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Recovery and Purification of Fumaric Acid from Fermented Oil Palm Empty Fruit Bunches Using a Simple Two-Stage Precipitation Procedure

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Abstract: Oil palm empty fruit bunches (EFBs) are an attractive lignocellulosic material that can be used as a cheap renewable feedstock to produce organic acids and many other value-added products. This research is aimed at investigating the potential of steam-exploded oil palm EFBs for the production of fumaric acid, a food additive widely used for flavor and preservation, through a separate hydrolysis and fermentation process using the selected fungal isolate *Rhizopus oryzae* K20. To develop an efficient method for the recovery and purification of fumaric acid from fermented oil palm EFBs, a two-stage precipitation protocol was employed, followed by an activated carbon-mediated polishing step to remove contaminants. After these two processes were accomplished, a recovery yield of 81.2% and a purity of 83.5% were achieved.

Keywords: activated carbon; lignocellulosic material; *Rhizopus oryzae*; separate hydrolysis and fermentation

Citation: Boondaeng, A.; Khanoonkon, N.; Vaithanomsat, P.; Apiwatanapiwat, W.; Trakunjae, C.; Janchai, P.; Niyomvong, N. Recovery and Purification of Fumaric Acid from Fermented Oil Palm Empty Fruit Bunches Using a Simple Two-Stage Precipitation Procedure. *Fermentation* **2022**, *8*, 121. https://doi.org/10.3390/fermentation8030121

Academic Editor: Alessio Siciliano

Received: 19 February 2022
Accepted: 7 March 2022
Published: 10 March 2022

1. Introduction

The development of modern technologies and industries requires more natural resources. Petroleum, one of the main energy sources under constant demand, is limited. Moreover, petroleum usage negatively impacts the environment. Therefore, renewable resources are the focus of sustainable energy technologies. Each renewable energy source, such as biomass, has unique benefits over fossil fuels in reducing global warming. In the past, many chemical compounds have been produced from petroleum via chemical processes. Advances in biotechnology have led to the production of many chemical compounds from renewable resources, such as lignocellulosic biomass [1]. Researchers from the National Renewable Energy Laboratory (NREL) have demonstrated the potential of chemical compounds derived from lignocellulosic biomass to act as important building blocks in producing valuable chemical substances [2].

Fumaric acid (FA), one of the top ten building-block chemicals, has broad-range applications in chemical, pharmaceutical, cosmetic, and food industries. Among its many applications, FA is traditionally employed as a food additive, for producing rosin-sized sheathing paper and unsaturated and alkyd polyester resins, as well as in personal care and cosmetics. In 2020, the global FA market was reported to reach 258.86 kilotons, valued at USD 660.9 million, and is expected to grow at a CAGR of more than 4% from 2021 to 2026 [3]. FA is commercially produced via chemical synthesis from maleic anhydride, which is in turn produced from butane, a non-renewable resource that contributes to global warming. A gradual distancing from petroleum usage as a conventional resource for producing

chemicals, materials, and liquid fuels is expected in years to come. A few microbial species are able to produce FA, including those belonging to the genera *Rhizopus*, *Mucor*, *Cunninghamella*, and *Circinella*. Among them, species belonging to the genus *Rhizopus* are considered the most convenient microorganisms for FA production [4,5]. Studies have investigated FA production using daily manure, starchy materials, and glycerol [5–7]. However, the utilization of oil palm empty fruit bunches (EFBs) for FA production is still limited. Oil palm EFBs have been widely exploited in the production of ethanol and aromatic compounds, and in the preparation of lignin, etc. [8–10]. They are an agricultural residue, disposed of in large quantities worldwide following the extraction of palm oil, and have been reported to contain approximately 51.7% cellulose and hemicellulose [8]. Therefore, oil palm EFBs represent a very promising resource for FA production.

During the fermentation process, other byproducts such as succinic, citric, and lactic acids are produced using *Rhizopus* strains [11]. It is also reported that *Escherichia coli* can produce FA from glucose with acetic acid as a by-product [12]. The present study examines the possibility of FA recovery and purification from fermented oil palm EFBs using *Rh. oryzae* K20. A simple two-stage precipitation procedure followed by the elimination of contaminants using an activated charcoal polishing step to produce high-purity FA crystals was investigated. The activated charcoal can be recovered and reused in the polishing step of subsequent purification cycles.

2. Materials and Methods

2.1. Raw Material and Pretreatment

Oil palm EFB, collected from Suksomboon Palm Oil Company Limited in Chonburi Province, Thailand, was used as a raw material in this study. The EFBs were sun-dried, ground to particles of approximately 2.5 cm, and kept in a sealed plastic bag until further use.

Approximately 200 g of oil palm EFBs was steam-exploded at 20 MPa (210 °C) for 4 min in a 2.5 L stainless steel batch digester (Nitto Koatsu Co., Ltd., Tokyo, Japan) [13]. The material was then separated into a solid residue and a liquid phase by filtration through a cheesecloth. The obtained solid residue was soaked for 60 min in water heated to 80 °C and was subsequently washed with tap water until reaching a neutral pH. The steam-explosion-pretreated material was delignified by soaking in a 15% (*w*/*v*) NaOH solution and incubated at 90 °C for 30 min. The obtained suspension was filtered and the solid residue was washed with tap water until reaching a neutral pH. The delignified solid residue was air-dried and stored in a sealed plastic bag until use in separate hydrolysis and fermentation (SHF).

2.2. Microbial Culture

Rhizopus oryzae K20, isolated from a soil sample collected in Nakhon Ratchasima Province in Thailand, was grown on PDA plates at 30 °C for 7 d. The spores were subsequently washed twice with sterile water to obtain a spore suspension. After filtration through layers of sterilized cotton, spores in the suspension were counted under a microscope using a hemacytometer (Boeco Germany, Hamburg, Germany) and were diluted to a desired concentration. For the seed culture, 1 mL of spores suspended in distilled water (1×10^7 spores/mL) was inoculated into the growth medium containing 30 g/L of glucose, 0.6 g/L of KH_2PO_4, 0.0176 g/L of $ZnSO_4 \cdot 7H_2O$, 1.5 g/L of urea, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, and 0.0005 g/L of $FeSO_4 \cdot 7H_2O$; and its pH was set to 7.0. After a 24 h incubation, the obtained cell pellet was washed with autoclaved distilled water and used as the seed culture for further experiments.

2.3. Separate Hydrolysis and Fermentation

Steam-exploded and delignified oil palm EFBs were used for FA production through SHF. They were enzymatically saccharified for 24 h using Cellic CTec 2 (185 FPU/mL, Novozyme A/S, Basgsværd, Denmark) in 50 mM of citrate buffer (pH 4.8) at 50 °C and

150 rpm. The enzyme loading was fixed at 15 FPU/g raw material. The released sugar concentration was determined using the Nelson–Somogyi method [14]. The obtained enzymatic hydrolysate (EFB-derived glucose) supplemented with the seed culture was further used for FA production in a 250 mL Erlenmeyer flask. The optimized production medium at pH 5.0 contained the following components: 75.5 g/L of EFB-derived glucose, 0.6 g/L of KH_2PO_4, 0.04 g/L of $ZnSO_4 \cdot 7H_2O$, 0.05 g/L of urea, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.29 g/L of $(NH_4)_2SO_4$, and 3.07 g/L of Na_2CO_3, as suggested in a previous report [15]. Fermentation was carried out at 30°C for 72 h.

2.4. Source of Glucose

To compare the efficiency of FA production from different glucose sources, *Rh. oryzae* K20 was cultivated in a medium containing either glucose or EFB-derived glucose under previously optimized conditions [15]. Fermentation was carried out at 30 °C for 72 h.

2.5. Upscale Production of Fumaric Acid in an Air-Lift Fermenter

The production of FA was upscaled by batch fermentation using *Rh. oryzae* K20 in a 3 L air-lift fermenter (MCI-6C, B.E. Marubishi Co., Ltd., Bangkok, Thailand) containing 1 L of the optimized medium. The initial EFB-derived glucose was varied from 80 g/L through 100 g/L to 120 g/L, the fermentation temperature was varied from 25 °C through 30 °C to 35 °C, the aeration rate was varied from 1.0 through 1.5 to 2.0 volume versus mass (VVM), and the initial pH was varied from 3.0 through 3.5 to 4.0. The culture was sampled at 24 h intervals for a total of 168 h, and the FA concentration was determined using high-performance liquid chromatography (HPLC) with a Bio-Rad (Richmond, VA, USA) Aminex HPX-87H column (300 mm × 7.8 mm).

2.6. Fumaric Acid Precipitation

A two-stage precipitation protocol was employed according to Figueira et al. [16]. The fermentation broth (500 g) was acidified to pH 0.75 by the addition of 5 N H_2SO_4 and left at 4 °C overnight. The obtained solids were collected by vacuum filtration. The recovered solids were rinsed with a known amount of a cold 0.4 M H_2SO_4 solution. The solids were added to 250 g of distilled water, heated at 80 °C in a shaking flask to resuspend the solids, and finally, left at room temperature to cool. The solution was acidified to pH 0.75 following the addition of 5 N H_2SO_4. The experiment was repeated until the washed FA was obtained. The precipitate containing FA was dried and weighed at various stages. After being resuspended in distilled water and heated at 80 °C, the percentage of FA was determined using HPLC. The FA concentration was also determined in the filtrate using HPLC.

2.7. Removal of Contaminants from the Fumaric Acid Solution

Activated charcoal granules, 4–10 mm in length and 4 mm in diameter (Sigma–Aldrich, Saint Louis, MO, USA), were washed by sonication to remove the black carbon powder and subsequently dried at 37 °C for further use. The precipitate obtained from the two-stage precipitation described above was dissolved by adding 10 N NaOH until the pH reached 10. The concentration of FA in the solution was adjusted to 30 g/L. Activated charcoal was added to the solution in an amount of 0.02 g/g FA solution, and the mix was incubated at 35 °C for 60 min with shaking at 150 rpm [16]. The activated charcoal was subsequently removed by vacuum filtration. The filtrate volume was measured and the FA concentration was analyzed using HPLC. The filtrate was acidified to pH 0.75 by adding 5 N H_2SO_4 and left at 4 °C overnight. The precipitate was recovered and dried until a constant weight was reached, as described above.

2.8. Recovery of Residual Fumaric Acid from Dilute Solutions

The diluted FA solution, obtained by two-stage precipitation and removal of contaminants as described above, was poured into a shake flask. Activated charcoal was added

in the amount of 0.1 g/g FA solution and the mix was incubated at 35 °C for 60 min with shaking at 150 rpm to adsorb the FA. The activated charcoal was vacuum-filtered and the residual FA concentration was detected by HPLC. The recovered activated charcoal was added into 1 N NaOH at a 1:1 ratio, and shaken for 60 min at 150 rpm and 35 °C for desorption. After vacuum filtration, the filtrate was assessed by HPLC. To recover FA from the filtrate, the solution was acidified to pH 0.75 by the addition of 5 N H_2SO_4, and left at 4 °C overnight. After vacuum filtration, the filtrate was analyzed by HPLC. The solid was rinsed, dried, and weighed. FA concentration was determined as described above.

2.9. Characterization of Bio-Synthesized Fumaric Acid

Absorption spectra of bio-synthesized FA were determined using Fourier-transform infrared spectroscopy (Nicolet IR200 FTIR, Thermo Scientific, Waltham, MA, USA). The spectra were scanned in the range from 400 to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1} and plotted as intensity versus wave number. Each evaluated spectrum represents a mean of 32 scans.

Determination of the thermal properties of FA was analyzed using the differential scanning calorimetry technique (DSC; DSC 1 STARe, Mettler Toledo, Greifensee, Switzerland). For the analysis of both commercial and bio-synthesized FA, measurements were performed from to 0 °C to 350 °C with a heating rate of 10 °C/min under nitrogen flow (flux rate of 50 mL/min).

2.10. Analytical Methods

A HPLC Instrument Shimadzu LC-20A system (Shimadzu, Tokyo, Japan), coupled with an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad, Richmond, VA, USA), was used for the quantitative measurement of FA. The mobile phase was 0.005 N H_2SO_4 and it was eluted at a flow rate of 0.6 mL/min and a temperature of 40 °C.

3. Results and Discussion

3.1. Source of Glucose

The comparison of FA production from glucose and EFB-derived glucose showed no clear difference between the two glucose sources, as shown in Table 1. In addition, yield and productivity obtained from the initial 75.5 g/L of glucose during the 72 h cultivation were approximately 0.07 g/g and 0.55 g/L/h for pure glucose, and 0.07 g/g and 0.56 g/L/h for EFB-derived glucose, respectively. These results indicate that the ability of *Rh. oryzae* K20 to produce FA from EFB-derived glucose was of a similar value as compared to pure glucose, which is consistent with the research by Liao et al. [17], who compared the effect of pure glucose and manure-fiber-derived glucose as raw materials for FA production. In the cited study, manure fiber was hydrolyzed to produce monosaccharides using two different pretreatments: concentrated acid hydrolysis and enzymatic hydrolysis. The FA fermentation of pure glucose and of glucose obtained from enzyme-pretreated hydrolysate of manure fiber was comparable (33% and 29%, respectively), whereas that of glucose from acid-pretreated hydrolysate was clearly lower (18%) during the entire course of fermentation. It was reported that the hydrolysate obtained after the acid pretreatment produced some byproducts (e.g., furfural from xylose, hydroxymethylfurfural from glucose, and some phenols from lignin), which had a negative effect on microbial metabolism. Moreover, the concentration of FA from fermentation of the hydrolysate obtained by enzymatic hydrolysis was similar to that from glucose. This result indicates that there was no significant difference between glucose and enzymatic hydrolysate of lignocellulosic materials on FA fermentation.

Table 1. Fumaric acid production from pure glucose and from glucose derived from steam-exploded oil palm EFB.

Carbon Source	Total Sugar (g/L) [ns]	Fumaric Acid (g/L) [ns]	Yield (g/g) [ns]	Productivity (g/L/h) [ns]
Pure glucose	75.5 ± 0.153	5.42 ± 0.072	0.07 ± 0.002	0.056 ± 0.002
Glucose derived from EFB	75.5 ± 0.100	5.3 ± 0.030	0.07 ± 0.002	0.055 ± 0.002

[ns] in the same row means that values are not significant at $p > 0.05$.

3.2. Upscale Fermentation of Fumaric Acid in an Air-Lift Fermenter

The optimized culture conditions acquired from a previous report [15] were further studied in a 3 L air-lift fermenter. The effects of initial glucose concentration, temperature, initial pH, and aeration rate on FA production by *Rh. oryzae* K20 are displayed in Figure 1. The highest FA concentration, 44 g/L, was obtained at an aeration rate of 1.5 VVM, an initial pH of 3.5, a temperature of 30 °C, and an initial glucose concentration of 100 g/L within a 72 h fermentation. The yield and productivity were 0.44 g/g and 0.61 g/L/h, respectively, being 8.32-fold higher than those recorded in a volumetric flask, which gave the highest FA production, 5.3 g/L, after a 72 h cultivation. Das et al. [18] claimed that the efficiency of fumaric acid production was affected by many factors including aeration rate, initial pH, temperature, initial glucose, etc. A high C/N ratio can increase the conversion of medium glucose to FA [19]. A higher initial glucose concentration may inhibit glucose utilization and decrease FA production efficiency [18]. However, the factors affecting FA production need to be optimized with each new medium composition of the individual fungus. The scored values corroborated the results obtained from fermentation media containing either lignocelluloses [17,20,21] or glucose [4,11,22–24]. The results of the present study show that upscaling the production of FA induces a higher concentration and improves fermentation productivity as well as fermentation yield.

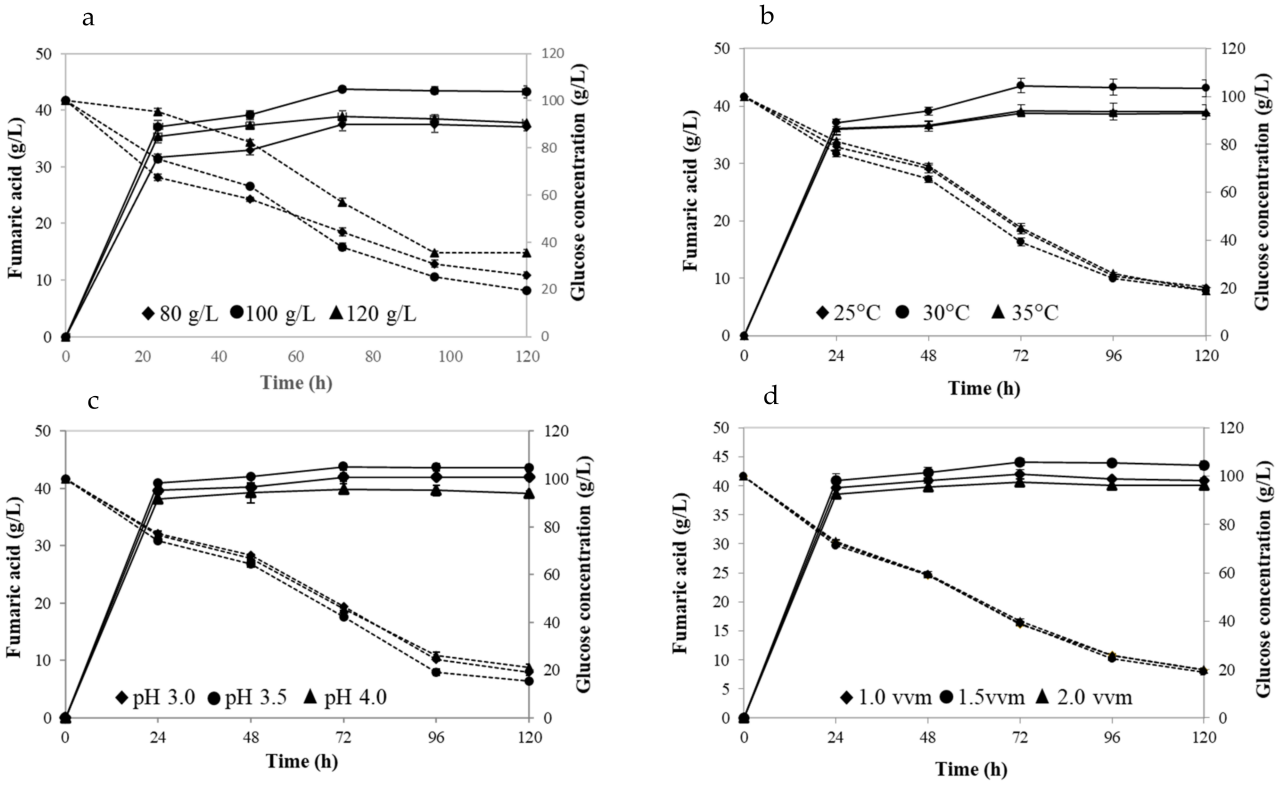


Figure 1. Fumaric acid concentration (solid line) and remaining glucose (dash line) at various initial glucose concentrations (g/L) (**a**), temperature (**b**), pH (**c**), and aeration rate (**d**).

3.3. Recovery of Fumaric Acid from Fermented Oil Palm Empty Fruit Bunches

The FA purification procedure from fermented oil palm EFBs was achieved using a two-stage precipitation process that included polishing the recovered FA with activated charcoal and the recovery of residual FA from liquid waste streams. Figure 2 represents a diagram of these processes with both mass balance and concentration in each stream. An FA concentration of 44 g/L obtained from fermented oil palm EFB broth was obtained using a two-stage precipitation process. In the first stage (streams 1–6), the fermentation broth was acidified with H_2SO_4, cooled, and filtered to gain a precipitated solid. The precipitated material was washed with a chilled H_2SO_4 solution to improve its purity. A recovery yield of 93.2% FA was obtained in the first precipitation stage. In the second precipitation stage (streams 7–15), the obtained solid was dissolved in water heated to 80 °C and then acidified with H_2SO_4, cooled to 4 °C, and filtered out. The recovered precipitate was washed with a chilled H_2SO_4 solution, dried, and weighed. After the described two-stage precipitation, a recovery yield of 81.2% and a purity of 83.5% were obtained in a fermentation broth containing 44 g/L FA. It was observed that the recovered precipitate had a low purity due to its dark color.

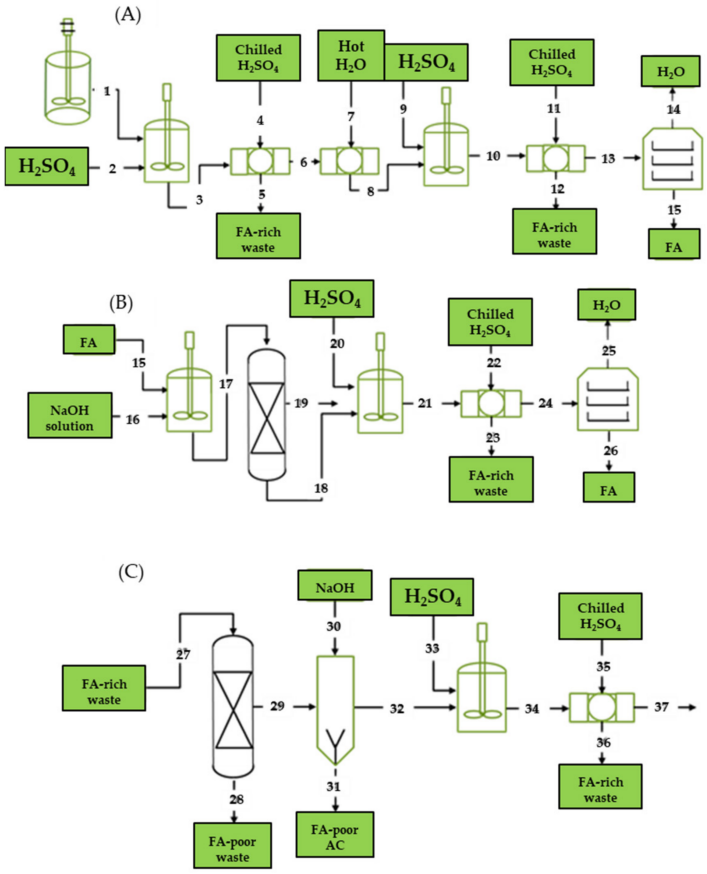
3.4. Removal of Contaminants from Fumaric Acid with Activated Charcoal

The results clearly show that the precipitated solid was still abundant in the impurities gained from the fermentation broth. Therefore, it was concluded that the improvement of FA purity using activated charcoal had to be performed, since this reagent is commonly used as an adsorbent for the removal of contaminants from a product. By studying the effects of pH and temperature on FA adsorption capacity, Figueira et al. [16] showed that it tended to decrease with an increased pH. To remove contaminants from the precipitated solid in order to reduce losses of FA production, the crystalline solid was dissolved in 10 N NaOH solution and adjusted to a concentration of ca. 30 g/L FA at pH 10, in which activated charcoal was poorly adsorbed on FA. After adding activated charcoal in the amount of 0.02 g/g FA solution and a subsequent shaking for 60 min at 35 °C, activated charcoal was removed by vacuum filtration. The obtained filtrate was acidified and left at 4 °C to allow FA to precipitate. The precipitate was then recovered, washed, dried, and weighed. After the removal of contaminants using activated charcoal, a cream-colored powder with a recovery yield of 81.2% and a purity of 83.5% was obtained.

3.5. Recovery of Residual Fumaric Acid from Dilute Solutions

After the two-stage precipitation process and the activated charcoal polishing step, residual FA from liquid waste streams was recovered by activated charcoal under acidic conditions. FA-rich waste from streams 5, 12 and 23, which contained a loss of 18.77% of FA, was pooled and supplemented with 0.1 g/g of FA solution at 35 °C for 60 min [16]. This was a point of a high adsorption of activated charcoal on FA. Activated charcoal was collected by vacuum filtration. After desorption of FA from activated charcoal, the filtrate was acidified and precipitated at 4 °C overnight. A recovery yield of 42.9% and a purity of 84.6% were obtained from the FA-rich waste, and the overall yield increased from 73.2% to 81.2% from previous steps. The streams 28 and 36 still contained 12.9% FA that could be recovered by redirecting both streams back to the two-stage precipitation process under acidic conditions. From the mass balances, FA losses found in the streams 19 and 31 are considered to be associated with activated charcoal adsorption on FA. As stream 19 still contained contaminating materials together with FA adsorbed onto activated charcoal, it was difficult to separate FA from these contaminants. In stream 31, a low amount of residual FA remained in the activated charcoal after its desorption, implying that some amount of activated charcoal might have been reused, which should be subject to further studies. Another experiment conducted by Figueira et al. [16] reported a recovery FA yield of 77.8% and a purity of 89.6% after the two-stage procedure. This procedure allowed an increase of the recovery yield of FA from 68.3% to 81.4%. Similarly, Zhang et al. [25]

reported a recovery yield of 93% with activated carbon followed by desorption by acetone, and a purity of >98% after water sweeping.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
FA (g)	44.0	0.0	44.0	0.0	2.8	41.0	0.0	41.0	0.0	41.0	0.0	2.6	38.0	0.0	38.0	0.0	38.0	35.4	2.5
FA (%)	5.0	0.0	3.4	0.0	0.2		0.0	7.6	0.0	7.0	0.0	0.4	-	-	86.4	0.0	3.0	2.8	-
	20	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	
FA (g)	0.0	35.4	0.0	2.8	32.2	0.0	32.2	8.3	2.1	6.1	0.0	1.4	4.9	0.0	4.9	0.0	1.3	3.5	
FA (%)	0.0	2.6	0.0	0.2	84.7	0.0	100.0	0.3	0.1	1.0	0.0	-	0.8	0.0	0.7	0.0	0.2	0.9	

Figure 2. Process flow diagram and mass balance of fumaric acid (FA) obtained after (**A**) a two-stage precipitation process, (**B**) a polishing step, and (**C**) recovery of FA residue from liquid waste streams.

3.6. Characterization of Bio-Synthesized Fumaric Acid

The bands observed in the region 4000–550 cm^{-1} arose from the vibrations of protons in the hydrogen bonds. The FTIR spectrum of the bio-synthesized FA was consistent with that of the commercial FA (Figure 3). The FTIR spectrum of FA was characterized by absorption bands at 1655 cm^{-1}, corresponding to a COO– antisymmetric stretching vibration, and 1419 cm^{-1}, corresponding to a symmetric stretching vibration. The band at 1209 cm^{-1} can be attributed to a C-O (H) stretching vibration. Figure 3 shows the DSC curves of both commercial and bio-synthesized FA, ranging from room temperature to 350 °C at a heating rate of 10 min^{-1} under an N_2 flow. The endothermic peak of the bio-synthesized FA appeared at 252.52–300.44 °C, and its melting point was 297.83 °C, which was in good agreement with the endothermic peak (254.28–306.38 °C) and the melting point (298.17 °C) of commercial FA, as shown in Figure 4.

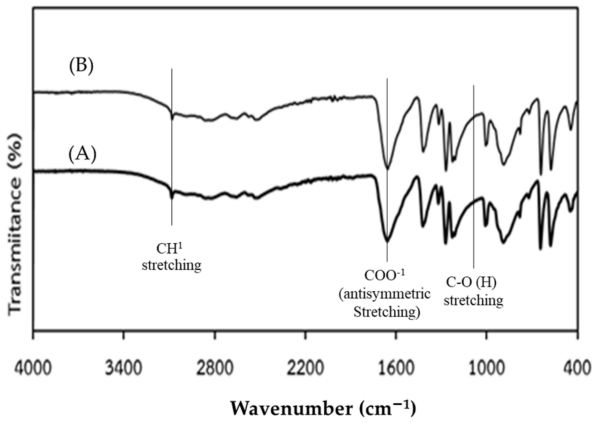


Figure 3. FTIR spectra of (A) commercial fumaric acid (FA) and (B) biosynthesized FA measured in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans.

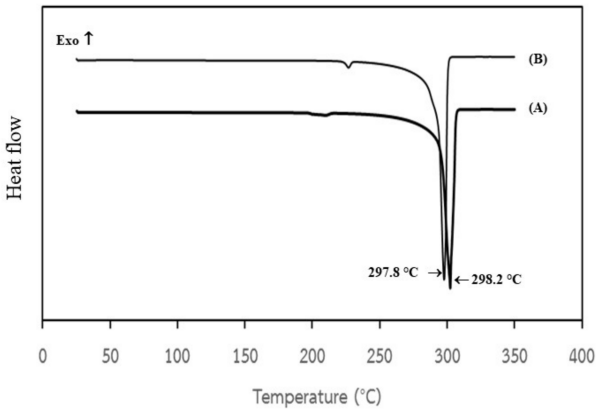


Figure 4. DSC scans of (A) commercial fumaric acid (FA) and (B) bio-synthesized FA.

This result corresponds to that reported by Saganowska and Wesolowski [26], who found the melting point of FA to be 295.81 °C using DSC measurements taken up to 350 °C. This implies that FA recovered from fermented EFB shows fairly similar properties to the commercial FA. This allows for the consideration of further development and commercialization of the reported technology process.

4. Conclusions

In this study, the implemented simple two-stage precipitation process for FA recovery resulted in an 81.2% recovery yield. After the activated charcoal polishing step, the obtained FA had a paler color; however, it was still somewhat cream-colored. However, the obtained FA had a fairly similar FTIR spectrum and melting point to the commercial FA. Based on this work, the obtained FA powder can be recommended for use in developing either antimicrobial packaging films or active packaging to improve both the microbiological safety and quality of fruits and vegetables during refrigerated storage.

Author Contributions: Correspondence author, supervision, writing—review and editing, and publication process, N.N.; project lead, conceptualization, funding acquisition, and writing—original draft, A.B.; project administration, N.K.; resource contribution, P.V.; validation, formal analysis, and investigation, W.A. and C.T.; and methodology, P.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data supporting the conclusions of this article are included in the manuscript.

Acknowledgments: Deep appreciation is expressed to the Agricultural Research Development Agency (Public Organization) (ARDA; Bangkok, Thailand), the Faculty of Science and Technology, Nakhon Sawan Rajabhat University (NSRU, Nakhon Sawan, Thailand) and the Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI; Bangkok, Thailand) for their support.

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

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