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Production of Biobutanol by *Clostridium* Species using Liquid Pineapple Waste

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Abstract
Biobutanol production by *Clostridium* species UPM-A1 strain was conducted with liquid pineapple waste as carbon source. Design Expert® Software was employed for generating the experimental design which consisted of three significant parameters, including temperature, yeast extract, and pH. The design consisted of twenty (20) different experiments including six (6) replicates at central points. The experiments were conducted in accordance with the conditions provided by the software, during which the highest biobutanol production was attained at run three (3) consisting, temperature 42.50 ^{0}C, yeast extract 2.14 g/L, and pH 5.5, with a total biobutanol concentration value of 0.441 g/L. After all, a model was generated to see the effects of the variables on the biobutanol produced. Furthermore, the software suggested optimum values for each parameter as temperature 49.35 ^{0}C, yeast extract 1.80 g/L, and pH 4 with a biobutanol concentration of 0.405 g/L. A laboratory experiment was conducted to test the predicted variables and eventually 0.417 g/L of biobutanol concentrations was achieved, which is slightly higher than the predicted. The research, eventually, provided a bottom-up method towards reducing the number of lignocellulose wastes from environments through the production of valuable products.

Keywords: *Clostridium* species, Biobutanol, Central Composite Design (CCD).

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Introduction
Butanol is an alcohol (C_nH2_n+1 OH), colorless liquid with odor closely similar to that of fossil oil, containing four carbon atoms and has molecular formula C_4H_9OH. Other families of alcohol apart from biobutanol are methanol with a carbon atom CH_3OH, ethanol with 2 carbon atoms C_2H_5OH, and propanol with 3 carbon atoms C_3H_7OH. Therefore, the term "Biobutanol" is the butyl alcohol produced from plant raw materials by means of microbial processes. Biobutanol obtained via microbial culture is highly fascinating nowadays. Since it has great achievements including automobiles as biofuels and many other applications, particularly in chemical industries which is used as solvent (Durre, 2007). As a result of global increasing stipulate for the production of renewable fuels, more interests in

microbial production of biobutanol are generated. This opened great opportunities to the biologists, because anaerobic bacteria particularly *Clostridium* species are capable of converting carbohydrates into a variety of solvents such as acetone, butanol, ethanol and more the like.

The production of solvents by *Clostridium acetobutylicum* is increasing interest worldwide due to its potential significance commercially. One of the research interests intended for raw materials cost reduction towards solvent production is the use of starch and other related materials due to their availability, and can be obtained at reasonable prices (Madihah*et. al*., 2000). Qureshi and Blaschekb (2001) reported that "the cost of substrate (raw materials) is an integral part for the overall cost of biobutanol production". It is equally important to note that biobutanol production with *Clostridium* species had been successful via Ethanol-Acetone-Biobutanol (EAB) culture processes (Durre, 2007). In earlier generation, substrates used for biobutanol productions include sugar cane, raw potatoes, cereal and grains. It can be seen that these materials are food related which could cause a competition between utilizing them for food and at the same time for biobutanol production (Durre, 2007). As time goes on, food shortage became pronounced, because more interest was given to biobutanol production utilizing the food-related materials. This, therefore, necessitated the exploration of crop wastes and Agricultural residues, including husks, shells, stovers, hulls, cobs, leaves, molasses, bagasse (Thaddeus *et al*., 2007), and many other materials that are non-food related, and at the same time, they are cost effective and relatively abundant. This research confined on the utilization of pineapple waste towards production of biobutanol using *Clostridium* species.

Materials and Methods

Microorganism and Inoculum Preparation

Clostridium species was used for this research towards biobutanol production. Prior to the production processes, 10 % (v/v) of the *Clostridium* species UPM-A1 strain were transferred from stock culture and inoculated into a freshly prepared Reinforced *Clostridium* Medium (RCM).

Reinforced *Clostridium* Medium was prepared anaerobically, using Hungate technique by making spurge of the media with nitrogen gas (Miller and Wolin, 1976). Moreover, the stock culture was heat shocked by putting into a water bath at approximately 90 ^{0}C for one minute to dissolve the spores formed by the bacteria, before inoculation (Ennis et al., 1986; Kim and Weigand, 1992). The stock culture was removed out of the water bath and allowed to cool at room temperature, then 10 % (v/v) was inoculated into the RCM medium and incubated at 37 ^{0}C. Samples were withdrawn hourly until the optical density (OD) reached approximately 1.0 at 600nm wavelengths. The culture was then removed out of the incubator and used for the subsequent fermentation processes.

Medium preparation.

Growth Medium (RCM)

RCM medium was used to initiate the growth of the Clostridium species UPM-A1 in the preparation of the inoculum. The medium has a distinctive quality, because it inhibits the growth of other bacteria that are not capable of producing biobutanol (Dürre, 2007). Table 1 shows the composition of the RCM medium. During preparation of the medium, the substances in Table 1 were individually added (except rasuzurin) into a 500mL beaker consisting 300mL distilled water and then allowed to dissolve completely while stirring continuously with a magnetic stirrer on magnetic hot plate. After being dissolved completely, the contents were then transferred into a 1000mL Dura Schott bottle and top up to 500mL with distilled water.

Table 1: Reinforced *Clostridium* Medium (RCM) Growth Medium composition

Contents	g/L
Sodium acetate	1.5

Sodium chloride	2.5
Soluble starch	0.5
Yeast extract	1.5
Meat extract	5.0
Peptone	5.0
Glucose	5.0
Rasuzurin	0.5

Rasuzurin (used as an indicator), 0.5mL was then added and immediately the color completely changed to blue. The medium was then sparged continuously with an oxygen-free nitrogen gas using Hungate technique to provide an anaerobic condition, until the color changed to purple and this is an indication that the medium is free from oxygen (Miller and Wolin, 1976; Nafizah and Madihah, 2010). Serum bottles, 50 mL were equally sparged for 3 minutes and then 40mL of the deoxygenated medium were gently transferred into the serum bottles with the aid of pipette while sparging to maintain the anaerobic condition. The bottles were immediately closed with a steel cap consisting of butyl rubber stoppers which allowed and resisted numerous needle entrances. Adjustment of pH to 6.8 was done by preparing one molar of hydrochloric acid and a molar of sodium hydroxide and then sterilized as described by Nafizah and Madihah (2010). In addition, other vital substances, vitamin and cysteine that were used to supplement the medium towards better performance of the bacteria were added. The composition of both the cysteine and vitamin was presented in Table 2. These substances, cysteine and vitamin, were newly prepared, filter-sterilized with 0.2µm pore size membrane filter, and finally sparged with oxygen-free nitrogen using Hungate technique to provide an anaerobic environment in the medium.

Table 2:Vitamin and Cysteine Composition

Contents	g/L
Amino benzoic acid	2.5
Cysteine	1.5
Biotin	0.5

Biobutanol Production Medium

The enhancement of biobutanol production was performed in growth and production medium (M2) under anaerobic condition using Hungate technique as described above. The fundamental composition of this medium as recommended by Green and Stephens (1996) is provided in Table 3. The nutrient composition of the medium was supplemented with 100 % (v/v) liquid pineapple waste used as a carbon source for the biobutanol production.

Table 3:Constituents of Production Medium

Contents	g/L
$MnSO_4.H_20$	0.001
NH_4NO_3	0.200
Sodium Chloride	0.050
K_2HPO_4	0.075
Yeast extract	0.500
$FeSO_4.H_20$	0.001
Liquid Pineapple Waste (LPW)	100 % (v/v)
KH_2PO_4	0.075
$MgSO_4.7H_20$	0.040

The liquid pineapple waste was collected from Lee Pineapple Company Limited Jalan Skudai 81300 Johor Bahru, Johor, Malaysia, in 2 liter Schott bottles. It was immediately autoclaved at 121^0C for approximately 50 minutes and stored in a fridge at 4^0C (to avoid any microbial growth) for further use. At the commencement of the fermentation process, the medium was removed from the fridge, mesh-filtered to remove solid debris, and then treated to provide an anaerobic environment using Hungate technique. After the attainment of the anaerobic condition, the medium was gently transferred into serum bottles (110 mL) with the aid of a peristaltic pump and the pH was adjusted accordingly. The components of the medium listed in Table 3were added using syringes and needle, and then inoculated with 10 % (v/v) of *Clostridium* species UPM-A1 strain.

Research Design and methodology

The whole experiments conducted in this study comprised of different phases. One of the phases concentrated in exploring the use of multi-factorial design with the aid of ntral Composite Design (CCD) to optimize biobutanol production. Design

Expert® Software Version 7.0.1 was employed for generating the experimental design using three significant parameters, including temperature, yeast extract, and pH. The whole design, therefore, consisted of twenty (20) different experiments including six (6) replicates at central points as categorically shown in Table 5, while Table 4 depicts the values for each parameter used for designing the experiment. The experiments were then conducted in accordance with the conditions provided by the software as presented in Table 5.

The research then went further on the fermentation processes, during which the *Clostridium* species UPM-A1 were grown in batch culture with Reinforced *Clostridium* Medium (RCM) to maintain a good condition of the bacteria.

Table 4:Values of the parameters used for Experimental design

			Values	
Parameters	**Variables**	**Units**	**Low**	**High**
Temperature	A	^{0}C	35	50
Yeast Extract	B	g/L	0.8	1.8
pH	C	-	4.0	7.0

Table 5:Experimental conditions provided by the Design Expert software

Std	Run	Temperature (^{0}C)	Yeast (g/L)	pH
1	17	35.00	0.80	4.00
2	12	50.00	0.80	4.00
3	14	35.00	1.80	4.00
4	9	50.00	1.80	4.00
5	20	35.00	0.80	7.00
6	1	50.00	0.80	7.00
7	10	35.00	1.80	7.00
8	19	50.00	1.80	7.00
9	13	29.89	1.30	5.50
10	7	55.11	1.30	5.50
11	16	42.50	0.46	5.50
12	3	42.50	2.14	5.50
13	8	42.50	1.30	2.98
14	5	42.50	1.30	8.02
15	6	42.50	1.30	5.50
16	18	42.50	1.30	5.50
17	11	42.50	1.30	5.50
18	2	42.50	1.30	5.50
19	4	42.50	1.30	5.50
20	15	42.50	1.30	5.50

Analytical Procedures

The fermentation was conducted in batch culture and samples were withdrawn in four hours intervals from (0 hr to 72 hrs) with sterilized syringes and needles (Terumo Corporation, Philippines) and centrifuged at 4000 rpm for 15 minutes. 1.5 mL from the supernatants was used for the biobutanol determination using Gas Chromatography (Agilent Technologies, 6890N network GC system) with a capillary column (HP-5 (30 m x 0.32 mm x 0.25um)). The column maximum temperature, detection mperature, and air flow rate were set at 325

°C, 250 °C and 450 mL/min, respectively. The pellets (after the centrifuge) were utilized for the determination of the bacterial growth via dry-cell weight method. Prior to the determination of the biobutanol, external standards were prepared by addition of 50µL of the butanol into a 15 mL test tube consisting 5 mL nano pure water. 1.5 mL of the mixture was transferred into a vial after vortexing for homogenization, and was eventually positioned into the GC machine to detect the standard peak values and their corresponding retention times.

Results and Discussion

Biobutanol production Optimization using the CCD

Laboratory experiments were conducted in accordance with the conditions provided by the software (Design Expert®, Version 7.0.1) using the optimized values of the variables (Nafizah and Madiha, 2010) (Table 5. After all, highest biobutanol produced from each run was determined and the results were presented in Table 6.

The results from Table 6 shows maximum production was attained at run 3 with value 0.441g/L, consisting 42.50 ^{0}C, 2.14 g/L, and 5.5 values of temperature, yeast extract, and pH, respectively. However, least production was observed at run 5 with 0.002 g/L consisting the combination of temperature 42.50 ^{0}C, yeast extract 1.30 g/L, and pH 8.02. This clearly showed and indicated that the studied parameters are highly significant towards biobutanol production. It can now clearly deduce from these two runs that increase in pH value with decrease in yeast extract concentration have tremendously contributed to the lower yield of biobutanol at run 5 and the opposite, as in run 3, led a better result. Though, at the six replicates consisting runs 6, 18, 11, 2, 4, and 15; 0.242 g/L, 0.131 g/L, 0.148 g/L, 0.209 g/L, 0.115 g/L, and 0.197 g/L of biobutanol productions were recorded, respectively.

Table 6 Biobutanol Produced by *Clostridium* species UPM-A1 under optimized conditions using CCD

Std	Run	Temperature (0C)	Yeast (g/L)	pH	Biobutanol (g/L)
1	17	35.00	0.80	4.00	0.132
2	12	50.00	0.80	4.00	0.047
3	14	35.00	1.80	4.00	0.224
4	9	50.00	1.80	4.00	0.426
5	20	35.00	0.80	7.00	0.170
6	1	50.00	0.80	7.00	0.000
7	10	35.00	1.80	7.00	0.076
8	19	50.00	1.80	7.00	0.165
9	13	29.89	1.30	5.50	0.020
10	7	55.11	1.30	5.50	0.018
11	16	42.50	0.46	5.50	0.258
12	3	42.50	2.14	5.50	0.441
13	8	42.50	1.30	2.98	0.132
14	5	42.50	1.30	8.02	0.002
15	6	42.50	1.30	5.50	0.242
16	18	42.50	1.30	5.50	0.131
17	11	42.50	1.30	5.50	0.148
18	2	42.50	1.30	5.50	0.209
19	4	42.50	1.30	5.50	0.115
20	15	42.50	1.30	5.50	0.197

Whereas, productions from the remaining runs ranged from 0.018 – 0.426g/L with the exception of run 1 with no any production being recorded and this could be as a result of high values of both temperatures (50 ^{0}C) and pH (7). Ahmed and Holland (1995) reported that "temperature increment supports microbial growth, but when the value is so high it would affect the metabolic activities of the bacteria and this could eventually lead to a process failure".

Graphical plots for optimization of biobutanol production

The graphic illustration of equations from data analysis is what expressed the essence of Response Surface Methodology (RSM) (Ooijkaas*et. al.*, 1999). Additionally, RSM is highly essential in assessing the implication of numerous parameters, particularly those relating composite relations. The complexity has a direct relationship with the number of parameters, because complication among the parameters rises with the values of the factors (Azaliza *et. al*., 2009).

Figures 1 to 3 are the graphical plots that were created with the software. They are expressing the relationships of two variables towards biobutanol yield while holding the other one at constant state Figure 1 depicted the three-dimensional effects of both yeast extract and temperature towards biobutanol production in an optimized condition. The figure clearly shows that biobutanol yield increased to climax point with an increase in both yeast extract and temperature, then decreased with further increase in temperature or decrease in yeast extract concentration

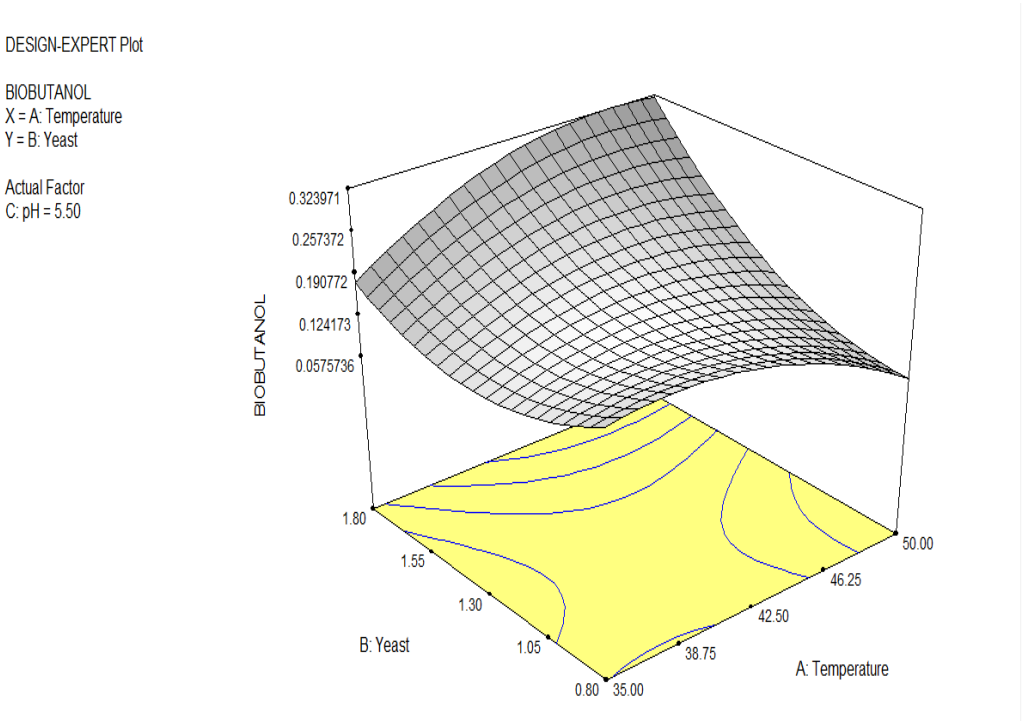

Figure 1: Graphical (3-D) representation for biobutanol yield; effects of temperature and yeast extract with initial pH value = 5.50.

Figure 2 also depicted the three- dimensional effects of both pH and temperature towards biobutanol production in an optimized condition. The figure evidently shows that biobutanol yield increased to high point (0.07 g/L) with a pH value of 4 and an increase in temperature to a value close to 50 ^{0}C, ther decreased with further increase in either pH or temperature (Figure 2). It can now be seen that optimum biobutanol yield was best observed in acidic condition, indicating that the bacterial strain could functions in an acidic environment towards biobutanol production (acid-tolerant). However, the

strain of Yi and Blaschek (2011) produced high yield of biobutanol at a pH value of 6.7 probably as a result of utilizing different bacterial strain with different carbon sources.

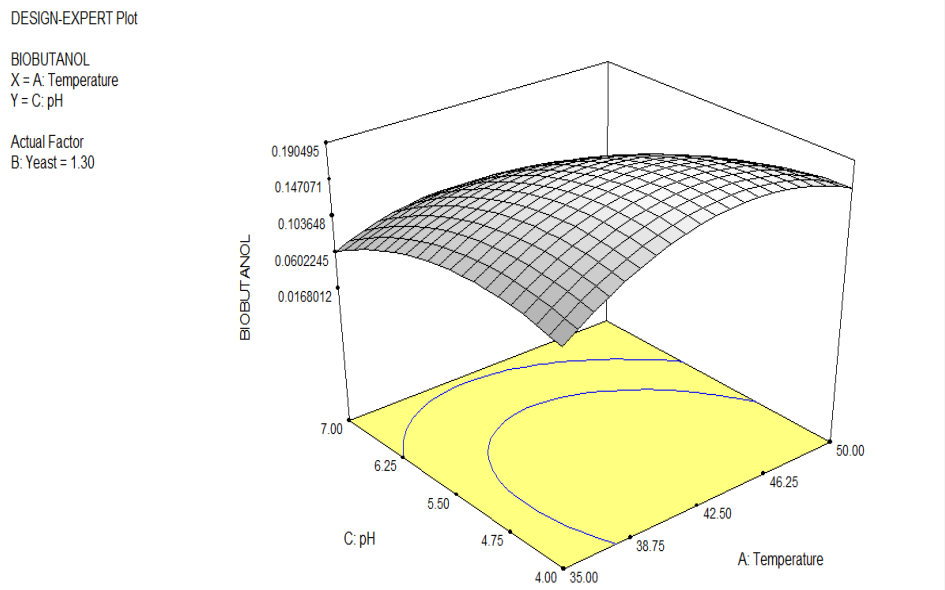

Figure 2: Graphical (3-D) representation for biobutanol yield; effects of temperature and pH (yeast extract = 1.3 g/L).

The effects of yeast extract and pH towards biobutanol yield is presented in Figure 3. In the figure 3, biobutanol yield was observed to increase with increase in yeast extract concentration, which could be due to a declined in the organic acids accumulated in the medium as observed by Li et al., (2012), and rapid biomass yield with a pH value of 4, then the biobutanol amount decreased with a decrease in yeast extract concentration and an increase in the amount of pH during the fermentation processes.

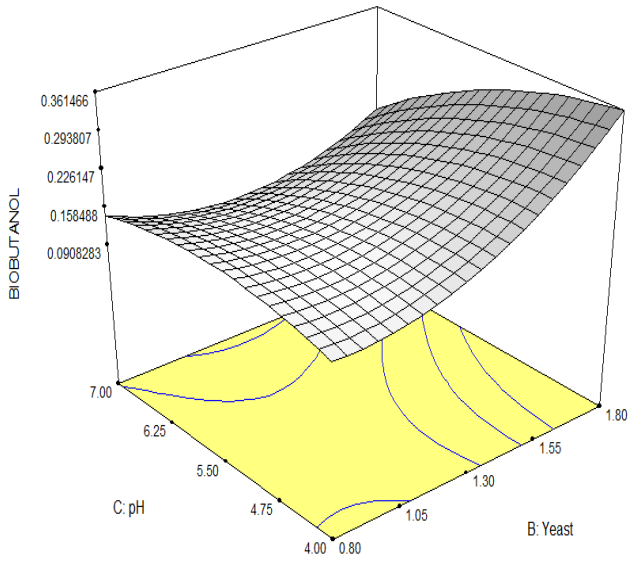

Figure 3: Graphical (3-D) representation for biobutanol yield; effects of yeast extract and pH (Temperature = 42.50)

Moreover, the software suggested optimum numerical value for each variable towards optimization of biobutanol. Figure 4 lepicted the suggested values with

temperature 49.35^{0}C, yeast extract 1.80, and pH 4 giving a predicted value of biobutanol 0.405 g/L with 91.8 % desirability. After all, the experiment was conducted to compare the result with the one predicted by the software. The result obtained was 0.417 g/L which is slightly higher than the predicted value (0.405 g/L) suggested by the software, but lower than the obtained value at run 3 with 0.441 g/L, with a difference of 0.06 %. This concluded that the model provided by the software is highly accurate, because values of both actual and predicted are closely similar (Kuehl, 2000). The smaller the value of the difference between actual and predicted (ranged between 0.010 % - 0.100 %), the accurate and consistent the experiment will be in justifying the model accuracy (Kuehl, 2000; Nafizah, and Madihah, 2010).

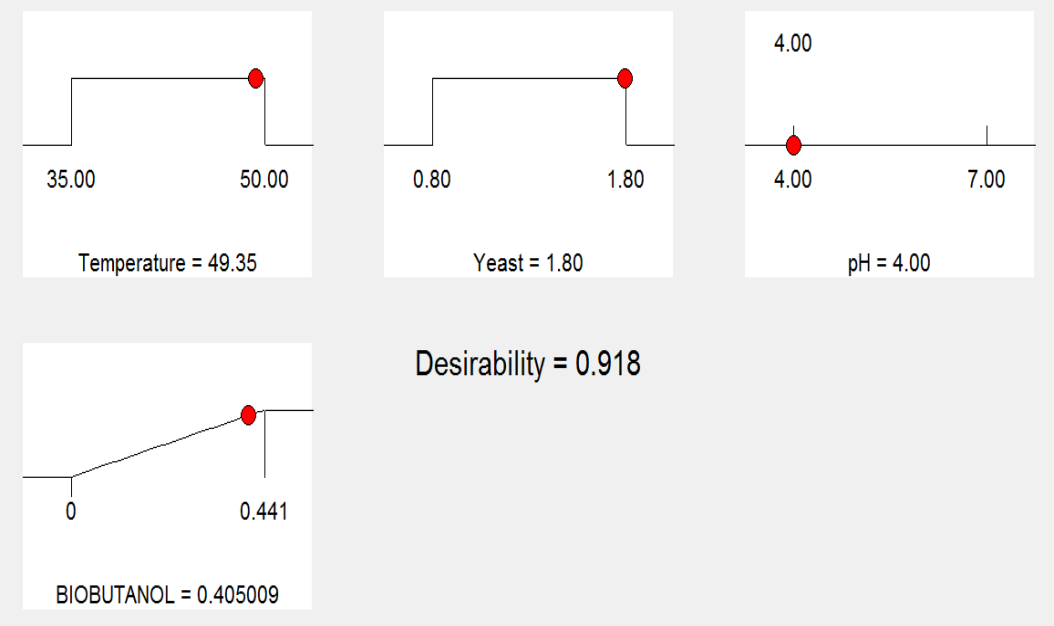

Figure 4: Optimum numerical values for each parameter towards Biobutanol Optimization suggested by the software.

Conclusion

Fermentation process with isolated *Clostridium* species was fruitfully conducted using Liquid Pineapple waste as carbon source. The highest biobutanol concentration attained was 0.441 g/L with optimum variable's value of temperature 42.50°C, yeast extract 2.14 g/L, and pH 5.5. The model generated showed that the interacting effects of both yeast extract and pH are highly significant, while that of temperature, though insignificant as a single entity, but yet significant when acted together with the other parameters. The study eventually established that the liquid pineapple waste can be utilized as a carbon source for microbial growth, which provided a bottom-up method towards reducing the quantity of lignocellulose wastes from environments through production of valuable products.

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Conflict of interest

Authors affirmed that there is no conflict of interest amongst them at all forms.

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