fermentation

MDPI

Article

Bio-Succinic Acid Production from Palm Oil Mill Effluent Using *Enterococcus gallinarum* with Sequential Purification of Biogas

Pooja Vilas Nagime, Apichat Upaichit, Benjamas Cheirsilp and Piyarat Boonsawang *

Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90110, Thailand; poojanagime1010@gmail.com (P.V.N.); apichat.u@psu.ac.th (A.U.); benjamas.che@psu.ac.th (B.C.)
* Correspondence: piyarat.b@psu.ac.th; Tel.: +66-74286372

Abstract: Bio-succinic acid production using microorganisms has been interesting as an environmentally friendly process. Palm oil mill effluent (POME) was considered as a cheap substrate to lower the cost of production. It was revealed that 2-fold diluted POME produced more succinic acid than undiluted and 5-fold diluted POME. In addition, the effects of various neutralizing agents on succinic acid production utilized to manage pH and CO_2 supply indicated that the utilization of $MgCO_3$ as a neutralizing agent produced succinic acid of 11.5 g/L with a small amount of by-product synthesis. Plackett–Burman Design (PBD) was used to screen the most significant nutrients for bio-succinic acid production from 2-fold diluted POME using *E. gallinarum*. From the Pareto chart, $MgCO_3$ and peptone presented the highest positive effect on the production of succinic acid. In addition, Box–Behnken Design (BBD) was conducted to increase bio-succinic acid production. Experiments showed the highest production of succinic acid of 23.7 g/L with the addition of 22.5 g/L $MgCO_3$ and 12.0 g/L peptone in 2-fold diluted POME. Moreover, the experiment of replacing $MgCO_3$ with CO_2 from biogas resulted in 19.1 g/L of succinic acid, simultaneously creating the high purity of biogas and a higher CH_4 content.

Keywords: succinic acid; palm oil wastewater; neutralizing agents; Plackett–Burman design; Box–Behnken design

Citation: Nagime, P.V.; Upaichit, A.; Cheirsilp, B.; Boonsawang, P. Bio-Succinic Acid Production from Palm Oil Mill Effluent Using *Enterococcus gallinarum* with Sequential Purification of Biogas. *Fermentation* **2023**, *9*, 369. https://doi.org/10.3390/fermentation9040369

Academic Editor: Hui Yun

Received: 17 March 2023
Revised: 1 April 2023
Accepted: 10 April 2023
Published: 12 April 2023

1. Introduction

With a global shortage of energy sources and a deteriorating ecology, bio-based synthesis of chemicals [1], fuels [2], and polymers [3] from sustainable and low-cost resources has sparked the public's attention. Succinic acid is a fundamental platform chemical with a huge spectrum of applications and a high economic value [1,4]. It is acknowledged as one of the most significant production components for a variety of industries, such as agriculture, medicine, food, and chemical manufacturing products, including surfactants, coloring agents, medications, flavoring agents, and chemicals [5,6]. Recently, scientists have been searching for an alternative process to produce succinic acid with a minimal greenhouse effect and environmental contamination. Succinic acid was produced using a variety of biomass types, including lignocellulosic sources [6–10]. However, pretreatment is required to make this lignocellulosic material available to microbes in the simplest form possible. Chemical, physical, and enzymatic approaches are used in these pretreatment methods. Nevertheless, these technologies are far more expensive when it comes to large-scale commercialization. To discover a solution to this problem, researchers examined using wastewater without pretreatment.

One of Thailand's important economic sectors is the palm oil industry, which accounts for 3.9% of global production [11]. During the production of crude palm oil, a massive amount of colored wastewater, known as palm oil mill effluent (POME), was generated. POME is the colloidal final discharge mixture of oil (0.6–0.7%), water (95–96%),

and solids (4–5%), which results in a high chemical oxygen demand (COD) [12]. The discharge of POME without suitable treatment causes environmental impacts as well as water pollution and greenhouse gas emissions [11–13]. There have been reports on the possibility of using POME as the substrate for environmentally friendly bio-products, including biogas, biohydrogen, polyhydroxyalkanoates, microbial oil, biodiesel, organic acids, and fertilizer [13–18]. The use of POME to produce succinic acid was reported for the first time by our research group. We preliminary investigated the production of succinic acid from 2-diluted POME, yeast extract (5 g/L), and $MgCO_3$ (15 g/L) using *Enterococcus gallinarum* [19]. It was found that succinic acid at 11.5 g/L could be obtained. However, a low amount of succinic acid was produced. The ideal nutrients for microbial culture are required to promote succinic acid synthesis. Moreover, most studies have investigated the production of succinic acid using *Actinobacillus succinogenes* [4,6–10]. In our earlier research, we discovered a novel isolated strain of *Enterococcus gallinarum* with strong succinic acid synthesis and low generation of undesirable acids [19].

The majority of microorganisms use the reductive branch of the tricarboxylic acid (TCA) cycle to produce succinic acid in anaerobic environments. In this pathway, CO_2 plays a significant role in controlling the generation of metabolite products. Phosphoenolpyruvate (3C) and CO_2 are converted into succinic acid (4C) via intermediate compounds, including oxaloacetate (OAA), malate, and fumarate [20]. Many studies have shown that the addition of salts such as $MgCO_3$ and $CaCO_3$ increased the availability of CO_2 and enhanced succinic acid synthesis [21]. However, adding these salts to the fermentation process results in an increase in the fermentation cost, and any precipitate remaining after fermentation undoubtedly raises the cost of downstream processing. Biogas generally consists of carbon dioxide (CO_2) (25–50%) and methane (CH_4) (50–75%), which can be employed as CO_2 sources in the synthesis of succinic acid [22,23]. As a result, biogas could improve in quality with higher CH_4 and lower CO_2. Figure 1 presents the proposed palm oil mill industry containing succinic acid and biogas purification. Consequently, the palm oil mill sector might implement this strategy to increase the sustainability of palm oil production in a cost-efficient manner with value-added products, a highly energy-efficient manner (from the high quality of biogas), and an environmentally friendly manner.

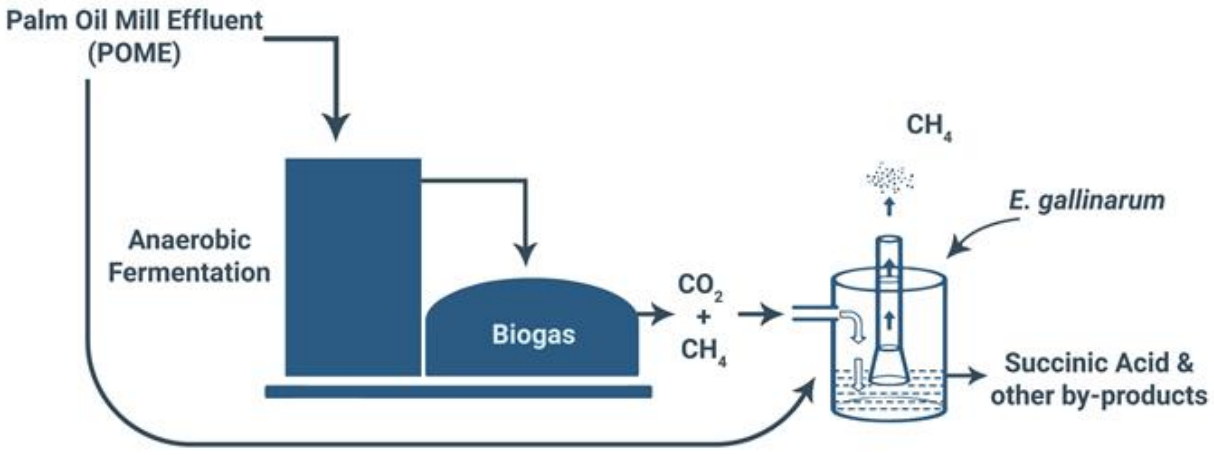


Figure 1. The proposed idea containing succinic acid and biogas purification for the sustainability of the palm oil mill industry.

The goal of this work was to investigate the efficiency of *Enterococcus gallinarum* for the production of succinic acid from POME as an inexpensive substrate. To increase succinic acid production and less undesirable product synthesis, response surface methodology (RSM) can be used for the maximizing succinic acid production. The impacts of crucial additional components on succinic acid production were also examined using the Plackett–Burman design (PBD). The optimum values of significant variables were also

explored using the Box–Behnken Design (BBD). Moreover, the utilization of CO_2 from biogas for the production of succinic acid was also studied.

2. Materials and Methods

2.1. Palm Oil Mill Effluent (POME)

POME was collected from the first pond of the wastewater treatment system located at the palm oil industry in Trang Province, Thailand. It was stored in a cold room (4 °C) until use. POME contained a high COD concentration (170 g/L) along with the dark brown liquid, a total reducing sugar of 13.9 g/L, and a low pH of 4.32. Centrifugation of POME was carried out at 8000 rpm (7455× *g*) for 10 min to remove debris before use.

2.2. Microorganisms and Inoculum Preparation

Enterococcus gallinarum (MW931746) was isolated from the rumen and tested for its ability to produce succinic acid. For inoculum preparation, the bacteria strain was prepared in Gifu Anaerobic Broth (GAM Broth) (Nissui Pharmaceutical Company, Tokyo, Japan). GAM broth contained 10 g/L peptic digest of animal tissue, 3.0 g/L papaic digest of soya bean meal, 10 g/L protease peptone, 13 g/L digested serum, 5.0 g/L yeast extract, 2.2 g/L beef extract, 1.2 g/L liver extract, 3.0 g/L dextrose, 2.5 g/L potassium dihydrogen phosphate, 3.0 g/L sodium chloride, 5.0 g/L starch-soluble, 0.3 g/L l-cysteine hydrochloride, and 0.3 g/L sodium thioglycolate, with a final pH adjustment of 7.0 [24]. The first preculture was conducted in a test tube with a volume of 5 mL at 37 °C and 120 rpm under anaerobic conditions for 24 h. For the second preculture, 2.5 mL of the first preculture was added to 22.5 mL of GAM and cultured at 37 °C and 120 rpm under anaerobic conditions for 24 h. Maintenance of the bacterial strain was performed in 20% glycerol at −80 °C.

2.3. Effect of Dilution of POME and Neutralizing Agents on Succinic Acid Production

POME was used as a substrate in undiluted, 2-fold diluted, and 5-fold diluted concentrations with the addition of 0.2 g/L of $MgCl_2$, 0.2 g/L of $CaCl_2$, 0.31 g/L of Na_2HPO_4, 1.16 g/L of NaH_2PO_4, and 5 g/L of yeast extract (YE) [24]. The effects of neutralizing agents, including $CaCO_3$, NaOH, Na_2CO_3, and $MgCO_3$ at 15 g/L, were investigated. The medium was sterilized at 121 °C for 15 min and the initial pH of 7.0 was adjusted. Fermentation was carried out in 120-mL serum bottles (a working volume of 100 mL) with 10% inoculum for 72 h at 37 °C and 120 rpm under anaerobic conditions. The samples were taken every 12 h for the determination of dry biomass weight, pH, and acid production. The COD of the fermentation broth at the beginning and end of the experiment was also analyzed. All experiments were conducted in triplicate.

2.4. Screening Parameters for Succinic Acid by PBD

Fermentation medium contained POME with 2-fold dilution with different 11 variables, including $MgCl_2$ (A), $CaCl_2$ (B), $MnCl_2$ (C), Na_2HPO_4 (D), NaH_2PO_4 (E), $MgCO_3$ (F), pH (G), YE (H), $(NH_4)_2SO_4$ (J), Peptone (K), and NH_4Cl (L) at two levels, including maximum coded as (+1) and minimum coded as (−1) (Table 1). The 12 experiment runs were conducted in 120-mL serum bottles. The medium was sterilized at 121 °C for 15 min, and the initial pH of 7.0 was maintained. The 10% of inoculum was added to the fermentation medium after it was autoclaved. Fermentation was observed in the anaerobic condition using 120-mL serum bottles at 120 rpm and 37 °C for 60 h. The experiments were carried out in triplicate. The samples were taken before and after fermentation to determine succinic acid production, other acids, dry biomass weight, COD, and pH.

Table 1. Experimental definition for PBD for succinic acid production by *E. gallinarum*.

Variables	Keys	Low Level (−1)	High Level (+1)
$MgCl_2$ (g/L)	A	0.2	2.0
$CaCl_2$ (g/L)	B	0.2	1.5
$MnCl_2$ (g/L)	C	0.05	0.07
Na_2HPO_4 (g/L)	D	0.3	4.4
NaH_2PO_4 (g/L)	E	1.5	4.4
$MgCO_3$ (g/L)	F	15	30
Initial pH	G	6.0	8.0
Yeast extract (YE) (g/L)	H	5.0	10
$(NH_4)_2SO_4$ (g/L)	J	1.4	3
Peptone (g/L)	K	3.0	12
NH_4Cl (g/L)	L	2.5	5.3

2.5. The Optimization of Succinic Acid Production by BBD

The significant factors from the PBD analysis were selected to investigate the optimized values using BBD with three levels. The 17 trial runs were conducted in 120-mL serum bottles. The medium was sterilized for 15 min at 121 °C and the initial pH of 7.0 was adjusted. Afterward, 10% of the inoculum was added to the fermentation medium. The fermentation in triplicate was carried out under anaerobic conditions at 120 rpm and 37 °C for 60 h. The sample was taken before and after fermentation to determine succinic acid production. Design-Expert® software, version 13 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for statistical analysis. Succinic acid was used as a response in the mathematical model based on the second-order polynomial Equation (1).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 \quad (1)$$

where Y is the response; X_1, X_2 are viable parameters; β_0 is the intercept; β_1, β_2, β_{12}, β_{11}, β_{22}, are coefficient estimates for succinic acid production.

2.6. Biogas Preparation

Biogas was generated from POME (pH of 4.3) with the inoculation of 20% anaerobic sludge (20 g VS/L) in a 1 L serum bottle. Nitrogen gas was purged into the bottle for 3 min to maintain an anaerobic condition. The fermentation was conducted for 2 weeks without pH regulation at 30 ± 2 °C. The biogas was continuously collected from the top of the bottle and kept in the gas bag.

2.7. Succinic Acid Production Coupling with CO_2 Removal from Biogas

The medium, which contained 2-fold diluted POME and 10 g/L of peptone with and without 23 g/L $MgCO_3$, was sterilized at 121 °C for 15 min, and the initial pH of 7.0 was adjusted. The 10% of inoculum was added to the fermentation medium in the 120-mL serum bottle with a working volume of 100 mL. Control was performed without the inoculation of microorganisms. For the experiment using CO_2 from biogas, the biogas was purged through the medium in the 120-mL serum bottle at 30 mL every 12 h. Fermentation was conducted in triplicates with each experiment under anaerobic conditions at 37 °C and 120 rpm for 72 h. The samples were taken every 12 h for the determination of growth, pH, and acid production. The COD removal of fermentation broth at the beginning and end of the experiment was also calculated. After 72 h of fermentation, the biogas in the headspace of the serum bottle was withdrawn by syringe. The biogas composition was determined using gas chromatography. The CH_4 and CO_2 contents of biogas at the initial stage and at the end of fermentation were compared.

2.8. Analytical Methods

The concentrations of succinic acid and other organic acids were determined using high-performance liquid chromatography (HPLC) that was well-equipped with a refractive index detector and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad Chemical Division, Hercules, CA, USA). Around 5 mM H_2SO_4 was included in the mobile phase, which had a flow rate adjustment of 0.60 mL/min. The injection volume was 20 µL, and the temperature was 50 °C [25]. To remove the particles of $MgCO_3$ from the sample, 0.2 M of HCL was added. Samples were then centrifuged for 15 min at 10,000 rpm, and the supernatant was filtered with a nylon syringe and a 0.22 µm membrane filter before being filled into a vial. Dry cell weight (DCW) was examined by centrifugation of broth for 10 min at 10,000 rpm followed by heating at 103 °C overnight.

The composition of biogas was determined using gas chromatography (GC) (Shimadzu GC-8A, Shimadzu, Kyoto, Japan) equipped with a Porapak Q capillary column and a thermal conductivity detector. Reducing sugar was determined using the 3,5-dinitrosalicylic acid (DNS) method [26]. COD was determined according to the standard method [27].

3. Results and Discussion

3.1. Effect of Dilution and Netralizing Agents on Succinic Acid Production from POME

POME was studied at three different dilutions (undiluted, 2-fold, and 5-fold). According to this study, *E. gallinarum* grew well and produced more biomass in 2-fold diluted POME than in undiluted and 5-fold diluted POME. Furthermore, the production of succinic acid from 2-fold diluted POME was found at 11.5 g/L, which was significantly higher than that from undiluted and 5-fold diluted POME (Table 2). The inaccessibility of nutrients to the bacterial culture may be due to more complex nutrients as well as the high phenolic compounds in undiluted POME, which adversely affected microbial growth [12]. In addition, less nutrients were available in 5-fold POME, resulting in less growth and biomass.

Table 2. Succinic acid and biomass productions from POME at the various dilution and neutralize agents (15 g/L) by *E. gallinarum* at the cultivation period of 60 h.

Dilution	Neutralizing Agents	Biomass (g/L)	SA (g/L)	FA (g/L)	LA (g/L)	AA (g/L)	% COD Removal	pH
Undilted POME	$MgCO_3$	1.53 ± 0.06	5.62 ± 0.02	0	0	0	23.3	6.82 ± 0.05
2-fold dilution	$MgCO_3$	2.61 ± 0.09	11.5 ± 0.43	0.021	0.442	0.278	50.0	6.24 ± 0.03
2-fold dilution	$CaCO_3$	2.03 ± 0.10	7.23 ± 0.67	0.314	0.863	1.31	-	5.36 ± 0.03
2-fold dilution	NaOH	1.24 ± 0.03	3.31 ± 0.35	0	0.325	0	-	4.51 ± 0.05
2-fold dilution	Na_2CO_3	1.48 ± 0.08	4.53 ± 0.28	0	0	0.442	-	4.85 ± 0.18
5-fold dilution	$MgCO_3$	2.39 ± 0.01	8.25 ± 0.01	0	0.554	0.293	40.0	6.64 ± 0.01

Note: SA = succinic acid; FA = formic acid; LA = lactic acid; AA = acetic acid.

It has been reported that the succinic acid producers have a significant impact on the redox phase of the fermentation medium. In this study, $MgCO_3$, $CaCO_3$, NaOH, and Na_2CO_3 were used as neutralizing agents. The highest biomass of 2.61 ± 0.09 g/L was obtained in MgCO3-supplemented media. The lower biomass was found in the media supplemented with NaOH and Na_2CO_3 with the values of 1.24 ± 0.03 g/L and 1.48 ± 0.08 g/L, respectively (Table 2). It can be seen that the neutralizing agents with Na^+ have a negative effect on the synthesis of succinic acid. The addition of a large amount of Na^+ to the fermentation medium causes cell flocculation, which tends to precipitate immediately in the fermentation broth. This could lead to a deterioration of cells and a disturbance in nutrient consumption [28]. However, the use of Na_2CO_3 produced more succinic acid than the use of NaOH. This result agreed with the finding of Andersson et al. [29], who reported that alkali carbonate was a more suitable neutralizing agent than alkali hydroxide in the production of succinic acid. Moreover, the fermentation with Na^+ failed to regulate the pH of the medium (pH < 6.0), which severely suppressed cell growth.

With the addition of Ca^{2+}, biomass was formed at 2.03 ± 0.10 g/L and succinic acid was formed at 7.23 ± 0.67 g/L. Although microbial cultivation in $CaCO_3$ medium resulted in a higher concentration of succinic acid than in Na+-supplemented medium, it was much lower than in $MgCO_3$-supplemented medium. It was caused by the lower solubility of $CaCO_3$ when compared to $MgCO_3$ [30]. This result agreed with the findings of Liu et al. [31], who found that high concentrations of sodium and calcium ions inhibited cell growth and succinic acid production but not magnesium ions.

It is believed that $MgCO_3$ enhanced dissolved CO_2 and PEP carboxykinase activity, which significantly involved the C4 pathway to synthesize succinic acid [30]. The succinic acid concentration of 11.5 ± 0.43 g/L obtained from 2-fold diluted POME with nutrients and $MgCO_3$ supplementation was still low. In addition, no report has investigated the optimum nutrient for the production of succinic acid from POME.

3.2. Screening Parameters for Succinic Acid Production by PBD

Table 3 shows the succinic acid production as a response to PBD. The highest concentration of succinic acid (23.06 g/L) was obtained from Run No. 12 with medium containing 2-fold diluted POME, 2.0 g/L of $MgCl_2$, 1.5 g/L of $CaCl_2$, 0.05 g/L of $MnCl_2$, 4.4 g/L of Na_2HPO_4, 4.4 g/L of NaH_2PO_4, 30 g/L of $MgCO_3$, pH = 6, 5 g/L of YE, 1.4 g/L of $(NH_4)_2SO_4$, 12 g/L of peptone, and 2.5 g/L of NH_4Cl.

Table 3. Plackett–Burman design variables in code levels with succinic acid as a response.

Run	A	B	C	D	E	F	G	H	J	K	L	Succinic Acid (g/L)
1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	10.11
2	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	22.46
3	−1	+1	+1	+1	−1	−1	−1	+	−1	+1	+1	15.50
4	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	14.99
5	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	22.08
6	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	18.56
7	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	17.24
8	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	16.76
9	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	15.79
10	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	15.48
11	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	17.67
12	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	23.06

Note: $MgCl_2$ (A), $CaCl_2$ (B), $MnCl_2$ (C), Na_2HPO_4 (D), NaH_2PO_4 (E), $MgCO_3$ (F), pH (G), YE (H), $(NH_4)_2SO_4$ (J), Peptone (K), NH_4Cl (L).

Table 4 represents the PBD analysis for the main effects of variables on the production of succinic acid. The negative effects were found in $MnCl_2$ (C), YE (H), and NH_4Cl (L). The contributions of C and L were 0.02% and 0.51%, respectively. Therefore, these two factors were excluded from the model. From the ANOVA analysis, it reveals an *F*-value of 42.4 with a *p*-value of 0.023 and an R^2 of 0.99. It was implied that the model was significant. The Pareto chart categorizes the effects from greatest to least (K > F > G > B > H > J > A > D > E > L > C) (Figure 2). However, the significant variables with a greater effect than the t-value limit (Figure 1) and a low *p*-value (0.05) were peptone (K), $MgCO_3$ (F), pH (G), $CaCl_2$ (B), YE (H), and $(NH_4)_2SO_4$ (J).

Peptone (K) was discovered to be the most important factor in the production of succinic acid. Among nitrogen sources, YE (H), $(NH_4)_2SO_4$ (J), peptone (K), and NH_4Cl (L), J and K presented the positive effects, but H and L gave the negative effects. Although YE was the most common nitrogen source used by *Actinobacillus succinogenes* for the synthesis of succinic acid, *E. gallinarum* preferred peptone as a nitrogen source in this study. This result agrees with the finding of Agarwal et al. [32], who reported that peptone as a nitrogen source supplemented in cane molasses produced a higher concentration of succinic acid than YE, $(NH_4)_2SO_4$, and NH_4Cl. The interaction between the carbon in POME and the nitrogen sources supplemented may have an impact on the microbial pathway and the

synthesis of metabolite products [33]. According to the Pareto chart, K and F represented the highest level of confidence, with contributions of 43.8% and 14.8%, respectively. However, these two factors were selected for further experimentation in the investigation of the optimum values by BBD.

Table 4. Standardized effect and ANOVA analysis for PBD.

	Effect	Sum of Squares	% Contribution	Mean Square	*F*-Value	*p*-Value **	Significant **
Model	-	149.4	-	16.6	42.4	0.023	Yes
A	1.26	4.79	3.19	4.79	12.2	0.073	No
B	1.90	10.8	7.21	10.8	27.7	0.034	Yes
C	−0.087	(0.023) *	0.02	-	-	-	-
D	1.24	4.59	3.06	4.59	11.7	0.076	No
E	1.14	3.92	2.61	3.92	10.0	0.087	No
F	2.72	22.5	14.8	22.5	56.9	0.017	Yes
G	2.38	17.0	11.3	17.0	43.6	0.022	Yes
H	−1.84	10.2	6.76	10.2	26.0	0.036	Yes
J	1.83	10.0	6.67	10.0	25.6	0.037	Yes
K	4.68	65.8	43.8	65.8	168.1	0.006	Yes
L	−0.50	(0.76) *	0.51	-	-	-	-

Note: $MgCl_2$ (A), $CaCl_2$ (B), $MnCl_2$ (C), Na_2HPO_4 (D), NaH_2PO_4 (E), $MgCO_3$ (F), pH (G), YE (H), $(NH_4)_2SO_4$ (J), Peptone (K), NH_4Cl (L). * C and L were excluded and not used in the model. ** The significant variables were considered at the 95% of confidence level (*p*-value < 0.05).

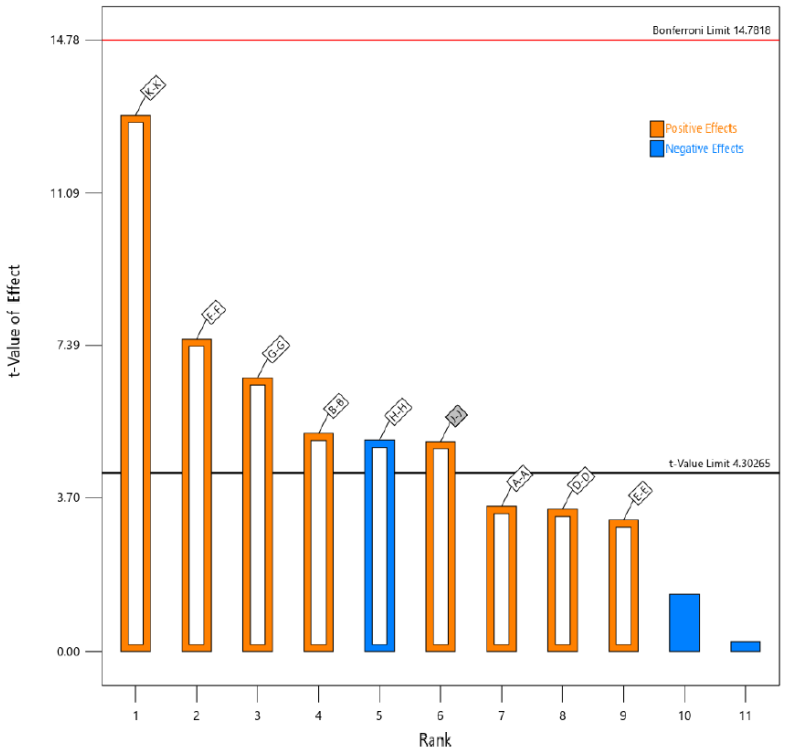


Figure 2. The Pareto chart of PBD analysis for the production of succinic acid (A = $MgCl_2$, B = $CaCl_2$, D = Na_2HPO_4, E = NaH_2PO_4, F = $MgCO_3$, G = pH, H = YE, J = $(NH_4)_2SO_4$, K = Peptone).

3.3. The Optimum Nutrient Supplementation for Succinic Acid Production by PBD

In order to analyze the optimum concentration of medium components and their interaction effect on succinic acid production by *E. gallinarum*, BBD with response surface methodology was implemented. $MgCO_3$ and peptone were the factors selected for BBD at three levels. The production of succinic acid with each run is presented in Table 5. A second-order polynomial model was constructed from the experimental data by Design-

Expert software (version 13.0). The mathematic model for the production of succinic acid was developed using the following Equation (2):

$$Y = 26.75 - (0.675\ X_1) + (4.65\ X_2) + (2.45\ X_1X_2) - (4.48\ {X_1}^2) - (4.13\ {X_2}^2) \quad (2)$$

where Y = succinic acid (g/L), X_1 = $MgCO_3$ (g/L) and X_2 = peptone (g/L).

Table 5. Experimental and predicted values of succinic acid by *E. gallinarum* using BBD.

Run	$MgCO_3$ (g/L) (X_1)	Peptone (g/L) (X_2)	Succinic Acid (g/L)		% Error
			Actual Values	Predicted Values	
1	15	3	16.9	16.6	1.69
2	15	7.5	24.1	22.9	4.79
3	15	7.5	23.3	22.9	1.52
4	15	12	19.2	21.0	−9.45
5	22.5	3	16.3	18.0	−10.2
6	22.5	3	17.9	18.0	−0.391
7	22.5	7.5	26.4	26.8	−1.33
8	22.5	7.5	27.2	26.8	1.65
9	22.5	7.5	25.4	26.8	−5.31
10	22.5	7.5	27.0	26.8	0.926
11	22.5	7.5	27.0	26.8	0.926
12	22.5	12	28.3	27.3	3.64
13	22.5	12	28.7	27.3	4.98
14	30	3	11.8	10.4	12. 2
15	30	7.5	21.3	21.6	−1.38
16	30	7.5	21.1	21.6	−2.35
17	30	12	23.9	24.6	−2.78

The highest concentration of succinic acid was obtained at about 28.7 g/L when $MgCO_3$ of 22.5 g/L and peptone of 12.0 g/L were added to the 2-fold diluted POME. However, the maximum succinic acid production was calculated to be 28.1 g/L with the addition of $MgCO_3$ at 23.1 g/L and peptone at 10.1 g/L. The ANOVA analysis results for the response surface quadratic model are shown in Table 6. The p-value < 0.05 and high F-values proved that the model equation (2) was significant and could be used to predict acid production. In this study, the F-value of 50.9 for lack of fit was not significant, which indicates that the model was good. In this study, the variability of the dependent factors was 95.9%, which was explained by an R^2 value of 0.9586. All factors significantly affected succinic acid production, except $MgCO_3$ (X_1) (Table 6). However, the double amount of $MgCO_3$ (${X_1}^2$) significantly influenced the acid synthesis. In addition, the interaction between $MgCO_3$ and peptone significantly impacted the succinic acid production by *E. gallinarum* (Figure 3).

Table 6. ANOVA analysis for succinic acid production by *E. gallinarum* using BBD.

Sources	Sum of Squares	df	Mean Square	*F*-Value	*p*-Value	Significant
Model	366.7	5	73.3	50.9	<0.0001	Yes
X_1-$MgCO_3$	3.64	1	3.64	2.53	0.1399	No
X_2-Peptone	172.9	1	172.9	120	<0.0001	Yes
X_1X_2	24.0	1	24.0	16.7	0.0018	Yes
${X_1}^2$	84.8	1	84.8	58.9	<0.0001	Yes
${X_2}^2$	72.1	1	72.1	50.1	<0.0001	Yes
Residual	15.8	11	1.44			
Lack of Fit	13.7	7	1.95	3.62	0.1157	No
Pure Error	2.16	4	0.54			
R^2	0.9586					
%C.V.	5.29					

Note: The significant variables were considered at the 95% of confidence level (p-value < 0.05).

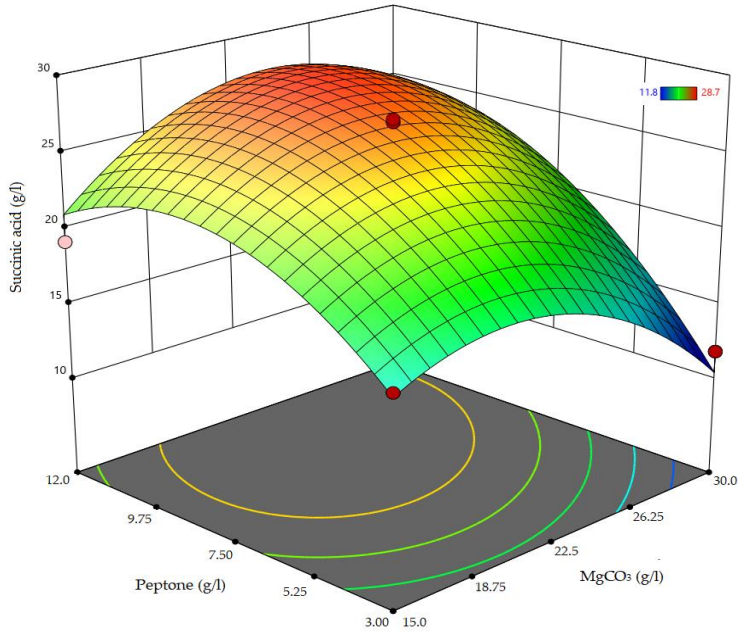


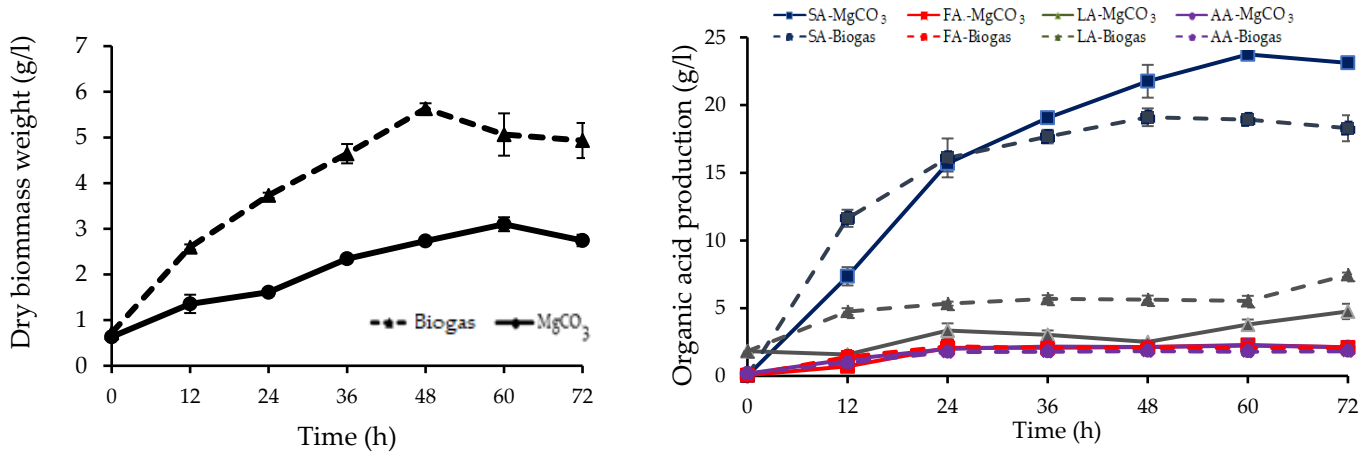
Figure 3. Response surface plot represents the effect of $MgCO_3$ and peptone on succinic acid production by *E. gallinarum*.

3.4. Succinic Acid Production Coupling with CO_2 Removal from Biogas

The production of succinic acid from 2-fold diluted POME supplemented with biogas purging in the fermentation broth was investigated. Biogas produced from POME without pH regulation was kept in the gas bag, which contained CO_2 at 69.8% and CH_4 at 30.2%. After the cultivation of *E. gallinarum*, bacterial growth increased gradually and reached an amount of 5.63 ± 0.15 g/L after 48 h. In comparison, the bacterial growth rate is slightly lower with the addition of $MgCO_3$ as a form of carbonate to fermentation broth (Figure 4a). This might be due to the different amounts of CO_2 provided in the broth. Biogas was supplied every 12 h whereas $MgCO_3$ was added once at the beginning. In addition, there would be insoluble $MgCO_3$ when the quantities of dissolved CO_2 reached the threshold level, which might result in turbid broth and reduce the mass transfer of nutrients to the bacteria culture. The pH of the system decreased to about 6.13 and 6.44 with the addition of biogas and $MgCO_3$, respectively. It was observed that $MgCO_3$ was better at regulating the pH of the medium than CO_2 from biogas. However, the pH in both cases was still in the acceptable pH range (6.0–7.2) for the production of succinic acid [31,34].

In this experiment, the highest succinic acid concentration of 23.7 ± 0.31 g/L was produced at 60 h from the fermentation of POME with the addition of $MgCO_3$ (Figure 4b). In comparison, succinic acid production was rapidly initiated at 11.64 ± 0.63 g/L at 12 h from the fermentation of POME with the sparging of biogas. Although biogas purging into POME enhanced the growth of microbial cells, the production of succinic acid (18.9 ± 0.39 g/L) was lower than that with the addition of $MgCO_3$ at 60 h. The higher succinic acid synthesis might be due to the availability of the Mg^{2+} ion, which acts as a cofactor to enhance the activity of phosphoenolpyruvate carboxylase [30]. In addition, the gaseous CO_2 from biogas also improves the production of lactic acid. The higher lactic acid production was found in the experiment with biogas purging than in the experiment with $MgCO_3$ addition (Figure 4b). The amount of CO_2 is recognized as an important factor in succinic acid metabolism. Phosphoenolpyruvate is the primary intermediate, which acts as a junction between the C3 and C4 pathways. The key step in the formation of succinic acid via the C4 pathway is phosphoenolpyruvate carboxylation, whereas ethanol, lactic acid, and acetic acid are produced via the C3 pathway. The levels of CO_2 affected the phosphoenolpyruvat carboxylase, resulting in the formation of metabolite acids [30]. Kuglarz and Rom [35] reported that the supplement of biogas (25% of CO_2) in the fermentation of

Miscanthus hydrolysates resulted in a lower succinic yield and a higher by-product yield than the supplement of 15–20 g/L $MgCO_3$. They suggested that combining CO_2 from biogas with 15–20 g/L $MgCO_3$ increased sugar utilization by 67–70% and succinic acid yield by 37–40%. Although the succinic acid concentration and yield for the fermentation with sparging biogas were lower than those with $MgCO_3$ addition (Table 7), it is economically feasible to replace $MgCO_3$ with CO_2 from biogas on a large scale. This study demonstrates that *E. gallinarum* is a good candidate for the synthesis of succinic acid. Furthermore, when compared to other wastes, POME is a promising substrate for producing succinic acid without the need for pretreatment and hydrolysis.



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

Figure 4. Biomass (**a**) and organic acid (**b**) production from POME supplemented with sparging of biogas (dash line) and $MgCO_3$ (solid line).

In terms of the environment, the COD of POME decreased by 28.1% and 37.1%, respectively, with the addition of biogas and MgCO3. Low COD removal was caused by COD generation during organic acid production. The COD equivalents of formic acid, acetic acid, lactic acid, and succinic acid were 16, 64, 96, and 112 g COD/mol of acid, respectively [36]. In the case of biogas purification, this experiment showed that the methane content increased from 30.2 ± 1.11% to 72.8 ± 1.40% and the CO_2 was removed from 69.8 ± 4.82% to 26.6 ± 1.05% after 72 h of fermentation. Therefore, it is advantageous to obtain succinic acid coupling with CO_2 removal from biogas, resulting in lower CO_2 emissions.

Table 7. Production of the desirable and undesirable products during fermentation with CO_2 supplement in a form of pure gas or biogas.

Microbial Strains	Main Substrates	CO_2 Sources	Time [1] (h)	Succinic Acid Production			By-Products [3] (g/L)	% Succinic acid [4]	Ref.
				Conc. (g/L)	Yield [2] (g/g)	Prod. (g/L-h)			
Enterococcus gallinarum	POME	Biogas	48	19.1 ± 0.65	1.37	0.398	9.52	66.7	This Study
Enterococcus gallinarum	POME	$MgCO_3$	60	23.7 ± 0.30	1.71	0.395	8.31	74.1	This study
A. succinogenes 130Z	Crude glycerol	$MgCO_3$	96	6.5 ± 0.1	2.1	1.3	N/A	N/A	[21]
A. succinogenes 130Z	Sugars-rich industrial waste	$MgCO_3$	24	22.6 ± 0.5	0.64	0.94	20.9	N/A	[23]
		$MgCO_3$ + biogas	24	25.5 ± 2.4	0.64	1.06	20.0	N/A	[23]

Table 7. *Cont.*

Microbial Strains	Main Substrates	CO_2 Sources	Time [1] (h)	Succinic Acid Production			By-Products [3] (g/L)	% Succinic acid [4]	Ref.
				Conc. (g/L)	Yield [2] (g/g)	Prod. (g/L-h)			
A. succinogenes 130Z	Whey	CO_2 gas	N/A	13.98 ± 0.1	0.6067	0.840	7.85	N/A	[37]
Enterobacter aerogenes LU2	Whery permeate	$MgCO_3$	168	57.7	0.62	0.34	N/A	N/A	[38]
Basfia succinici-producens	OFHKW hydroly-sate	$MgCO_3$	12	5.5 ± 0.2	0.39	N/A	N/A	N/A	[39]
		$MgCO_3$ + biogas	12	3.8 ± 0.8	0.25	N/A	N/A	N/A	[39]
A. succinogenes 130Z	OFHKW hydroly-sate	$MgCO_3$	24	42.3 ± 2.5	0.705	N/A	N/A	N/A	[40]
		Biogas	24	21.7 ± 1.7	0.630	N/A	(>30%)	N/A	[40]
		$MgCO_3$ + biogas	24	45.7 ± 2.5	0.754	N/A	(24–25%)	75.6	[40]

Note: [1] Fermentation time; [2] Yield was calculated from succinic acid dividing by the initial reducing sugar; [3] By-products are formic, acid, acetic acid, and lactic acid; [4] succinic acid was calculated from the amount of succinic acid divided by the total acid; N/A = Data are not available; OFHKW = organic fraction of of household kitchen waste.

4. Conclusions

This work demonstrates the efficient use of POME for succinic acid production using a straightforward procedure and a few additional nutrients. The use of RSM was beneficial in identifying the most crucial substances to supplement into POME for succinic acid synthesis, which were $MgCO_3$ at 22.5 g/L and peptone at 12.0 g/L. To our knowledge, this is the first report to produce succinic acid from POME using *E. gallinarum* (MW931746) and sequential purification of biogas produced from POME. This experiment successfully integrated succinic acid with CO_2 removal from biogas for the palm oil industry. The highest succinic acid concentrations of 23.7 and 19.1 g/L were obtained with the addition of $MgCO_3$ and with biogas sparging (no $MgCO_3$), respectively. To improve succinic formation and yield, the combination of $MgCO_3$ and biogas sparging under the optimal conditions should be further studied. This study could be a biorefinery concept with a useful strategy to reduce environmental pollution with the generation of bio-based products in the wastewater treatment plant of the palm oil mill industry.

Author Contributions: Conceptualization, P.B.; investigation, P.V.N.; writing—original draft, P.V.N.; writing—review and editing, P.B.; supervision, A.U., B.C. and P.B.; funding acquisition, P.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Prince of Songkla University (Grant No. AGR6502070S-0) and Thailand Research Fund (Grant No. RTA6280014).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The first authors gratefully acknowledge the Higher Education Research Promotion and Thailand's Education Hub for Southern Region of ASEAN Countries (TEH-AC) Scholarship from Prince of Songkla University. The third and fourth authors would like to acknowledge Thailand Research Fund (Grant No. RTA6280014).

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

References

1. Allais, F.; Coqueret, X.; Farmer, T.; Raverty, W.; Rémond, C.; Paës, G. Editorial: From biomass to advanced bio-based chemicals & materials: A Multidisciplinary perspective. *Front. Chem.* **2020**, *8*, 131.
2. Malode, S.J.; Prabhu, K.K.; Mascarenhas, R.J.; Shetti, N.P.; Aminabhavi, T.M. Recent advances and viability in biofuel production. *Energy Convers. Manag. X* **2021**, *10*, 100070. [CrossRef]
3. Moshood, T.D.; Nawanir, G.; Mahmud, F.; Mohamad, F.; Ahmad, M.H.; AbdulGhani, A. Sustainability of biodegradable plastics: New problem or solution to solve the global plastic pollution? *Curr. Res. Green Sustain. Chem.* **2022**, *5*, 100273. [CrossRef]
4. Omwene, P.I.; Yağcıoğlu, M.; Öcal-Sarihan, Z.B.; Ertan, F.; Keris-Sen, Ü.D.; Karagunduz, A.; Keskinler, B. Batch fermentation of succinic acid from cheese whey by *Actinobacillus succinogenes* under variant medium composition. *3 Biotech* **2021**, *11*, 389. [CrossRef]
5. Lu, J.; Li, J.; Gao, H.; Zhou, D.; Xu, H.; Cong, Y.; Zhang, W.; Xin, F.; Jiang, M. Recent progress on bio-succinic acid production from lignocellulosic biomass. *World J. Microbiol. Biotechnol.* **2021**, *37*, 16. [CrossRef]
6. Putri, D.N.; Sahlan, M.; Montastruc, L.; Meyer, M.; Negny, S.; Hermansyah, H. Progress of fermentation methods for bio-succinic acid production using agro-industrial waste by *Actinobacillus succinogenes*. *Energy Rep.* **2020**, *6*, 234–239. [CrossRef]
7. Song, Y.; Lee, Y.G.; Ahn, Y.S.; Nguyen, D.T.; Bae, H.J. Utilization of bamboo as biorefinery feedstock: Co-production of xylo-oligosaccharide with succinic acid and lactic acid. *Bioresour Technol.* **2023**, *372*, 128694. [CrossRef]
8. Thuy, N.T.H.; Kongkaew, A.; Flood, A.; Boontawan, A. Fermentation and crystallization of succinic acid from *Actinobacillus succinogenes* ATCC55618 using fresh cassava root as the main substrate. *Bioresour. Technol.* **2017**, *233*, 342–352. [CrossRef]
9. Anwar, N.A.K.K.; Hassan, N.; Yusof, N.M.; Idris. A. High-titer bio-succinic acid production from sequential alkalic and metal salt pretreated empty fruit bunch via simultaneous saccharification and fermentation. *Ind. Crops Prod.* **2021**, *166*, 113478. [CrossRef]
10. Xi, Y.L.; Dai, W.Y.; Xu, R.; Zhang, J.H.; Chen, K.Q.; Jiang, M.; Wei, P.; Ouyang, P.K. Ultrasonic pretreatment and acid hydrolysis of sugarcane bagasse for succinic acid production using *Actinobacillus succinogenes*. *Bioprocess Biosyst. Eng.* **2013**, *36*, 1779–1785. [CrossRef]
11. Ma, J.J.; Fujino, T.; Lim, Y.; Niyommaneerat, W.; Chavalparit, O. Greenhouse gas emission from palm oil industry in Thailand and its countermeasures. *Int. J. Earth Environ. Sci.* **2021**, *6*, 184.
12. Krishnan, S.; Homroskla, P.; Saritpongteeraka, K.; Suttinun, O.; Nasrullah, M.; Tirawanichakul, Y.; Chaiprapat, S. Specific degradation of phenolic compounds from palm oil mill effluent using ozonation and its impact on methane fermentation. *Chem. Eng. J.* **2023**, *451*, 138487. [CrossRef]
13. Ng, D.K.S.; Wong, S.L.X.; Andiappan, V.; Ng, L.Y. Mathematical optimisation for sustainable bio-methane (Bio-CH_4) production from palm oil mill effluent (POME). *Energy* **2023**, *265*, 126211. [CrossRef]
14. Mahmod, S.S.; Takriff, M.S.; AL-Rajabi, M.M.; Abdul, P.M.; Gunny, A.A.N.; Silvamany, H.; Jahim, J.M. Water reclamation from palm oil mill effluent (POME): Recent technologies, by-product recovery, and challenges. *J. Water Process Eng.* **2023**, *52*, 103488. [CrossRef]
15. Akhbari, A.; Ibrahim, S.; Ahmad, M.S. Optimization of up-flow velocity and feed flow rate in up-flow anaerobic sludge blanket fixed-film reactor on bio-hydrogen production from palm oil mill effluent. *Energy* **2023**, *266*, 126435. [CrossRef]
16. Liew, P.W.Y.; Jong, B.C.; Sudesh, K.; Najimudin, N.; Mok, P.S. Characterization of P(3HB) from untreated raw palm oil mill effluent using *Azotobacter vinelandii* Δ*Avin_16040* lacking S-layer protein. *World J. Microbiol. Biotechnol.* **2023**, *39*, 68. [CrossRef]
17. Louhasakul, Y.; Treu, L.; Kougias, P.G.; Campanaro, S.; Cheirsilp, B.; Angelidaki, I. Valorization of palm oil mill wastewater for integrated production of microbial oil and biogas in a biorefinery approach. *J. Clean. Prod.* **2021**, *296*, 126606. [CrossRef]
18. Shaw, K.M.; Poh, P.E.; Ho, Y.K.; Chan, S.K.; Chew, I.M.L. Predicting volatile fatty acid synthesis from palm oil mill effluent on an industrial scale. *Biochem. Eng. J.* **2022**, *187*, 108671. [CrossRef]
19. Nagime, P.V.; Upaichit, A.; Cheirsilp, B.; Boonsawang, P. Isolation and screening of microorganisms for high yield of succinic acid production. *Biotechnol. Appl. Biochem.* 2022, in press. [CrossRef]
20. Liu, X.; Zhao, G.; Sun, S.; Fan, C.; Feng, X.; Xiong, P. Biosynthetic pathway and metabolic engineering of succinic acid. *Front Bioeng. Biotechnol.* **2022**, *10*, 843887. [CrossRef]
21. Kanchanasuta, S.; Champreda, V.; Pisutpaisal, N.; Singhakant, C. Optimization of bio-succinic fermentation process from crude glycerol by *Actinobacillus succinogenes*. *Environ. Eng. Res.* **2021**, *26*, 200121. [CrossRef]
22. Gunnarsson, I.B.; Alvarado-Morales, M.; Angelidaki, I. Utilization of CO_2 fixating bacterium *Actinobacillus succinogenes* 130Z for simultaneous biogas upgrading and biosuccinic acid production. *Environ. Sci. Technol.* **2014**, *48*, 12464–12468. [CrossRef] [PubMed]
23. Vigato, F.; Angelidaki, I.; Woodley, J.M.; Alvarado-Morales, M. Dissolved CO_2 profile in bio-succinic acid production from sugars-rich industrial waste. *Biochem. Eng. J.* **2022**, *187*, 108602. [CrossRef]
24. Pinkian, N.; Phuengjayaem, S.; Tanasupawat, S.; Teeradakorn, S. Optimization of succinic acid production by succinic acid bacteria isolated in Thailand. *Songklanakarin J. Sci. Technol.* **2018**, *40*, 1281–1290.
25. Olajuyin, A.M.; Yang, M.; Thygesen, A.; Tian, J.; Mu, T.; Xing, J. Effective production of succinic acid from coconut water (*Cocos nucifera)* by metabolically engineered *Escherichia coli* with overexpression of *Bacillus subtilis* pyruvate carboxylase. *Biotechnol. Reports* **2019**, *24*, e00378. [CrossRef]
26. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]

27. APHA; AWWA; WEF. Chemical Oxygen Demand. In *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washington, DC, USA, 2005; Part 5220.
28. Wang, C.C.; Zhu, L.W.; Li, H.M.; Tang, Y.J. Performance analyses of a neutralizing agent combination strategy for the production of succinic acid by *Actinobacillus succinogenes* ATCC 55618. *Bioproc. Biosyst. Eng.* **2012**, *35*, 659–664. [CrossRef]
29. Andersson, C.; Helmerius, J.; Hodge, D.; Berglund, K.A.; Rova, U. Inhibition of succinic acid production in metabolically engineered *Escherichia coli* by neutralizing agent, organic acids, and osmolarity. *Biotechnol. Progress* **2009**, *25*, 116–123. [CrossRef]
30. Bumyut, A.; Champreda, V.; Singhakant, C.; Kanchanasuta, S. Effects of immobilization of *Actinobacillus succinogenes* on efficiency of bio-succinic acid production from glycerol. *Biomass Convers. Biorefin.* **2022**, *12*, 643–654. [CrossRef]
31. Liu, Y.P.; Zheng, P.; Sun, Z.H.; Ni, Y.; Dong, J.J.; Wei, P. Strategies of pH control and glucose-fed batch fermentation for production of succinic acid by *Actinobacillus succinogenes* CGMCC 1593. *J. Chem. Technol. Biotechnol.* **2008**, *83*, 722–729. [CrossRef]
32. Agarwal, L.; Isar, J.; Meghwanshi, G.K.; Saxena, R.K. A cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by *Escherichia coli*. *J. Appl. Microbiol.* **2006**, *100*, 1348–1354. [CrossRef] [PubMed]
33. Yu, J.; Li, Z.; Ye, Q.; Yang, Y.; Chen, S. Development of succinic acid production from corncob hydrolysate by *Actinobacillus succinogenes*. *J. Ind. Microbiol. Biotechnol.* **2010**, *37*, 1033–1040. [CrossRef]
34. Tan, J.P.; Luthfi, A.A.I.; Manaf, S.F.A.; Wu, T.Y.; Jahim, J.M. Incorporation of CO_2 during the production of succinic acid from sustainable oil palm frond juice. *J. CO2 Util.* **2018**, *26*, 595–601. [CrossRef]
35. Kuglarz, M.; Rom, M. Influence of carbon dioxide and nitrogen source on sustainable production of succinic acid from *Miscanthus* hydrolysates. *Int. J. Environ. Sci. Dev.* **2019**, *10*, 362–367. [CrossRef]
36. Nualsri, C.; Kongjan, P.; Reungsang, A.; Imai, T. Effect of biogas sparging on the performance of bio-hydrogen reactor over a long-term operation. *PLoS ONE* **2017**, *12*, e0171248. [CrossRef] [PubMed]
37. Louasté, B.; Eloutassi, N. Succinic acid production from whey and lactose by *Actinobacillus succinogenes* 130Z in batch fermentation. *Biotechnol. Rep.* **2020**, *27*, e00481. [CrossRef]
38. Szczerba, H.; Komoń-Janczara, E.; Dudziak, A.; Waśko, A.; Targoński, Z. A novel biocatalyst, *Enterobacter aerogenes* LU2, for efficient production of succinic acid using whey permeate as a cost-effective carbon source. *Biotechnol. Biofuels* **2020**, *13*, 96. [CrossRef]
39. Babaei, M.; Tsapekos, P.; Alvarado-Morales, M.; Hosseini, M.; Ebrahimi, S.; Niaei, A.; Angelidaki, I. Valorization of organic waste with simultaneous biogas upgrading for the production of succinic acid. *Biochem. Eng. J.* **2019**, *147*, 136–145. [CrossRef]
40. Kuglarz, M.; Angelidaki, I. Succinic Production from source-separated kitchen biowaste in a biorefinery concept: Focusing on alternative carbon dioxide source for fermentation processes. *Fermentation* **2023**, *9*, 259. [CrossRef]