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Factorial analysis of Ferulic Acid production from biowaste

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Abstract

There were countless attempts on applying biowaste from agriculture activities as a feedstock for renewable energy and other various biomaterials, since it abundantly possessed complex carbohydrate and aromatic polymer structure called lignocellulose which has been available around the world. Ferulic acid (FA) recognized to be great anti-oxidant compounds are a sought-after product and desired by healthcare, pharmaceutical and food industries around the globe. This study employed enzymatic hydrolysis of feruloyl-polysaccharide from banana stem waste (BSW) by a novel mixed culture from soil to produce FA using 2^5 full factorial design (FFD). The effect and interaction of five factors affecting FA production were investigated, namely; fermentation temperature (A; ^oC), agitation (B; rpm), water-to-BSW ratio (C;v/v), substrate-to-inoculums ratio (D;v/v), and time (E; days). The linear model was well fitted at R^2=0.8019 with factors contribution percentages in the order of E > C > A > D > B. Time had 27.37% contribution indicating the importance of cell growth activities during incubation that highly affected product yield. Meanwhile, interaction of DE was highly significant showing the trend of substrate utilization throughout the microbe feeding time closely related to the process's mechanism behavior. The most FA output produced was 1.2187 mg FA/g BSW with parameters at ambient (26 ^0C) temperature, 150 rpm agitation, 1:1 water-to-BSW ratio, 1:1 substrate-to-inoculums ratio, and one day. The hydrolysis process applied in this study was found to be affected by various factors, yet could be a great option for the production of FA as the highly valuable bio material. Furthermore, BSW was proven significantly feasible and great for producing FA naturally.

Keywords: Ferulic acid; Agricultural Waste; Experimental Design; Soil mixed culture; Environmental Biotechnology

1. Introduction

Ferulic acid (FA) is one of phenolic compound, which possess antioxidant properties used worldwide. It also has numerous physiologic functions, including anti-microbial, anti-oxidant, and anti-cancer activities, which are used in many applications, particularly the food, cosmetic, and pharmaceutical industries [1]. FA has been investigated to potentially prevent coronary vessel disease, increase sperm vitality [2], and lower cardiovascular compilations [3]. FA structure is abundantly available in lignocellulosic materials comprised of cellulose, hemicelluloses, and lignin [4]. Due to the low cost and high availability, lignocellulosic materials such as barley bran, corncobs, corn leaves, and oat fiber have been investigated as a good source of phenolic acid for consumption [5].

One of the potential sources of lignocellulose is banana stew waste (BSW). Every 60 kg of banana harvested is estimated to produce 200 kilograms of discarded waste stems [6]. In Malaysia alone, banana plantations comprise an estimated 34,000 ha [7]. Most common practice by farmers are leaving the stems where they were chopped to

replenish nutrients back into the soil but this could lead to an ecosystem disruption such as eutrophication [8]. Some farmers typically burn the stems, building up environmental pollution and causing serious global warming [9]. This practice seems wasteful because banana stem was discovered to be a potential source of lignocellulose [10], hence well-suited as the substrate for producing FA especially with the abundantly available in the tropical Malaysian agriculture soil.

An enzyme assisted extraction commonly known as enzymatic hydrolysis is the preferred technique to produce bio compound such as FA [11]. The process utilized a specific biocatalyst, could work under mild reaction condition, did not produce undesirable by-products and most of all, environmentally friendly. To break the chain of FA from the polysaccharide complex of lignocellulose, the feruloyl polysaccharide chain is hydrolyzed using targeted enzyme, which is feruloyl esterase or ferulic acid esterase (FAE) [12]. This type of enzyme can be produced by numerous kinds of bacteria cultures found in soil [13]. Using mixed culture of soil is a novel alternative approach for generating source of crude enzyme to be incorporated in the hydrolysis process.

The one factor at-a-time method is conventionally used to screen and optimize parameters upon producing organic compounds. Unfortunately, this method is time-consuming and generates misleading factor interactions for new raw materials and processes. A statistical approach such as factorial analysis, varies all of the factors simultaneously, estimating the combined effect of the selected variables and also the significance percentage. Full factorial design (FFD) employing factorial analysis is based on the statistic's fundamental principle, randomization, replication, and duplication [14]. It simplifies the screening of factors affecting material's production by statistically assessing the interactions between multiple parameters over a range of values. Previously, FFD was successfully employed to extract FA from lignocellulose such as maize bran [15], brewer's spent grain [16], and paddy straw [17].

This study is the first to explore BSW as a biomass source for FA production by enzymatic hydrolysis using soil mixed culture (SMC). Two-level FFD was applied to assess production effects by screening the selected factors while observing the interactions between these factors on the output.

2. Materials and Method

The following are the materials used and the methods involved in this research. No human and animals were harmed during the experiments.

2.1. Raw materials and chemicals

Banana stem regarded as biowaste was used as the substrate, obtained from banana plantation area of Kuantan, Pahang, specifically in the Gambang area. Once compiled, the preparations took place immediately to prevent further degradation of the stems. FA 99% was purchased from Sigma Aldrich (Malaysia). Acetonitrile HPLC grade used as a mobile phase purchased from Fisher Scientific (Malaysia).

2.2. Characterization

The characterization of banana stem revealed the content of compound potentially bounded within the material's structure to discover its capability as a substrate for producing FA. The stems were chopped into uniform cubes and oven-dried until dry weight was constant. Then, the residues were analyzed according to the method of Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) [18] using a Fiber Analyzer (Fibertech system, USA). The percentage of lignocellulose were calculated based on the expressions of [19];

$$\%\text{Cellulose} = \%\text{ADF} - \%\text{Lignin} \quad \text{(Eq.1)}$$

$$\%\text{Hemicellulose} = \%\text{NDF} - \%\text{ADF} \quad \text{(Eq.2)}$$

2.3. Substrate Preparation

The stem was chopped and grinded with distilled water at 1:1 v/v ratio until become homogenous slurry. Then, the slurry was packed accordingly in plastic seals, and stored in a freezer at -20 ^{o}C. This slurry was used during the entire experiment and referred as BSW hereafter. Prior to use, frozen BSW was thawed completely in ambient temperature.

2.4. Soil mixed culture

The source of inoculums for hydrolysis reaction in this study was obtained from mixed culture of soil. Soil sample was taken from the same banana plantation where stem waste was gathered. It was collected using plastic pipes (polyvinyl chloride) under 10cm depth from the surface. SMC was prepared by mixing obtained soil with the BSW in 1:4 v/v ratios according to the inlet substrate per day for an entire one month. After a month, the mixed culture was ready to be incorporated.

2.5. Feruloyl polysaccharide analysis

The hydrolysis experiments were conducted in a 250 ml conical flask on an incubator shaker. Reaction mixture was performed by fermentation of BSW with SMC at 1:1 ratio (v/v) according to the conditions set by the experimental design. The enzymatic hydrolysis was stopped by immediate centrifugation at 10000 rpm for 5 min and chilled. The supernatants were separated to conduct the analytical assays.

2.6. Experimental design

An experimental design requires fewer measurements than the classical one-at-a-time experiment to properly investigate relationship between multiple variables. Design-Expert software (Stat-Ease, version 8.0.6) was used as the tool for the design of factorial analysis. It was done by screening process employing a two-level FFD involving five factors; fermentation temperature (A;^{o}C), agitation (B;rpm), water-to-BSW ratio (C;v/v), substrate-to-inoculums ratio (D;v/v), and time (E;days). These factors were selected based on the objective of achieving an eco-biotechnology process, where minimal to none chemical wastage will be discarded into the environment. Besides that, the selected factors were ensured to lead a hassle-free experiment, yet efficiently tackled the essential ingredient towards optimum product generated, maintaining an effective technique that would become a novel feasible approach of enzymatic hydrolysis to be adapted in the future.

The yield of FA (mg FA/g BSW) was set as the dependent variable. A total of 44 sets of hydrolysis experiments, including center points, were carried out. The order of the running experiments was restrictedly randomized to eliminate possible bias [20]. Table 1 contains the list of ranges for each factor, which were selected by preferring mainly straightforward factors, which do not need the use of chemical treatments or complex fermentation preparation. In the experimental design, low and high factors coded as −1 and +1; the midpoint coded as 0 (for numerical factors). The standard approach to the analysis of the experimental design data is to evaluate a list of the main and interaction effects supported by an analysis of variance (ANOVA) following a linear regression model as in Eq.3, indicating which effects are significant [21].

$$\hat{y}_i = b_0 + \sum_{i=1}^{n} b_i X_i \quad \text{(Eq.3)}$$

In Eq.1, $\hat{y}_i$ represents the value of the response or dependent variable, b_o is the interception coefficient, b_i the linear coefficients, n the number of variables studied, and X_i represents the coded independent variables.

Table 1. The value of level for each factor in FFD.

Variable	Symbol	Real value of levels		
		-1	0	+1
Temperature (^{o}C)	A	Ambient (26)	-	35
Agitation (rpm)	B	no agitation	-	150

Water-to-BSW ratio (v/v)	C	1	1.5	2
Substrate-to-inoculums ratio (v/v)	D	1	1.5	2
Time (day)	E	1	3	5

2.7. High-Performance Liquid Chromatography (HPLC) Analysis

The amount of FA was measured by the HPLC system (Agilent, HP-1100) with a diode array detector (DAD) at 280 nm wavelength and Agilent Zorbaq SB-AQ C18 analytical column. The condition of the column controlled at 30 ^{o}C. Acetonitrile and water (55%:45%) used as the mobile phase at 1 mL/min flow rate, and the volume of injection for every vial was 10 µL. A set of standard dilutions prepared at a FA concentration in the range of 0.1 to 0.5 g/L.

3. Results

3.1. BSW characteristics

Banana stem which most of the time being unused and discarded was a highly potential biowaste as a source of lignocellulose that comprised of cellulose, hemicellulose, and lignin. Cellulose is the main component of a plant cell, which holds the formula of $(C_6H_{10}O_5)n$ consists of a linear homopolymer. It is composed of hundreds to several thousand D-glucose unit linked by β-1,4-glycosidic bonds [22].

Three types of cellulose, namely 1) α-cellulose, 2) β-cellulose, and 3) γ-cellulose. The α-cellulose represents the purity of cellulose, β-cellulose and γ-cellulose together are called hemicellulose, while the sum of cellulose and hemicellulose refers to holocellulose. Hemicellulose is derived from polysaccharides of plants which is a heterogeneous polymer of pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and sugar acids. While cellulose is crystalline and more resistant, hemicellulose's structure is random, amorphous, and easily hydrolyzed. It is generally believed that hemicellulose is the glucan in the matrix of the cell, and the main components are xylan, xyloglucan, glucomannan, manna, galactomannan, callose, and so on [23].

Lignin is the exclusive chemical composition of gymnosperm and angiosperm, a polymer of aromatic subunits usually derived from phenylalanine. Three main monomers nonlinearly and randomly linked in a lignin complex are coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. It serves as a matrix around the polysaccharide components of some plant cell walls, providing additional rigidity and compressive strength as well as rendering the walls hydrophobic and water impermeable [24]. A simplified chemical structure of banana stem composition is represented in Fig 1.

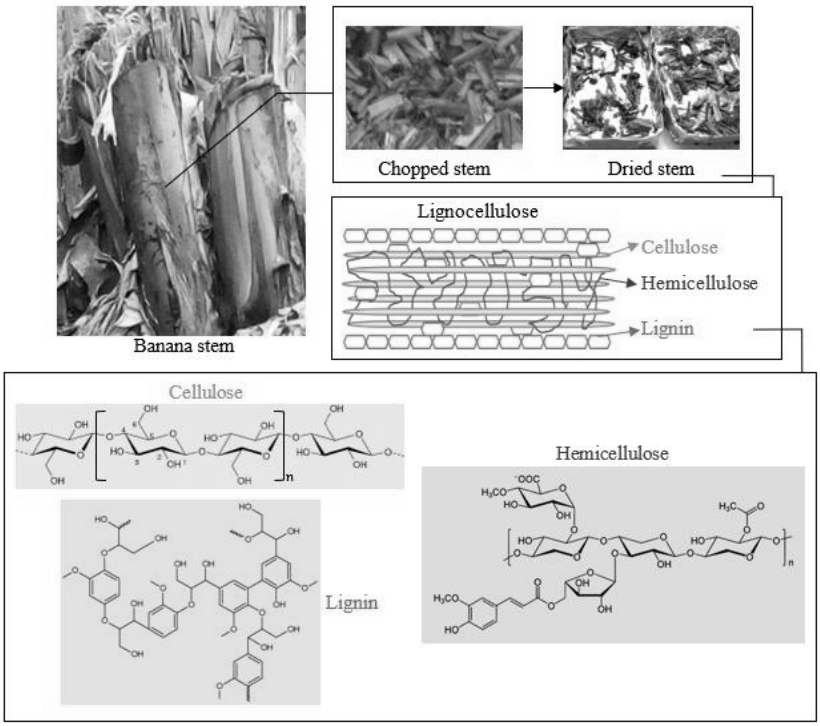

Fig. 1. The diagram of banana stem structure breakdown.

The data of BSW sequential analysis were presented as weight percent for each parameter, which were 10.76% lignin, 30.36% cellulose, and 17.98% hemicelluloses. These values were comparable with characteristics of other banana stem from various places as listed in Table 2. The varied percentage amount of lignocellulose content from each stem were due to the different species of banana tree, climate as well as geographical factor [25].

Table 2. Comparison of banana stem's composition.

Author	Lignin %	Hemicellulose %	Cellulose %
This study	10.8	18.0	30.4
[26]	17.0	5.0	62.1
[27]	14.7	27.0	37.0
[28]	3.3	45.7	43.5
[29]	17.3	19.6	60.8

The composition of lignocellulose in banana stem used in this study met the proportion of three main biopolymers, which were 40 – 60% cellulose, 20 – 40% hemicellulose, and 10 – 25% lignin [30]. FA structure was mostly attached among hemicellulose, esterified to arabinose chain of arabinoxylans [31] and also randomly linked in a lignin monomers complex, exclusively in softwood stem [32]. Hence, with the present of 18.0% hemicellulose and 10.8% lignin detected, it was revealed that BSW was suited to be the substrate for enzymatic hydrolysis in producing FA.

3.2. Experimental data

The overall result of FFD experiments is presented in Table 3. A high yield of FA obtained was 1.2187 mg FA/g BSW. It was achieved within the parameters' range, which was at ambient temperature, 150 rpm agitation, 1:1 (v/v) water-to-banana stem waste (BSW) ratio, 1:1 (v/v) substrate-to-inoculums ratio, and one day. It indicates that the range selected was suitable for this study. Discussions will focus thoroughly on the main effects and their interactions.

Table 3. Experimental data for FFD of FA production

Std	Run	A	B	C	D	E	Y (mg/g)	Std	Run	A	B	C	D	E	Y (mg/g)
39	1	-1	+1	0	0	0	0.8114	2	23	+1	-1	-1	-1	-1	0.9666
20	2	+1	+1	-1	-1	+1	0.6366	31	24	-1	+1	+1	+1	+1	0.5686
21	3	-1	-1	+1	-1	+1	0.6101	41	25	-1	-1	0	0	0	0.6300
15	4	-1	+1	+1	+1	+1	0.6073	33	26	-1	-1	0	0	0	0.6062
19	5	-1	+1	-1	-1	+1	0.6507	17	27	-1	-1	-1	-1	+1	0.6204
9	6	-1	-1	-1	+1	-1	0.8161	12	28	+1	+1	-1	+1	-1	0.7619
18	7	+1	-1	-1	-1	+1	0.6176	1	29	-1	-1	-1	-1	-1	0.8164
32	8	+1	+1	+1	+1	+1	0.6793	37	30	-1	-1	0	0	0	0.5818
24	9	+1	+1	+1	-1	+1	0.5702	5	31	-1	-1	+1	-1	-1	0.6818
22	10	+1	-1	+1	-1	+1	0.6674	35	32	-1	+1	0	0	0	0.6268
28	11	+1	+1	-1	+1	+1	0.7009	27	33	-1	+1	-1	+1	+1	0.6471
6	12	+1	-1	+1	-1	-1	0.7992	30	34	+1	-1	+1	+1	+1	0.6547
13	13	-1	-1	+1	+1	-1	0.6379	34	35	+1	-1	0	0	0	0.9123
11	14	-1	+1	-1	+1	-1	0.7561	3	36	-1	+1	-1	-1	-1	1.2187
36	15	+1	+1	0	0	0	0.8477	4	37	+1	+1	-1	-1	-1	0.8736
25	16	-1	-1	-1	+1	+1	0.8459	7	38	-1	+1	+1	-1	-1	0.8223
40	17	+1	+1	0	0	0	0.9097	42	39	+1	-1	0	0	0	0.8736
23	18	-1	+1	+1	-1	+1	0.6032	16	40	+1	+1	+1	+1	-1	0.8139
38	19	+1	-1	0	0	0	0.8685	8	41	+1	+1	+1	-1	-1	0.7537
10	20	+1	-1	-1	+1	-1	0.8885	14	42	+1	-1	+1	+1	-1	0.8295
44	21	+1	+1	0	0	0	0.7985	43	43	-1	+1	0	0	0	0.8782
29	22	-1	-1	+1	+1	+1	0.5817	26	44	+1	-1	-1	+1	+1	0.6268

4. Discussions

4.1. Analysis of variance (ANOVA)

Only contribution above 1% was selected except for main factors in order to construct a significant model as shown in Fig. 1. The linear regression equation was as in Eq. 4.

FA = 0.7425 + 0.325 A + 0.0092 B - 0.0488 C - 0.0154 D - 0.0863 E - 0.0255 AB + 0.0298 AC + 0.0198 AD - 0.0217 BD + 0.0232 `CE + 0.0359 DE + 0.0325 ABD + 0.0294 ABE - 0.0191 ADE + 0.0201 BCD + 0.0181 BDE - 0.0233 CDE - 0.0220 ABCE (Eq. 4)

In Eq. 4, FA represents ferulic acid concentration, A is temperature, B is agitation, C is water-to-banana stem waste ratio, D is substrate-to-inoculums ratio, E is time, AB, AC, AD, BD, CE, DE, ABD, ABE, ADE, BCD, BDE, CDE, and ABCE are the interactions involved in the model.

The significance of the model was analysed by the analysis of variance (ANOVA), as shown in Table 4. The F-value and P-value were 5.6212 and <0.0001, respectively, signifies that the estimated model fits the experimental data reasonably [33]. The value of R^2 obtained was 0.8019. The previous study stated that the R_2 value above 0.6 is considerably accepted [33, 34]. Adjusted R^2 and predicted R^2 values were 0.6592 and 0.5246, respectively. It justified that, if the model is significant, lack of fit not significant, and adjusted and predicted R^2 values are within 0.2 of each other, the model provides good predictions for average outcomes [35].

Table 4. ANOVA of feruloyl polysaccharide hydrolysis from BSW using SMC.

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F
Model	0.6455	0.0359	5.6212	<0.0001 significant
A	0.0466	0.0466	7.3040	0.0122
B	0.0037	0.0037	0.5789	0.4538
C	0.0764	0.0764	11.969	0.0020
D	0.0076	0.0076	1.1873	0.2863
E	0.2384	0.2384	37.376	< 0.0001
AB	0.0285	0.0285	4.4723	0.0446
AC	0.0284	0.0284	4.4558	0.0450
AD	0.0125	0.0125	1.9648	0.1733
BD	0.0151	0.0151	2.3697	0.1363
CE	0.0172	0.0172	2.6943	0.1132
DE	0.0413	0.0413	6.4759	0.0175
ABD	0.0339	0.0339	5.3144	0.0297
ABE	0.0277	0.0277	4.3461	0.0475
ADE	0.0117	0.0117	1.8321	0.1880
BCD	0.0129	0.0129	2.0348	0.1661
BDE	0.0105	0.0105	1.6412	0.2119
CDE	0.0174	0.0174	2.7276	0.1111
ABCE	0.0155	0.0155	2.4363	0.1311
Residual	0.1595	0.0064		
Lack of Fit	0.1170	0.0069	1.2980	0.3667 not significant
Pure Error	0.0424	0.0053		
Cor Total	0.8049			
R^2	0.8019			
Adj R^2	0.6592			
Pred R^2	0.5246			

4.2. Main effects contribution

The percentage contribution of main factors and selected interactions were shown in Fig. 2. It is observed that factor time, E, had the highest significant effect, followed by water-to-BSW ratio, C, temperature, A, substrate-to-inoculums ratio, D, and lastly factor agitation, B. The time of inoculation found to be the most crucial factor, with a 27.37% contribution. The process needs a suitable period to enter stages of inoculums growth and consumption of substrate (metabolism). The pattern has been reported by other previous studies where they stated that contact time between the mixtures is crucial to ensure the increased yield of product [36, 37].

The ratio of water-to-BSW was second most important contributed 8.76%. Water is known as a universal solvent and very important in the biological system. It has a solvent function for organisms and cells to dissolve nutrients and scavenges wastes or metabolites. Water also stabilizes the structure of molecules and cells. It has been stated by in other research that insufficient water during fermentation can cause poor diffusion of solutes and gas. It can disrupt cell metabolism due to the lack of substrates in or near the cell [38]. Factor temperature had a 5.35% contribution became the third-highest significant factor for the process. It is vital as enzyme activities depend on the surrounding medium. Because of this, the most suitable temperature is essential to ensure the enzyme is at its best

possible condition. This behavior also proved by the previous study where they investigate the temperature effect on the enzyme activation [39].

The substrate-to-inoculums ratio had a lesser contribution, which is 0.87%. The different feeding amounts of the substrate could affect the consumption rate of the process. Because of this, the enzymatic reaction could decline or increase hence affecting the production of FA [40]. Factor agitation was the least contributed effect with only 0.42%. The agitation helps the mixture to disperse efficiently to increase their contact area and ensure full coverage during the reaction. Al-Zuhair et al. (2003) also stated in their work that a large interfacial area allowed the enzyme to penetrate the interface at higher agitation speeds. It could enhance the hydrolysis and increase the production of FA.

Term	Stdized Effects	Sum of Squares	% Contribution
Intercept			
A-Temperature	0.065	0.047	5.35
B-Agitation	0.018	3.693E-003	0.42
C-Water to Banana Stem ratio	-0.083	0.076	8.76
D-Substrate to Inoculum ratio	-0.026	7.574E-003	0.87
E-Time	-0.15	0.24	27.37
AB	-0.051	0.029	3.27
AC	0.051	0.028	3.26
AD	0.034	0.013	1.44
AE	-0.019	2.895E-003	0.33
BC	-5.704E-003	2.603E-004	0.030
BD	-0.037	0.015	1.74
BE	-0.021	3.604E-003	0.41
CD	0.014	1.547E-003	0.18
CE	0.040	0.017	1.97
DE	0.061	0.041	4.74

Fig. 2. The percentage contribution of each main factors and their interaction throughout screening experiments in FFD.

4.3. Interaction of factors

Since FFD is a linear model, hence only two-way interaction factors will be discussed. This is because, in a linear model, a linear response can only exist between two factors interactions. By observing the interactions, factors affecting the response could be interpreted according to the significance percentage and behavior it represents. Whereas, for a higher number of factors interactions demand an orthogonal model design such as response surface methodology, that could also detect the curvature in the response function [42]. The two highest contributions (based on Fig. 2) were the interaction between substrate-to-inoculums ratio and time with 4.74% and the interaction between temperature and agitation with 3.27%.

Fig. 3 (a) shows the plot of a high and low amount of substrate-to-inoculums ratio (D) at a different time (E). It indicates that a more extended time FA increased with the amount of substrate, whereas for a short period, the yield of FA slightly decreased when the substrate amount increased. This was due to the limiting substrate phenomenon and inoculums' growth rate. The highly saturated broth caused an overwhelmed feeding for the bacteria that lead to inefficient enzyme dispersion.

Meanwhile, the plot in Fig. 3 (b) illustrates the interaction between temperature, A, and agitation, B. Yield of FA was seen to have maintained a high rate even in an ambient temperature (26 ^{o}C) when movement was incorporated. As temperature was slightly raised, FA yield also increased gradually. This showed an efficient dispersion of the process, and proved that the enzyme involved for greatly released FA were mesophilic type [43]. Because of this behavior, it can be said that this process was sufficient when applying agitation and without needing to increase temperature, hence reducing the cost of power consumed as well [44]. Although agitation could beneficially increase the contact area, it might cause an increment of air rate during the reaction. Hence, careful use of agitation rate is practiced to ensure non-inhibition of FA yield occur in the reaction mixture.

(a) (b)

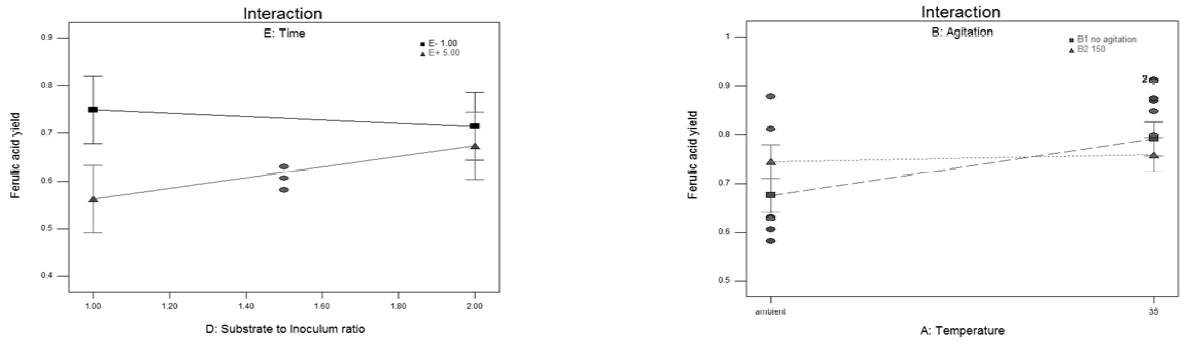

Fig. 3. (a) Interaction plot between substrate-to-inoculums ratio (D) and time (E); (b) Interaction plot between temperature (A) and agitation (B).

5. Conclusion

The production of FA from BSW using a novel approach of enzymatic hydrolysis via SMC was well performed in this study. As factorial analysis of FFD was employed for parameters screening, it was revealed that time had the highest contribution percentage among other factors studied. The highest FA yielded was 1.2187 mg FA/ g BSW at ambient (26 ^{o}C) temperature, 150 rpm agitation, 1:1 (v/v) water-to-BSW ratio, 1:1 (v/v) substrate-to-inoculums ratio, and one day. The contribution for all factors following their significant order was 27.37% for time, 8.76% for water-to-BSW ratio, 5.35% for temperature, 0.87% for substrate-to-inoculums ratio, and 0.42% for agitation. Although these main factors play a significant role in the process, their interactions proved to affect the reaction mechanism differently and could increase or inhibit the reaction. These data could be further used for future enhancement of FA production such as process optimization and kinetic analysis.

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