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Assessment of Acidified Fibrous Immobilization Materials for Improving Acetone-Butanol-Ethanol (ABE) Fermentation

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Abstract: Acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum* is a process that can be used to produce butanol, which can be utilized as an alternative to petroleum-based fuels. Immobilization of the bacteria using three different fibrous materials was studied in order to see how to improve the ABE fermentation process. The results were compared to those of non-immobilized bacteria. Modal and charcoal fibers had OD levels below one at 72 h with the butanol concentration reaching 11.0 ± 0.5 and 10.7 ± 0.6 g/L, respectively, each of which were close to the free cell concentration at 11.1 ± 0.4 g/L. This suggests that bacteria can be efficiently immobilized in these fibrous materials. Although an extended lag phase was found in the fermentation time course, this can be easily solved by pre-treating fibrous materials with 3.5% HCl for 12 h. From comparisons with previous studies, data in this study suggests that a hydrophilic surface facilitates the adsorption of *C. acetobutylicum*.

Keywords: immobilization; modal fibers; acid treatment; *Clostridium acetobutylicum*; Acetone-Butanol-Ethanol (ABE) fermentation

1. Introduction

After the industrial revolution, the consumption of fossil fuels such as natural gas, coal and gasoline increased dramatically. The industrial revolution also created lots of greenhouse gases such as CO_2 that brought about global warming and climate change. In order to reduce the consumption of petrol chemical fuels, people looked to develop alternatives to fossil fuels.

Acetone-butanol-ethanol (ABE) fermentation, developed during World War II, is an anaerobic fermentation process used to produce butanol. The strain of bacteria used in ABE fermentation is *Clostridium acetobutylicum*. ABE fermentation was replaced in favor of petrochemical production in the late 1960s due to having a lower productivity and yield. With the pollution generated by petrochemicals growing every day, people looked to ABE fermentation as an alternative and tried to find a way to improve the process. The key to increasing butanol production is improving the survivability of the bacteria. Thus, researchers have utilized extract separation and immobilization to maintain the bacterial count and improve ABE fermentation. Separation of butanol can decrease its toxicity to bacteria whereas immobilization can enhance the tolerance of toxicity via improved cell density [1–3]. The immobilization technique also facilities the downstream butanol separation while it reduces the amount of carbon needed for biomass formation. Multiple immobilization materials for increasing ABE fermentation, such as brick [2,4], polyvinyl alcohol [5], metals [6], agriculture wastes [7], and weaving fibers [8], were used in earlier studies. Weaving fibers, such as vegetable fibers, are highly malleable hydrophilic materials

that can be mixed with other materials to improve various characteristics. Animal fibers made from protein can even act as nitrogen sources. Various kinds of fibrous materials are acquired from weaving industry wastes, which would result in lower costs. With these advantages, weaving fibers have the potential to make ABE fermentation more economical and environmentally friendly.

This study aims to observe the performance of cotton balls, modal fibers, and charcoal fibers. Cotton is a readily available fibrous material, and so can easily be looked into as an immobilization material for the bacteria. Modal fiber is a cellulose-based fiber originating from renewable wood pulp. Its high water absorbance, strength, and availability make it ideal for immobilization testing. Charcoal fiber consists primarily of bamboo fiber, which is harvested from bamboo plants and then burned in an oven. As a result, charcoal is made almost entirely of carbon, giving it high tensile strength. Like modal, its ability to absorb water and its renewability (due to the ease of harvesting from bamboo plants) make it ideal as an immobilization material. Some of these fibers received an acid pretreatment to generate more surface area. Overall butanol production, kinetic performance, and in vitro performance will be analyzed for the cotton balls, modal fibers, and bamboo charcoal fibers. The morphology of the *C. acetobutylicum* adsorbed on these three materials was scanned by field-emission scanning electron microscope (FESEM) and the specific surface area was determined by the Brunauer–Emmett–Teller (BET) method [9].

2. Experimental Section

2.1. Microorganism and Culture Environment

C. acetobutylicum ATCC 824 was the bacteria used in this study for ABE fermentation. A detailed description of *C. acetobutylicum* ATCC 824 cultivation can be found in this previous study [10]. While preculturing the microorganism, the bacterial stock was injected into Reinforced Clostridial Medium (RCM) at a concentration of 5% and heat-shocked at 80 °C for 5 min. The bacteria were then inoculated into a batch bottle containing 100 mL LB-s medium, which consisted of NaCl at 10 g/L, tryptone at 10 g/L, yeast extract at 5 g/L, $MgSO_4 \cdot 7H_2O$ at 0.6 g/L, $FeSO_4 \cdot 7H_2O$ at 0.11 g/L, and $CaCl_2$ at 0.008 g/L. Glucose was added to the bottle at an initial concentration of 60 g/L. The bottles were incubated at 37 °C and 200 rpm, and underwent ABE fermentation for 96 h. Note that the cultivation of *C. acetobutylicum* was achieved under anaerobic condition as previously described [10]. Then 3 g of each immobilization material were used when applied.

2.2. Immobilization Materials and Pretreating Process

The immobilization materials used in this study were cotton balls (CSD Ltd., Changhua, Taiwan), modal fiber (Shing-Long Ltd., Yunlin, Taiwan), and charcoal fiber (composed of 20% of bamboo charcoal fiber and 80% of cotton, Shing-Long Ltd.). For the acid pretreating process, all fibrous materials were soaked in 3.5% $HCl_{(aq)}$ for 12 h and washed with deionized (DI) water.

2.3. Analytical Methods

An UV-VIS spectrophotometer (GENESYS 10S, Thermo Scientific, Waltham, MA, USA) was used to determine the optical density of the bacterial culture at 600 nm. The ABE concentrations for the three materials was determined by gas chromatography (Hewlett Packard HP 5890 Series II, Agilent Technologies, Santa Clara, CA, USA) [11,12]. The DNS method was used to determine the concentration of the glucose remaining [13]. Samples were diluted so that the above measurements were in the dynamic range of calibration curve.

The surface of the three materials was characterized by a field-emission scanning electron microscope (FESEM, model JSM-6700F, JEOL Ltd., Tokyo, Japan). Before pictures were taken, the materials were washed with DI water and put into a drying oven to eliminate moisture. The specific surface area of each immobilization material was measured by the BET method. A micromeritics ASAP 2010 porosimeter was used to absorb nitrogen and the de-gassed condition was controlled at 80 °C.

2.4. In Vitro Performance of the Materials

Bacterial solutions containing *C. acetobutylicum* was prepared by first filling test tubes with 9 mL of the Reinforced Clostridial Medium (RCM, OXOID, Hampshire, UK), capping them with rubber septa, and then sealing them with aluminum. The head space of the test tubes was flushed for 10 min with nitrogen filtered in a 0.2 μm syringe filter. Test tubes containing an oxygen-free headspace were sterilized by autoclaving at 121 °C for 20 min. After cooling the test tubes to room temperature, a mineral stock solution (60 g/L $MgSO_4 \cdot 7H_2O$, 11 g/L $FeSO_4 \cdot 7H_2O$, 0.8 g/L $CaCl_2$) and the glucose solution of 600 g/L were added to them to create a primary medium for growing the bacteria. 1 mL of the bacteria was transferred to each test tube and cultivated at 37 °C and 200 rpm for 72–96 h. Different amounts of modal and charcoal supports were added into test tubes containing 5-mL bacterial solutions to start the in vitro immobilization experiments at 100 rpm. These experiments were performed in an aerobic environment at room temperature to prevent excessive bacterial growth. The performance of in vitro immobilization was determined by calculating the difference between the initial and final OD_{600} of the liquid phase. The OD_{600} was converted into cell dry weight (g/L) by a calibration curve with the conversion factor of 0.748 $g \cdot L^{-1} \cdot OD_{600}{}^{-1}$.

3. Results

3.1. The Performance of Immobilized Materials for ABE Fermentation

The growth curves and butanol production of non-immobilized bacteria and immobilized bacteria were compared with each other. It can be seen in Figure 1 that at a fermentation time of 48 h, the OD_{600} of non-immobilized bacteria reached 7.7 ± 2.9 with a butanol concentration of 8.9 ± 2.5 g/L. On the other hand, the OD_{600} of bacterial cultures in cotton reached 6.0 ± 1.3 with a butanol concentration of 7.0 ± 4.4 g/L. The OD_{600} of cultures in modal and charcoal fiber reached lower numbers of 1.2 ± 0.4 and 3.0 ± 1.8 while the butanol concentrations were still at high values of 4.2 ± 2.5 and 2.5 ± 3.4 g/L, respectively. Overall, OD_{600} of the immobilized bacteria was lower than that of the non-immobilized bacteria.

Modal and charcoal fibers had OD levels below one during the period of 72 h and beyond. The highest butanol concentration was 11.8 ± 0.6, 11.0 ± 0.5, and 10.7 ± 0.6 g/L for cotton, modal, and charcoal materials, respectively, each of which were close to the non-immobilized bacterial concentration at 11.1 ± 0.4 g/L. This suggests that bacteria can be effectively immobilized in the fibrous materials tested in this study, with modal and charcoal fibers displaying the best results. Additionally, a slow onset of butanol production for bacterial cultures in these three materials was observed in Figure 1b, indicating a noticeable mass transfer resistance. This, on the other hand, reflects the effectiveness of the immobilization capability of the fibrous materials tested.

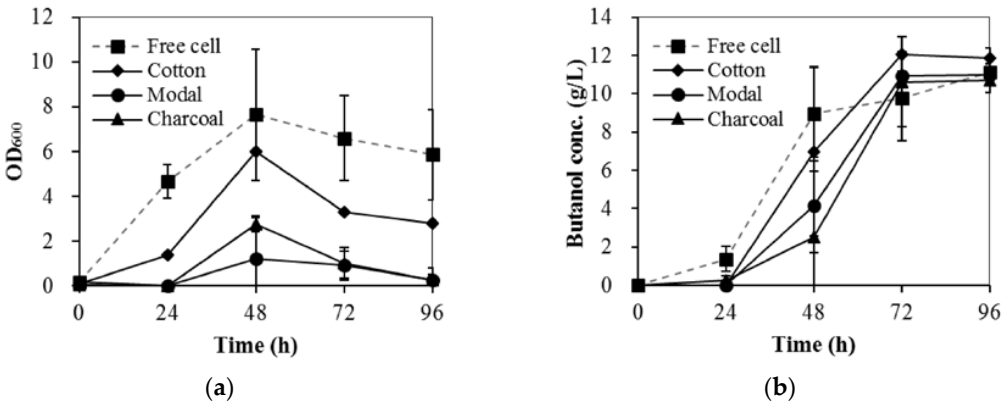


Figure 1. (**a**) Growth curve and (**b**) butanol production of *C. acetobutylicum* on different immobilized materials with 60 g/L glucose. The standard deviation was used for the error with $n = 3$.

3.2. The Effects of Acid Pretreatment

Samples of the three fibrous materials used were treated with hydrochloric acid (HCl). ABE fermentation performance values using these acidified fibrous materials can be seen in Figure 2. At a fermentation time of 48 h, the acidified cotton, modal fibers, and charcoal fibers had OD_{600} values of 6.1 ± 1.7, 1.5 ± 0.3, and 5.0 ± 1.7, respectively, while the normal cotton, modal fibers and charcoal fibers had OD_{600} values of 6.0 ± 1.3, 1.2 ± 0.4, and 2.7 ± 1.8 (Figure 3). This indicates that the immobilization capabilities of three acidified fibrous materials were kept intact. The butanol concentration increased with the acidified modal fibers (with a *p*-value of 0.01). The acidified modal fibers had a butanol concentration level of 10.4 ± 0.0 g/L, respectively, whereas the non-acidified modal fibers had a concentration level of 4.2 ± 2.5 g/L. The elevated butanol concentrations, even higher than those of non-immobilized bacteria at 48 h as shown in Figure 1, indicate that the mass transfer resistance was significantly reduced while keeping the immobilization capabilities. On the other hand, acidified cotton and charcoal fibers had no significant impact on the butanol concentration compared to non-acidified fibers. In summary, evidence demonstrated in terms of kinetics points to acidifying modal fiber as a method for improving butanol productivity, whereas no statistical evidence points to acidifying cotton and charcoal fibers having the same effect.

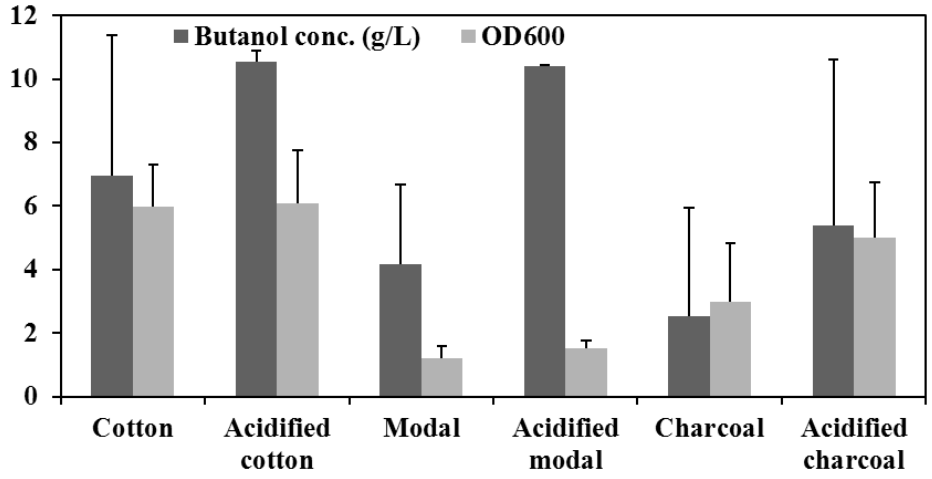
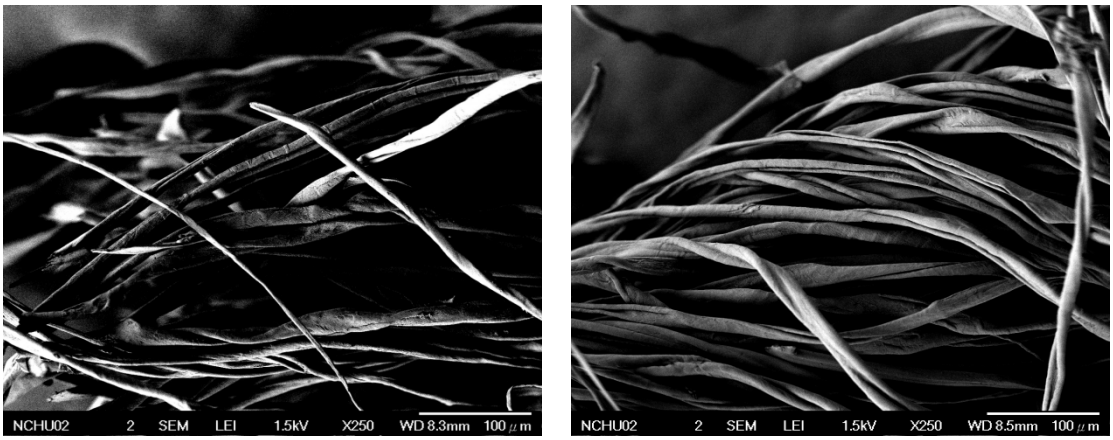


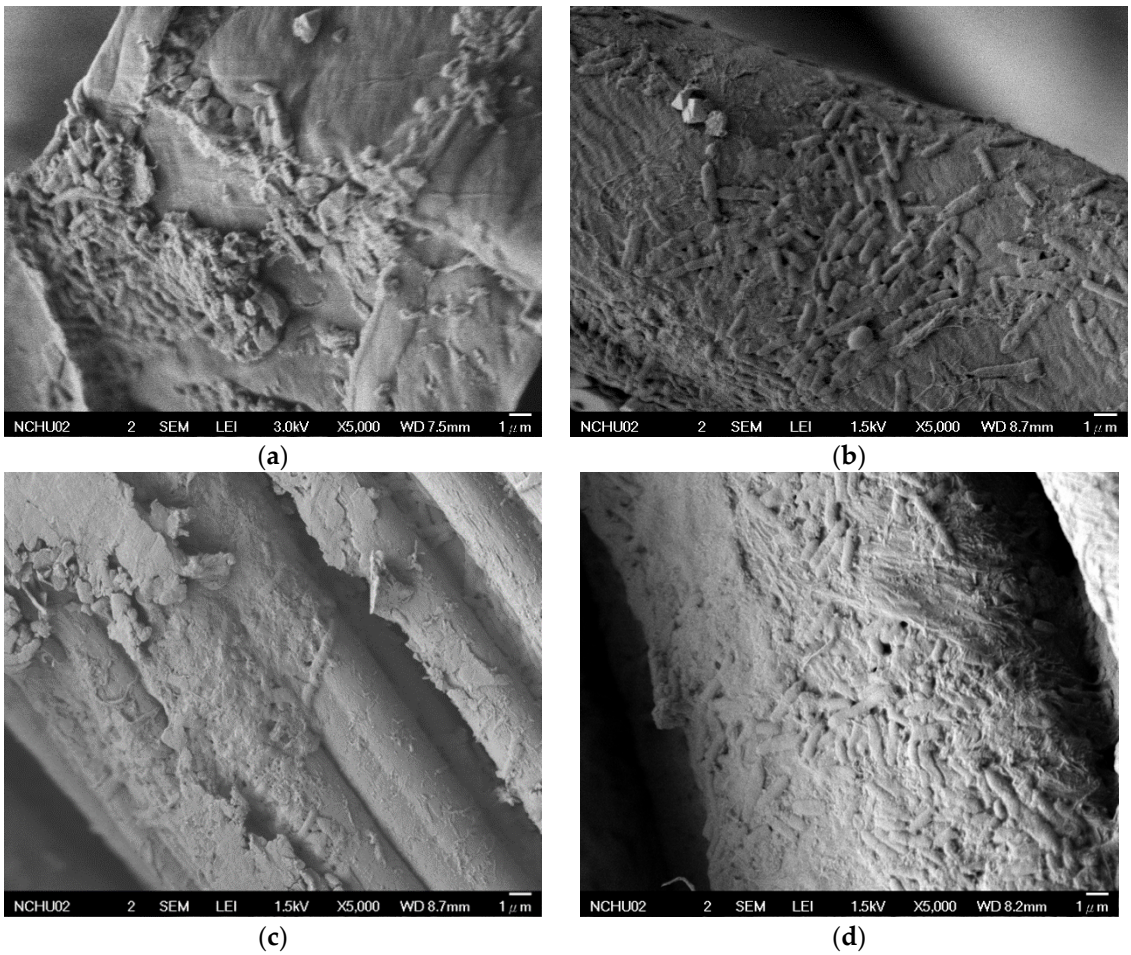
Figure 2. Butanol concentration and cell growth of *C. acetobutylicum* on normal and acidified immobilization materials with 60 g/L glucose at 48 h. The standard deviation was used for the error with $n = 3$.

Figure 3a,b show FESEM images of the modal and charcoal fibers. These fibrous materials had diameters of 5–10 μm. The surface porosity, defined as the distance between each fiber, was greater than 50 μm. Figure 4a,b reveal FESEM images of bacteria on cotton and acidified cotton, respectively, with a more homogeneous distribution of the immobilized bacteria seen in Figure 4b. This may reflect that the stability of butanol production can be increased by using acidified cotton where the standard deviation of the butanol concentration was decreased (Figure 2). It can be seen in Figure 4c that only some areas on the modal fibers were occupied by bacterial colonies. This drawback can be solved by using acid pretreatment so the whole surface area on an acidified fiber can be fully employed for bacterial immobilization (Figure 4d). Figure 4e,f show no significant difference in how much bacteria were immobilized. The effects of acid pretreatment on modal fiber can be perceived by measuring its specific surface area. It can be seen in Figure 5 that there was a nine-fold increase in the specific area for the acidified modal material. This increase in the specific area provides excellent immobilization while minimizing the mass transfer resistance.



(**a**) (**b**)

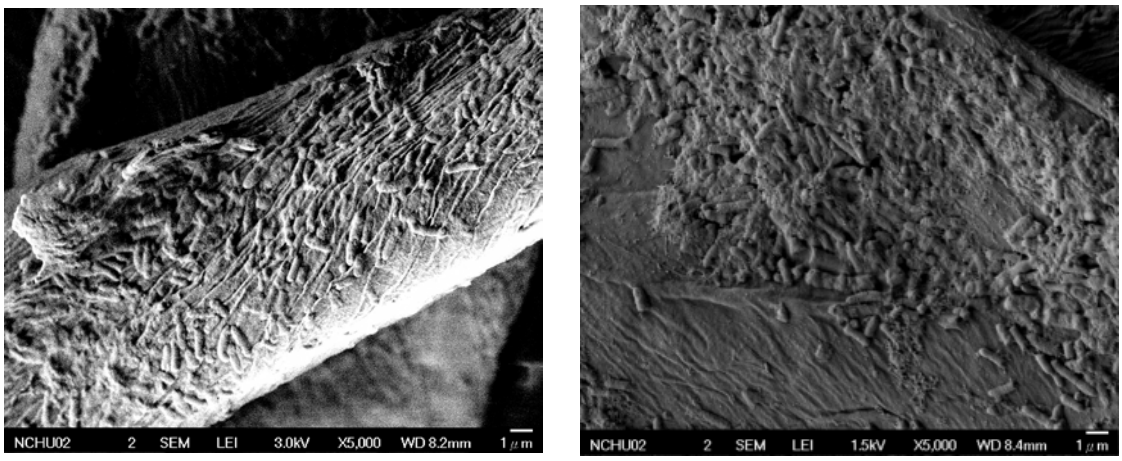
Figure 3. Field-emission scanning electron microscope images of (**a**) modal and (**b**) charcoal fibers.



(**a**) (**b**)

(**c**) (**d**)

Figure 4. *Cont.*



(**e**) (**f**)

Figure 4. FESEM images of *C. acetobutylicum* bacteria immobilized on supports. (**a**) Bacteria on cotton fiber; (**b**) bacteria on acidified cotton fiber; (**c**) bacteria on normal modal fiber; (**d**) bacteria on acidified modal fiber; (**e**) bacteria on charcoal fiber; (**f**) bacteria on acidified charcoal fiber.

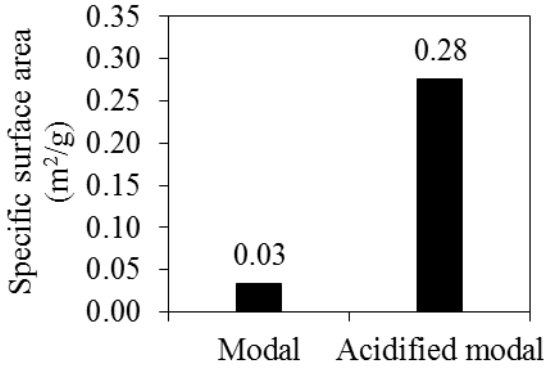


Figure 5. Specific surface area of modal and acidified modal materials.

3.3. In Vitro Performance of the Materials

Figure 6 indicates that modal fiber has an immobilization capability of 17.1 ± 0.3 mg-biomass/g-modal while charcoal fiber has a capability of m. Note that one unit of OD_{600} is equivalent to 0.748 mg-biomass/L.

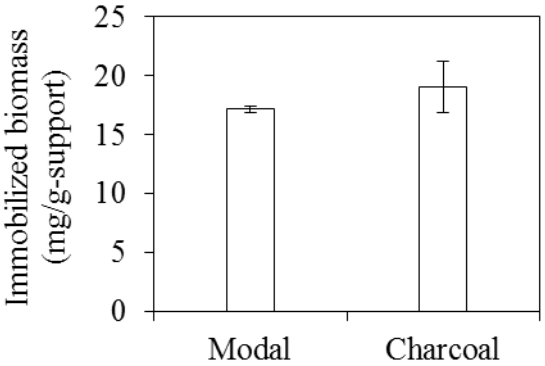


Figure 6. In vitro performance of the modal fiber and charcoal fiber. The standard deviation was used for the error with $n = 3$.

4. Discussion

In this study, experiments using the materials cotton, modal fiber, and charcoal fiber for bacterial immobilization were conducted. It was found that modal and charcoal fibers were suitable bacterial immobilization materials for ABE fermentation. We propose that a hydrophilic surface facilitates more adsorption of *C. acetobutylicum*. This can be argued as follows. When poly(3-hydroxybutyrate) (PHB) materials were fabricated into a fibrous structure, it could in vitro immobilize up to 90 mg-biomass/g-PHB with a surface area of 18.3 m^2/g-support (unpublished data). The immobilization capabilities for modal fiber or charcoal fiber were significantly lower than that of the PHB-based material because of the low specific surface area. The modal fibers have a specific surface area of 0.03 m^2/g-modal while the PHB-based material has a specific surface area of 18.3 m^2/g-PHB (unpublished data). Thus, modal fibers in vitro can immobilize 570 mg-biomass/m^2 while PHB-based supports in vitro can immobilize 4.9 mg-biomass/m^2. This difference in two orders of magnitude can be attributed to surface hydrophobicity, where modal fibers with a hydrophilic surface favor adsorption of the bacteria. The good adsorption of bacteria on the modal or charcoal fibers can be seen from FESEM images.

By pre-treating the modal fiber materials with 3.5% HCl for 12 h, the kinetics of ABE fermentation with the acidified modal fibers were significantly enhanced. It can be argued that the structure of modal fibers was etched by the acids so that the mass transfer resistance was decreased. Therefore, the long lag phase of batch ABE fermentation cultivated with modal fibers was solved. Furthermore, the acid treatment not only presumably increased the adsorption of *C. acetobutylicum* by increasing the specific surface area of acidified modal fibers (Figure 5), but also provided a correct scale of geometry for the formation of homogenous biofilms (Figure 4c,d). This facilitated the entrapment of *C. acetobutylicum*. Therefore, an improved ABE fermentation in terms of kinetics was shown in Figure 2. To have a maximum bacterial immobilization capacity, both adsorption and entrapment should be considered. We propose that a hydrophilic surface on the immobilization material facilitates the adsorption of *C. acetobutylicum*. To further improve the immobilization capability, we need to consider a support structure, which in turn takes into account entrapment.

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Conflicts of Interest: The authors declare no conflict of interest.

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