Global NEST Journal, Vol 18, No X, pp XX-XX, 2016

PERFORMANCE, YIELD AND CHARACTERISTICS OF BIOFLOCCULANTS (UPMBF13) PRODUCED BY *BACILLUS SUBTILIS* UPMB13 DURING SUBMERGED AND SOLID-STATE FERMENTATION

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Received: 26/02/2016
Accepted: 11/04/2016
Available online: 24/06/2016

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ABSTRACT

This study compares the distinctive performance, yield and characteristics of bioflocculants (UPMBF13) derived from submerged (SmF) and solid-state fermentation (SSF) of *Bacillus subtilis* UPMB13. The bioflocculants were subjected to morphological characterization, functional groups determination, molecular weight measurement, protein content quantification and specific compound identification. The SmF and the SSF strategies yielded an average of 2.70 g l^{-1} and 1.25 g kg^{-1} of UPMBF13, respectively. The submerged UPMBF13 resulted in a clear suspension with visible flocs formation, while flocs from the SSF treatment were less apparent. Higher total proteinaceous contents of UPMBF13 from the SSF led to the inferiority in flocculating performances. The produced UPMBF13 consisted of hydroxyl, carboxyl, methoxyl and carbonyl functional groups which contributed to their flocculating abilities. The molecular weights of UPMBF13 were around 10-50 kDa, characterizing them into low-molecular weight bioflocculants. The submerged UPMBF13 were more fibrous in nature than the SSF, giving the submerged UPMBF13 the upper hand in flocculation. SmF was the best fermentation method yielding high performing bioflocculants at a faster rate by the utilization of non-elaborative techniques. SSF, on the other hand, was proven feasible but further improvements are needed.

Keywords: *Bacillus subtilis*, Bioflocculants, Solid state fermentation, Submerged fermentation

1. Introduction

Bioflocculants are extracellular-polymeric substances with flocculating capabilities, produced naturally by microorganisms during growth (Subramanian *et al.*, 2009). They are recognized as an environmentally friendly alternative in suspended solid treatment applications compared to chemical flocculants that are being scrutinized for their harmful by-products (Muthulakshmi *et al.*, 2013).

Zulkeflee Z., Shamsuddin Z.H., Aris A.Z., Yusoff M.K., Komilis D. and Sánchez A. (2016), Performance, yield and characteristics of bioflocculants (UPMBF13) produced by *Bacillus subtilis* UPMB13 during submerged and solid-state fermentation, *Global NEST Journal*, **18**(X), XX-XX.

Limitations in commercial application of bioflocculants are mainly due to the implementation costs required in all aspects of their production (Christenson and Sims, 2011). Studies have compared the potentials of submerged (SmF) and solid-state fermentations (SSF) with emphasis on their advantages and disadvantages. Submerged fermentation, due to the minimal hassles required in its handling, monitoring, and overall process and control, is generally preferred (Singhania *et al.*, 2010). However, the SmF process could be more expensive due to the cost of the liquid substrate required. Solid-state fermentation (SSF) is accepted in the production of biopolymers (Thomas *et al.*, 2013) in general, as a substitute to the SmF, but documented evidence on bioflocculant productions specifically through SSF are still lacking. Implementation of SSF aims at curtailing production cost by utilizing readily abundant organic wastes as substrates (Dhillon *et al.*, 2013). Nonetheless, the SSF process could also be a challenge due to the complexity of handling bulk quantities of solid substrates (Abraham *et al.*, 2013). These issues raised uncertainties in controlling and standardizing the fermentation process for regular and continuous bioflocculant production in the long run.

The current study investigated the production of novel bioflocculants named; UPMBF13, by a locally isolated rhizobacterium *Bacillus subtilis* UPMB13 through both the SmF and SSF processes. To the best of our knowledge, no studies comparing submerged and solid-state fermentation on bioflocculant production have been published. The performance, yield, rate of production and the distinctive characteristics of the bioflocculants produced from both fermentations were compared to understand their flocculating potential and the efficacy of the fermentation strategy. The extraction mechanism for the bioflocculants produced from both the SmF and SSF will be described and their characterizations determined to reflect their flocculating potentials. This information will contribute to the overall understanding of the flocculating abilities of the produced bioflocculants so that to better select the best fermentation strategy to be implemented for continuous production of high quality bioflocculants during suspended solids treatment and commercialization.

2. Materials and methods

2.1. Bacterial strain

The bioflocculant-producing strain *B. subtilis* UPMB13 were obtained from the culture collection of the Department of Land Management, Universiti Putra Malaysia (Amir *et al.*, 2003). The rhizobacteria isolated from oil-palm root is an endospore former, Gram positive, motile bacteria with the optimum culture pH and temperature of 6-7 and 25-40^{o}C, respectively, when grown in a liquid media of tryptic soy broth (Merck). The strain produces mucoid and ropy colonies on tryptic soy agar (Merck) media but when cultured on nutrient agar (Merck) the colonies formed were devoid of these characteristics. The liquid culture broth bearing the strain is viscous in nature, indicative of the bacterial growth and UPMBF13 production. The validation of the strain using Basic Local Alignment Search Tool (BLAST) program analysis of the nucleotide sequence of the amplified product based on the 16S ribosomal ribonucleic acid (rRNA) gene sequencing verified the strain as *B. subtilis* at 99% similarity. Additional biochemical identification test based on 29 biochemical and enzymatic reaction tests (BBL Crystal Gram-Positive ID System) also proved the strain as *B. subtilis* (99%).

2.2. Submerged fermentation

Batch cultures of the submerged fermentation utilizing 100 ml tryptic soy broth (in triplicates using 250 ml Erlenmeyer flasks) were prepared (Merck, Spain) at 1% (v/v) inoculation level at the optimal conditions of initial pH 7.0, incubation temperature 25±2 °C to 30±2 °C, and shaking speed of 130 rpm for 24, 48 and 72 h. The flocculating capacities of each batch culture were verified through kaolin assays prior to the extraction and purification of UPMBF13 from the cell-free supernatant of the fermentation broth.

2.3. Solid-state fermentation

About 20 g of substrate in triplicates with 500 ml Erlenmeyer flasks were used for the batch cultures of the solid-state fermentation. The substrate consisted of solely soybean fiber residues (Natursoy, Spain) with the addition of wood chips as the non-nutritive bulking agent at a ratio of 2:1 (w/w). The optimal fermentation condition was at 5% (v/v) inoculation level with an initial pH of 6.5-7.0, initial moisture content at 60-65% and was incubated in a control temperature chamber at 25 °C±2 °C for 24, 48 and 72 h under static mode. Nothing less than 5% inoculum level was used to decrease the lag phase in UPMB13 growth on the solid substrate, while the level was also ensured not to exceed the optimal initial moisture content level needed for the fermentation. The flocculating capacities of each batch culture were verified through kaolin assays prior to the extraction and purification of the bioflocculants from the filtered substrate suspension. The preparation of the filtered substrate suspension is discussed elsewhere (Zulkeflee and Sánchez, 2014).

2.4. Extraction and purification

There is no unified method for bioflocculant extraction (Subramanian *et al.*, 2009). In this study, UPMBF13 produced through the submerged and solid-state fermentations were extracted through ethanol precipitations of the cell-free supernatant and the filtered substrate suspension, respectively at a ratio of 2:1 (v/v) of the ice cold ethanol (96% v/v) to the bioflocculant source. The mixed solution was left to precipitate overnight at 4 °C and then collected by centrifugation at 12,000 x g for 15 min at 4 °C before being re-suspended in ultra-pure Milli-Q water. The suspensions were further purified through dialysis against ultra-pure water with a visking tube with a molecular weight cut-off (MWCO) of 12-14 kDa (Kawaguchi and Decho, 2002) at a minimum of three times water change in 24 h at 4 °C to separate any impurities. The purified UPMBF13 were then collected through lyophilisation.

2.5. Flocculation assay

Flocculating activities of UPMBF13 derived from both fermentation strategies were measured according to the methods explained elsewhere (Zulkeflee and Sánchez, 2014). The flocculating activity was considered both via the clarity of the treated suspension, based on equation (1), and through visual observations of the visible floc formation.

$$\text{Flocculating activity (\%)} = [(A - B)/A] \times 100 \quad (1)$$

where: A is the optical density of the blank and B is the optical density of the treated kaolin suspension at 550 nm using a spectrophotometer. The pH at the start of the reaction is 5.5, which is the natural pH of the kaolin suspension without any amendments. Bioflocculants produced by UPMB13 were proven to have a wide pH tolerance from pH 4.0-8.0 (Zulkeflee *et al.*, 2012). All measurements were done in triplicates with the mean and standard deviation determined. Significant differences were analyzed through analysis of variance (ANOVA). Differences were considered significant at a 5% significance level ($p<0.05$).

2.6. Total protein content and amino acid derivatives

The total protein content of the purified UPMBF13 was determined through Bradford assay with bovine serum albumin (BSA) (2 mg ml^{-1}) (Sigma, Spain) as the protein standard. Amino acid derivatizations were determined using Waters Acc-Q Tag method, together with High Performance Liquid Chromatography (HPLC) (Waters, USA). The method utilizes the derivatization reagent named Acc-Q Fluor™ reagent which consists of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) that derivatizes primary and secondary amines in samples and produces fluorescently labeled amino acid for detection by the HPLC (Samicho *et al.*, 2013). About 0.1 g UPMBF13 samples were digested with 5.0 ml of 6 N HCl for 24 h at 110℃. The resulting hydrolysates were added with 4.0 ml of 2.5 mM alpha amino butyric acid (AABA) and 100 ml of de-ionized water and were filtered using Whatman No.1 filter paper and through a syringe filter. A volume of 10 µl aliquot from the filtrates was added with 100 µl AQC reagent and subsequently about 5 µl from the mixed solution were injected into the HPLC for each sample. The Waters Acc-Q Tag column

(3.9 mm x 150 mm) (Waters, USA) was used as the column for the HPLC. The column temperature was set at 36℃. Acc-Q Tag Eluent A concentrate and 60% acetonitrile Acc-Q Tag Eluent B were used as the mobile phase at a flow rate of 1 ml min^{-1}. The fluorescence detector excitation wavelength was 250 nm, the emission wavelength was 395 nm, gain and filter set were 1.0 and 1.5 seconds, respectively.

2.7. Molecular weight determination

The molecular weights of the extracted UPMBF13 were determined by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis (Yong *et al.*, 2011) using a vertical mini gel electrophoresis setup; Bio-Rad Mini-PROTEAN® Tetra cell (Bio-Rad, Spain). The Mini-PROTEAN® TGX™ precast gel was mounted on the cassette and 1x running buffer was added and allowed to overflow into the mini tank. UPMBF13 samples were diluted at a ratio of 3:1 (v/v) in the sample buffer (Laemmli loading dye) which was priorly added with β-mercaptoethanol (BME) and then denatured by heating at 95 °C for 10 min. The denatured samples were then loaded into the respective wells; with the UPMBF13 of the SSF loaded in the second to the fifth well (quadruplicates), followed by the SmF UPMBF13 in the sixth to the ninth well (quadruplicates). The Precision Plus Protein™ All Blue Standard and Precision Plus Protein™ Dual Color Standard (Bio-Rad, Spain) were inserted into the first and the tenth well, respectively, as the molecular weight markers. The electrophoresis was run at 85 V constant for 1 h and a half at room temperature. The gel collected was washed with de-ionized water and subsequently fixed with 40% methanol and 10% acetic acid mixture for 30 min, followed by 50 ml of Coomassie G-250 staining solution (Bio-Rad, Spain). The gel was left soaked in the staining solution overnight and the visible bands were finally observed when the gel was submerged in de-ionized water.

2.8. Functional group determination and morphological characterization

The functional groups of UPMBF13 were determined as pressed potassium bromide, KBr disks using Fourier Transform Infrared (FT-IR) Bruker Tensor 27 (Bruker Optics, Germany) spectroscopy and the morphological characterization through scanning electron microscope (SEM) EVO-MA10 (Carl Zeiss, Canada). The samples attached onto carbon stubs were gold-coated twice and then examined at an accelerating voltage of 20.0 Kv (Zulkeflee and Sánchez, 2014). Further qualitative determinations of polysaccharide content in UPMBF13 were also determined through the FT-IR analysis.

3. Results and discussion

3.1. UPMBF13 yield, rate of production and flocculating performances

About 0.90 g l^{-1} of purified UPMBF13 can be collected from the liquid broth of the SmF at a minimum 24 h of incubation and about 1.25 g kg^{-1} from the fermented substrate of the SSF at a minimum of 72 h of incubation under optimized fermentation conditions. The amount of UPMBF13 recovered in SmF is comparable to the amount of bioflocculant PX collected from *Bacillus circulans* at 0.78 g l^{-1} reported by Li *et al.* (2009). A notably lower recovery was obtained in SSF when compared to the biopolymer poly-γ-glutamic acid collected from *Bacillus licheniformis*, at 9.84 g kg^{-1} (Bajaj *et al.*, 2008).

On a relative basis of the amount of substrate used, the production through SSF yielded approximately 30% higher UPMBF13 production compared to the SmF. Higher yield obtained in the SSF compared to the SmF are common in other comparative studies on the SmF and SSF bioproduction of enzymes (Sandhya *et al.*, 2005; Singhania *et al.*, 2010). However, in this study the rate of UPMBF13 production from the SmF was notably faster. This was particularly due to the nature of the liquid substrate which is naturally easier to assimilate for growth, while the solid substrate of the SSF would take a longer period to biodegrade and promote growth and bioflocculant production. It is extrapolated that the SmF can produce an average of 2.7 g l^{-1} of UPMBF13 in 72 h; which is two-fold the value of the UPMBF13 produced by the SSF in the same duration. Therefore, time-wise, it was concluded that UPMBF13 production by *B. subtilis* UPMB13 was more feasible through the SmF strategy.

Figure 1 depicts the flocculating performances of UPMBF13 of the SmF and the SSF in terms of percentage suspension clarity and flocs formation. The SmF UPMBF13 led to a maximum activity of 95% with large visible flocs formed while the SSF UPMBF13 had a maximum activity of 71% with less apparent flocs were observed. The performances comparison was done under optimized flocculation conditions (Zulkeflee *et al.*, 2012).

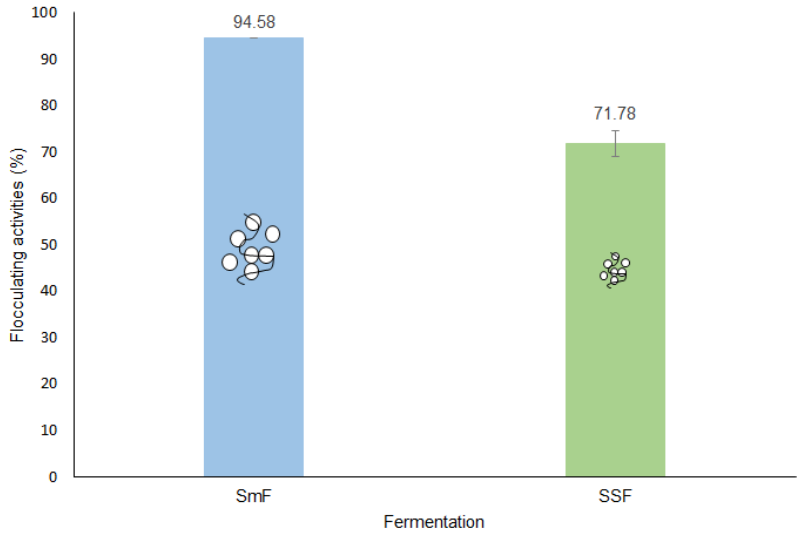

Figure 1. Flocculating activities of UPMBF13 of the SmF and the SSF. The indicates the visualization of the flocs formed upon treatment with the bioflocculants.

3.2. Total proteinaceous content

About 16 amino acids peaks can be detected through the Waters Acc-Q Tag method, including the internal standard used, AABA, with the exception of cysteine, asparagines and glutamines. Table 1 summarizes the results for free amino acids derivatized from both the SmF and SSF.

UPMBF13 were found to be composed of 6.9% (w/w) and 3.6% (w/w) protein and of 17.1% (w/w) and 46.8% (w/w) amino acids from both the SmF and SSF, respectively. Charged anions of weak carboxylic groups are always present on the surfaces of any intra and extracellular proteins (Hess and van der Vegt, 2009). The higher amount of protein and amino acid present would contribute to the additional negative charges in the flocculation system. Therefore, the higher amount of total proteinaceous content (protein and amino acids) of the SSF could contribute to a lower flocculating performance as compared to the UPMBF13 from the SmF due to the overbearing negative charges it possessed. This was proven true in comparing the general performances of UPMBF13 of the SSF that were inferior to that of the SmF (Figure 1).

Glutamic acid was the major fraction of the total amino acid derivatized from UPMBF13 of the SmF at a percentage of 44.7% (w/w) followed by lysine at 9.91% (w/w). The SSF differed, since the major fraction was found to be lysine, at a percentage of 15.4% (w/w) whilst glutamic acid was found second at a 5% lower quantity. Scrutinizing more, it could be seen that the percentage amount of other free amino acids present in UPMBF13 from SSF were higher compared to the corresponding amounts from the SmF. For example: aspartic acid (7.76%), glycine (6.17%), arginine (4.02%), threonine (5.23%), alanine (4.44%), proline (2.62%), tyrosine (2.46%), valine (5.16%), isoleucine (4.48%), leucine (7.31%), phenylalanine (5.22%) and serine (4.90%).The high levels of glutamic acid measured in both UPMBF13 sources prove the occurrence of repeated unit of the amino acid forming the poly-γ-glutamic acid (γ-PGA). The γ-PGA is the only biopolymer produced by *B. subtilis* strains that has been reported to have flocculating capabilities (Bhunia *et al.*, 2012). Therefore, the bioflocculants from *B. subtilis* UPMB13 was confirmed to be consisted of γ-PGA as one of the important flocculating fractions.

Table 1. Free amino acid composition of 0.1 g UPMBF13 of the SmF and SSF

Amino Acid	SmF amount (ng)	SSF amount (ng)
Glutamic acid	7.67	5.65
Lysine	1.70	8.42
Aspartic acid	0.91	4.25
Serine	0.83	2.68
Threonine	0.82	2.86
Glycine	0.81	3.37
Leucine	0.70	4.00
Valine	0.64	2.82
Alanine	0.58	2.43
Arginine	0.55	2.20
Phenylalanine	0.49	2.86
Isoleucine	0.47	2.45
Proline	0.35	1.43
Tyrosine	0.33	1.34
Methionine	0.27	-
Histidine	0.16	-

3.3. Molecular weight

Molecular weight is one of the most important determinants of flocculating activity of a polymeric flocculant (Ji *et al.*, 2010). The visualizations of the molecular bands showed that the molecular weight of UPMBF13 of the SmF was between 10-50 kDa while for the SSF the molecular weight was around 10-37 kDa (Figure 2); characterizing the SmF UPMBF13 as a higher molecular weight bioflocculant than the SSF. The difference in colour intensities of the bands from the SSF samples could be due to interference from the buffer used or even from the dye when it comes in contact with the proteins of the sample. This is because proteins interact differently with protein stains even at identical protein loads (http://www.bio-rad.com/en-sg/applications-technologies/sds-page-analysis). However, this is not observed for the SmF samples. Samples giving lower intensity bands than the other might be due to the protein forming soluble aggregates. Moreover, different staining intensities could also be due to different cell densities of the samples (Greer *et al.*, 2010).

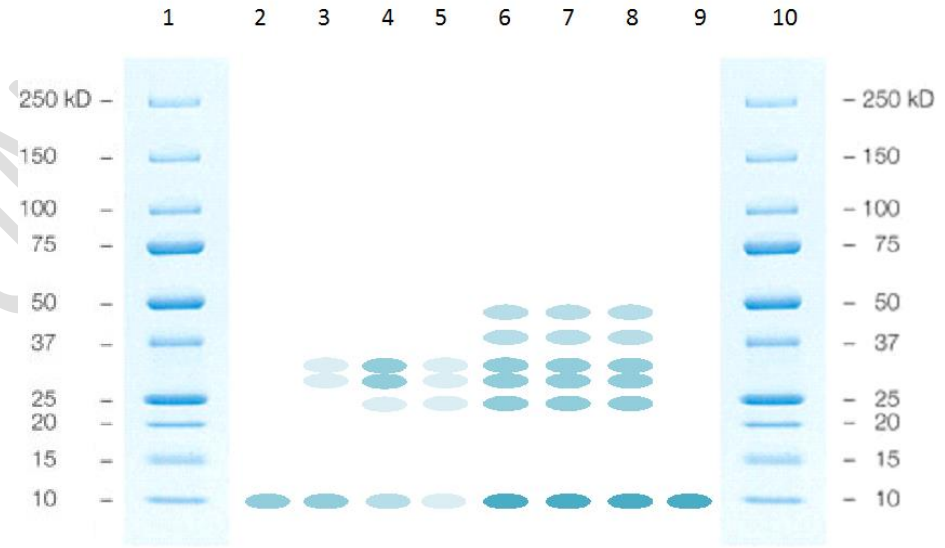

Figure 2. Molecular weight of UPMBF13 of the SSF (well number 2 to 5) and the SmF (well number 6 to 9) left overnight in de-ionized water, re-drawn from the stained gel obtained from the electrophoresis

The increase in polymer molecular weight would lead to a higher molecular polymeric adsorption capacity (O'Shea *et al.*, 2010), hence better flocculating performances. Therefore, the relatively higher molecular weight of the SmF UPMBF13 would lead to a better flocculating performance as compared to the SSF. Nevertheless, UPMBF13 from both sources are still considered to be low-molecular weight bioflocculants with high flocculating capacities in comparison to other reported low-molecular weight bioflocculants produced by *Agrobacterium* sp. M-503 at 8.1×10^4 Da. (Li *et al.*, 2010).

3.4. Functional groups and polysaccharide presence

Overall interpretations of the infrared spectra are in accordance with the approach by Coates (Coates, 2000). Referring to Figure 3(a) of the infrared spectrum of UPMBF13 from the SmF, the broad absorption peak at 3255 cm^{-1} exhibits the characteristic of O-H band of the hydroxyl functional group (Ntsaluba *et al.*, 2013). The weak peak at 2923 cm^{-1} indicates the presence of aliphatic C-H stretching from methylene group, while the peaks at 1574 cm^{-1} and 1447 cm^{-1} showed the stretching band of the carboxyl group (COO-) originated from phenols or tertiary alcohols (Ntsaluba *et al.*, 2013). The 1316 cm^{-1} and 1262 cm^{-1} peaks imply the occurrence of C-O bond of the carboxyl group and P=O bend of the phosphoramide group, respectively.

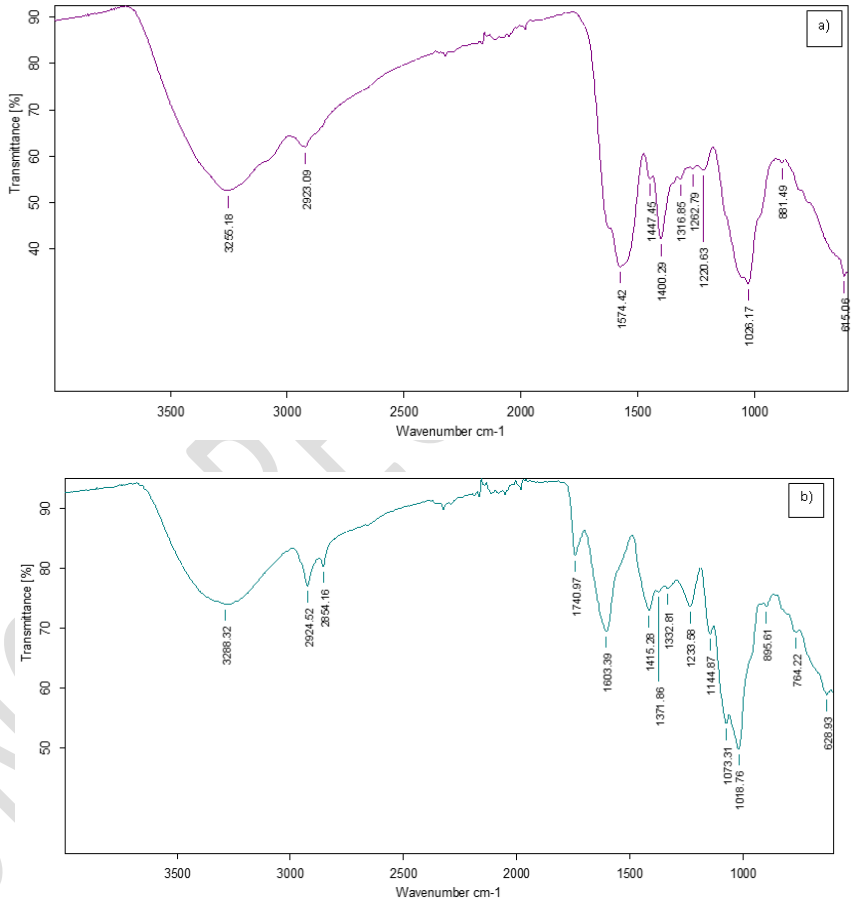

Figure 3. Infrared spectra of UPMBF13 derived from (a) SmF and (b) SSF

The infrared spectrum of UPMBF13 from the SSF in Figure 3(b) showed the wide stretching at 3288 cm^{-1} and the sharp spike at 2924 cm^{-1} are characteristics of hydroxyl and methylene functional groups, respectively. The small peak at 2854 cm^{-1} shows the presence of methyl ether group (Coates, 2000). A modest spike at 1740 cm^{-1} indicates the presence of the carbonyl functional group (C=O) (Li *et al.*, 2009). The 1603 cm^{-1} and 1415 cm^{-1} stretch are naturally carboxyl in nature. Sulfur-oxy compounds presence could be detected by the weak bend at 1371 cm^{-1} and 1332 cm^{-1} in the form of dialkyl sulfones or organic sulfates (Coates, 2000). The 1233 cm^{-1} peak shows the occurrence of carboxyl group. Sharp spikes at 1073

cm^{-1} and 1018 cm^{-1} together with a smaller spike at 1144 cm^{-1} signify the presence of methoxyl groups (Aljuboori *et al.*, 2013). The occurrence of intense peaks in the area of 1200 cm^{-1} to 800 cm^{-1}, the fingerprint region of sugars (Wu and Ye, 2007), portrayed in both spectra suggest the presence of polysaccharide in UPMBF13, concluding the missing flocculating fraction apart from the γ-PGA.

In general, the infrared spectra from both sources show the presence of hydroxyl, carboxyl, methoxyl, and carbonyl groups, which are the key functional groups in flocculation processes (Wu and Ye, 2007). These groups provide charged binding sites suitable for aggregation and flocs formation of the suspended colloidal particles by the added bioflocculant (Ntsaluba *et al.*, 2013).

3.5. Morphological characteristics

The microscopic images of the surface morphological characteristics of UPMBF13 obtained through the SmF and SSF are depicted in Figure 4(a) and Figure 4(b), respectively.

The images from both sources appeared identical with some distinct features which could be seen captured at different angles. Generally, UPMBF13 are naturally fibrous in nature with thin and broad crust-like flake structures observed through higher magnification. The fibrous nature of UPMBF13 is more apparent for the SmF compared to the SSF. It can also be noticed that smooth globular or cylindrical structures are present together and are interlinked between the crust-like formations at some angles. These notably are the attributes of γ-PGA (Yang *et al.*, 2011).

Figure 4. Microscopic images of UPMBF13 of (a) SmF: and (b) SSF

4. Conclusions

The SmF and SSF strategies yielded an average of 2.70 g l^{-1} and 1.25 g kg^{-1} of UPMBF13, respectively after 72 h fermentations. The compositional fraction of UPMBF13 includes protein at 6.9% (w/w) and 3.6% (w/w) and amino acids at 17.1% (w/w) and 46.8% (w/w) from both the SmF and SSF, respectively. The flocculating fractions of the bioflocculants are concluded to be polysaccharides and γ-PGA. The average molecular weights of the bioflocculants from both sources ranged between $10x10^3$ Da to $50x10^3$ Da, thus characterizing them to be high-performing low-molecular weight bioflocculants. Furthermore, the bioflocculants were justified to possess all four key functional groups in a good flocculation system, namely the hydroxyl, carboxyl, methoxyl and carbonyl groups. The bioflocculants were observed to be fibrous with broad and thin crust-like structure, which provide the bridging mechanism in bioflocculation. In general, the attributes of the SmF bioflocculants were superior to the SSF ones. Thus, the SmF is the best fermentation strategy to be implemented for continuous production of high performing bioflocculants in the long run. Further cost related studies could be done to improve the SmF strategy through non-elaborative techniques and through utilization of cheaper substrates to ensure cost-

effectiveness of its implementation. Furthermore, more studies are needed for the SSF strategy to improve the performances of the bioflocculant produced.

Acknowledgements

This work was supported by the Ministry of Higher Education, Malaysia under the Fundamental Research Grant Scheme [02/04/10/832FR], the Spanish Ministerio de Economía y Competitividad [Project CTM2015-69513-R] and the European Commission under the Erasmus Mundus Action 2 Plan (MAHEVA Project) scholarship program. Dimitrios Komilis thanks Techniospring for the financial support.

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