IOP Conference Series: Materials Science and Engineering

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To cite this article: N R Abu Sepian *et al* 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **991** 012007

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Characterization of gel beads used for immobilization of *Chlorella vulgaris* and enhancement its density for lipid production

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Abstract. The success of microalgae immobilized in gel beads for lipid production heavily relies on the characteristics of the matrices employed. This study aims to determine cell density and characteristics of immobilized *Chlorella vulgaris* cells that contributed to the viability of the cells within beads. The new combined matrices with volumetric ratios (Matrices:Microalgae) namely, (1) mixed matrices, $S_AC_{MC}C_A$ (0.3:1) (2) S_AC_A (0.3:1) (3) S_AC_{MC} (1:1) were proposed in this study and (4) S_A (1:1) (sodium alginate, S_A; calcium alginate, C_A; sodium carboxymethyl cellulose, C_{MC}) as a control experiment. The size of the beads, membrane thickness and chemical compound of these beads were examined. The cell density demonstrated that $S_AC_{MC}C_A$ beads presented the highest value of $1.72 \pm 0.5 \times 10^9$ cells/mL and lipid yield (30.43 ± 0.30 %) compared to S_AC_A (24.29 ± 0.50 %), S_AC_{MC} (13.00 ± 0.60 %) and S_A (6.71 ± 0.50 %). The characterization had provided important characteristics for a successful entrapment of *Chlorella vulgaris*. In addition, the combination of single matrices had improved the cell density and lipid production for future applications in the biofuel industry.

1. Introduction

During the past few years, microalgae has caught the world's attention as a potential feedstock for biofuel production and other industrial applications [1,2]. Biofuel derived from microalgae biomass has triggered significant interest among researchers, primarily attributable to the ability to rapidly grow greater lipid content and higher biomass yields, compared to conventional crops [3]. In addition, *C. vulgaris* is able to accumulate lipids which are suitable for biodiesel production and comparable to the composition of commercial diesel fuel. Due to its fast growth and ease of cultivation, *C. vulgaris* has attracted many researches as the optimal choice for biodiesel production [4–10]. However, the small size of microalgae cells (<50 μm) implies the difficulties in the recovery process when scaling up operations are implemented in a bioreactor [9,11–13]. Hence, a harvesting method with less energy consumption and applicable to most species are needed to resolve the problem [10,11]. Current

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technology involving the immobilization of microalgae has been extensively applied in harvesting microalgae in order to simplify the separation process. Immobilized microalgae have been used for various high-value applications such as production of photopigments, biohydrogen, biosensors, wastewater treatment and heavy metal removal [14,15]. However, the operating and production cost of the immobilization matrix is an important factor to produce high-value products, and to date, an economical process remains in research for a sustainable long term objective [3]. Thus, microalgae immobilized within beads should be designed innovatively to cover the costs and at the same time add profit, through generating valuable by-products.

Among the most employed natural matrices are agar, carrageenan and alginate [14,15]. Alginates are recognized as the most studied matrices for the immobilization of any living cells, including fungi, yeast, enzyme, and algae [16]. They are constituted in a group of linear copolymers that consist of β-D-mannuronic acid (M-blocks) and α-L-guluronic acid (G-blocks) in different sequences and blocks. Sodium carboxymethyl cellulose (C_{MC}) is linear and water-soluble, and has a long chain, anionic polysaccharide natural gum that is used in the food and pharmaceutical industries. It appears in a white-to-cream-color, and is odorless, tasteless, and visible in powder form [17]. Each matrix possesses diversity in its gel structure, formation, and cross-linking among polymers, which highly depends on the sources of the algal and its topographical location [18]. A matrix used for the immobilization process should possess a variety of chemical properties where the entrapment of the microalgae can be achieved through ionic or chemical covalent bonding. Additionally, the matrix should have a large capacity of forming bonds, a high porosity level, large areas inside the immobilized beads for the microalgae cells growth, non-toxic properties and stable for long-term cultivation period [14,15,19–21]. However, the immobilization of microalgae cells comprises certain restrictions. The immobilized beads are highly exposed to bead disruption, which inevitably led to the loss of the microalgae cells, mass transfer limitations of nutrients and CO_2 in and out of the inner cell, as well as free space for the microalgae movement and growth inside the matrix [12,19,20]. Thus, using alginate as an entrapment gel for *C. vulgaris* is a proper choice based on results from previous work, and the primary novelty to be proposed in this research is the mixing of sodium alginate with another natural matrix to yield the highest possible lipid production percentage [22].

The success of achieving a high cell concentration of beads does not only depend on the matrix, but is also influenced by the species of the microalgae [21]. Therefore, it is crucial to select matrices that allow for the diffusion of nutrients and CO_2 at rates that are sufficient for the viability and functionality of the microalgae cells [23,24]. The majority of prior work regarding immobilization only focused on using a single natural matrix. Hence, the emphasis of this study is to determine the most suitable combination of natural matrices which can potentially enhance lipid production and its potential for biodiesel production. This new method may contribute to the biofuel from renewable resources field in the harvesting process of microalgae. The experiment involves the characterization of the immobilized beads. This includes particle size, thickness of the membrane, and the chemical bonding within the structure, which were determined to further understand the basic constituents of the matrices, as well as the influence of these characteristics on cell growth and lipid production.

2. Materials and methods

2.1. Strain, medium and culture conditions

Microalgae strains (*Chlorella vulgaris* 211/11B) were purchased from Culture Collection of Algae and Protozoa (CCAP), United Kingdom. The microalgae was grown in modified Bold Basal Medium (BBM) with 3-fold Nitrogen and Vitamins consisting of :(1) 10 mL per litre BBM (I) consisting of: $NaNO_3$ (75 g/L), $CaCl_2.2H_2O$ (2.5 g/L), $MgSO_4.7H_2O$ (7.5 g/L), $K_2HPO_4.3H_2O$ (7.5 g/L), KH_2PO_4 (17.5 g/L), NaCl (2.5 g/L); (2) 6 mL per litre of BBM (II) consisting of: Na_2EDTA (0.75 g/L), $FeCl_3.6H_2O$ (0.097 g/L), $MnCl_2.4H_20$ (0.041 g/L), $ZnCl_2$ (0.005 g/L), $CoCl_2.6H_2O$ (0.002 g/L), $Na_2MoO_4.2H_2O$ (0.004 g/L); (3) 1 mL per litre BBM (III) consisting of 1.2 g/L Vitamin B_1 (thiaminhydrochloride); and (4) 1 mL per litre of 1 g/L BBM (IV) consisting of Vitamin B_{12} (cyanocobalamin) [25]. The stock culture was

prepared in an Erlenmeyer flask containing 250 mL sterile BBM medium. It was aerated at constant pressure under the illumination of a fluorescent lamp (Philip TL-D 36W/865, light output 3050 lm) for 24 hours, and cultured at 25 - 28 °C for 12 days of cultivation.

2.2. Preparation of immobilized microalgae beads

Sodium alginate (S_A), C_A (alginic acid calcium salt from brown algae) and Sodium carboxymethylcellulose (C_{MC}) were purchased from Sigma Aldrich Company. 0.06 g of each immobilized microalgae beads were prepared with a ratio of; (1) 1:1 for S_AC_A, (2) 1:1 for S_AC_{MC} (3) 1:1:2 for mixed matrices and S_A solution as a control experiment and mixed with 3 mL of BBM media. Next, 10 mL of *C. vulgaris* stock culture (approximately initial cell concentration of 8 x 10^8 cells/mL) was added at a volumetric ratio of 0.3:1 and 1:1 (Matrices:Microalgae (Mc)) for each combination of matrices solution. The mixture was stirred until it dissolved, and was slowly dripped using a micropipette (1 mL) into the $CaCl_2$ (2 w/v %) solution. About 130 of the microalgae beads were formed and stabilized in the $CaCl_2$ solution for 1 h. Next, the beads were filtered and rinsed twice with sterilized distilled water, prior to being used for growth and characterization.

2.3. Physical characterization

2.3.1. Chemical content analysis of immobilized beads. The immobilized beads as mentioned in Section 2.2 were cultivated until 10 days. Subsequently, all the immobilized beads were analyzed using the diamond crystal single bounce attenuated total reflectance (ATR) attached at the FTIR (Nicolet 6700, USA). The wavelengths of FTIR spectroscopy was conducted from ranging from 400 to 4000 cm^{-1}. Each measurement of the sample was repeated independent three times.

2.3.2. Size of the beads and membrane thickness. The size and membrane thickness of each immobilized bead of different matrices was measured using a light microscope (Axiostar plus, Germany) and Dino-Eye AM4023x Eyepiece Camera through Dino Capture 2.0 software, with 5x magnification.

2.3.3. Membrane Surface and Pore Size. The immobilized beads preparation for analysis was similar to Section 2.2. The image of the membrane surface and pore size of the beads were measured using a scanning electron microscope (FEI Quanta 450, USA) with 1000x magnification. The beads were attached on an aluminium plate, before being scanned [26].

2.4. Determination of cell density of immobilized microalgae beads

The cell density was calculated by taking five immobilized microalgae beads every day for a 10 days period and dissolved in 2 w/v % of sodium carbonate anhydrous. Then, the cell density of the dissolved beads was measured using a UV-VIS spectrophotometer (Varian Cary 50 Probe) at 600 nm [27,28]. The same dissolved solution was used to count the microalgae cells using counting chambers (Neubauer-Improved Haemocytometer Hirschmann®) through Dinoeye 2.0 software (Dino-Eye AM4023x Eyepiece Camera). The culture medium absorbance reading was also measured to determine the value of the cells lost of the microalgae cells into the medium. The growth curves of the immobilized microalgae were constructed from the pre-determined calibration curve of absorbance and cell density and cell lost. The experiments were made in triplicates.

2.5. Lipid extraction of immobilized microalgae

For lipid extraction purposes, the immobilized microalgae beads on day 10 were solubilized in sodium carbonate anhydrous (2 w/v %), and dried in an oven at 80 °C for 24 h. Subsequently, 0.07 g of the dried biomass was employed into screw-capped test tubes, and 5.5 mL of distilled water was added to the biomass. The test tubes were sonicated (sonicator Fisher brand FB15051) for about 15 min at 40 °C, until all the biomass was completely dissolved [29]. The lipid from the biomass was extracted using the Bligh and Dyer method, and 12 mL of methanol and chloroform mixture (2:1) was stirred for 24 h at 60

– 65 °C [30]. After the extraction process, the solution was centrifuged at 3000 rpm for 10 min, and the bottom layer was collected. Nitrogen gas flow was used to evaporate the lipid, and the weight of crude lipid was measured simultaneously. Each measurement of the sample was repeated three independent times.

2.6. Transesterification method

The transesterification method was continued using the highest lipid extracted from section 2.5. The process was carried out by using 4.25 mL of methanol, 5 mL of hexane and 215 µL of HCl (37% vol) in a screw-capped test tube. The mixture was stirred at 750 rpm at temperature 80 – 85 °C for 2 h. Then, the mixture was cooled down before it being centrifuged for 10 min at 3000 rpm [14]. The mixture formed two layers where the top layer contained the desired products (fatty acid methyl ester) and the bottom layer contained the excess remaining HCl, methanol and glycerol. Three replication of transesterification method were made to validate the experimental data.

3. Results and discussion

3.1. The effect of combination of single matrices on the cell density and polymeric film thickness of C. vulgaris cells immobilized within beads

Figure 1 and 2 show the cell density curves of four different immobilized microalgae with volume ratio 0.3:1 and 1:1, respectively. Figure 1 shows that the immobilized microalgae using $S_AC_{MC}C_A$:Mc (0.3:1) exhibited the highest cell density (1.72×10^9 cells/mL) followed by S_AC_A:Mc (0.3:1) (1.40×10^9 cells/mL), S_AC_{MC}:Mc (0.3:1) and S_A:Mc (0.3:1) on 10 days of cultivation period. Meanwhile, immobilized beads with volumetric ratio (1:1) in Figure 2 shows a contradict results whereby the highest cell density was performed by using S_AC_{MC}:Mc (1:1) matrix (1.36×10^9 cells/mL) and followed by S_A:Mc (1:1) (1.26×10^9 cells/mL), $S_AC_{MC}C_A$:Mc (1:1) and S_AC_A:Mc (1:1). As can be seen in Figure 2, the immobilized beads using $S_AC_{MC}C_A$:Mc (1:1) demonstrated a quite similar curve cells growth with S_AC_{MC}:Mc (1:1) in the first 7 days, but started to grow slowly at 8 days of cultivation period. This was maybe due to the oversaturated of the microalgae cells within the immobilized beads [27]. Thus, mixed matrices $S_AC_{MC}C_A$:Mc (1:1) was not suitable for a long cultivation period. For that reason, characterization focusing on the $S_AC_{MC}C_A$:Mc (0.3:1), S_AC_A:Mc (0.3:1), S_AC_{MC}:Mc (1:1) and S_A:Mc (1:1) immobilized beads were executed to determine the cause of this phenomenon for an efficient immobilization method prior lipid production process.

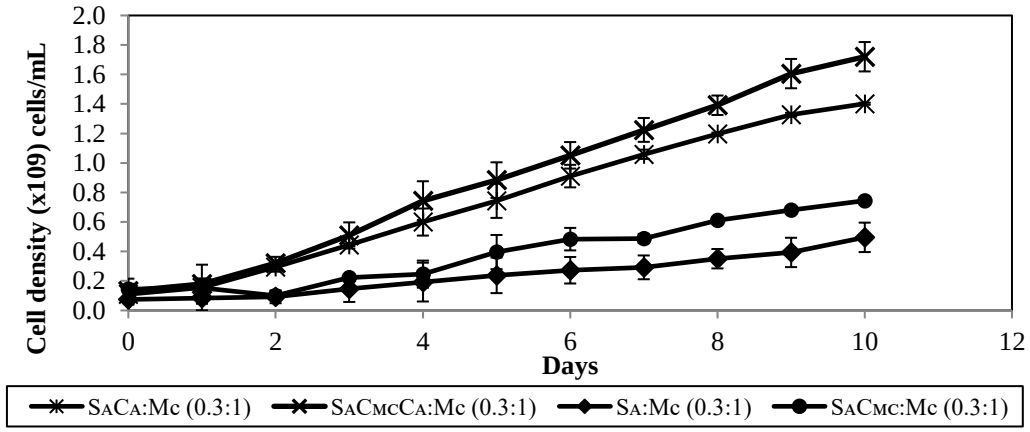

Figure 1. The cell density of *C. vulgaris* cells immobilized within beads using S_A, S_AC_A S_AC_{MC} and $S_AC_{MC}C_A$ matrices with volumetric ratio 0.3:1 (Matrices:microalgae) until 10 days of cultivation, constant pressure of aeration, 24 hours of photoperiod at room temperature. Data shown as the mean ± standard deviations of three replications (n=3).

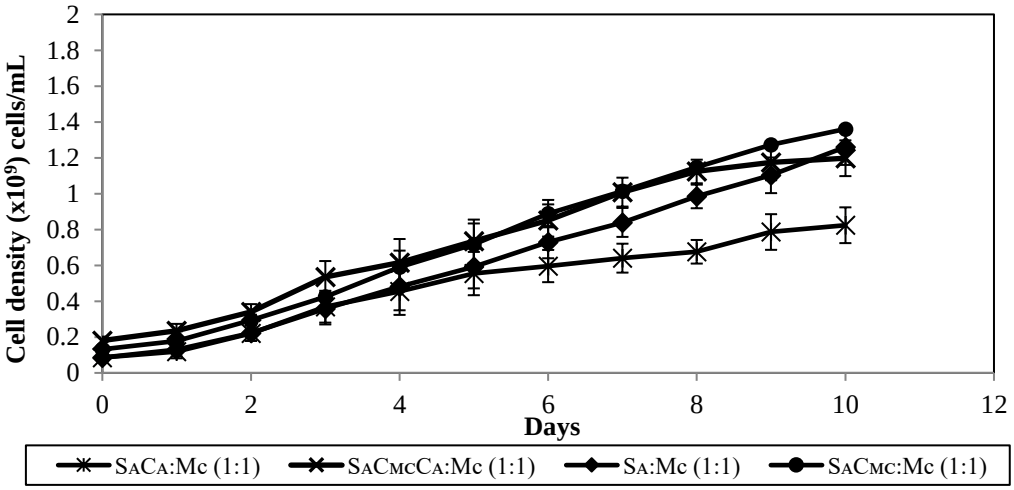

Figure 2. The cell density of *C. vulgaris* cells immobilized within beads using S_A, S_AC_A S_AC_{MC} and $S_AC_{MC}C_A$ matrices with volumetric ratio 1:1 (Matrices:microalgae) until 10 days of cultivation, constant pressure of aeration, 24 hours of photoperiod at room temperature. Data shown as the mean ± standard deviations of three replications (n = 3).

The thickness of the polymeric film layer plays an important role in influencing the mass transfer of nutrients and CO_2 within the beads and this depends on the concentration of $CaCl_2$ solution [21,27]. In this study, 2 w/v % of $CaCl_2$ concentration was employed, since it was justified in previous studies to be the most suitable concentration for the immobilization of microalgae [21]. The polymeric film membrane (approximately 0.206 ± 0.124 mm) in Figure 3 (a) formed by $S_AC_{MC}C_A$:Mc was thinner compared with the immobilized beads of S_AC_A:Mc (Figure 3 (b)) which demonstrated a higher membrane layer. According to Lam and Lee (2012), a thin layer matrix of polymeric film improved the movement of nutrients and CO_2 in and out of the immobilized beads and eventually promotes the metabolic activity of the microalgae cells. Thus, a thin membrane layer probably will increased the cell density as shown in Figure 1 which the highest cell density was obtained by immobilized bead using $S_AC_{MC}C_A$:Mc (0.3:1). However, a vice versa result was shown for microalgae immobilized within beads with volume ratio (1:1). Although the immobilized beads using S_A:Mc (1:1) shows a thinner membrane layer than the bead in Figure 3 (d), the cell density value was lower compared to the immobilized bead using S_AC_{MC}:Mc (1:1).

This proven that a high cell density of immobilized microalgae was not only influenced by the thickness of polymeric film layer but also on the matrices employed. The addition of other matrices with S_A had formed a different geometry structure that enhanced the microalgae cells growth. In addition, the different volumetric ratios (0.3:1 and 1:1) give insignificant impact to the cell density and thickness of the polymeric membrane. Among all the immobilized beads, the microalgae immobilized in the $S_AC_{MC}C_A$:Mc (0.3:1) beads was the best since it produced the highest cell density. Hence, this indicates that a combination of single matrices created a unique matrix structure which enhanced cell viability and provided a suitable space area for the microalgae cells growth compared to the immobilized bead using S_A matrix alone.

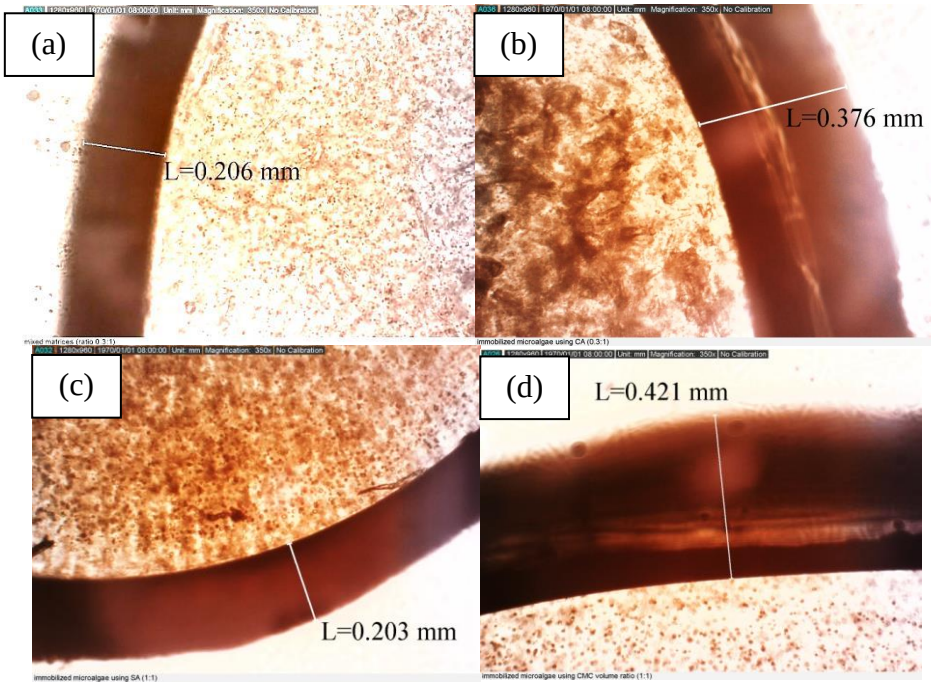

Figure 3. Images of the polymeric film thickness of *C. vulgaris* cells immobilized within (a) Mixed matrices ($S_AC_{MC}C_A$:Mc) (0.3:1) (b) S_AC_A:Mc (0.3:1) (c) S_A:Mc (1:1) and (d) S_AC_{MC}:Mc (1:1) Matrices:Microalgae (volume ratio) beads, at 5x magnification through Dino Capture 2.0 software.

3.2. Chemical content analysis of immobilized beads

In general, the crosslinking of the matrices is with divalent cations (Ca^{2+}) via carboxyl groups (COOH) by primary valences and hydroxyl groups (OH) by secondary valences [16,31,32]. The polymeric chains become folded and stacked under the bond interaction, which causes the structure to transform from the random coils to the organized ribbon-like structure linked via Ca^{2+}. This bonding of the matrices chains contributes to the hydrogel form referred to as the "egg-box" structure [33,34].

FTIR spectra were used to identify the chemical bonding in the structure of the immobilized beads. Figure 4 presents the FTIR spectra of the immobilized microalgae beads. All the beads with different combinations of matrices and volumetric ratios (0.3:1 and 1:1) offer a similar trend of FTIR analysis with the same absorption wavenumbers (between 1630 and 3300 cm^{-1}). This demonstrates that all the immobilized beads consist of the same chemical bonding which formed between Ca^{2+} and the matrices. The wavenumbers at 3284.09 cm^{-1} indicate that the bead consists of a polymeric hydroxy (OH) group. A broad distribution of absorption between 3200 and 3550 cm^{-1} shows that the OH stretching vibration region is rich and forms a strong dimeric stable structure in the immobilized bead [26,35]. This hydroxy group comprises the bonding between the alginate molecules (intramolecular hydrogen bonding) and alginate-water molecules (intermolecular hydrogen bonding) [36]. The bonding of alginate-water molecules (>95%) inside the structure provides an aqueous environment for the bioactivity of the entrapped microorganisms [37]. This chemical bonding is an important indicator for a successful entrapment and growth of microalgae cells within the crosslink polymer.

The peak of 1635.88 cm^{-1} signifies the symmetric stretching vibration of carboxylate ester (COO^-), and primary and secondary organic amide (C-N or N-H) group [35,38]. This result is in line with that by Chabane et al. (2017), who reported that the same absorption band also appeared around 3420 and 1636 cm^{-1} for S_A and other beads using aluminum-pillared montmorillonite (Al-PILMt), polyvinyl alcohol (PVA) and $CaCO_3$ [34]. Since the alginate was derived from various natural sources, many impurities such as proteins, carbohydrates, heavy metals and polyphenolic compounds were present within the chemical structure [20]. These results indicated that all the beads have suitable chemical

characteristics for the growth of microalgae cells which are entrapped between the crosslinking of the polymer and no significant difference of the chemical structure based on the FTIR result.

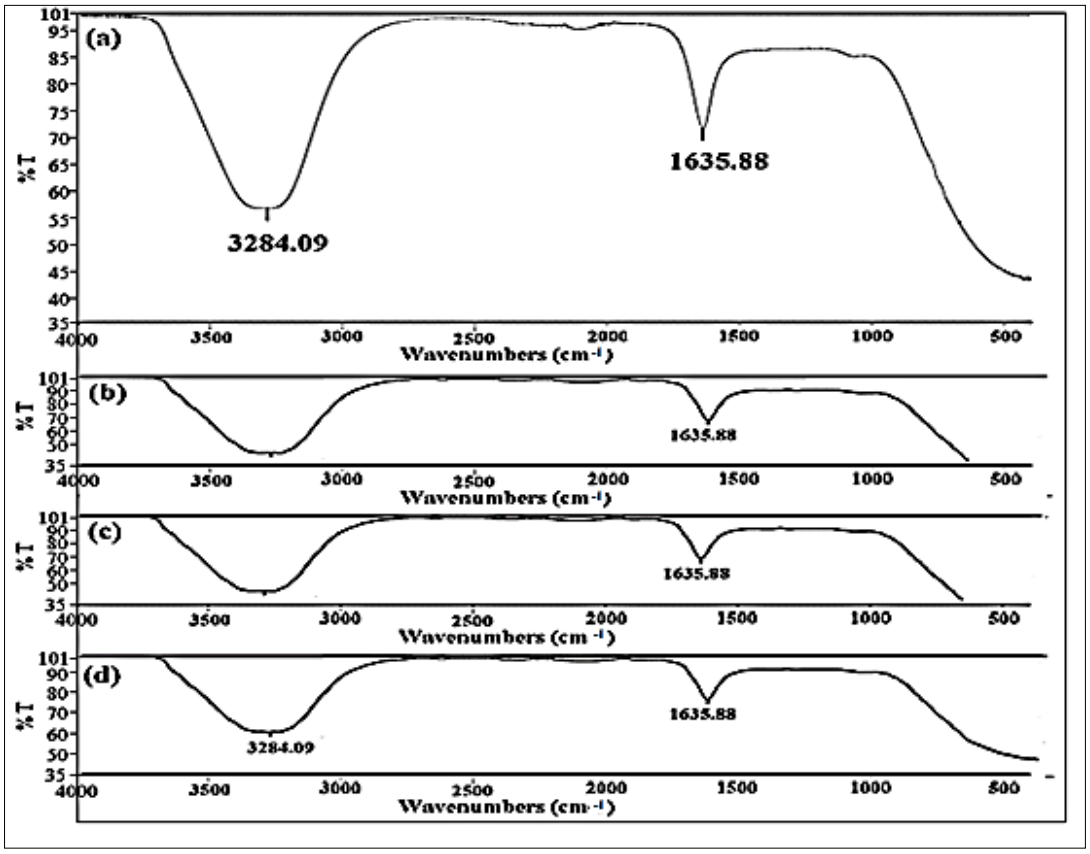

Figure 4. FTIR analysis ranging from 400 to 4000 cm^{-1} of *C. vulgaris* cells immobilized within (a) Mixed matrices ($S_AC_{MC}C_A$:Mc) (0.3:1) (b) S_AC_A:Mc (0.3:1) (c) S_A:Mc (1:1) and (d) S_AC_{MC}:Mc (1:1) Matrices:Microalgae (volume ratio) beads. The analysis were replicated three times (n=3).

3.3. Size of the immobilized beads

The size of immobilized beads can be influenced by several factors such as the chemical properties, rate of agitation, species of microalgae, and technique of preparation [16,39]. Figure 5 shows the range of beads size of various matrices. The immobilized beads using S_AC_{MC}:Mc (1:1) (Figure 5d) displayed the largest diameter. Most of these beads have a diameter of 3.2-3.4 mm. This result is in line with that produced by Joo et al. (1999), who obtained approximately 3.51 mm in diameter using C_{MC} as an encapsulation material [21]. About 50% of the immobilized beads of S_A and $S_AC_{MC}C_A$ (Figure 5a and 5c) have a size of 2.6 – 2.8 ± 0.05 mm and 2.2 – 2.4 ± 0.08 mm respectively. Among the immobilized beads, the size of the S_AC_A beads (Figure 5b) was the smallest, with most beads ranging from 1.6 mm to 1.8 mm ± 0.04.

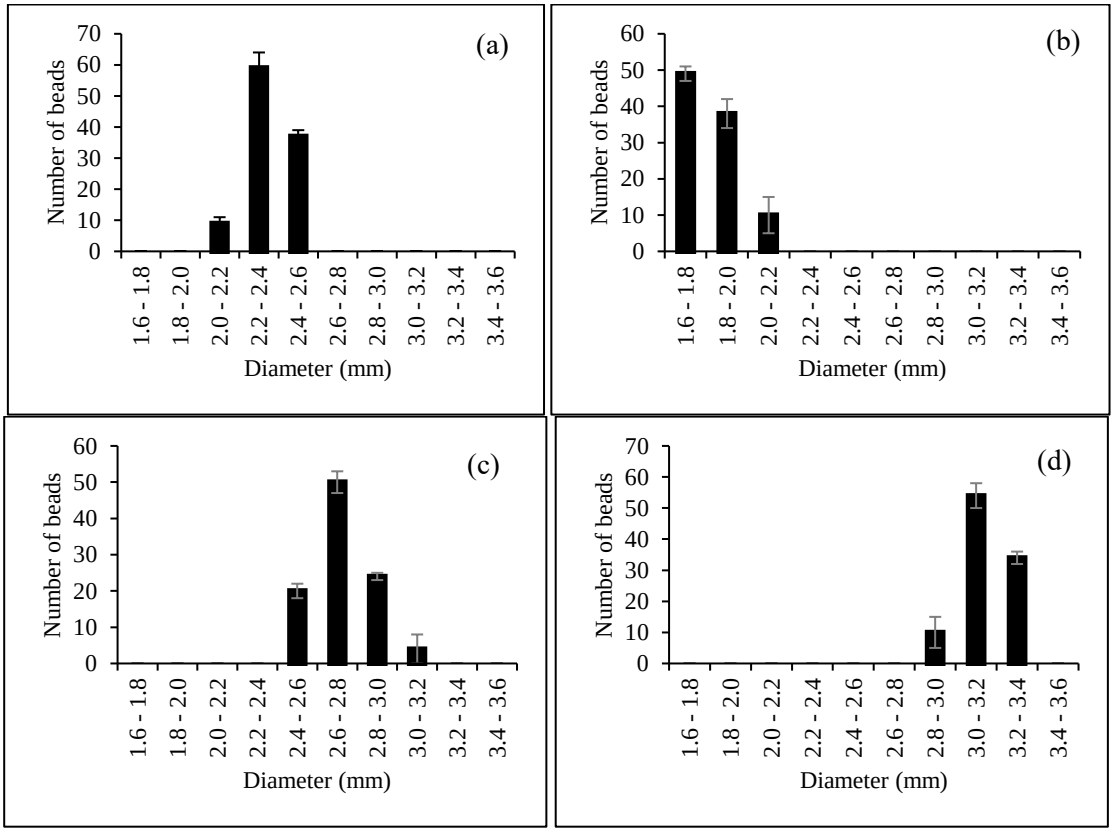

Figure 5. Size of the immobilized beads with matrices:Mc (volume ratio) using (a) Mixed matrices ($S_AC_{MC}C_A$:Mc) (0.3:1) (b) S_AC_A:Mc (0.3:1) (c) S_A:Mc (1:1) and (d) S_AC_{MC}:Mc (1:1). Data shown as the mean ± standard deviations of three replications (n=3).

Based on this trend, the size of the immobilized beads is larger for the volume ratio of 1:1 compared to 0.3:1. In this case, the size of the beads likely depends on the volumetric ratios of the matrix to microalgae, as well as the type of matrix used to mix with S_A. The advantage of a larger inner-size of beads or capsules is that it provides a wider space for the growth of microorganisms, and might in turn increase the accumulation of cells [40]. However, Nussinovitch (2010) claims that the bead size has no significant effect on the final concentration of cells [16]. This statement can be related to Figure 1 and 2, in which the highest cell density was attained by immobilized beads of $S_AC_{MC}C_A$:M_C with a volume ratio 0.3:1, whereas the beads of S_A:M_C with a volume ratio 1:1 exhibited the lowest cell density. Hence, choosing a suitable matrix for the immobilization of microorganisms is vital, since it supports the growth of the cells rather than focus on the size of beads, which has less influence on the growth of the cells.

3.4. Surface Images and Pore Size of Immobilized Microalgae

Figure 6 shows the SEM surface images of beads from four different matrices. A rough and wave-like surface image was observed in Figure 6 (a) with a large pore size, ranging between 10.8 ± 0.05 and 32.78 ± 0.05 µm. These pores increase the molecular diffusion of nutrients and CO_2 in and out of the cell, thereby increasing the growth of the microalgae cells [41]. Referring to the micrograph of sodium alginate in Figure 6 (c), the membrane surface of the immobilized beads was distributed by many pores in the range of 8.74 ± 0.05 to 29.01 ± 0.05 µm. While in Figure 6 (b) and (d), there were only a few pores visible on the surface of the beads. These types of surface image are similar to that observed in the work of Fan et al. (2017) for the immobilization of enzymes and yeast. The authors reported that a tight structure with a few pores on the surface may protect the cells inside the beads from the contamination of the culture medium, thus enabling the molecular diffusion of nutrients and CO_2 in and

out of the cells [23]. However, the result of the cell density (Figure 1 and 2) of immobilized beads using S_A deviated from the previous statement by Fan et al. (2017). This revealed that, to some extent, the membrane surface with too many pores is inconvenient for the growth of the cells inside the beads, due to the easy movement of the cells out of the matrix layer. Apart from that, the different volumetric ratios (0.3:1 and 1:1) of beads had no effect to the surface of the beads as can be observed on the SEM figures.

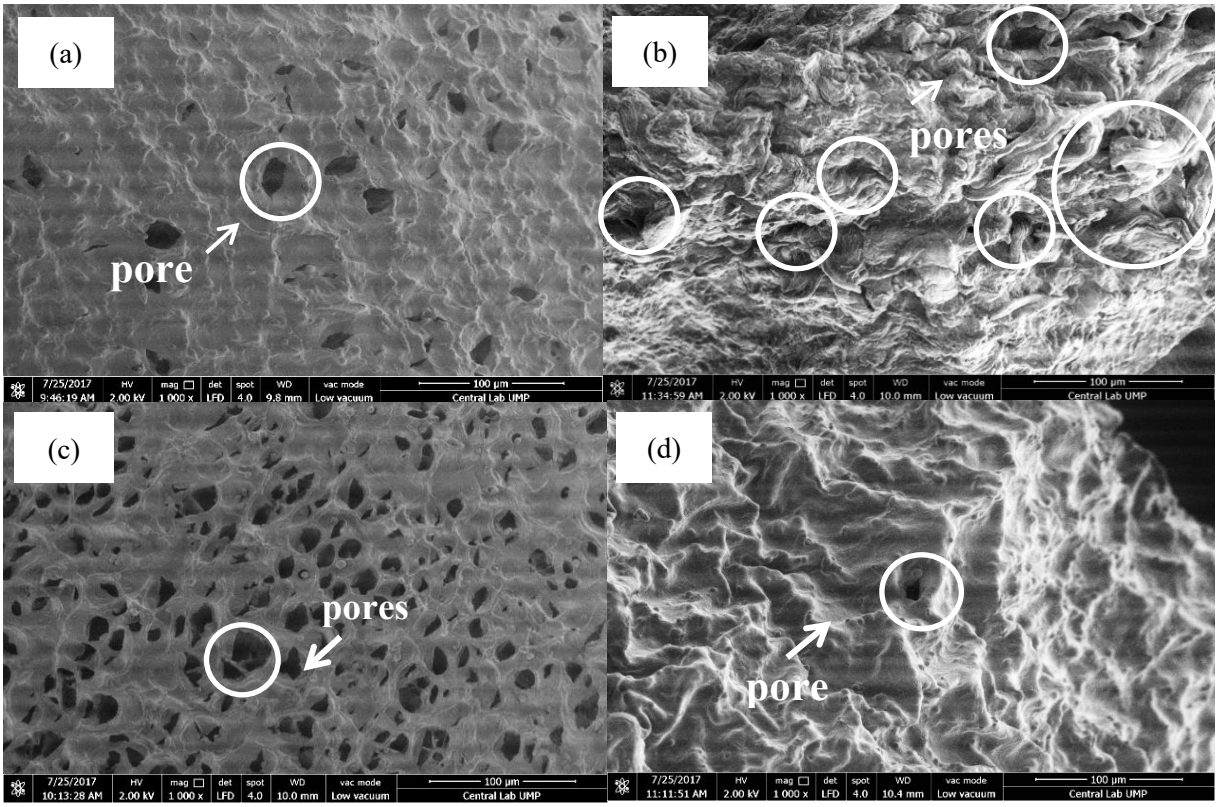

Figure 6. SEM membrane surface and pore images of immobilized microalgae at 1000 x magnification with matrices:Mc (volume ratio) (a) Mixed matrices ($S_AC_{MC}C_A$:Mc) (0.3:1) (b) S_AC_A:Mc (0.3:1) (c) S_A:Mc (1:1) and (d) S_AC_{MC}:Mc (1:1).

Figure 6 (b) and (d) display a rough and inhomogeneous structure surface with fewer pores of smaller sizes (6.75 ± 0.05 to 27.48 ± 0.05 µm). The inhomogeneous distribution of the polymer concentration on the surface towards the inner gel might probably result from the irreversible gelling reaction mechanism by the crosslinking ions. This irreversible gelling mechanism was controlled by the diffusion rate of Ca^{2+} ions and the polymer molecules to form gelling zone [41]. It can be observed in Figure 6 (a) that the combination of the three natural matrices changed the inhomogeneous surface to a rough surface, with some larger pores on the surface compared to other immobilized beads. These surface characteristics of the immobilized bead using $S_AC_{MC}C_A$ offer a successful combination of matrices which enhanced the growth of *C. vulgaris* cells based on the cell density result (Figure 1 and 2). Consequently, the efficiency of these immobilized beads was evaluated for lipid production through lipid extraction method.

3.5. Lipid extraction of immobilized beads and fatty acid methyl ester profile of $S_AC_{MC}C_A$

The characterization discussed in Sections 3.1, 3.2, 3.3 and 3.4 demonstrated that the immobilized beads using $S_AC_{MC}C_A$ had several supportive characteristics of a good matrix for the immobilization of microalgae. Via characterization, these physical and chemical properties show that, in order to influence the growth of the microalgae cells in which to obtain a high lipid production, the cell concentration and weight of the biomass should be relatively high [6]. This scenario is because the primary concern in the commercialization of microalgae as a biofuel feedstock is to enhance the biomass and lipid production. The microalgae biomass with volumetric ratio of 0.3:1 performed a higher lipid yield than the 1:1 volume ratio and the highest lipid percentage (30.43 ± 0.30 %) was obtained from immobilized biomass from mixed matrices [22]. This result was expected because of the higher cell density which contributes to the higher lipid yield. All the result values were in line with previous literature reported by Chen et al. (2011) in which the value of lipid extracted from *C. vulgaris* biomass cultivated under phototrophic condition lies between 5 – 40 % [42]. Moreover, this result was higher compared to Lam and Lee (2012) which obtained 12 % of lipid yield using S_A as immobilized matrix of *C. vulgaris* cells [27].

Figure 7 shows the fatty acid methyl ester (FAME) profile of oil extracted from microalgae cells immobilized within $S_AC_{MC}C_A$:Mc (0.3:1) bead. The oil was mainly consisted of C16:0 (palmitic acid methyl ester), C18:0 (stearic acid methyl ester), C18:1 (oleic acid methyl ester) and C18:2 (linoleic acid methyl ester). From the Figure 7, C18:0 performed the highest percentage with 35.59 ± 0.27 % of the whole composition and was followed by C16:0 (32.2 ± 0.12 %), C18:1 (6.78 ± 0.31 %) and C18:2 (25.42 ± 0.14 %). These fatty acid methyl esters are presence naturally in oil crops, such as sunflower, soybean, palm oil and cottonseed which had potential for biodiesel production [27]. The data shows that the extracted oil contained 67.8 ± 0.22 % of saturated fatty acid (SFA) and 32.2 ± 0.21 % of unsaturated fatty acids (UFA). The result was comparable with Lam and Lee (2012) in which the extracted oil from microalgae biomass immobilized using S_A consists a higher SFA (60.1 %) than UFA (38 %) [27]. The highly UFA leads to unstable of biodiesel as it will oxidize more rapid than commercial diesel, whereas a highly SFA in biodiesel resulted in poor cold flow properties [29,43]. Previous study by Gopinath et al. (2010) reported that biodiesel with high SFA had higher thermal efficiency and emits lower oxides of nitrogen compared to biodiesel with high UFA [44]. Thus, the oil extracted from microalgae biomass via immobilization technique has properties that suitable for biodiesel production.

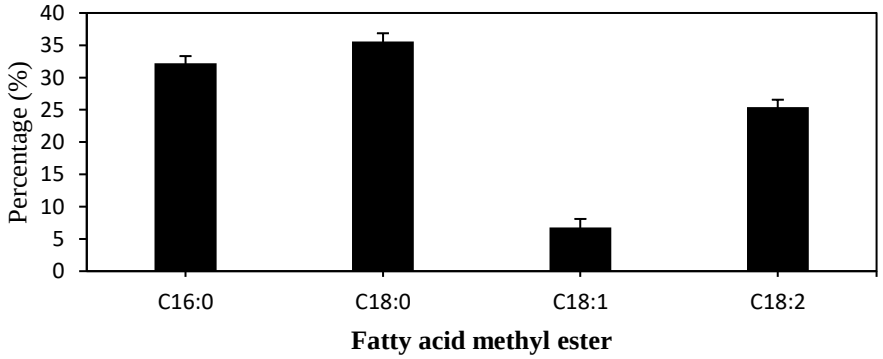

Figure 7. Fatty acid methyl ester profile of microalgae cells immobilized within $S_AC_{MC}C_A$:Mc (0.3:1) bead (C16:0 - palmitic acid methyl ester, C18:0 - stearic acid methyl ester, C18:1 - oleic acid methyl ester and C18:2 - linoleic acid methyl ester).

4. Conclusion

Based on these characterizations, it can be concluded that a high lipid production was significantly influenced by a thin layer of membrane thickness, a smaller of matrix:Mc volume ratio, as well as the type of matrix used. The size of immobilized beads had less influenced on the cell density and lipid production of immobilized microalgae. The combination of natural matrices revealed changes on the

membrane bead size and chemical content of the structure, which essentially influenced the growth of the microalgae cells. This observation was further verified through the result of cell density, where the mixed matrices exhibited the highest cell density of 1.72×10^9 cells/mL and 30.43v± 0.30 % of lipid yield, compared to S_AC_A (24.29 ± 0.50 %), S_AC_{MC} (13.00 ± 0.60 %) and S_A (6.71 ± 0.50 %). The results reveal the potential of mixed matrices ($S_AC_{MC}C_A$) as an appropriate choice for the immobilization of *C. vulgaris*, which is also sufficient for cell viability to produce lipids for larger-scale operations.

Acknowledgements
This work was supported by the Universiti Malaysia Pahang (UMP) internal grants (RDU180352), Faculty of Chemical and Process Engineering, UMP and MyBrain15 scholarship.

References
[1] Taparia T, Mvss M, Mehrotra R, Shukla P and Mehrotra S 2015 *Biotechnol. Appl. Biochem.* **63** 715–26
[2] Razon L F and Raymond R T 2011 *Appl. Energy* **88** 3507–14
[3] Chisti Y 2013 *J. Biotechnol.* **167** 201–14
[4] Concas A, Pisu M and Cao G 2013 *Chem. Eng. Trans.* **32** 1021–6
[5] Concas A, Pisu M and Cao G 2015 *Chem. Eng. J.* **263** 392–401
[6] Hamedi S, Mahdavi M A and Gheshlaghi R 2016 *Biofuel Res. J.* **3** 410–6
[7] Álvarez-Díaz P D, Ruiz J, Arbib Z, Barragán J, Garrido-Pérez M C and Perales J A 2017 *Algal Res.* **24** 477–85
[8] Al-Iwayzy S H, Yusaf T and Al-Juboori R A 2014 *Energies* **7** 1829–51
[9] Rajanren J R and Ismail H M 2017 *Biofuels* **8** 37–47
[10] Mohammad Mirzaie M A, Kalbasi M, Mousavi S M and Ghobadian B 2016 *Prep. Biochem. Biotechnol.* **46** 150–6
[11] Guisan J M 2013 *Methods in Molecular Biology* vol 1051 (New York: Springer Science+Business Media)
[12] Gani P, Mohamed Sunar N, Matias-Peralta H, Abdul Latiff A A and Mohamad Fuzi S F Z 2017 *Prep. Biochem. Biotechnol.* **14** 333–41
[13] Liu S, Xu J, Yu H, Zhao C, Chen W and Ma F 2018 *J. Environ. Eng. (United States)* **144** 1–7
[14] Ahmad A L, Mat Yasin N H, Derek C J C and Lim J K 2014 *Environ. Technol.* **35** 2244–53
[15] Eroglu E, Smith S M and Raston C L 2015 *Biomass and Biofuels from Microalgae* vol 2, ed N.R. Moheimani et al. (Switzerland: Springer International Publishing) pp 19–44
[16] Nussinovitch A 2010 *Bead Formation, Strengthening, and Modification* (Switzerland: Springer Science+Business Media)
[17] Glicksman M 1986 *Food Hydrocolloids* (Boca Raton, FL: CRC Press)
[18] Moreno-Garrido I 2008 *Bioresour. Technol.* **99** 3949–64
[19] Guiseley K B 1989 *Enzyme Microb. Technol.* **11** 706–16
[20] Lee K Y and Mooney D J 2012 *Prog. Polym. Sci.* **37** 106–126
[21] Joo D S, Cho M G, Lee J S, Park J H, Kwak J K, Han Y H and Bucholz R 2001 *J. Microencapsul.* **18** 567–76
[22] Abu Sepian N R, Mat Yasin N H, Zainol N, Rushan N H and Ahmad A L 2019 *Environ. Technol.* **40** 1110–7
[23] Fan Y, Wu Y, Fang P and Ming Z 2017 *Water Sci. Technol.* **75** 75–83
[24] Liu X D, Yu W Y, Zhang Y, Xue W M, Yu W T, Xiong Y, Ma X J, Chen Y and Yuan Q 2002 *J. Microencapsul.* **19** 775–82
[25] Culture Collection of Algae and Protozoa 2015 3N-BBM+V (Bold Basal Medium with 3-fold Nitrogen and Vitamins; modified)
[26] Md Sai'aan N H, Soon C F, Tee K S, Ahmad M K, Youseffi M and Khagani S A 2017 *IECBES 2016 - IEEE-EMBS Conference on Biomedical Engineering and Sciences* pp 611–6
[27] Lam M K and Lee K T 2012 *Chem. Eng. J.* **191** 263–8

[28] Dianursanti and Santoso A 2015 *Energy Procedia* **65** 58–66
[29] Ahmad A L, Mat Yasin N H, Derek C J C and Lim J K 2014 *Environ. Technol. (United Kingdom)* **35** 891–7
[30] Bligh E and Dyer W 1959 *Can. J. Biochem.* **37** 911–7
[31] Sugawara S, Imai T and Otagiri M 1994 *Pharm. Res.* **11** 272–7
[32] Draget K I, Skjåk Bræk G and Smidsrød O 1994 *Carbohydr. Polym.* **25** 31–8
[33] Rees D A 1981 *Pure Appl. Chem.* **53** 1–14
[34] Chabane L, Cheknane B, Zermane F, Bouras O and Baudu M 2017 *Chem. Eng. Res. Des.* **120** 291–302
[35] Coates J 2006 *Encyclopedia of Analytical Chemistry* ed R A Meyers (Chichester, United Kingdom: John Wiley & Sons Ltd) pp 1–23
[36] Sartori C 1997 *The characterisation of alginate systems for biomedical applications* (Brunel University)
[37] Wang W, Liu X, Xie Y, Zhang H, Yu W, Xiong Y, Xie W and Ma X 2006 *J. Mater. Chem.* **16** 3252
[38] Sarmento B, Ferreira D, Veiga F and Ribeiro A 2006 *Carbohydr. Polym.* **66** 1–7
[39] Delrieu P E and Ding L 2001
[40] Lee B H and Park J K 1996 *Korean J. Biotechnol. Bioeng.* **11** 398–404
[41] Smidsrød O and Skjåk-Bræk G 1990 *Tibtech* **8** 71–8
[42] Chen C Y, Yeh K L, Aisyah R, Lee D J and Chang J S 2011 *Bioresour. Technol.* **102** 71–81
[43] Nautiyal P, Subramanian K A and Dastidar M G 2014 *Fuel* **135** 228–34
[44] Gopinath A, Puhan S and Govindan N 2010 *Int. J. Energy Environ.* **1** 411–30