Surfactant, Nitrogen and Carbon Media Optimization for
*Trichoderma Asperellum* LBKURCC1 Laccase Production by
Flask Solid State Fermentation of Rice Straw

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Abstract. Laccase is an important industrial enzyme used in the paper, food and textile industry. It is produced by many different organisms, including filamentous fungi. *Trichoderma asperellum* LBKURCC1 is a strain isolated from Riau soil, which can produce laccase by solid state fermentation (SSF) of rice husk and rice straw. The aim of this work was to optimize SSF production of laccase from rice straw, through optimizing Nitrogen, Carbon and surfactant supplements to the fermentation media. Effect of surfactant, nitrogen supplement, and carbon supplement were evaluated by using a Central Composite Design (CCD) and surface response analysis. The concentration of the surfactant, Tween-20, at all concentration levels tested had no significant effect to the model. In contrast, the nitrogen and carbon supplement concentrations were significant factors (*P*-Value < 0.05) enhancing laccase production. Optimum conditions for laccase production were 23 g/L nitrogen and 1% carbon supplement, giving a maximum laccase activity of 56.8 U/L enzyme extracted, equivalent to 0.7 U per g rice straw fermented. Optimizing the nitrogen and carbon supplement increased yields up to 3 times the level obtained in a non-optimized media.

1. Introduction
Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are a family of enzymes that catalyse the mono electronic oxidation of aromatic compounds, including phenolics found in lignin, aromatic amines, benzenethiols, substituted phenols, hydroxyindoles, as well as a wide variety of inorganic compounds, using molecular oxygen as an electron acceptor [1]. Laccases are multicopper glycoprotein enzymes, having 10-45% glycosylation, with average molecular weights of 60 to 70 kDa [2]. Laccases are produced by a wide variety of organisms, from higher plants [3], insects [4], to microbes such as fruiting body fungi [5], filamentous fungi [6] and bacteria [7]. Laccases from different organisms have different redox potentials [8]. Fungal laccases have generally higher redox potentials, and are therefore more commercially favourable, than bacterial or plant laccases [9]. Having higher redox potentials, renders fungal laccase as better oxidators than bacterial and plant laccases [10]. Due to its ability to oxidise diverse substrates, the laccases have achieved a lot of interest for industrial and environmental applications, among others for textile-dye decolourization.
[11], biopulping and biobleaching [12], bioremediation of environmental pollutants [13], development of biosensors [14], biofuel cells [15], and synthesis of fine bioactive chemicals [16].

Trichoderma asperellum LBKURCC1 is a Riau, Indonesia, isolated strain from soil in a cacao plantation. It can produce laccase by submerge and solid state fermentation (SSF) of rice husk and rice straw, with higher yields in the SSF system, even when not optimized yet [17].

Production of T. asperellum LBKURCC1 laccase by SSF of rice straw still requires optimization to increase production levels to economic value. Rice straw is the substrate media of choice for laccase SSF production, due to its abundance as an agricultural waste in Indonesia and south-east Asia [18]. Optimising the concentration of media supplements for SSF is among the most frequently applied methods to get higher production of enzymes. SSF optimisation is done at the laboratory scale, and the simplest method is to optimize using a flask format fermentation system [19]. Central Composite Design (CCD) is an advanced set of mathematical and statistical technique applied to study the interactive effect of different factors at different levels with development of a proper model, which is suitable for optimisation of fermentation media, both in a submerged [20] or SSF system [19]. In this paper we report our findings on optimisation of three media supplements for SSF of rice straw by the filamentous fungi T. asperellum LBKURCC1. We limit our research to optimizing concentrations of the surfactant compound, nitrogen (N) and carbon (C) SSF media suplements.

2. Methodology

2.1. Preparation of Media and Fungal Inoculum

2.1.1. Potato Dextose Agar (PDA). PDA consist of 200 gr Potatoes, 20 gr Dextrose, 17 gr Agar, 1 L H₂O. Potato (Solanum tuberosum) was peeled and 200 g were washed and cut into cubes. The potatoes were then transferred into a pot containing one L of water and placed on a hot plate to boil until soft. After boiling, the remaining potato solids were strained through a sieve. The liquid obtained was transferred into a 1 L beaker glass and 20 g of dextrose was added to the mixture. This media was autoclaved for 15 minutes at 121°C. After cooling to 50°C, 5 mL of 1% sterile citric acid was added to every 1 L PDA to inhibit the growth of bacteria. 30 mL media were dispensed into petri dishes, and allowed to solidify.

2.1.2. Potato Dextrose Broth (PDB). Each liter of PDB in water consisted of the following ingredients: 200 gr Potatoes, 20 gr Dextrose, 17 gr Agar, 5 mL Citric Acid. 200 g of peeled potatoes were washed and cut into cubes. It was then transferred into a pot containing one L of water and placed on a hot plate with stirring to boil until soft enough to mash. After boiling, it was strained through a sieve. The liquid obtained was transferred into a beaker glass and 20 g of dextrose, together with 5 mL 1% citric acid was added to the mixture. After mixing, 30 mL aliquots of this PDB were dispensed into 100 mL Erlenmeyer flasks and steam sterilized with an autoclave for 15 minutes at 121°C. After sterilisation the PDB was allowed to cool to room temperature before inoculating it with fungi.

2.1.3. Basal Medium for Laccase Production. Each liter of basal medium for SSF contained the following: 1 g (NH₄)₂SO₄, 1 g MgSO₄, 0.6 g KH₂PO₄, and 0.05 g CuSO₄·5H₂O in water.

2.1.4. Substrate Preparation. SSF was performed using rice straw as the main substrate for laccase induction. The rice straw was washed then air-dried and cut into 1 to 2 cm length pieces.

2.1.5. Fungal Inoculum. 2 plugs with a diameter of 1 cm obtained from a five day lawn of the filamentous fungi T. asperellum LBKURCC1 on PDA petri dishes were inoculated aseptically to sterile 30 mL PDB in flasks. The inoculum was incubated at room temperature until reaching mid-exponential growth phase, before inoculating into the SSF rice straw media.
2.2. Production of Laccase by SSF of Rice Straw

Production of laccase was done in 500 mL Erlenmeyer flasks, and started by inoculating 30 mL of mid-exponential phase T. asperellum LBKURCC1 inoculum to sterilized 30 mL basal medium containing 40 g rice straw. The production of laccase was optimized based on varying concentration of the surfactant (tween-20), the nitrogen supplement (soybean meal), & the carbon supplement (glycerol) by the CCD methodology (Table 1) as part of the SSF conditions. The flasks were incubated at room temperature (±30 °C) for 8 days, with every day mixing by gentle shaking of the flasks for 1 to 10 minutes, to distribute the growing fungal mycelia evenly in the media.

2.3. Laccase Extraction From SSF Media and Enzyme assays

400 mL of 0.2 M sodium acetate buffer (pH 5.5) was added to the SSF flasks after the 8th day of incubation. The flasks were shaken at 150 rpm for 10 minutes on a rotary shaker at room temperature for the enzyme extraction. The mixtures were then cold centrifuged at 8000 rpm for 10 min, and the filtrate decanted to separate it from the solid media. The filtrate was vacuum filtered through a GF/C filter paper. The enzyme solution was washed three times with cold sodium acetate buffer using Corning ultra-spin 10 kD ultrafiltration membranes with centrifugation at 5-10°C. The final washed enzyme solution was brought to the original extraction volume. This enzyme solution was stored in small aliquots at -20°C until further needed. Laccase activity was determined using ABTS as the substrate following the method described by Kiiskinen et al.[21]. The enzyme activity of laccase was expressed as units. One unit of laccase activity (U) is defined as the amount of enzyme needed to oxidise 1 μmol of ABTS per minute.

2.4. Experimental design and media optimization for laccase production

A set of 20 experiments were designed using the Central Composite Design (CCD) Box-Wilson method for three different variables hypothesize to influence T. asperellum LBKURCC1 laccase production by rice straw SSF [22]. The three variables were the concentrations for Surfactant (Tween-20), Nitrogen supplement (Soybean meal), and Carbon supplement (Glycerol). Five different levels for each variable were assigned as -α, 1, 0, +1, +α (Table 1). As a starting point reference, we used as the central coded value at zero for all variables, the levels suggested by Gao et al. [20] as the optimum concentrations for T. harzianum ZF-2 laccase production in a submerged fermentation system. All twenty set CCD experiments of T. asperellum LBKURCC1 laccase production by rice straw SSF were performed in duplicates. The data obtained was analysed using the statistical software Minitab 17v1 in order to generate response surface graphs, and to obtain a second-order polynomial equation representing the systems characteristics.

| Variable (X)         | Range and Levels | 1.682 (-α) | -1 | 0 | 1 | 1.682 (α) |
|----------------------|------------------|------------|----|---|---|------------|
| Tween-20 (g/L) (X₁)  |                  | 0.16       | 0.5| 1 | 1.5| 1.84       |
| Soybean meal (g/L) (X₂) |                | 3.66       | 1  | 23| 34.5| 42.34     |
| Glycerol (g/L)       |                  | 0.16       | 1.5| 1 | 1.5| 1.84       |

2.5. Validation of Model

The theoretical optimized parameters were tested for actual production results in verification experiments, and compared to the CCD generated Response Surface Model (RSM) predicted production optimum level. Validation verification experiments were done in duplicate SSFs and three times repeated testing of the enzyme activity produced.
## 3. Results and Discussion

The *T. asperellum* LBKURCC1 inoculum in PDB media that was used for the SSF production of laccase was at the mid-exponential growth phase, because at this phase there is rapid growth in the size and number of cells, and the cells are at the most productive metabolic stage. Fungal mycelia cells at the mid-exponential growth phase will rapidly colonize the fermentation media, resulting in the rapid metabolism of available nutrients, important for the fermentation process. Inoculum quality in terms of the growth phase of the organism is an important factor to give optimal fermentation production results [23].

Concentration of surfactants (Tween-20) \((X_1)\), nitrogen supplements (Soybean meal) \((X_2)\), and carbon supplements (Glycerol) \((X_3)\) were chosen as variables to optimise laccase production by *T. asperellum* LBKURCC1 SSF of rice straw in a flask reactor. Table 2 shows the results of the ANOVA F-test to determine the statistical significance of interaction between the variables tested. The ANOVA results for the selected quadratic model showed that the model was significant with a Model \(F = 23.12\) and \(P\)-value of 0.0000. These results showed that the linear effects of soybean meal \((X_2)\) and glycerol \((X_3)\), quadratic effects of \(X_2^2\) and \(X_3^2\), and 2-ways interaction of \(X_1X_3\) were significant toward *T. asperellum* LBKURCC1 laccase production by SSF of rice straw \((P < 0.01)\). In contrast, the linear effects of the surfactant tween-20 \((X_1)\), quadratic effect, and 2-way interactions involving tween-20 in \(X_1X_2\) and \(X_1X_3\), had no significant effect \((P>0.05)\) to laccase production. The calculated coefficient \((R^2)\) value of 0.9541 indicates that the statistical model was able to explain the 95.41% response variability (Table 3). A regression equation (Equation 1) was generated based on *T. asperellum* LBKURCC1 laccase production response \((Y)\) to the three SSF variables tested.

\[
Y = -62.92 + 4.881X_2 + 107.8X_3 - 0.7130X_2^2 - 38.01X_3^2 - 1.072X_2X_3
\]  

(Equation 1)

### Table 2. ANOVA for production of *T. asperellum* LBKURCC1 laccase by SSF of rice straw in a CCD design with surfactant, nitrogen and carbon supplement concentration variables.

| Source           | DF | Adj SS | Adj MS | F-Value | P-Value | Statistical Interpretationa) |
|------------------|----|--------|--------|---------|---------|-----------------------------|
| Model            | 9  | 3444.44| 382.72 | 23.12   | 0.000   |                             |
| Linear           | 3  | 685.99 | 228.66 | 13.82   | 0.001   |                             |
| X1 (Tween-20)    | 1  | 5.21   | 5.21   | 0.32    | 0.587   | Not significant             |
| X2 (Soybean meal)| 1  | 506.61 | 506.61 | 30.61   | 0.000   | Significant                 |
| X3 (Glycerol)    | 1  | 174.61 | 174.16 | 10.52   | 0.009   | Significant                 |
| Square           | 3  | 2402.04| 800.68 | 48.37   | 0.000   |                             |
| X1X1             | 1  | 9.73   | 9.73   | 0.59    | 0.461   | Not significant             |
| X1X3             | 1  | 1259.36| 1259.36| 76.09   | 0.000   | Significant                 |
| X3X3             | 1  | 1278.97| 1278.97| 77.27   | 0.000   | Significant                 |
| 2-Way Interaction| 3  | 356.41 | 118.80 | 7.18    | 0.007   |                             |
| X1X2             | 1  | 44.42  | 44.42  | 2.68    | 0.132   | Not significant             |
| X1X3             | 1  | 8.30   | 8.30   | 0.50    | 0.495   | Not significant             |
| X2X3             | 1  | 303.69 | 303.69 | 18.35   | 0.002   | Significant                 |
| Error            | 10 | 165.52 | 16.55  |         |         |                             |
| Lack-of-Fit      | 5  | 106.83 | 21.37  | 1.82    | 0.263   |                             |
| Pure Error       | 5  | 58.69  | 11.75  |         |         |                             |
| Total            | 19 | 3609.95|        |         |         |                             |

Note: a) Significant interaction is interpreted for \(P\)-Values < 0.05. Not significant interaction is interpreted for \(P\)-Values \(\geq0.05\).
Table 3. Model summary for *T. asperellum* LBKURCC1 laccase production by rice straw SSF in a CCD design with surfactant, nitrogen and carbon supplement concentration variables.

|     | S       | R-sq     | R-sq adj  | R-sq (pred) |
|-----|---------|----------|-----------|-------------|
|     | 4.06837 | 95.41%   | 91.29%    | 73.20%      |

The response surface 3D graph (Figure 1) and the 2D contour plot (Figure 2) are the graphical representations of the regression equation 1 based on the dependent variables of soybean meal (Nitrogen) and glycerol (Carbon) supplement concentrations. The goal of the response surface method analysis is to efficiently determine optimum values of the variables tested to get maximum response. From the results shown in Figure 1 and 2, it can be concluded that soybean meal and glycerol supplements to the basal SSF rice straw medium can improve laccase production. This is in agreement with findings by Gao *et al.* [20], who showed that carbon and nitrogen supplements had significant effects in increasing laccase production by *Trichoderma harzianum* ZF-2 in a submerged fermentation system.

![Figure 1. Contour plot of laccase activity vs soybean meal (Nitrogen) and glycerol (Carbon) supplement concentrations](image1)

![Figure 2. Surface plot of laccase activity versus soybean meal (Nitrogen) and glycerol (Carbon) concentration supplements](image2)
The 3D response surface graph and 2D contour plots revealed that maximum laccase activity of 56.8 IU/L was achieved at soybean meal concentrations of 23 g/L; and glycerol 1% at 8 days SSF of rice straw by \textit{T. asperellum} LBKURCC1. This predicted maximum laccase activity is equivalent to laccase production of 0.7 U per g rice straw fermented, and is 3 times the level obtained without optimization. At higher concentrations of soybean meal and glycerol, there was a decrease in laccase enzyme activity, possibly through catabolyte repression or production of other substances that interfere with the analysis of laccase activity. For example, the presence of tannic acid can interfere with laccase activity determination using ABTS as the substrate [24]. Although the enzyme samples had been thoroughly washed to eliminate all molecules with molecular weights lower than 10 kD, some trace amounts of tannic polymers may still be present in the final enzyme solution when production of the tannic acids are increased.

The surfactant Tween-20 concentrations had no significant effect on laccase production. This is in agreement with findings by Gao et al. [20] for \textit{Trichoderma harzianum} ZF-2 laccase production in a submerged fermentation system. Apparently the type of surfactant used may have different effects on fungal laccase production. In a study by Zhou et al., production of laccase by SSF of rice straw, rice bran and sawdust by a \textit{Penicillium} species was significantly increased by addition of bio-surfactants such as tea saponin and rhamnolipid [25]. In this study we only investigated the effect of the surfactant Tween-20, which is a synthetic surfactant. For validation experiments, we still added Tween-20 at concentrations of 1 g/L. Further study should be done to analyse effects of other kinds of surfactants, synthetic or bio, on the SSF of rice straw by \textit{Trichoderma} species for the production of laccase.

The surface response generated model was validated by actual laboratory experiments using the optimum concentrations of soybean meal and glycerol suggested by the computer generated model, at Tween-20 surfactant concentrations of 1 g/L. The results of the model validation as shown in Table 4, showed a good agreement of the predicted laccase activity produced, with the results of the actual laboratory rice straw SSF experiments. Differences between the predicted and experimental values were less than 2%. According to Sahoo et al. [26] differences between predictive and experimental values less than 3% shows that the model has a good fit and has high predictive ability.

| Substrate   | Optimized concentration | Predicted response (U/L) | Experimental response (U/L) |
|-------------|-------------------------|--------------------------|-----------------------------|
| Tween-20 (X1) | 1 g/L                   | 56.8                     | 57.7                        |
| Soybean meal (X2) | 23 g/L                  |                          |                             |
| Glycerol (X3)   | 1 %                     |                          |                             |

4. Conclusion
In conclusion, among the three variable supplemental nutrients investigated by CCD and surface response, nitrogen supplement (soybean meal) and carbon supplement (glycerol) gave statistically significant and positive effects (P-Value< 0.05) on the production of laccase, while the surfactant Tween-20 had no significant effect. The final concentrations in the rice straw SSF medium optimized with CCD were 23 gr/L nitrogen supplement (soybean meal) and 1% carbon supplement (glycerol). Applying this optimized medium, the maximum experimental production of \textit{T. asperellum} LBKURCC1 laccase by SSF of rice straw was 57.7 U/l, and was achieved at room temperature after 8 days of fermentation in a flask reactor. The experimental results were in line with the predicted surface response analysis results.

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References
[1] Forootanfar H and Faramarzi M A 2015 Insights into laccase producing organisms, fermentation states, purification strategies, and biotechnological applications Biotechnol. Prog. 31 1443–63
[2] Pollegioni L, Tonin F and Rosini E 2015 Lignin-degrading enzymes FEBS J. 282 1190–213
[3] Wang J, Feng J, Jia W, Chang S, Li S and Li Y 2015 Lignin engineering through laccase modification : a promising field for energy plant improvement Biotechnol. Biofuels 8 145
[4] Dittmer N T and Kanost M R 2010 Insect multicipper oxidases: diversity, properties, and physiological roles Insect Biochem. Mol. Biol. 40 179–88
[5] Zheng F, An Q, Meng G, Wu X J, Dai Y C, Si J and Cui B K 2017 A novel laccase from white rot fungus Trametes orientalis: Purification, characterization, and application Int. J. Biol. Macromol. 102 758–70
[6] Baldrian P 2006 Fungal laccases-occurrence and properties FEMS Microbiol. Rev. 30 2215–42
[7] Chauhan P S, Goradia B and Saxena A 2017 Bacterial laccase: recent update on production, properties and industrial applications 3 Biotech 7 323
[8] Martinez A T et al. 2017 Oxidoreductases on their way to industrial biotransformations Biotechnol. Adv. 35 815–31
[9] Mate D M and Alcalde M 2017 Laccase: a multi-purpose biocatalyst at the forefront of biotechnology Microb. Biotechnol. 10 1457–67
[10] Jeon J R, Baldrian P, Murugesan K and Chang Y S 2012 Laccase-catalysed oxidations of naturally occurring phenols: From in vivo biosynthetic pathways to green synthetic applications Microb. Biotechnol. 5 318–32
[11] Adnan L A, Sathishkumar P, Yusoff A R M, Hadibarata T and Ameen F 2017 Rapid bioremediation of Alizarin Red S and Quinizarine Green SS dyes using Trichoderma lixii F21 mediated by biosorption and enzymatic processes Bioprocess Biosyst. Eng. 40 85–97
[12] Singh G and Arya S K 2019 Utility of laccase in pulp and paper industry: A progressive step towards the green technology Int. J. Biol. Macromol. 134 1070–84
[13] Viswanath B, Rajesh B, Janardhan A, Kumar A P and Narasimha G 2014 Fungal laccases and their applications in bioremediation Enzyme Res. 163242
[14] Kim J H, Hong S G, Sun H J, Ha S and Kim J 2016 Precipitated and chemically-crosslinked laccase over polyaniline nanofiber for high performance phenol sensing Chemosphere 143 142–7
[15] Kudanga T and Le Roes-Hill M 2014 Laccase applications in biofuels production: current status and future prospects Appl. Microbiol. Biotechnol. 98 6525–42
[16] Kudanga T, Nemadziva B and Le Roes-Hill M 2017 Laccase catalysis for the synthesis of bioactive compounds Appl. Microbiol. Biotechnol. 101 13–33
[17] Nugroho T T, Akbar I, Astina D, Helianty S and Saputra E 2018 Colour removal of an azo-textile dye and production of laccase by submerged cultures of Trichoderma asperellum LBKURCC1 J. Phys. : Conf. Ser. 1116 042027
[18] Bakker R, Elbersen W, Poppens R and Lesschen J P 2013 Rice straw and wheat straw: Potential feedstocks for the biobased economy (Wageningen: Wageningen UR, Food & Biobased Research/NL Agency Ministry of Economic Affairs) p 6
[19] Hanung C, Osmond R, Risdianto H, Suhardi S, and Setiadi T 2013 Optimisasi Produksi Enzim Lakase pada Fermentasi Kultur Padat menggunakan Jamur Pelapuk Putih Marasmius sp. : Pengaruh ukuran partikel, kelembapan, dan konsentrasi Cu Jurnal Selulosa 3 67–74
[20] Gao H, Chu X, Wang Y, Zhou F, Zhao K, Mu Z and Liu Q 2013 Media optimization for laccase production by Trichoderma harzianum ZF-2 using response surface methodology J. Microbiol. Biotechnol. 23 1757–64
[21] Kiiskinen L, Kruus K, Bailey M, Ylo E, Siika-aho M and Saloheimo M 2004 Expression of Melanocarpus albomyces laccase in *Trichoderma reesei* and characterization of the purified enzyme *Microbiology* **150** 3065–74

[22] Leiviska K 2013 *Introduction to experimental design* (Oulu: University of Oulu Control Engineering Laboratory) pp 22–6

[23] Krishna C and Nokes S E 2001 Predicting vegetative inoculum performance to maximize phytase production in solid-state fermentation using response surface methodology *J. Ind. Microbiol. Biotechnol.* **26** 161–70

[24] Terron M C Lopez-Fernandez M, Carbajo J M, Junca H, Tellez A, Yague S, Arana-Cuenca A, Gonzalez T and Gonzalez A E 2004 Tannic acid interferes with the commonly used laccase-detection assay based on ABTS as the substrate *Biochimie* **86** 519–22

[25] Zhou M F, Yuan X Z, Zhong H, Liu Z F, Li H, Jiang L L and Zeng G M 2011 Effect of biosurfactants on laccase production and phenol biodegradation in solid-state fermentation *Appl. Biochem. Biotechnol.* **164** 103–14

[26] Sahoo B K, Chakraborty U, Mukherjee J and Pal T K 2010 Optimization and Validation of Modulated Release Formulation of Ranitidine HCl by Response Surface Methodology *J. Biomed. Sci. Res.* **2** 76–85