Statistical optimization of the extraction of citric acid from the solid fermented substrate of empty fruit bunches

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Abstract
Optimization of the process parameters for the extraction of a product from a solid substrate after bioconversion is essentially important to maximize the yield. The extraction of citric acid from the solid substrate of oil palm empty fruit bunches (EFB) after bioconversion was initially optimized by following single factor variation. Following this, the extraction parameters were optimized statistically with the help of experimental design by Box-Behnken Design under Response Surface Methodology through the development of a second order regression model. The statistical analysis of the result showed that in the range studied all three factors, that is, shaking speed, solvent ratio, and shaking time, had a significant influence on the citric acid extraction. The highest amount of citric acid extracted was 337.34 ± 1.1 g/kg-dry EFB, for which the extraction parameters were a shaking speed of 125 rpm, a shaking time of 58.5 minutes, and a solvent ratio of 10.70. The coefficient of determination observed ($R^2$) from the analysis was .9921, indicating a satisfactory fit of the model with the response. The analysis showed that all the terms of the model were highly significant with the $P$-value <.05.

KEYWORDS
A. niger, citric acid, extraction, oil palm empty fruit bunches (EFB), optimization, solid state bioconversion

1 INTRODUCTION

Citric acid is the second largest fermentation product in the world and it is mainly produced from starch or sucrose-based media (sucrose or glucose syrups) using Aspergillus niger.1-5 A major portion of the citric acid produced is used in the food and beverage industry (75%)6,7 and the pharmaceutical industry (12%),8 while the remaining 13% is consumed by other industries.9 Other applications of citric acid include antioxidation, flavor enhancement, preservation, acidulation, and as a synergistic agent and plasticizer.10-13 Recently, citric acid produced from the A. niger metabolism was used to recover uranium from El-Sebaiya phosphate rock through its bioleaching.14 Citric acid is also a green solvent for edible oil extraction from seed crops.15 This multifunctional commodity chemical is also used for the synthesis of biopolymers for culturing a variety of human cell lines, drug delivery systems, and the bioremediation of heavy metals due to its powerful sequestering action with various transitional metals.16,17 The rapid increase of the world population and their
improving living standard will significantly increase the global demand for citric acid due to the consumption of food, beverage, and other applications. The gradual increase of the global demand for citric acid has made researchers interested in developing alternative processes for the production of citric acid from various solid substrates through solid state bioconversion.

The challenges that are faced by the citric acid production industry to meet the increasing global demand through fermentation or bioconversion processes include use of renewable substrates, the bioconversion process conditions, and the extraction of citric acid from the substrate after bioconversion. A number of studies have been conducted to discover new renewable substrates as well as on the optimization of media and process conditions for liquid state and solid state bioconversion for citric acid production. Recovery of citric acid is an important step after production through fermentation of the substrate. In the case of liquid state fermentation or submerged fermentation, direct recovery of citric acid is possible from the aqueous phase of the product. Nevertheless, another step of extraction from a solid fermented substrate is essential in the case of solid state fermentation before recovery. Several separation techniques for the recovery of citric acid from aqueous solutions that are low in concentration have been used in downstream processing. The conventional recovery process follows three steps: precipitation with calcium hydroxide, citric acid regeneration by adding sulfuric acid to the precipitated calcium citrate, and crystallization. Recently, reactive extraction was used with tri-n-butyl phosphate, tri-n-octylamine, and Aliquat 336 (A336), tertiary amine and supercritical carbon dioxide. Bipolar membrane electrodialysis is an effective process for recovery. Biological electrodialysis is also used with a bipolar membrane for citric acid recovery. Solvent extraction and ion exchange are alternative processes that eliminate the generation of the byproduct calcium sulfate by using calcium hydroxide and sulfuric acid in the precipitation process. A supported liquid membrane was used to extract citric acid directly from fruit juices by Chanukya et al. The macroporous adsorption process is also an effective method for citric acid recovery as it provides a good selectivity in extraction processes.

However, little research has been carried out for the improvement of yield through optimization of the extraction process parameters for solid state fermentation. Distilled water has mostly been used as a solvent for the extraction of citric acid produced from fermented Musa paradisiaca peels, Pongamia pinnata oil seed cake (known as Honge), cassava bagasse, fermented fruit waste, sugarcane bagasse, coffee husk and cassava bagasse, fermented carob pod, and mussel processing waste while Tran et al. used deionized water for fermented pineapple waste. For proper mixing and dilution, a rotary shaker was used by Monrroy et al., Chetan et al., Kumar et al., Roukas, and Tran et al. and centrifuging was used by Yadegary et al. while a magnetic stirrer was used by Prado et al. at varying speeds and durations.

Only Dhillon et al. has attempted to optimize the citric acid extraction from fermented apple pomace as a solid-substrate. However, the characteristics of lignocellulosic substrates like oil palm empty fruit bunches (EFB) are completely different from apple pomace due to the presence of cellulose and hemicellulose inside the core of lignin. Therefore, optimization of the citric acid extraction process, especially from fermented lignocellulosic substrates, bears the same importance as the optimization of the media as well as the process conditions for the maximization of product recovery. However, we have not found any published report on the statistical optimization of citric acid from fermented lignocellulosic substrate. The statistical approach for the design and analysis of the optimization experiment is widely used and is the best option in terms of saving experimental time, minimizing the cost of experiment, and to obtain the best solution for the combined effect of the extraction process parameters. Statistical optimization under Response Surface Methodology (RSM) is widely used for maximizing production. Therefore, citric acid extraction from the fermented solid substrate oil palm EFB was optimized in this study statistically by using Box-Behnken Design (BBD) under RSM.

2 | MATERIALS AND METHODS

2.1 | Substrate preparation

Oil palm EFB were used as the substrate for the production of citric acid through solid state bioconversion. The EFB was collected from Seri Ulu Langat palm oil mill in Dengkil, Selangor, Malaysia in polyethylene bags and stored in a cold room at 4°C to avoid unwanted biodegradation by microorganisms. The EFB sample was washed with tap water, to remove mud or any other dust, until clear water came out, and was dried at 105°C for 24 hours. The dried EFB was milled to obtain a 0.5 mm down grade particle size. The ground EFB was again dried at 60°C for 48 hours to obtain a constant dry weight for the experimental study.
2.2 | Strain and inoculums

The filamentous fungal strain *A. niger* IBO-103MNB (IMI396649) identified by CABI Europ-UK was used for this bioconversion experiment. The fungal strain was grown on 3.9% w/v of potato dextrose agar (Mark) plates at 32°C for 4 days and washed with 25 mL sterilized distilled water for the preparation of the inoculum. The washed spore suspension was filtered with Whatman No. 1 filter paper to remove the biomass and collected in a 100 mL Erlenmeyer flask. The concentration of the spore was counted with a hemocytometer to maintain the spores’ density of $1 \times 10^8$ spores/mL.

2.3 | Solid state bioconversion experiment

The substrate was prepared with 6 g of EFB with a particle size of $\leq 0.5$ mm (30% w/w of total moist substrate), 6.4% (w/w) (1.28 g in 20 g substrate) of sucrose as cosubstrate, 9% (v/w) of minerals as nutrient, and 2% (v/w) of methanol as stimulator. Mineral solution was prepared with MgSO$_4$$\cdot$7H$_2$O, ZnSO$_4$$\cdot$7H$_2$O, MnSO$_4$, and CuSO$_4$$\cdot$5H$_2$O at a concentration of 0.5 g/L, 6.6 mg/L, 5 mg/L, and 1.0 mg/L, respectively in distilled water. The substrate was inoculated with 15.5% (v/w) having spore concentration of $1 \times 10^8$ spores/mL. The moisture content of 70.3% (v/w) was adjusted with sucrose solution, mineral solutions, inoculums, methanol, and distilled water. The initial pH was maintained at 6.5. Sterilization of the substrate was done by autoclaving it at 121°C for 15 minutes according to Bari et al. The bioconversion experiment was carried out in a 250 mL Erlenmeyer flask by incubating it for 6 days at 33.1°C.

2.4 | Optimization of citric acid extraction process

The solvent ratio, shaking time, and shaking speed were considered to be the parameters for the extraction process of citric acid from the solid substrate after bioconversion. The parameters were optimized initially following single factor variation to determine the probable optimum level and finally by statistical approach according to the BBD under RSM.

2.4.1 | Experimental design by single factor variation

The influence of the solvent ratio, shaking time, and shaking speed was investigated through the variation of each factor individually while other factors were kept constant (Table 1). Distilled water was used as a solvent because citric acid is highly soluble in water as compared to any other solvent for industrial applications. Distilled water was added to the substrate after bioconversion as a ratio of dry substrate (EFB) and diluent. The diluted samples were shaken according to the experimental design in an orbital rotary shaker at the designed speed and duration. The total quantity of citric acid extracted was determined based on the concentration of citric acid in aqueous solution and total volume of extract obtained through the extraction process. Finally, the result was converted in terms of EFB fermented. Moisture content of fermented substrate was taken into consideration to add the additional water for maintaining EFB to solvent ratio.

2.4.2 | Box-Behnken experimental design under RSM

The highest levels of the extraction parameters from single factor experiments were considered to be the basal point for the design of the final optimization experiment through BBD. Two other points were considered to be the high level and low level in the experimental design. BBD gave a total of 17 experimental runs with 13 combinations of which five runs

| Table 1 | Experimental design for approximate media optimization by OFAT method |
|---|---|---|
| Parameter | Variation of parameters | Value of fixed parameters |
| Solvent ratio (water:EFB) | 6, 8, 10, 12, 14, and 16 | Shaking time, 60 (min) and shaking speed, 150 (rpm) |
| Shaking time (min) | 30, 45, 60, 75, and 90 | Solvent ratio, 10 (v/w) and shaking speed, 150 (rpm) |
| Shaking speed (rpm) | 100, 125, 150, 175, and 200 | Solvent ratio, 10 (v/w) and shaking time, 60 (min) |
### Table 2

Experimental design using BBD showing coded and actual values along with the experimental and predicted (using the model equation) values of citric acid extraction

| Run no. | Shaking speed (rpm), A | Solvent ratio (v/w), B | Shaking time (min), C | Extracted citric acid (g/kg) (g/kg of dry EFB) |
|---------|------------------------|------------------------|-----------------------|-------------------------------------------------|
|         | Experimental | Predicted |                     |                                                |
| 1       | 150 (0)   | 15 (−1) | 12 (+1) | 60 (0) | 313.83 | 309.21 |
| 2       | 150 (0)   | 10 (0)  | 60 (0)  | 340.05 | 333.56 |
| 3       | 150 (0)   | 10 (0)  | 60 (0)  | 335.54 | 337.04 |
| 4       | 250 (+1)  | 10 (0)  | 30 (−1) | 242.50 | 239.75 |
| 5       | 50 (−1)   | 10 (0)  | 90 (+1) | 267.40 | 267.02 |
| 6       | 50 (−1)   | 10 (0)  | 90 (−1) | 280.55 | 281.44 |
| 7       | 150 (0)   | 12 (+1) | 90 (−1) | 288.86 | 289.17 |
| 8       | 150 (0)   | 10 (0)  | 60 (0)  | 339.08 | 340.51 |
| 9       | 150 (0)   | 10 (0)  | 60 (0)  | 324.87 | 326.61 |
| 10      | 150 (0)   | 12 (+1) | 30 (−1) | 289.10 | 292.03 |
| 11      | 250 (+1)  | 8 (−1)  | 60 (0)  | 245.56 | 248.73 |
| 12      | 50 (−1)   | 8 (−1)  | 60 (0)  | 265.34 | 264.78 |
| 13      | 150 (0)   | 8 (−1)  | 90 (+1) | 271.33 | 268.79 |
| 14      | 250 (+1)  | 12 (+1) | 60 (0)  | 268.50 | 269.13 |
| 15      | 250 (+1)  | 10 (0)  | 90 (+1) | 264.81 | 264.07 |
| 16      | 150 (0)   | 10 (0)  | 60 (0)  | 338.00 | 339.99 |
| 17      | 150 (0)   | 8 (−1)  | 30 (−1) | 240.89 | 240.71 |

The extract was collected using a vacuum filtration apparatus. The extractability of the supernatant due to the variation of the parameters was determined to estimate the quantity of the citric acid extracted from 1 kg dry EFB after bioconversion. The extracted amount of citric acid was considered to be the response for the optimization using RSM.

The results obtained according to the experimental design were used to evaluate the developed second order regression model (Equation ((1))) by analyzing the analysis of variance (ANOVA), the value of $R^2$, $P$-value, and $F$-value. The coefficient of determination, $R^2$, represents the quality of fit of the polynomial model equation. The statistical software package Design-Expert11.1.2.0 (Stat Ease Inc., Minneapolis, Minnesota) was used to predict the effect of the variables on citric acid extraction with the generated regression model. The result of the optimization was validated by laboratory experiment.

### 2.5 Determination of citric acid

The concentration of citric acid in the extract was determined by the Waters HPLC instrument equipped with a refractive index detector and ShodexRSpak KC 811 column (inner dia. 8 × 300 mm, Shodex, Japan). Phosphoric acid solution (0.1%) was used as the eluent for the analysis of the acid solution. The HPLC was operated with a pump flow of 1 mL/min, column temperature of 40°C, and sample volume of 5 μL for the analysis of citric acid. The concentration was determined by the
integration method and peak area. The Breeze software (version 3.3), Waters Corporation, Milford, MA automatically calculated the concentrations of citric acid. The extracted citric acid was expressed as g/kg of dry solid substrate.

2.6 Determination of extractability and extract recovery

According to the proportions determined in the design, the solvent distilled water was added to the fermented substrate and this was diluted with a spatula manually. The diluted fermented substrate was then shaken in an orbital rotary shaker at the speed and duration of agitation as determined in the design. The thoroughly diluted fermented substrate was filtered by a Whatman No. 1 filter with a vacuum filtration unit. Extractability was determined as a percentage of the total volume of the solvent. The total quantity of citric acid extracted was determined through the multiplication of the citric acid concentration in the aqueous solution and the total volume of the extract obtained through the extraction process. The moisture content of the fermented substrate was taken into consideration when adding additional water for maintaining the EFB to solvent ratio. Finally, the extract recovery was converted into terms of fermented EFB and expressed as g/kg-EFB.

3 RESULTS AND DISCUSSION

The extraction of the citric acid from the solid substrate after bioconversion was optimized for the maximum recovery of the product. Three parameters, namely, solvent ratio, shaking time, and shaking speed, were considered for optimization. Distilled water was used as a suitable solvent because citric acid is highly soluble in water. The optimization study was divided into two steps: single factor variation and the statistical approach using RSM considering the parameters.

3.1 Single factor variation method

The effect of the solvent ratio on extract recovery was determined by varying the solvent ratio as 6, 8, 10, 12, 14, and 16 while shaking time and shaking speed were maintained at 60 minutes and 150 rpm, respectively. Figure 1 shows that the concentration of the citric acid was inversely proportional to the solvent ratio within the limit of the experiment. On the other hand, the increment of the citric acid extractability and citric acid extract recovery was observed with the increase of the solvent ratio of up to 10, after which it became almost steady. About $326.78 \pm 1.5$ g/kg-EFB of citric acid with extractability of 79% was obtained when 10 times the solvent of dry EFB was used. The highest extractability and extract recovery were 84.55% and $350.23 \pm 2.3$ g/kg-EFB, respectively at solvent ratio of 16. From these analysis it is observed that only 23.45 g/kg-EFB (7.18% of $326.78 \pm 1.5$ g/kg-EFB) more citric acid can be extracted by adding 60% more solvent. In consideration of solvent content and product extraction, the water to EFB ratio of 10 should be suitable because less solvent content is desirable considering the downstream processing cost.

The effects of shaking duration and shaking speed on citric acid recovery were also determined independently. The experiments were carried out according to the design shown in Table 1. The effects of shaking time and shaking speed on the extraction of citric acid are presented in Figures 2 and 3.

From the plotted figure it is clearly observed that the extraction of citric acid is following the same trend of the concentration of citric acid in the extracted solvent. It means that extraction of citric acid is dependent on the concentration

**Figure 1** Effect of the solvent ratio on extractability, citric acid concentration, and extract recovery

![Figure 1](image-url)
of citric acid in the extracted solvent. The highest concentration and the highest extracted citric acid of 41.47 ± 0.74 g/L and 326.22 ± 3.4 g/kg-EFB, respectively were obtained at 60 minutes of shaking duration. The extract was 78.61 ± 0.5% of the total solvent. The concentration of citric acid as well as the citric acid recovery was reduced at higher duration of shaking. The reduction of citric acid concentration in the extracted solvent at higher shaking duration might be due to the adsorption of citric acid in the fermented substrate. The influence of shaking time on adsorption was also observed by Said et al. The adsorption phenomenon is influenced by the active side of the adsorbent and the presence of the electrostatic field around the adsorbent. Furthermore, extract recovery decreased at higher shaking period due to the reduction of citric acid concentration and less volume of solvent recovery.

Figure 3 shows that the effects of shaking speed on citric acid concentration, solvent extractability, and citric acid recovery are following similar trends like shaking time. The extract (citric acid) recovery is gradually increasing together with the concentration of citric acid in the solvent and the solvent extractability (in terms of percentage of total solvent used) as the shaking speed increases to 150 rpm, and then all are decreasing. At higher shaking speeds, solvent retaining in the fermented substrate was increased which means the water holding capacity of fermented substrate was increased. It might be due to the particles of the fermented substrate coming in closer contact which led to the percentage of bound water increasing. This phenomenon may happen in case of longer shaking at constant speed which was observed during the time variation of shaking. Moreover, EFB is a lignocellulosic fibrous material which has not completely fermented and this partially fermented fibrous material might act as an adsorbent for citric acid in aqueous phase. The adsorption phenomena might also influence the concentration of citric acid in the extract. Similar variation at higher and lower speed of shaking was also observed by Hossain et al. Again, reduction of citric acid at higher agitation speed might be due to more adsorption of citric acid on fermented solid substrate that can be explained by the phenomenon of how shaking speed escalates the mobility of the adsorbate particle to the adsorbents.

Thus, substrate to solvent ratio of 10, shaking time of 60 minutes, and shaking speed of 150 rpm can be considered as center points for further optimization by statistical approach using BBD under RSM.

### 3.2 Optimization of extraction parameters by BBD under RSM

The extraction experiments were conducted as per BBD (Table 2) in which the solvent ratio, shaking time, and shaking speed were considered to be independent variables for the maximum extraction of citric acid from EFB after bioconversion. The amount of extracted citric acid for each experimental design matrix was used as the response. The experimental
results within the experimental constrains were fitted to a second order polynomial model for the prediction of the optimum level by the Design Expert software. The developed model is presented as follows:

\[
Y (\text{Citric acid, g/kg – EFB}) = -736.079 + 1.065095A + 155.9685B + 5.809037C - 0.03004AB - 0.002896AC - 0.12894BC - 0.00356A^2 - 6.75963B^2 - 0.03992C^2
\]  

(2)

where, the citric acid extract \((Y)\) is a function of shaking speed \((A)\), solvent ratio \((B)\), and shaking time \((C)\).

The coefficient of determination \((R^2 = .9921)\) indicates a high degree of correlation between the experimental and the predicted values. It can be said that 99.21% of the variables satisfied the response of the model. The high significance of the model is also proven by the adjusted coefficient of determination (98.19%).\(^{53,54}\) The very high value of adequate precision (signal to noise ratio) of 26.82 compared to the desirable value (greater than 4) indicates that this model can be used to navigate the design space.

It is observed from the corresponding analysis of variance (ANOVA) that the developed quadratic regression model was highly significant with a very low probability value \((P_{\text{model}} > F < .0001)\) from the Fisher’s \(F\)-test (Table 3). Furthermore, the model terms A, B and interactive term AC as well as all quadratic terms were highly significant at the level of \(P < .01\) except the model terms C and interactive term AB and BC which were also significant at the level of \(P < .05\). The significance of the linear, quadratic, and interactive effects of the parameters indicates that a little variation in their levels would considerably affect the extraction of the product.\(^{45}\)

The ANOVA also shows that the lack of fit of the model was insignificant which is highly desirable for the model, along with the significance of all model terms. The lack of fit test is performed to check whether the selected model is adequate to describe the observed data or a more complicated model is required. It is the comparison of the variability of the current model residuals and the variability of replicate settings of the factors. From the lack of fit test it can be mentioned that the model is adequate for the observed data at a confidence level of 95% with the \(P\)-value > .05.

The regression equation can be graphically presented as a 3D response surface and 2D contour plots to determine the optimum values of the variables within the experimental ranges.\(^{55}\) The cyclic interactions of two variables among the three variables are presented with 3D response surface and 2D contour plots in Figures 4-6 to predict the extraction. The response surface is mainly used to efficiently find the optimum values of the variables for the maximum response.\(^{55}\) An infinite number of combinations of the two test variables are presented by each contour curve while the other variable remains at the level of zero (center). A perfect interaction between the independent variables is indicated by the elliptical contours.\(^{56}\) The surface confined by the smallest ellipse in the contour diagram shows the maximum predicted value.

| Source        | Sum of squares | Mean squares | \(F\)-value | Prob > \(F\) |
|---------------|----------------|--------------|--------------|-------------|
| Model         | 19990.15       | 2221.13      | 97.06        | <.0001**    |
| Shaking speed, A | 1372.23        | 1372.23      | 58.52        | <.0001**    |
| Solvent ratio, B | 2329.90        | 2329.90      | 99.35        | <.0001**    |
| Shaking time, C | 191.28          | 191.28       | 8.16         | .0245*      |
| \(A^2\)       | 5334.14        | 5334.14      | 234.38       | <.0001**    |
| \(B^2\)       | 3066.41        | 3066.41      | 134.74       | <.0001**    |
| \(C^2\)       | 5420.57        | 5420.57      | 238.18       | <.0001**    |
| \(AB\)        | 144.43         | 144.43       | 6.35         | .0399*      |
| \(AC\)        | 301.82         | 301.82       | 13.26        | .0083**     |
| \(BC\)        | 239.41         | 239.41       | 10.52        | .0142*      |
| Lack of fit   | 28.93          | 9.64         | .2959        | .8276       |

\(R\)-squared \((R^2)\): .9921  Predicted \(R^2\): .9669  Adequate precision: 26.8242

* Significant, ** highly significant.
The response surface from the second order quadratic equation for the interaction of the shaking speed and solvent ratio was found to be elliptical throughout the entire region (Figure 4). The figure depicted that the extraction of citric acid was considerably affected by both the variables of shaking speed and solvent ratio. The intersection point of the major and minor axes of the ellipse indicates the maximum extraction of citric acid for the given ranges of both variables. The extract decreased at higher and lower values of both parameters within the experimental ranges. About 335.65 g/kg-EFB of citric acid extraction was predicted from the response surface at the shaking speed and solvent ratio of about 150 rpm and 10 (v/w), respectively, while the shaking time was 60 min.

The interactive effect of the shaking speed and shaking time for citric acid extraction was also indicated by an elliptical surface plot (Figure 5). The predicted citric acid extraction decreased with an increase or decrease of the values of both parameters within the ranges.

Figure 6 shows the response surface plot of citric acid extraction as a function of shaking time and solvent ratio in which the shaking speed was kept constant at 150 rpm. The extraction of citric acid was affected by both the parameters of shaking time and solvent ratio. The extraction of citric acid increased by increasing the shaking time up to 60 min and decreased upon further increase from 60 to 90 minutes. Similarly, citric acid extraction increased upon increasing the solvent ratio from 8 to 10 (v/w) and decreased upon further increases of the solvent ratio from 10 to 12 (v/w). The maximum extraction of citric acid (335.65 g/kg-EFB) was obtained when the shaking time was 60 minutes, the solvent ratio was about 10 (v/w) and the shaking speed was about 150 rpm.

A set of experiments with three replications was performed in order to verify the optimization results and to validate the model developed. The validation experiments with predicted value by using developed model equation and the actual experimental results are shown in Table 4. The highest citric acid extraction of 339.89 g/kg-EFB was predicted from the model equation while experimental extract of 337.34 ± 1.1 g/kg-EFB was obtained from the validation experiment at optimum conditions (shaking speed of 125 rpm, solvent ratio of 10.70, and shaking time of 58.50 minutes), which is close to the predicted value. For all experimental cases actual values are slightly less than the predicted values.
According to the knowledge of the researcher, Dhillon et al.\textsuperscript{42} optimized the extraction of citric acid by statistical approach using central composite design. Dhillon et al.\textsuperscript{42} found that citric acid extraction increased up to 20 minutes of agitation and 200-240 rpm agitation rate. And the extraction decreased at further increase of both variables showing the negative effect on the response. Interactive forces between the citric acid and fermented substrate influence the recovery of product from fermented solid substrate. The variation of extract from fermented solid substrate depends on variation of these interactive forces. The hydrophobic/hydrophilic nature of fungal mycelium, Vander Waals forces, and ionic and hydrogen bonds also determine the efficacy of the product recovery.\textsuperscript{42}

Other than that several researchers have followed some extraction procedures that also can be discussed here. Prado et al.\textsuperscript{35} used 50 mL distilled water with 5 g fermented cassava bagasse, where the solvent to substrate ratio was 10, and mixed this with a magnetic stirrer for 10 minutes. Kumar et al.\textsuperscript{36} added 100 mL of distilled water to 5 g dried fermented fruit waste and agitated this on a rotary shaker for 2 hours, where the solvent to dried substrate ratio was 20. Vandenberghhe et al.\textsuperscript{37} also used 10 times more of the solvent compared to the fermented sample for the extraction of citric acid from sugarcane bagasse, coffee husk, and cassava bagasse. Roukas\textsuperscript{38} mixed the fermented carob pod with 200 mL distilled water (10 times the dry carob pod) by using a rotary shaker at 250 rpm for 30 minutes to extract citric acid. A 10 mL of deionized water was added to 1 g of fermented pineapple waste and agitated for 60 minutes on a rotary shaker by Tran et al.\textsuperscript{40} Pintado et al.\textsuperscript{39} used nine times more distilled water than substrate for the extraction of citric acid from fermented mussel processing waste.

From the discussion, it is observed that the optimized parameters for citric acid extraction from EFB after bioconversion produce better results than the parameters of citric acid extraction that have been used by other researchers when considering the solvent ratio, shaking speed, and shaking time. The optimum values of the solvent ratio, shaking speed, and shaking time for the extraction of citric acid from fermented EFB are satisfactory based on the usual practice by the researchers.
CONCLUSIONS

In this study, the solvent ratio, shaking speed, and shaking time were considered as the significant parameters for citric acid extraction from the solid substrate after bioconversion. Possible optimum levels for the solvent ratio, shaking time, and shaking speed were found from single factor variation experiments to be 10, 60 min, and 150 rpm, respectively. The highest amount of citric acid extract obtained from the single factor variation experiment was 326.78 ± 1.5 g/kg dry EFB. Through the developed quadratic model from the statistical optimization experiment designed by BBD, it was predicted that the highest extraction is 339.89 g/kg dry EFB. Each variable of the developed quadratic model showed significant influence on the extraction of citric acid. The model also showed the significant interactive effects among the parameters which are clearly observed from the contour plots. The predicted extraction was validated at the optimum conditions of a shaking speed of 125 rpm, solvent ratio of 10.70, and shaking time of 58.50 minutes and obtained extracted citric acid of 337.34 ± 1.1 g/kg-EFB. The validated extract amount is slightly less (0.75% of 339.89 g/kg dry EFB) than the predicted value. Therefore, it can be mentioned that the statistical method of optimization gives the better solution.
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Md. Bari contributed to the data curation, formal analysis, investigation, methodology, and writing the original draft, review, and editing. Md. Alam contributed to the conceptualization, funding acquisition, project administration, resources, supervision. Abdullah Mamun contributed to the conceptualization, supervision, validation. Hanufa Khatun contributed to the data curation, visualization, writing the original draft, review, and editing.

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