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OPTIMIZATION OF BIOVANILLIN PRODUCTION OF LEMONGRASS LEAVES HYDROLYSATES THROUGH PHANEROCHAETE CHRYSOSPORIUM

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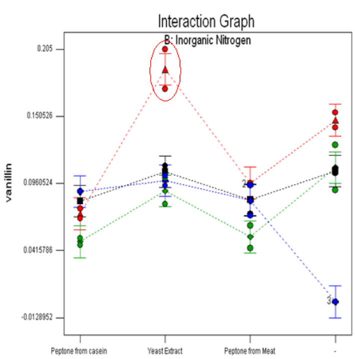
Graphical abstract



Abstract

The recent study has demonstrated the effects of different nitrogen sources on vanillin production by *Phanerochaete chrysosporium*. Primary screening supported maximum biotransformation of ferulic acid (from lemongrass leaves hydrolysate) to vanillin by using ammonium chloride and yeast extract as inorganic and organic nitrogen source, respectively. With the 2-level factorial analysis, the optimum conditions of vanillin production from ferulic acid by *P. chrysosporium* was achieved at 0.192g/L with a molar yield of 24.5%.

Keywords: Economic Transformation Programme, Agricultural Production, Biomass, Biovanillin, Ferulic acid, *Phanerochaete chrysosporium*

Abstrak

Kajian terkini telah menunjukkan kesan dari kepelbagaian sumber nitrogen ke atas penghasilan vanillin oleh *Phanerochaete chrysosporium*. Saringan awal telah menyokong biotransformasi maksimum asid ferulik (daripada bendalir daun serai) kepada vanillin dengan penggunaan ammonium klorida dan ekstrak yis, masing-masing sebagai bahan nitrogen bukan organik dan organik. Dengan menggunakan analisis 2-Peringkat Faktorial, keadaan optimum penghasilan vanillin daripada asid ferulik oleh *P. chrysosporium* telah dicapai pada 0.192g/L dengan hasil molar sebanyak 24.5%.

Kata kunci: Program Transformasi Ekonomi, Hasil Pertanian, Biomas, Biovanillin, Asid Ferulik, *Phanerochaete chrysosporium*

1.0 INTRODUCTION

Vanilla or vanillin is one of the common flavoring agents used in many parts of the world. It is crystallized as white or light yellow solid form, prismatic needles that sublime in vacuum without any changes. It has sweet aroma that gives odor and flavor as well as food preservation [1, 2].

Vanilla is produced naturally from vanilla pod. It can be artificially synthesized from lignin or guaiacol of paper industry as well as biological fermented by different types of strains and fungi using agricultural waste biomass [3].

Varied biomass had been utilized before to produce vanillin from oil palm empty fruit bunch (OPEFB), rice bran oil, maize bran, corn cobs and sugar beet pulp as well. These agricultural wastes contain high hydroxycinnamic acid of phenolic group with ferulic acid compound as main precursor of biovanillin conversion [4, 5]. Massive study of biovanillin especially from oil palm biomass; ferulic acid (53% w/w) have been conducted and established by most Education Institutions with the collaboration of Malaysia Research Bodies [6, 7, 8]. Estimated 70% of the agricultural production is discarded as waste and ended up with conventional clearing (burning activities) that cause the air pollution. One of the alternative ways to solve this problem is to convert agricultural biomass (substrate) into value added product (biovanillin) through green technology (eco-friendly) using microbial or enzymatic reaction.

In Malaysia, one of the abundant agricultural wastes which have high lignocellulose content is lemongrass leaves. Lemongrass leaves consisted of 28.5-47% (w/v) of hemicelluloses, 29.9-35% (w/v) cellulose and 7-11% (w/v) lignin.

Lemongrass plant is second major contributor in spices listing on Food-Agro after ginger followed by musk limes, limes, hot chilli, galangal, turmeric and nutmeg in Malaysia (Table 1). In 2012, the total production of lemongrass leaves was 7,612 metrics tonnes whilst estimated 5328 metrics tonnes of these productions were discarded as waste. Therefore, lemongrass leaves was chosen due to its easy access with abundant resources of ferulic acid (0.1% w/v) and potentially contributed into vanillin production [9].

There are very few studies reported regarding optimization of vanillin productions done statistically in submerged fermentation (SLF). In previous study, primary experiments regarding the screening of fungi in one-step biovanillin production suggested that *Phanerochaete chrysosporium* is a potential vanillin producer from ferulic acid precursor [10]. In general, the vanillin production may influence by different physical and chemical factors such as cultivation temperature, moisture content, inoculum size, and supplement medium composition [11]. Thus, there is a need to identify the optimal process conditions to ensure maximum enzymes production yields as well as most economical processing strategies. With this objective, a two-step statistical strategy combining general factorial design (GFD) to determine the most suitable nitrogen supplement in medium, followed by 2-level factorial design (2LFD) to select the key parameters that influence vanillin production. Nutrients, especially those that provide good and appropriate nitrogenous source, are an extremely important factor to induce vanillin production as well as provide the balanced growth conditions for mycelium formation [12].

Studies have described the use of one-factor-at-a-time method (OFAT) for medium optimization on vanillin production [13]. However, this approach is time-consuming and does not depict the interaction among the experimental factors [14] ref. Statistical optimization through respond surface methodology (RSM) can eliminate these limitations by identifying the ideal combination of factors with any levels that can affect the process and indicates the optimal value of the selected parameters [12] ref. The RSM statistical method is preferred due to its reliability as well as ability to quickly identify the effects of factors at different concentration and significantly reduce the number of experiments needed [11].

Table 1 List of Spices in Malaysia, 2012 [15]

Plant	Production Area (hectares)	Total Production (metrics tonnes)
Ginger	1,148	9,017
Lemongrass	1,180	7,612
Musk Limes	1,185	6,794
Limes	807	4,485
Hot chilli	321	1,415
Galangal	241	1,467
Turmeric	174	1447
Nutmeg	33	392

2.0 EXPERIMENTAL

2.1 Microorganism and Inoculum

In this study, the fungal strain *P. chrysosporium* (ATCC 24725), was grown in potato dextrose plates (PDA) at 27°C for seven days. The spores were harvested using 1% (v/v) Tween-80 and collected by centrifugation at 4000 rpm for 20 min. The spores were diluted with sterile distilled water into a suitable concentration according to the design matrix.

2.2 Screening Chemical and Physical Factors by 2 Level Factorial Design (2LFD)

Figure 1 shows the schematic diagram for the experimental design. All experiments were carried out in shake flasks, using *P. chrysosporium* for production of vanillin from lemongrass leaves hydrolysate. Preliminary experiment employed the general factorial design to study the effect of nitrogen sources added into modified Tilay's medium [13]. Based on the results of the general factorial design, the modified Tilay's medium consisted the best nitrogen source (organic and inorganic nitrogens) used in the next experimental strategy. The full fractionate 2-Level Factorial Design was used to screen the significant variables from the modified Tilay's medium and physical cultivation conditions that would affect the vanillin production in SLF. This design was conducted to investigate the statistical significance of the factors on vanillin production during fermentation of *P. chrysosporium* from lemongrass leaves hydrolysate. Seven variable factors or parameters with respect to vanillin production; inorganic nitrogen (65 mM to 85mM), organic nitrogen (2mM to 6mM), incubation temperature (25°C to 45°C), pH (4.0 to 8.0), incubation time (12 hr to 48hr), agitation (100rpm to 200rpm) and inoculum size (6% (v/v) to 9(v/v). Each independent variable was investigated at a high (+1), a low (-1) level and centre points (0). A total of 70 sets of experiments containing six replicates at centre points were employed. Each combination was run in triplicate and statistically analysed using the Design-Expert 6.0.4 (Stat-Ease Inc. Minneapolis, USA) software to evaluate the model's interaction, R^2, curvature and statistical significance in terms of the F-test.

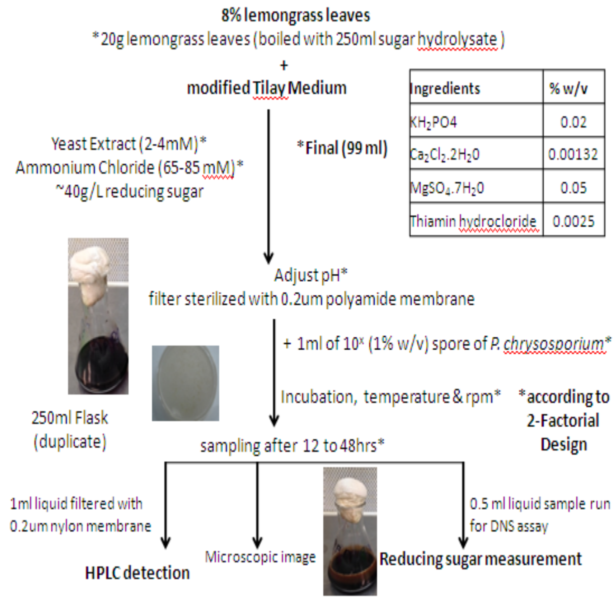


Figure 1 Schematic diagram for experimental design

2.3 Determination of Vanillin Production through High Performance Liquid Chromatography (HPLC); Capillary Column

The phenolic compounds and products (ferulic acid and vanillin) were analyzed by high performance liquid chromatography (HPLC) at a flow rate of 20μl/min and detection at 254, 284 and 320 nm. Two mobile phases used in ratio 50:50. The mobile phase used were; A: water: acetic acid (98:2 v/v) and B: water:acetonitrile:acetic acid (78:20:2 v/v/v). The capillary column was from Agilent eclipse XDB-C18 column (5 μm, 0.5 x 150 mm) [16].

3.0 RESULTS AND DISCUSSION

3.1 Screening of Nitrogen Source by General Factorial Design (GFD)

The most suitable combination of nitrogenous sources, with all concentrations fixed at 78.708 mM, was screened using GFD approach for their individual and combined effects on vanillin production by *P. chrysosporium*. The reason to modify nitrogen source in the medium without changing the original Tilay's medium concentration (with total nitrogen content 78.708 mM) was to compare the best combination and/ or sole nitrogenous supplement that can significantly affect the vanillin production (Figure 2). This was done since the original Tilay's medium was claimed to be able to induce optimal vanillin production. It should be reminded that the sample without nitrogen sources was kept as the control sample. Other media components and physical cultivation parameters were kept identical. The data from general factorial design has similar finding to the work done by Tilay and co-worker on the basis of one factor at a time method. They have reported that ammonium chloride as the best among the different inorganic nitrogen sources screened for vanillin production (44.67% molar yield). While, it is contradict to the finding of organic source, where corn steep liquor supported maximum of vanillin production (30% molar yield) [13]. However, this finding has supported by Lessage-Meessen and co-worker that reported yeast extract as organic nitrogen to support maximum vanillin production [17]. To date no specific reason has been reported on the selective effect of nitrogen sources into maximum vanillin production [12].

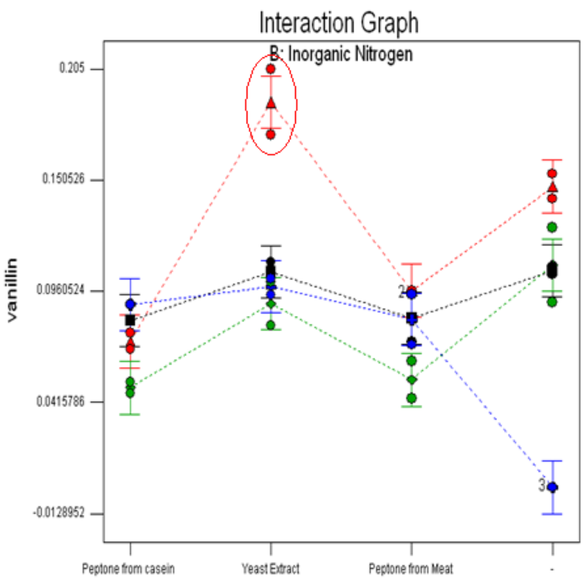


Figure 2 Result of preliminary study of general factorial

3.2 Screening Chemical and Physical Factors by 2 Level Factorial Design (2LFD)

Initially, literatures suggested that the selected seven variables such as temperature, inoculum sizes, inorganic and organic nitrogen concentration, incubation time and agitation parameters would give some general influences on vanillin production [10, 11, 12, 13, 14]. However, no information is available for vanillin production from lemongrass leaves hydrolysates by *P. chrysosporium*. Thus, a half-full factorial 2LFD for the seven independent variables was appropriate to investigate the main effects of individual factors were ammonium chloride (A), yeast extract (B), incubation temperature (C), pH (D), incubation time (E), agitation (F), and inoculum sizes (G) and gives unbiased estimation on all two-factor interaction (AB, AC, AD, BA, BA and etc.) as well as multi-factor interaction (BCE, BCFG, and etc.) A total of 70 trials with two concentrations for each variables and six replicates at the centre points on vanillin production were conducted.

The significance of the selected factorial model was checked by p-value to denote the significant of the coefficients and useful to understand the pattern of mutual interaction between the independent variables [11]. The model was significant as the p-value is less than 0.05 while value less than 0.0001 representing the model is highly significant. Our statistical analysis revealed that the model is highly significant. This is proved by a F-value 58.02 with P-value of the model (P<0.0001), as it is evident from the low p-value together with high Fischer's F-test values. The result indicates that there was only 0.01% chances that the value of "Model F-value" would appear due to noise.

The regression coefficients and determination coefficient R^2 for the linear regression model of vanillin production was 0.9956. These values indicate that the model comprehended a 99.56% of data variability. The adjusted determination coefficient (Adj R^2= 0.9784) was also satisfactory to confirm the significance of the model. The Pred R^2 of 0.8343 agreement with that of Adj R^2 of 0.9298. Under statistically optimum conditions vanillin production from ferulic acid by *P. chrysosporium* was 0.192g/L with a molar yield of 24.5%.

Residual analysis for diagnostic plots including half-normal plots (Fig. 3) was used to confirm whether the mentioned assumptions for the ANOVA studies were well fitted. In general, half normal plot is used to select the significant factors that are appropriate to be included in the model. The points which fall well out from the diagnostic line are said to have significant effect on the responses and vice versa [18] Based on the result (Figure 3 and 4) most of the factors for vanillin production have considerable variation in the types significant variables at the respective responses.

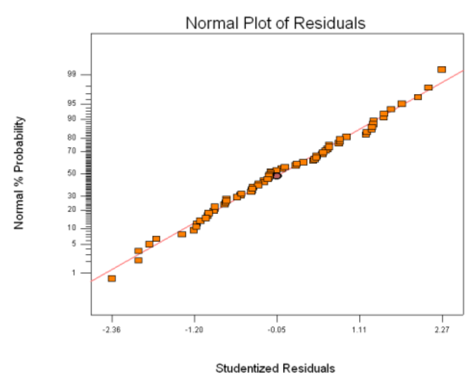


Figure 3 Normal probability analysis of residuals plot for vanillin production

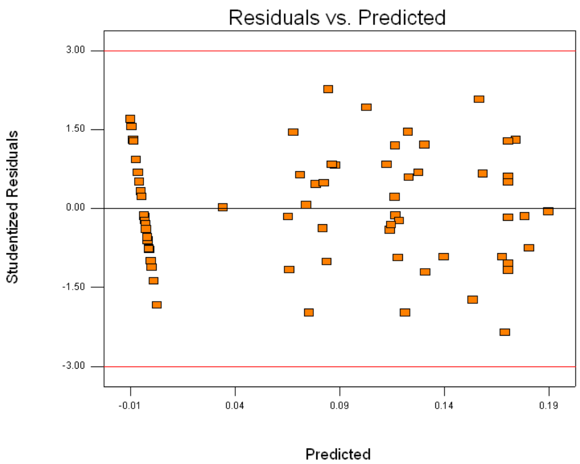


Figure 4 Parity plots of distribution actual versus predicted values of vanillin production

3.3 Localization of Optimum Conditions

The three dimensional (3-D) response surface plots and contour graph (Figure 5a, 5b, 5c) were also used to determine the optimum process conditions as well as to evaluate the model significant. These are indicative of the infinite combined effects of any two independent variables towards maximum vanillin production with all other independent variable fixed at zero level. To date, several parameters are indirectly involved into maximum vanillin production [11]. Figure 5a demonstrated the interaction between ammonium chloride (inorganic nitrogen) and yeast extract (organic nitrogen) on vanillin production. The figure showed that when fermentation is conducted with combination of both inorganic and organic nitrogen, the vanillin production yield is induced by higher ammonium chloride concentration (86 mM) yet only lower of yeast extract concentration needed (2 mM).

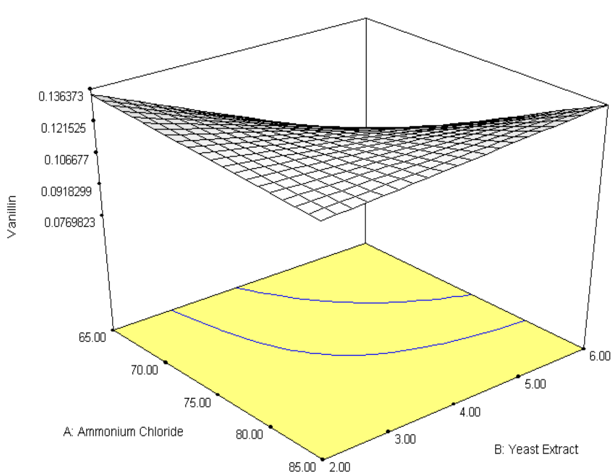


Figure 5a 3D response surface plots showing relative effect of two variable which between concentration of ammonium chloride and yeast extract on vanillin production

Figure 5b demonstrated the interaction between ammonium chloride (inorganic nitrogen) and incubation temperatures on vanillin production. The figure showed that when fermentation is conducted using higher inorganic nitrogen concentration (ammonium chloride) and further incubated at temperature below 45°C, the production of vanillin is enhanced. The vanillin production peaked at temperature between 35°C to 45°C, but decreased above this limit. This is because, at high temperature, the enzymes of metabolic pathway undergo thermal-denaturation which causes the maintenance energy required for cellular growth to increase and therefore channelizes the microorganisms to synthesize only minimum amount of essential protein for growth and other physiological process required to minimum energy maintenance [20]. However, at low temperature, the transport rate of substrate across the cells is suppressed, which subsequently declines the enzymes production yield [11,12]

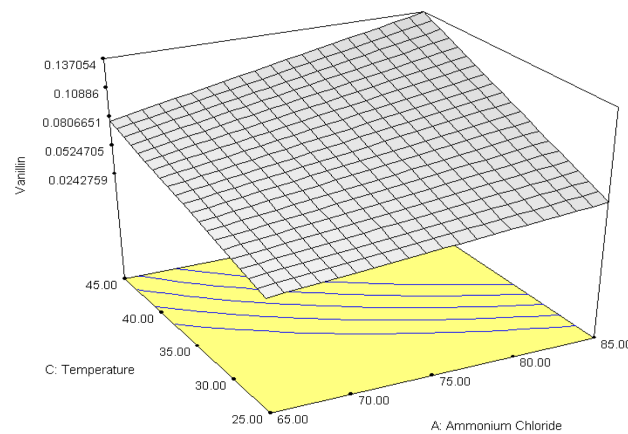


Figure 5b 3D response surface plots showing relative effect of two variable which between incubation temperature and ammonium chloride concentration on vanillin production

Figure 5c demonstrated the interaction between incubation temperatures and pH value on vanillin production. The vanillin production peaked at temperature between 30°C to 35°C whilst pH value between 5 to 7. However, the vanillin production is decreased above this limit. The results demonstrated that intermediate combination were adequate for optimal vanillin production. A reduction in vanillin production at higher incubation temperature and pH value was due to declines enzymes production yield [12,13].

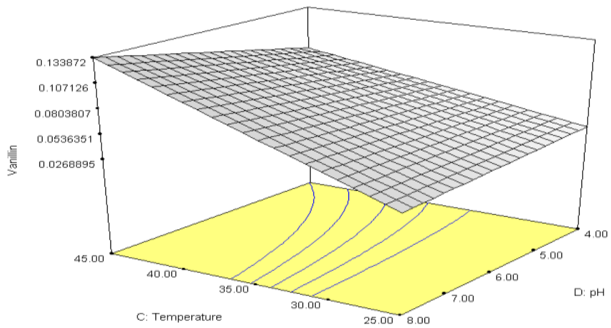


Figure 5c 3D response surface plots showing relative effect of two variable which between incubation temperature and pH value on vanillin production

The 3D and contour plots were used to present the predicted response as a function of process factors [19]. However, only the effects of two factors could be interpreted in each graph, Thus, perturbation plots (Figure 6) which could illustrate the effects of changing each factors towards the response while keeping all other constants were used [21]. Several crucial factors such as ammonium chloride (A), incubation temperature (C), and pH (D) have combined standout influence on the responses while lacking of these factors can appreciably lower the vanillin production (Figure 6). The graph further depicted that higher ammonium chloride concentration and incubation temperature had increased vanillin production gradually up to the center value. These factors have been collectively and widely recognized as essential to induce enzymes synthesis as well as balanced fungi growth [10, 11, 13, 17]. Ammonium chloride (A) and incubation temperature (C) (Figure 6) are the most obvious factors that lead to significant different of vanillin production. Vanillin production was not strongly affected by pH value but an adequate pH value had contributed to optimal vanillin production.

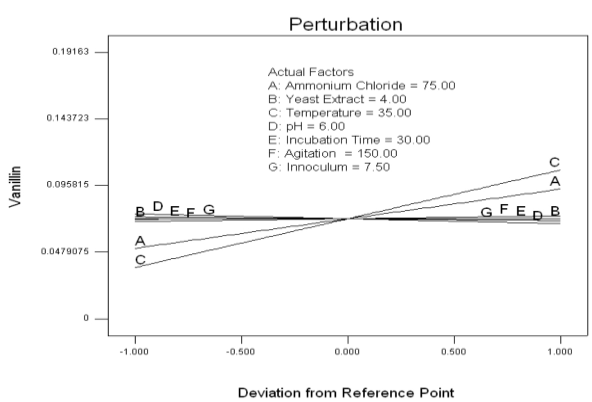


Figure 6 Perturbation graph showing the effect of each independent variable towards vanillin production at their respective middle point; A, ammonium chloride; B, yeast extract; C, incubation temperature; D, pH value; E, incubation time; F, agitation; G, inoculum sizes

4.0 CONCLUSION

Based on the results, inorganic nitrogen (ammonium chloride), and temperature are the most affecting parameters that gave the highest vanillin production at 0.192g/L which increased 92% as compared to unoptimized experiment.

Acknowledgement

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