Solid-state fermentation of soybean residues for bioflocculant production in a pilot-scale bioreactor system

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Abstract

An innovative approach using soybean residues for the production of bioflocculants through solid-state fermentation was carried out in 4.5 L near-to-adiabatic bioreactors at pilot scale level. An added inoculum of the strain *Bacillus subtilis* UPMB13 were tested in comparison with control reactors without any inoculation after the thermophilic phase of the fermentation. The flocculating performances of the extracted bioflocculants were tested on kaolin suspensions and crude bioflocculants were obtained from 20 g of fermented substrate through ethanol precipitation. The production of bioflocculants was observed to be higher during the death phase of microbial growth. The bioflocculants were observed to be granular in nature and consisted of hydroxyl, carboxyl and methoxyl groups that aid in their flocculating performance. The results show the vast potential of the idea of using wastes to produce bioactive materials that can substitute the current dependence on chemicals, for future prospect in water treatment applications.

Keywords: *Bacillus subtilis*; bioflocculant; pilot scale; solid-state fermentation; soybean wastes.

Introduction

The current interest of producing biologically-derived products of environmental importance has led to the emergence of green and environmentally friendly technologies in bioprocesses for the substitution of the present dependence on chemically-based approaches. These bioprocessing technologies are likewise devoted to the development of cost-effective measures through the utilization of cheaper fermentation substrates to meet the current market demand for the interested bioproducts (Oner, 2013). In this framework, solid-state fermentation (SSF) using readily available wastes as sources of carbon and energy for the production of bioactive compounds are currently seek over as alternative to other conventional fermentation methods that are more costly and mostly chemically driven (Zimbardi *et al*., 2013). According to Mitchell *et al.* (2006), the shift of interest to SSF is due to the fact that some microorganisms may produce bioactive products only under solid-state conditions or in relatively higher amounts when compared to submerged fermentation. In addition to this, SSF based on wastes as substrates will not only curtail production cost but also help the environment through the minimization of waste disposal (Dhillon *et al.*, 2013).

The basic principle of SSF is the culturing of microorganisms in the absence or near-absence of free water on humid substrates; commonly agricultural residues or food processing by-products, with just the adequate moisture to sustain the biological growth (Pandey, 2003; Abraham *et al.*, 2013).

Soybean pulp is a part of the soybean fibres leftover from the production of soy-based products especially tofu (Shurtleff and Aoyagi, 2013). This part of soybean is partially humid and corresponds to the oldest and most insoluble part of the soybean fibres, which are available in bulk quantities. This makes them suitable as zero-cost substrate for a SSF system.

Bioflocculants are extracellular polymeric substances produced by microorganisms that act as aid for flocculating suspended particles in water treatment applications (Kimura *et al.*, 2013). The interest in producing bioflocculants that are more environmentally friendly (Muthulakshmi *et al*., 2013) is as an alternative to the commonly used commercial flocculants which are reported to be potent carcinogen; due to their toxic monomers, that pose threats to human health and the ecosystem (Buraimoh and Ojo, 2013). The research conducted on bioflocculant production by microorganisms were mostly based on the usage of the conventional submerged fermentation (SmF) processes (Buthelezi *et al*., 2010; Luvuyo *et al*., 2013). However, the production of bioflocculants by SSF using wastes are scarcely studied and thus, it can be considered a novel approach. Hence, this study addresses the interest of producing bioflocculants through SSF using solely soybean fibres as substrate at an easily scalable level that can be a base for a complete scale-up of the SSF system. The usefulness of a specific inoculum such as *Bacillus subtilis* UPMB13, which is a well-known producer of bioflocculants under sterilized SmF conditions (Zulkeflee *et al*., 2012), is also studied. The study highlights not only the SSF conditions during the microbial growth and the production of bioflocculants, but also addresses the extraction mechanism and the characterization of the extracted bioflocculant for future production and commercialization.

Methods

Solid-state fermentation (SSF)

The fermentation conditions for the bioflocculant production by SSF were as reported by the authors in Zulkeflee and Sánchez (2014). Soybean residues were obtained from the leftover production of tofu by Natursoy (Barcelona, Spain) and were used as the sole substrate for the fermentation. Wood chips were added to the soybean fibres at a volumetric ratio of 2:1 (soybean fibres:wood chips) resulting in a substrate mixture with an initial moisture content of 60% (wet weight). The main characteristics of the substrate mixture are presented in Table 1. The wood chips acted as bulking agent that promotes porosity and allows aeration and oxygen diffusion to the entire system (Ruggieri *et al*., 2009; Santís-Navarro *et al*., 2011). Another important property of the mixture is its biodegradability that, measured as dynamic respiration index, shows a relatively high level of microbial activity (Table 1). The determination of the dynamic respiration index and its interpretation can be found elsewhere (Ponsà *et al*., 2010; Barrena *et al*., 2011).

Table 1 General characteristics of the substrates mixture.

Substrate mixture	Soybean fibres:Wood chips (2:1)
Water content (% db)	60.3 ± 0.6
Organic matter (% db)	97 ± 1
pH	5.37 ± 0.04
Dynamic respiration index (g O_2 kg^{-1} DM h^{-1})	2.2 ± 0.2

db: dry basis; DM: dry matter

The mixtures were then processed in 4.5 L pilot-scale thermally isolated bioreactors (Dewar® vessels) resulting in approximately 1200 g of total mass per batch. The experimental setup for the bioreactors can be found elsewhere (Santís-Navarro *et al*., 2011; Abraham *et al*., 2013). Briefly, air was continuously supplied at a rate of 0.1 L/min and leachates were recovered from the bottom of the reactor where a mesh is placed to separate the leachate and substrate and acts as an air sparger. Temperature and oxygen were continuously recorded (Santís-Navarro *et al*., 2011; Abraham *et al*., 2013).

The flocculating activities of the bioflocculants produced were tested out by sampling about 20 g of fermented substrate from each reactor at 24, 48, 72 and 96 h after inoculation of the inoculated reactor. All the material in each reactor was mixed before sampling. The fermentation was run for a total of 11 days. During SSF, two strategies were tested. The first one consisted of inoculating a reactor with *B. subtillis* UPMB13 cultured in tryptic soy broth at a level of 5% (v/w) after the thermophilic phase of the SSF, while the other reactor was remained un-inoculated, with only the presence of the autochthonous microorganisms of the wastes. Three replications were carried out of each strategy in different moments. The results of each strategy were very similar and the deviation was less than 5% in the level of bioflocculant produced, although typical differences in temperature were observed. For this reason, only one example of each strategy is presented.

Extraction of bioflocculants

The recovery method of the bioflocculants produced through SSF was in accordance with the procedure described by Chen *et al.* (2005) with some minor modifications. About 20 g of fermented solid material were diluted with 10 volumes of distilled water and then shaken at 25°C in an orbital shaker at 130 rpm for 1 h. The mixture was then filtered through a double layer of muslin cloth to remove the bulk wood chips and soybean residues. The filtered liquid was then repeatedly (3 times) centrifuged at 10,000xg for 10 min to remove any residual fibres to get a clear supernatant. The supernatant was then vacuum-filtered through a 4.5 µm Whatman filter paper. The resulting filtrate was then added to two volumes of ice-cold ethanol (96% v:v) and allowed to precipitate overnight at 4^{o}C. The precipitated bioflocculants were then collected through centrifugation at 12,000xg for 15 min and redissolved in ultrapure Milli-Q water. This further product served as the crude bioflocculant suspension used in the flocculation assay.

Flocculation assay

Flocculation assays using kaolin clay with an average diameter of 4-5µm (Kaolin (M) Sdn. Bhd., Malaysia) as suspended particles were conducted in accordance with the method explained in Zulkeflee *et al*. (2012), slightly modified for the determination of the flocculating performance of the crude bioflocculants derived from SSF, as explained: 50 mL of the kaolin clay suspension were pipetted in a 100 mL conical flask. 0.5 mL of the crude bioflocculant suspension was added to the kaolin suspension together with 4.5 mL of 0.1% $CaCl_2$. The flask was then shaken for 30 s and left to settle for 5 min. The flocculation activity was expressed by measuring the absorbance in optical density of the upper phase of the suspensions after settlement using a spectrophotometer at the wavelength of 550 nm. The flocculation activity (in percentage) was calculated according to the Equation (1) by Kurane and Matsuyama (1994):

$$\text{Flocculating activity (\%)} = [(A-B)/A]\ (x100) \qquad (1)$$

where A is the optical density (OD) of control (no bioflocculant added) at 550 nm and B is the OD of the sample at 550 nm.

Characterization of the bioflocculants

Functional group determination

The functional groups of the dry bioflocculants were determined using the potassium bromide disks method. KBr disks were obtained through FT-IR spectrometry using a bench-top FTIR spectrometer Bruker Tensor 27 (Bruker Optics, Germany) with a Golden Gate™ MKII Single Reflection Attenuated Total Reflectance (ATR) (Specac, England) accessory. Spectra between 500-4000 cm^{-1} were recorded (Espargaró *et al*., 2012). The determination of the functional group of the bioflocculant was done at room temperature (25^{o}C).

Surface morphological characteristics

The surface morphology of the dry bioflocculant was observed using a scanning electron microscope (SEM) EVO-MA10 (Carl Zeiss, Canada). The samples attached to carbon stubs as the conductive bridge, were gold-coated twice through sputtering to eliminate any charge effect and then examined using the microscope at an accelerating voltage of 20.0 kV.

Routine analytical methods

Dry matter, organic matter and pH were measured using standard methods according to the US Composting Council (2001).

Results and Discussion

Temperature and oxygen profiles

The profiles of temperature and oxygen levels during the solid-state fermentation of the soybean residues are depicted in Figure 1a and 1b for the control bioreactor (with no added inoculum) and the bioreactor inoculated after the thermophilic phase, respectively. This figure is selected as an example of both strategies.

Overall both fermentations have similar general trends where the initial increment of temperatures were observed at the second day of fermentation, which were basically due to the rapid biodegradation of the substrate by the indigenous microorganisms, and later a gradual decrease before rising up again due to the unstable nature of the organic matter degradation process (Abraham *et al*., 2013). The temperature of the inoculated bioreactor (Figure 1b) experienced an increase after inoculation and was maintained around 35^{o}C while the un-inoculated reactor temperature continued its gradual decrease after a slight increase due to sampling. This can be due to the additional activity and proliferation that occurs after the inclusion in the reactor of the *B. subtilis* UPMB13 strains. The observed downward spike of the temperature and upward spike of the oxygen levels throughout the curve were due to sampling, where the reactors were briefly disconnected from aeration to allow the homogenisation process during sampling. It could be also seen that after each sampling a slight increase in temperature occurred, especially in the earlier days of the sampling from 6th to the 8th day. This is normally observed after each homogenisation process (Abraham *et al*., 2013). Furthermore, the oxygen levels were observed to be maintained above 10% in concentration in air, demonstrating that the bioreactor system remained well aerated throughout the fermentation process and the prevalence of aerobic conditions.

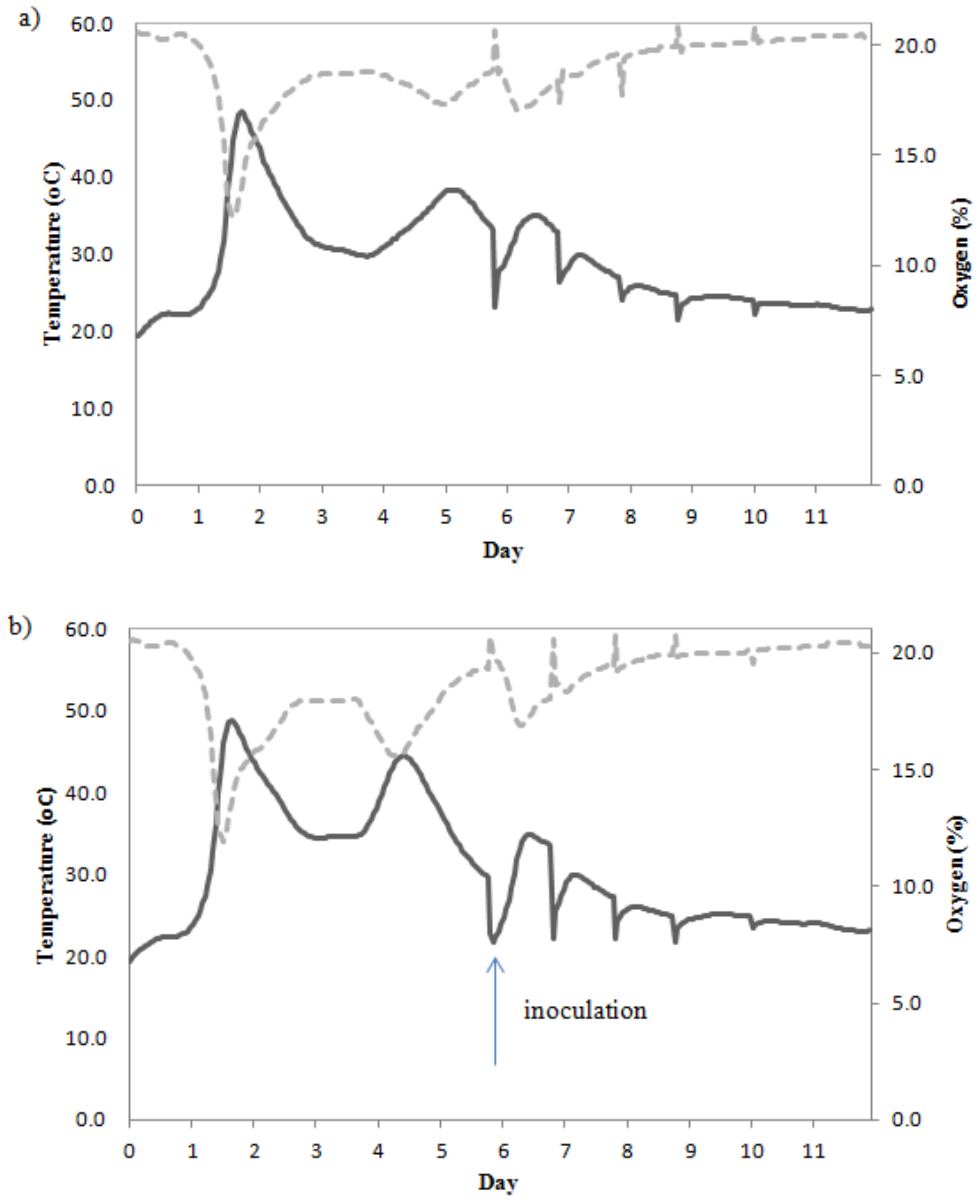

Figure 1. Temperature (solid line) and oxygen (dotted line) profile of the (a) control bioreactor and (b) the inoculated bioreactor with 5% *Bacillus subtilis* UPMB13.

Flocculating activity

The production of bioflocculants during SSF was determined by measuring the flocculating activities. The production took place later during the fermentation process, towards the ceasing of bacterial growth, which suggests that the excretion of bioflocculants by the consortia of indigenous microbes together with the added inoculums occurs during the cell autolysis in the death phase. This result is similar to the findings reported by Salehizadeh *et al.* (2000) and Liu *et al*. (2010), where it was reported that the bioflocculants production increased sharply towards the end of growth while being relatively lower anytime earlier than the death phase.

Figure 2 shows the production of bioflocculants from the samples fermented after the thermophilic phase. Basically, it can be observed that the indigenous microorganisms,

living in the substrate mixture, have the potential to produce the bioflocculants. This can be demonstrated as in the inoculated bioreactor at initial sampling time just before inoculation, the flocculating activity achieved was already around 70%. In contrast, at the same initial time the bioflocculant from the control reactor was lower. The control reactor continued an increasing trend of the bioflocculant production towards the end of the fermentation while the inoculated reactor fluctuated with time. A possible explanation for this fact could be that *B. subtilis* UPMB13 presence poses a competitive environment to the indigenous microbes, especially in terms of food availability. Therefore, as a survival mechanism, the excreted bioflocculants might have been consumed back by them as substitute for food. This is in accordance with the findings of Kimura *et al*. (2004), where it is reported that in the later stationary phase of growth, the produced bioflocculants may be a source of food during starvation.

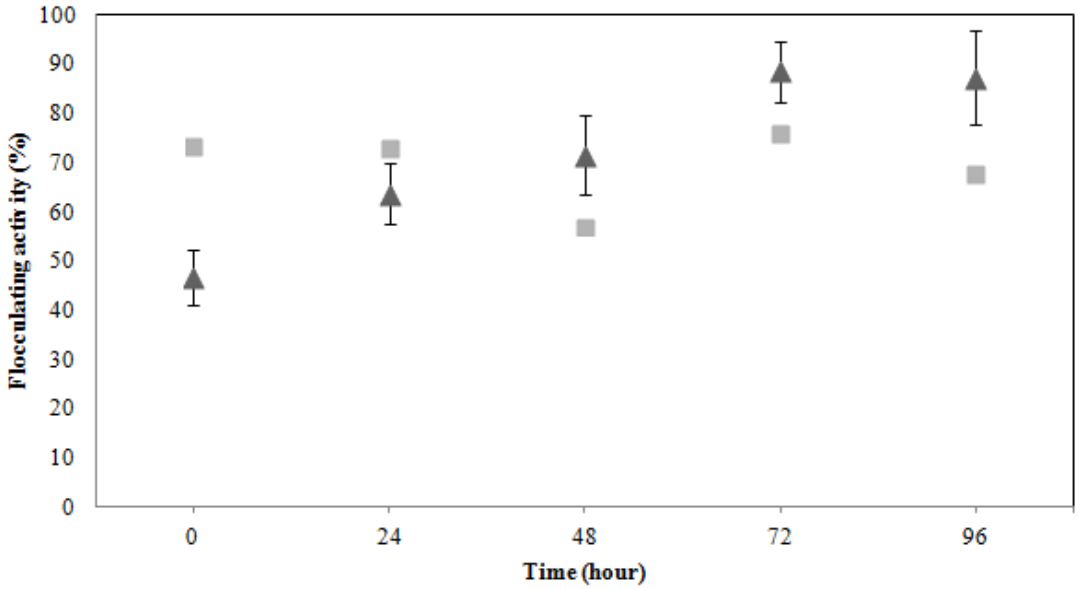

Figure 2. Flocculating activities measured at the sixth day of fermentation at the end of the thermophilic phase, denoted by time zero, followed by the activity registered at 24, 48, 72 and 96 h after inoculation. Symbols: control bioreactor (▲) and inoculated bioreactor (■).

According to Abraham *et al.* (2013), scaling-up a SSF process could be a challenge due to the intrinsic complexity of managing bulk quantities of solid substrate. Nevertheless, in this study, the production of bioflocculants was proven practical at a pilot scale level. The nature of the pilot scale setup using readily available soybean fiber wastes incurred minimal cost (only transportation) and was non-elaborative and incomplex without any modification to the substrate system, thus making it a promising low-cost production process that would be profitable for future prospect and further scale-up at an industrial level for mass production of the bioflocculants for commercialization.

Infrared spectra

Figure 3a and 3b presents the infrared spectra of the crude bioflocculants collected both from the control and the inoculated reactor, respectively. Both spectra look generally similar with very slight differences as the one from the control bioreactor presents more

peaks.

The absorption peak observed around the 3200-3289 cm^{-1} region exhibits the characteristic of O-H band (Liu *et al*., 2010). The weak vibration band at 2923-2929 cm^{-1} indicates the presence of aliphatic C-H bands (He *et al*., 2010), while the 1542-1548 cm^{-1} showed the stretching band of the COO- group (Liu and Cheng, 2010). The 1412-1443 cm^{-1} bands imply the presence of uronate, carboxyl and methoxyl group (Nwodo and Okoh, 2012). The peaks between 1066 cm^{-1} and below generally refer to sugar derivatives (Wu and Ye, 2007).

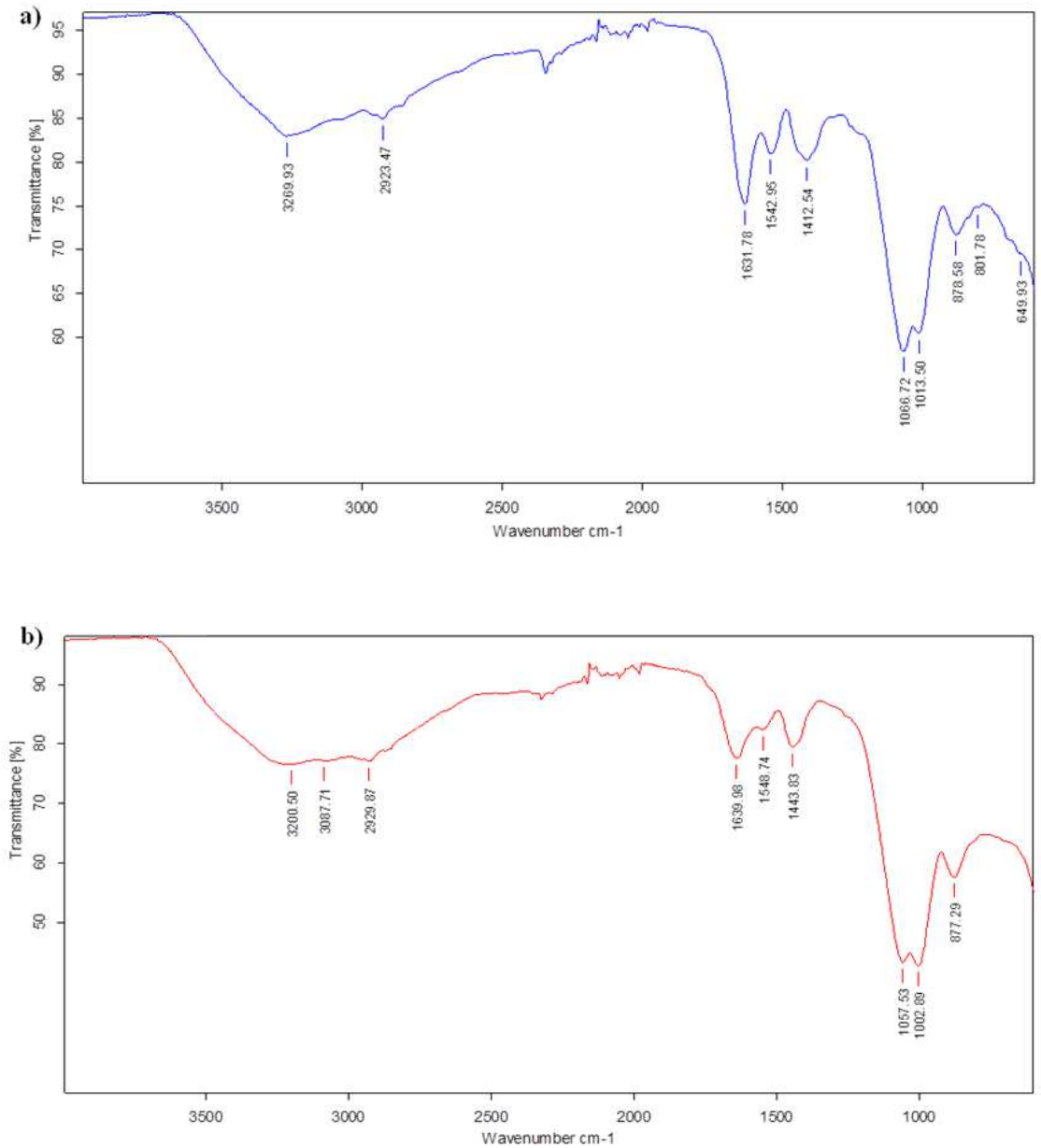

Figure 3. Infrared spectrum of the bioflocculants collected from the (a) control reactor and (b) the reactor inoculated with 5% *Bacillus subtilis*.

Overall the spectra suggest the presence of hydroxyl, carboxyl and methoxyl groups, which are the key functional groups in the flocculation processes (Zheng *et al*., 2008).

SEM images

The SEM images of the crude bioflocculant collected from the un-inoculated and the inoculated reactors are shown in Figure 4a and 4b, respectively.

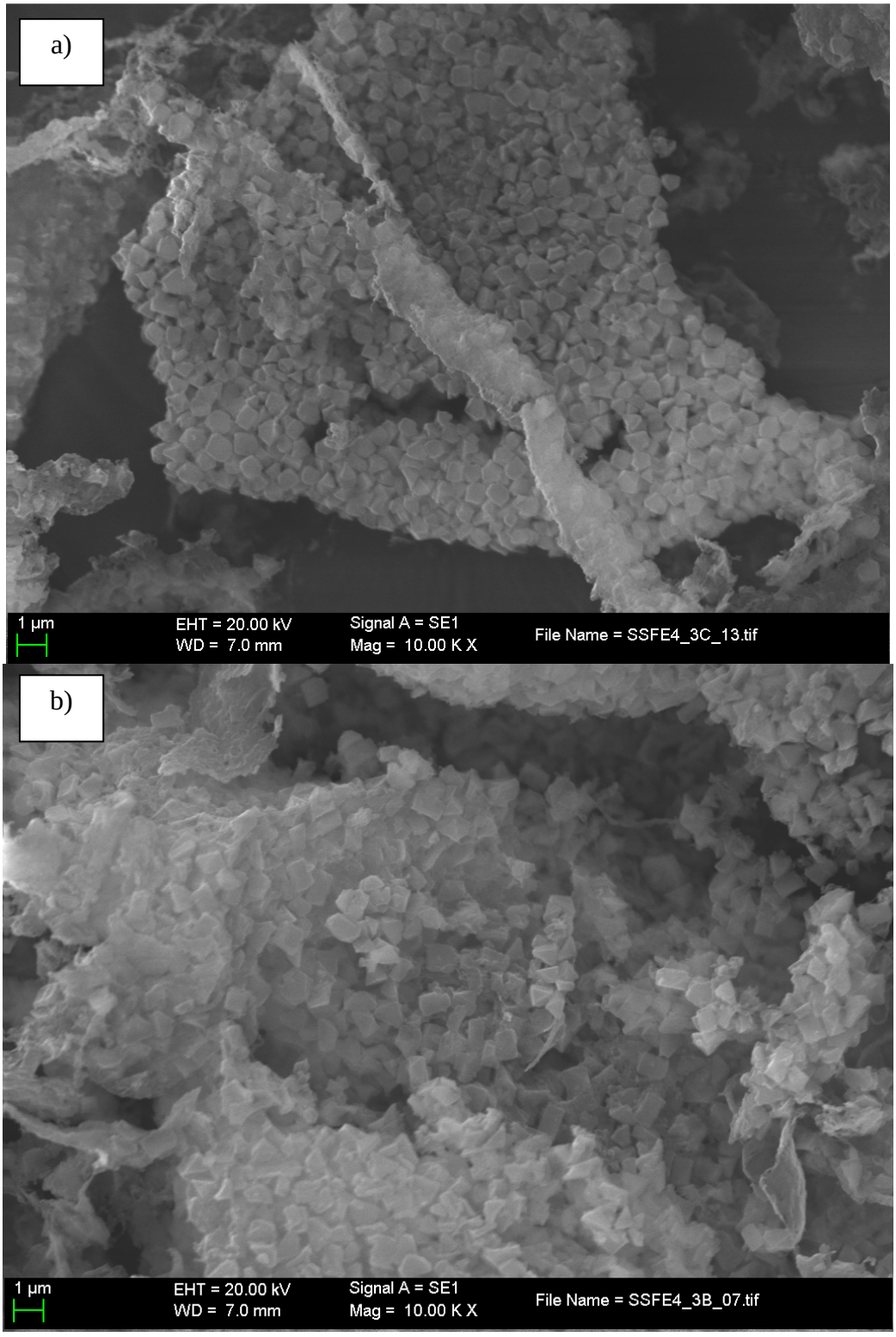

Figure 4. SEM images of the crude bioflocculants extracted from the control bioreactor (a) and the bioreactor inoculated with *Bacillus subtilis* (b).

The images portray the characteristic of the extracted bioflocculants to be granular in nature with uniformity in shape and sizes. This is similarly reported by Xiong *et al.* (2010) on the morphology of the bioflocculant produced by *Bacillus licheniformis* which was observed to be coarse-grained granular in structure.

Although both (Figure 4a and 4b) seem similar, a different feature could be noticed as the bioflocculants resulted from the inoculated reactor were observed to be coated or aggregated together forming a more amorphous structure (Wang *et al.*, 2011), whereas the ones from the un-inoculated reactor were better defined with visual borders between the granules. The coating layer observed the inoculated samples could be originated from the presence of *B. subtilis* UPMB13 extracellular substances.

Conclusion

Production of bioflocculants through SSF using solely soybean residues as substrate was proven feasible in near-to-adiabatic bioreactors at pilot scale level. Production of the bioflocculants was observed to occur later during the fermentation towards the decrease of bacterial growth. High flocculating activities were observed by the bioflocculants extracted with or without additional inoculation. The crude bioflocculants extracted were granular in nature and consisted of hydroxyl, carboxyl and methoxyl groups. Even though the effect of inoculation was not proven to be synergistic, more research is necessary to extract conclusive results from the effect of inoculation on SSF processes for the production of bioflocculants.

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