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The use of factorial design for ferulic acid production by co-culture

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ABSTRACT

Several factors can influence the use of co-culture for ferulic acid production from banana stem waste. The aim of this study was to analyse factors of temperature, pH, agitation, water-to-substrate ratio, volume of inoculum, fermentation time, and type of co-culture by employing a 2^4 fractional factorial design (FFD). The results showed the order of contribution effects towards ferulic acid production: pH > type of co-culture > volume of inoculum > agitation > fermentation time > temperature > water-to-substrate ratio. Four factors including pH, agitation, type of co-culture, and volume of inoculum were shown to have significant effects on ferulic acid production. Among the determining factors, the main factor of pH and the interaction of temperature and fermentation time had the strongest effect on ferulic acid production. The result indicated that FFD was useful to improve the ferulic acid production by considering all the interactions of variables involved.

1. Introduction

Ferulic acid (FA) was first isolated from *Ferula foetida* and it presents in plant cell wall components as covalent side chains. Component of lignocelluloses is found with dihydroferulic acid collectively. It gives rigidity to the cell wall by making the crosslink between polysaccharides and lignin (Kumar and Pruthi, 2014). FA was discovered in most monocot plant such as wheat bran, corn kernel, sugar beet pulp, and rice of bran oil, with varying quantity from 5 to 28 g/kg (Fazary and Ju, 2007). Extensive studies have been performed on conversion of lignocellulosic material to FA. Agricultural residues are sources of lignocellulosic material. Banana stem waste (BSW) is a lignocelluloses waste, which consists of 15.42% lignin, 53.45% cellulose, and 28.56% hemicellulose (Silveira et al., 2008).

Microorganisms are used to degrade lignin and hemicellulose in waste material by producing lignin-degrading enzyme to produce ferulic acid (Hasyierah et al., 2008). A few microorganisms have been identified to be able to hydrolyse FA from the materials of cell wall (Faulds and Williamson, 1995). Several enzymes such as ferulic acid esterase (FAE) and xylanase produced by microorganisms act synergistically in maximising the degradation of plant cell wall to release FA (Huang et al., 2011). *Bacillus* spp. was found as the most potent extracellular enzyme producer (Balasubramanian and Simoes, 2014). Donaghy et al. (1998) successfully screened some *Bacillus* spp. as FAE producers, while Heck et al. (2002) found that *Bacillus subtilis* contributed the highest xylanase activity in their research. The utilisation of multi-enzyme by co-culture provides a promising method to improve the product yield. However, best condition of a biological process should be determined to ensure a maximum yield of a product.

Design expert software (DX) is a tool that can be used efficiently for design of experiment (DOE). The software provides a function known as full factorial design which is useful to study and determine the influences of several variables necessary in a process (Golshani et al., 2013). A few research works have been accomplished by employing factorial design for ferulic acid production effectively. Ismail and Zainol (2014) proved that, by employing factorial design, the best variable of temperature was determined in producing the highest content of FA from BSW using sugarcane press machine. In addition, Mussatto et al. (2007) also successfully applied the factorial design approach in maximising the FA extraction from brewer spent grain through alkaline hydrolysis method. They found that temperature had the greatest effect, followed by the reaction time and NaOH concentration. Currently, the application of factorial design on the production of FA by co-culture through fermentation has never been reported. Therefore, this research is a value added to expand the knowledge on the production of FA by microorganisms. In this study, FFD was applied to determine the significant factors involved in fermentation of BSW by co-culture to produce FA. The effects of seven factors on FA production were analysed using 2^4 fractional factorial design. FFD is a

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good alternative to full factorial design, due to many factors were involved in this research.

2. Materials and methods

2.1. Microorganism

In this study, three strains of *Bacillus* spp. were isolated from soil, which were *Bacillus thuringiensis* Bt407, *Bacillus cereus* strain CCM 2010 and *Bacillus pumilus* SAFR-032. The bacterial strains were acclimatised for a month in BSW before the isolation. The strains were identified by 16S rRNA sequence analysis method. All the strains were grown and maintained on nutrient agar plate.

2.2. Substrate

BSW was obtained from banana plantation in Kuantan, Pahang. The stem was cleaned to remove any dirt attached before being used as substrate. The stem was cut into a cube (1 cm). The substrates were prepared at different ratios by mixing them with water and blended. Initial pH was adjusted according to Table 1. The substrate was prepared in 250-ml Erlenmeyer flask for each set of experiment and sterilised at 121 °C for 15 min.

2.3. Preparation of co-culture

Before starting the experiment, bacterial strains of *Bacillus pumilus* SAFR-032, *Bacillus cereus* strain CCM 2010 and *Bacillus thuringiensis* Bt407 were resuscitated by plating onto nutrient agar plate and incubated at 37 °C for 24 h. A single colony of each strain was scraped and inoculated into a universal bottle containing 10 ml of nutrient broth. The cultures were grown for 22 h at 37 °C in an incubator. A total of 10% of the enriched cultures were added to 50 ml sterile nutrient broth in 100-mL Erlenmeyer flask and incubated further for 22 h at 37 °C. Co-culture of A (*B. thuringiensis, B. cereus*, and *B. pumilus*) and B (*B. thuringiensis* and *B. cereus*) were prepared. *B. thuringiensis, B. cereus,* and *B. pumilus* were grown as co-culture by adding a total of 50 ml stationary phase inoculum of single culture at equal volume into 1000-mL Erlenmeyer flask containing 500 ml of nutrient broth. Inoculum was incubated in an incubator shaker with mild shaking for another 22 h at 37 °C.

2.4. Experimental set-up

Fermentation of BSW involving co-culture was performed under aseptic technique. Inoculum of co-culture at stationary phase was inoculated into 250-ml Erlenmeyer flask containing substrate. Incubation was conducted in an incubator shaker at different temperatures and agitation speed according to Table 1. Sample was collected at various times (Table 1) and centrifuged. The FA content in the supernatant was quantified using high performance liquid chromatography (HPLC). The outputs of the experimental design were analysed with Design Expert software to evaluate the effects of each factor involved.

2.5. Factorial design methodology

In this study, Design Expert software (Version 8.0.6, State-Ease) was used. The screening was performed through fractional factorial design (FFD) to determine the experimental variables and interactions that have significant influence on the production of ferulic acid. The experimental data were analysed by FFD to fit the following first order polynomial equation (Eq. (1)):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i \quad (1)$$

where Y represents the value of the response, β_0 is the constant coefficient, n is the number of variables, β_i represents the coefficient of the linear parameters, and X_i represents the coefficient of the interaction parameters.

A 2^4 fractional factorial design consisting of 16 runs were performed for all the parameters, as shown in Table 2.

2.6. Analytical method

Samples were analysed according to Chamkha et al. (2001) with modification using HPLC (Agilent 1100 system). It was equipped with Agilent Zorbaq SB-AQ C18 analytical column with an isocratic mobile phase consisted of acetonitrile, distilled water, and acetic acid (30:69.5:0.5, v/v). The flow rate was set at 0.6 ml/min. Ferulic acid was injected via 25-ml injection loop and measured using a diod array detector (DAD) at 280 nm. Prior to the analysis, samples were prepared by centrifugation at 5800 rpm for 15 min. The supernatant were filtered into vial using 0.45 μm nylon syringe filter.

3. Results and discussion

3.1. Statistical analysis for ferulic acid production from BSW

Table 2 shows the amount of ferulic acid obtained within the range of 40.22 to 488.41 mg/kg. The highest content of FA (488.41 mg/kg) was obtained when the fermentation was performed for 24 h at 26 °C, initial pH of 9.5, agitation at 150 rpm, water-to-substrate concentration one of 2:1, with 2% inoculum of co-culture B (*B. cereus* and *B. thuringiensis*) as shown in standard order 7.

The analysis of variance (ANOVA) analysis for FA production was done to determine the significance of the model (Table 3). The statistical significance of regression equation can be checked using F-values, while the p-values were used to examine the significance of each coefficient. From the model, the F-value is 171.31 and the p-value is very low ($p < 0.0001$). There was only 0.01% chance that the model F-value this large could occur due to noise. Generally, a good model is implied by the greater of the calculated F-value several times compared to the tabulated value (Hamzaoui et al., 2008). The smaller p-values show the more the significance of the corresponding variable (Masoumi et al., 2011). The model term effect of B, C, E, G, AC, AE, AF, and BD were statistically significant in affecting the ferulic acid production. While, the model term of A, D, and F were not significant, as their p-values are larger than 0.05.

The R^2 from the ANOVA is used to indicate how close the data to the regression line. A good fitting model shows R^2 of more than 80% (Karazhiyan et al., 2011). The satisfactory R^2 value was obtained from this analysis (0.9979), which indicates that the model fits the experimental and predicted values well. The final equations in term of actual factors were determined as follows:

$$FA_A = 120.64 - 5.24A + 77.73B + 1.22C + 29.87D - 16.09E - 8.69F - 0.03AC + 0.37AE + 0.27AF - 4.02BD \quad (2)$$

$$FA_B = 164.58 - 15.24A + 77.73B + 1.22C + 29.87D - 16.09E - 8.69F - 0.03AC + 0.37AE + 0.27AF - 4.02BD \quad (3)$$