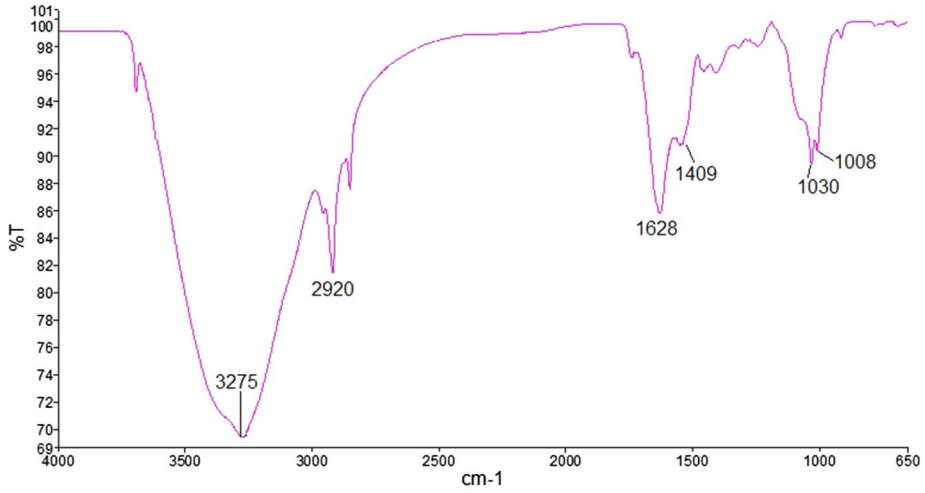

Fig. 4 FTIR spectrum of crude bioflocculant produced by *Bacillus marisflavi* NA8 from palm oil mill effluent

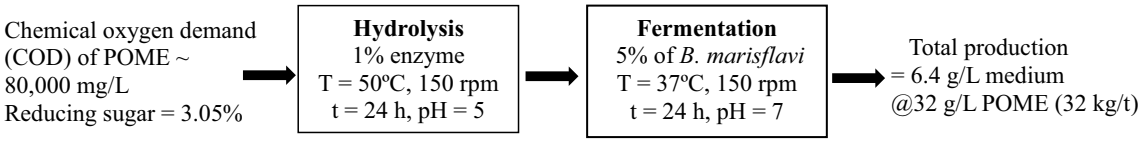

Fig. 5 Process summary of bioflocculant production from palm oil mill effluent (POME)

bioflocculant by *Aspergillus niger* with at an average flocculating rate of 77.2 % (Aljuboori et al. 2014). In this study, the hydrolysed POME contained a higher TOC of 23,250 mg/L; thus, higher bioflocculant production could be achieved. Using optimized culture conditions, approximately 6.4 g of crude bioflocculant could be recovered per litre fermentation broth of *B. marisflavi* culture. From Fig. 5, 32 g of bioflocculant could be produced out of 1.0 L POME. This translated into 32 kg/t bioflocculant from POME. The cost of POME hydrolysate is lower as compared to commonly used C sources (i.e. per litre fermentation medium—USD 0.06 for POME medium; USD 0.36, 0.90 and 2.25 for glucose, fructose and L-glutamate, respectively. This showed that POME could be a potential low-cost substrate for bioflocculant production.

Conclusion

POME is of potential use in a bioconversion process to provide sugar hydrolysates that can serve as a fermentation substrate for bioflocculant production. The cultivation and use of bioflocculant-producing bacteria from POME has both environmental and economic benefits. POME can potentially produce 32 g/L crude bioflocculant under the optimum conditions. This study demonstrated another feasible way to recover POME for value addition.

Authors' contributions

BN-A carried out the experiments, analysis and interpretation of data and drafted the manuscript; AB-N and S-KL have been involved in as co-researchers of the project and critically revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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