The Sustainable Production of a Novel Laccase from Wheat Bran by *Bordetella* sp. JWO16: Toward a Total Environment

John Onolame Unuofin 1,2

1 SA-MRC Microbial Water Quality Monitoring Centre, University of Fort Hare, Private Bag X1314, Alice 5700, South Africa; junuofin@gmail.com
2 Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Private Bag X1314, Alice 5700, South Africa

Abstract: Laccase is increasingly adopted in diverse industrial and environmental applications, due to its readily accessible requirements for efficient catalytic synthesis and biotransformation of chemicals. However, it is perceived that its industrial production might incur some unfavorable overhead, which leads to expensive market products, and the corresponding negative environmental feedback, due to the use of capital-intensive and precarious chemicals. To this end, this study was designed to evaluate the performance indicators of the valorization of wheat bran by a novel Jb1b laccase and its subsequent application in waste minimization and water management, on a laboratory scale. Optimal Jb1b laccase was produced in submerged fermentation medium containing wheat bran, an agroindustrial residue, through response surface methodology (RSM) algorithm, and was applied in dye decolorization and denim bioscouring, respectively. Results showed that the resultant enzyme manifested unique biochemical properties, such as enhanced tolerance at certain physicochemical conditions, with a residual activity of at least ca. 76%. Furthermore, phenomenally high concentrations of synthetic dyes (0.2% w/v) were decolorized over 56 h, and a 6 h mediator-supported simultaneous denim bleaching and decolorization of wash effluent was observed. The sustainability of the production and application processes were inferred from the reusability of the fermentation sludge as a potential biofertilizer, with subsequent prospects for the biostimulation and bioaugmentation of contaminated soils, whereas the decolorized water could be adopted for other uses, amongst which horticulture and forestry are typical examples. These phenomena therefore authenticate the favorable environmental feedbacks and overhead realized in this present study.

Keywords: environmental sustainability; environomics; dye decolorization; denim bleaching; laccase

1. Introduction

The production of fine bulk biochemicals and other value-added bioproducts for a robust bioeconomy is beginning to derive attention from many capitalist-directed countries worldwide. This is done in a bid to accommodate a more environmentally friendly production process, as various governments are beginning to tighten environmental policies with regard to industrialization and industrial production. As a result, industry and academia must identify sustainable solutions to overarching problems that could stimulate the anticipatable inertia faced by industries, when undergoing partial or total transition, in conforming to newly inaugurated policies. Moreover, a critical perspective of the environmental economics of production must be considered, as it is expected that the assets must outweigh the liabilities, in this case. In a critical study by Sheldon [1], the all-encompassing metrics regarding the manufacture of a certain product was appraised, where cost and environmental benefits were pivotal in the ratification of a production process. Similarly, a previous review had elucidated the environomics of a production process, with particular regard to laccase production, which presumed the adoption of environmental wastes as more beneficial, when compared to conventional feedstock [2].
Laccases are becoming increasingly sine qua non for a greener community, due to their need for only basic molecular atmospheric oxygen, as a vehicle for the catalytic breakdown of a wide array of aromatics, organics and inorganics. The prospects of its production from inexhaustible, cheap substrates and environmental wastes [3,4] have given its production much credence in recent years, as it supports cleaner production. Moreover, the employment of laccases in the modification of some industrial processes, such as denim bleaching, dye decolorization, delignification of biomass for biofuel production, and degradation of other aromatic environmental pollutants, which contributes significantly to waste minimization and recycling, has warranted its adoption and resultant production on a large scale. One of a few approaches to ensure the optimal production of laccases is the response surface methodology (RSM), which is a computational algorithm that grants the engineer insight into the combinatorial effects of certain process parameters, to obtain a better product yield. However, in order to ascertain the overall sustainability of laccase production and its application, an economic and lifecycle environmental effect assessment should be conducted. A typical example of this is the ‘cradle-to-gate’ assessment of five characteristic enzyme products produced by Novozymes, Denmark, where different production chains, feedstocks, and final products were juxtaposed [5]. Wheat bran (Triticum aestivum), which is mostly stripped off during milling processes as a result of its perception as just a byproduct, and often stockpiled as waste, could present an environmental burden if not properly managed. However, there has been a recent adoption of wheat bran in certain food formulations, since it is rich in fiber as well as therapeutic phytochemicals and micronutrients. Interestingly, its inherent phenolic compounds and elements have been suggested to induce the production of lignocellulolytic enzymes, including laccase. It is also imagined that the use of wheat bran as feedstock for laccase production will enable a circular bio-based economy of agricultural industry with regard to the utilization of lignocellulosic wastes. In that regard, this study was conducted with the aim of mostly assessing the environmental implications of valorizing wheat bran residues for novel laccase production through fermentation by an arid climate bacterial denizen, and its application in biodecolorization. The study was based on the hypothesis that the production and application of JWO16 laccase would be economically practicable on an industrial scale.

2. Results and Discussion

The towering concern and cynicism about the environmental footprints which are anticipated from the large-scale production of industrially relevant biochemical, the offshoots from their catalytic reactions, and the disposal of feedstock at the end of their lifecycle, has necessitated the initiation and maintenance of environmental sustainability. In this fashion, a remarkable laccase-producing bacterial strain, Jb1b, was adopted for the sustainable cleaner production of laccase, and its subsequent application in the biodecolorization of some synthetic dyes.

2.1. Laccase Production and Process Optimization

Laccase production was screened under various cultural and nutritional conditions, in a unifactorial approach, where independent parameters such as pH, nitrogen and carbon source, inorganic and aromatic compounds were observed to be crucial to the high laccase yields. Jb1b recorded maximum laccase production at pH 5, where an estimate of 0.202 U/mL was observed (Figure S1). This was closely followed by pH 6, which elicited an output of 0.194 U/mL. Overall, the slightly acidic pH optima observed in this has been reported in earlier investigations, using different proteobacteria [3,4,6], where an inference on the possible influence of the cellular transport processes was made. Among the nitrogenous sources assessed, NH$_4$NO$_3$, elicited the highest laccase yield (ca. 28.1 U/mL), while the second-best nitrogen source was the organic yeast extract (ca. 20 U/mL) (Figure S2). In a recent study, NH$_4$NO$_3$ was observed as the optimal source of nitrogen, and enhanced maximum production of laccase (31.521 U/mg and 31.523 U/mg) during the degradation of some agro-residues by Stenotrophomonas sp. CFb-09 [7]. Agro-industrial
residues served as an excellent source of carbon for laccase production. However, among the tested residues, wheat bran (WB) elicited the maximum laccase yield overall (ca. 28.9 U/mL) (Figure 1a). WB has been observed to contain 27% total carbohydrate, 14% protein, 6% lipids and approximately 64% digestible organic nitrogen, and has no stringent requirement for pre-treatment when used for enzyme production [8,9]. Interestingly, on assessment of different inorganic and aromatic compounds, which are generally believed to be pollutants in wastewater polluted environments, acetaminophen elicited the maximum laccase activity (ca. 37.5 U/mL); however, there was no significant difference when compared to the induced vanillic acid (V.A), which was ca. 37.2 U/mL (Figure 1b). A similar trend was observed for a strain of *Stenotrophomonas maltophilia*, which was isolated from wastewater influent [4]. However, its maximum laccase activity was recorded in V.A (ca. 37.3 U/mL), while a non-significantly different alternative was observed in acetaminophen (ca. 36.5 U/mL). The profile exhibited by this bacterial isolate would be especially beneficial in the adoption of hospital and olive oil mill wastewater, among other potential polluted environments, as matrices for economically feasible, large-scale laccase production. An agitation speed of 100 rpm was recorded to be optimal for laccase production, which has been recorded in other proteobacteria [3,4].

Selected optima for laccase production were input into the RSM algorithm, which was chosen to assess the interaction effects of the individual cultural and nutritional factors (pH, agitation speed, wheat bran and NH$_4$NO$_3$). This was done to minimize the skewedness and the insufficient reliability of the OVA5 system of process optimization, and also simulate the most probable combinations for enhanced production on a large scale. In this regard, outcomes from CCD generated experimental runs were incorporated in a second-order model (Table 1), which generated a noise-free polynomial regression equation, Equation (4), to further elucidate laccase production.

![Figure 1](image-url)
Figure 1. (a): Effects of agroindustrial residues on Jb1b laccase production. Asterisks indicate significant difference ($p < 0.05$) among treatments. MP = mandarin peelings, GS = grape stalks, MS = maize stover, WB = wheat bran; (b): Effects of inorganic and aromatic compounds on Jb1b laccase production. Asterisks indicate significant difference ($p < 0.05$) among treatments.

Table 1. CCD template for laccase production in Jb1b.

| Run Order | pH | Wheat Bran (g/200 mL) | Agitation Speed (rpm) | NH$_4$NO$_3$ (g/200mL) | Actual Value | Predicted Value |
|-----------|----|----------------------|-----------------------|-------------------------|--------------|-----------------|
| 1         | 3  | 2                    | 100                   | 0.1                     | 31.85        | 33.43           |
| 2         | 3  | 2                    | 50                    | 0.1                     | 32.78        | 30.92           |
| 3         | 5  | 1                    | 100                   | 0.2                     | 15.66        | 16.20           |
| 4         | 4  | 1.5                  | 75                    | 0.15                    | 22.01        | 23.62           |
| 5         | 3  | 1                    | 50                    | 0.1                     | 21.47        | 21.07           |
| 6         | 3  | 2                    | 50                    | 0.2                     | 33.17        | 33.10           |
| 7         | 5  | 1                    | 50                    | 0.2                     | 16.43        | 19.05           |
| 8         | 5  | 1                    | 100                   | 0.1                     | 16.28        | 18.72           |
| 9         | 4  | 1.5                  | 75                    | 0.1                     | 22.09        | 23.71           |
| 10        | 3  | 1.5                  | 75                    | 0.15                    | 26.89        | 28.34           |
| 11        | 4  | 1.5                  | 75                    | 0.15                    | 23.02        | 23.62           |
| 12        | 4  | 1.5                  | 75                    | 0.15                    | 21.31        | 23.62           |
| 13        | 4  | 1.5                  | 75                    | 0.15                    | 23.33        | 23.62           |
| 14        | 4  | 1.5                  | 75                    | 0.2                     | 24.96        | 23.54           |
| 15        | 4  | 1                    | 75                    | 0.15                    | 22.09        | 21.15           |
Table 1. Cont.

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|-----------|----|----------------------|-----------------------|---------------------------------|--------------|-----------------|
| 16        | 4  | 1.5                  | 100                   | 0.15                            | 25.34        | 23.53           |
| 17        | 4  | 1.5                  | 75                    | 0.15                            | 26.20        | 23.62           |
| 18        | 5  | 1                    | 50                    | 0.1                              | 28.29        | 21.58           |
| 19        | 5  | 2                    | 100                   | 0.2                             | 19.45        | 16.22           |
| 20        | 4  | 1.5                  | 75                    | 0.15                            | 23.17        | 23.62           |
| 21        | 5  | 2                    | 50                    | 0.1                              | 19.07        | 21.60           |
| 22        | 3  | 1                    | 50                    | 0.2                             | 22.48        | 23.26           |
| 23        | 3  | 1                    | 100                   | 0.2                             | 26.04        | 25.77           |
| 24        | 3  | 2                    | 100                   | 0.2                             | 36.12        | 35.61           |
| 25        | 5  | 1.5                  | 75                    | 0.15                            | 16.82        | 18.90           |
| 26        | 4  | 2                    | 75                    | 0.15                            | 26.89        | 26.09           |
| 27        | 5  | 2                    | 100                   | 0.1                             | 18.91        | 18.74           |
| 28        | 5  | 2                    | 50                    | 0.2                             | 19.38        | 19.07           |
| 29        | 4  | 1.5                  | 50                    | 0.15                            | 22.63        | 23.71           |
| 30        | 3  | 1                    | 100                   | 0.1                             | 24.49        | 23.58           |

Jb1b laccase production (U/mL)

\[
Y = 23.62 - 4.72A + 2.47B - 0.087C - 0.086D - 2.46AB - 1.34AC - 1.18AD \quad (1)
\]

where \( Y \) is the perceptible laccase activity (U/mL), and \( A, B, C \) and \( D \) are pH, carbon source, agitation speed and nitrogen source, respectively.

Correspondingly, the ANOVA for laccase production, as portrayed in Table 2, revealed that the chosen model was suitable, with an \( F \)-value of 17.96, a significant model algorithm \((p < 0.0001)\) and a non-significant lack-of-fit \((0.2076)\).

Table 2. ANOVA for Response Surface Reduced 2FI model.

| Source            | Sum of Squares | df | Mean Square | \( F \) Value | \( p \)-Value | Prob > \( F \) |
|-------------------|----------------|----|-------------|---------------|--------------|---------------|
| Model             | 658.63         | 7  | 94.09       | 17.96         | < 0.0001     | significant   |
| A-pH              | 401.39         | 1  | 401.39      | 76.61         | < 0.0001     | -             |
| B-Wheat Bran      | 109.47         | 1  | 109.47      | 20.89         | 0.0001       | -             |
| C-Agitation Speed | 0.14           | 1  | 0.14        | 0.026         | 0.8738       | -             |
| D-\( \text{NH}_4 \text{NO}_3 \) | 0.13       | 1  | 0.13        | 0.025         | 0.8754       | -             |
| AB                | 96.48          | 1  | 96.48       | 18.41         | 0.0003       | -             |
| AC                | 28.81          | 1  | 28.81       | 5.50          | 0.0285       | -             |
| AD                | 22.21          | 1  | 22.21       | 4.24          | 0.0516       | -             |
| Residual          | 115.27         | 22 | 5.24        | -             | -            | -             |
| Lack of Fit       | 101.23         | 17 | 5.95        | 2.12          | 0.2076       | not significant|
| Pure Error        | 14.03          | 5  | 2.81        | -             | -            | -             |
| Cor Total         | 773.89         | 29 | -           | -             | -            | -             |

\( R^2 = 0.8511; \text{Adj } R^2 = 0.8037; \text{Pred } R^2 = 0.6066; \text{Adeq Precision} = 16.427 \).
At \( p < 0.05 \), the interaction of pH and wheat bran (AB) was observed to contribute beneficially to laccase production (Figure 2a), as did their individual effects (Table 2). This confirms their status as crucial to the organism’s metabolism and corresponding secretion of laccase. In particular, this observation of the positive interaction of pH and wheat bran was reported in a previous study [3]. Consequently, the interaction effects that gave optimal response (36.12 U/mL) were recorded as pH 3, 2 g/200 mL wheat bran, 100 rpm and 0.2 g/200 mL NH\(_4\)NO\(_3\). These optima were further verified during numerical optimization, which produced a desirability coefficient of 0.975 (Figure 2b). The closeness of this value to 1 infers the appropriateness of the fermentation conditions toward optimal laccase yield. The model was further used to forecast the response of interaction of factors outside the boundary for this study: pH 3, 2.5 g/200 mL wheat bran, 100 rpm and 0.25 g/mL NH\(_4\)NO\(_3\), which elicited the maximum laccase yield, overall (42.1 U/mL).

2.2. Biochemical Novelty of the Laccase

The laccase secreted by Jb1b exhibited some tremendous physicochemical properties, which ranged from thermostolerance to halo- and surfacto-tolerance. First, the laccase activity was optimal at pH 7, and a similar outcome was observed by Boruah et al. [10]. However, relative activities of ca. 76%-98% were observed at pH regimes 3–11 (Figure S4). This trend of adaptability was observed earlier [11], where exudates from *Citrobacter* sp. were assessed at extreme pH regimes. Moreover, enhanced stability was observed for over 455 min at the aforesaid pH regimes (Figure 3a), with minimum residual activity estimates greater than the initial laccase activity (100 %). Although remarkable stabilities were [12], they were observed at different experimental conditions. Interestingly, the durability of the laccase was emphasized by stability at 4 \(^{\circ}\)C, in pH 3–11-buffered solutions, over 23 weeks, where at least 85% residual activities were recovered after pre-incubation (unpublished data).

![Design-Expert® Software](image)

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Maximum laccase activity was observed at 80 °C; even so, remarkable relative activities were generally recorded at high-temperature regimes (Figure S5). Similar thermophilic laccases have been reported by several authors [3,6,13]. During stability studies, Jb1b exhibited a trend of robustness for over 400 min at the temperature profiles assessed (40–90 °C) (Figure 3b). Instinctive checks at 1140 and 1440 min corroborated its tremendous residual activities, especially at regimes from 40 °C to 70 °C. A recombinant laccase, LAC_2.9, expressed no detectable loss of activity after it was incubated at 60 °C for 16 h; however, only 80% of laccase activity was recoverable at 70 °C [14]. Other interesting properties of Jb1b have been outlined in Table 3, which portrayed its distinct metalo-, halo-, and surfacto-tolerance and, most importantly, its ability to transform considerable amounts of substrate (ABTS) to products in the presence of different concentrations of a renowned halide laccase inhibitor (NaF) [15]. Only a few studies have reported enhanced halotolerance of laccase [16–19]. Conversely, the impressive range of residual activities observed at high concentrations of solvents (20% and 40%) suggests its adeptness at reaction conditions that require the dissolution of substrates for easy miscibility. Additionally, its metalo-, halo-tolerance implies that it could be used synchronously with eutectic solvents for several applications, which may ultimately contribute to a reduction in the global clean-water footprint.

Of all substrates evaluated in this study, the non-phenolic chemicals were more pliable to Jb1b laccase oxidation; this observation is corroborated by Unuofin et al. [16] and might be due to the precipitous formation of radicals, which serve as vehicles for continuous reaction. This observation might be useful in the catalysis of chemical compounds, which are of synthetic orientation.

Furthermore, values from a kinetic study using ABTS as substrate were fitted to a Lineweaver–Burk plot, from which remarkable kinetic trademarks, such as high specificity \(K_{\text{cat}}/K_{\text{m}}: 1.28 \times 10^4 \, \mu\text{M}^{-1} \, \text{s}^{-1}\), substrate affinity \(K_{\text{m}}: 0.5909 \, \mu\text{M}\) and catalytic constant \(K_{\text{cat}}: 7.57 \times 10^3 \, \text{s}^{-1}\), were obtained for Jb1b laccase. This outcome was portrayed by a member of the firmicutes, especially its affinity for ABTS [20]. The kinetic statistics portrayed by this laccase imply faster reaction times and increased substrate sensitivity, which is ideal for time-lag-free applications.
Figure 3. (a): Effect of different pH regimes on the stability of Jb1b laccase; (b): Effect of different temperature profiles on the stability of Jb1b laccase.

2.3. Laccase-Encoding Genes

A snapshot molecular analysis suggested that laccase expression might be regulated by a single homologous sequence (Figure 4). This observation is antithetical to an earlier study, which attributed robust biochemical novelty and laccase polyextremotolerance to the expression of multiple homologous genes [16]. Although there are studies corroborating the occurrence of multiple laccase genes and its associated influence on enhanced laccase robustness and versatility [21,22], it is assumed that Jb1b laccase might be a yellow laccase with high redox potentials, since it was extracted from a lignocellulose-supplemented medium. Moreover, it had been surmised that yellow/white laccases possess some endogenous lignin-generated phenolic compounds [23].
Table 3. Tolerance of Jb1b laccase to concentrations of metals, surfactants, halides and solvents.

| Metals and Surfactants | Concentration (mM) | Residual Activity (%) | Halides and Solvents | Concentration (% wv⁻¹) | Residual Activity (%) |
|------------------------|--------------------|-----------------------|----------------------|------------------------|-----------------------|
| None                   | -                  | 100                   | None                 | -                      | -                     |
| Mn²⁺                  | 1                  | 110.53 ± 2.2⁺         | NaCl                 | 5                      | 108.40 ± 3.38⁺        |
| -                      | 2.5                | 105.22 ± 1.4 b         | -                    | 10                     | 100.35 ± 2.72 a        |
| -                      | 5                  | 101.84 ± 2.6 a         | -                    | 20                     | 110.97 ± 2.06 d        |
| -                      | 7                  | 103.40 ± 0.8 b         | -                    | 40                     | 110.33 ± 1.86 d        |
| Fe³⁺                  | 1                  | 109.02 ± 1.6 b         | NaF                  | 2                      | 109.41 ± 2.37 f        |
| -                      | 2.5                | 111.36 ± 3.0 c         | -                    | 5                      | 98.89 ± 1.16 c         |
| -                      | 5                  | 111.13 ± 2.6 c         | -                    | 10                     | 91.03 ± 1.64 a         |
| -                      | 7                  | 107.46 ± 1.5 a         | -                    | 20                     | 90.15 ± 1.33 a         |
| Ba²⁺                  | 1                  | 108.40 ± 2.4 b         | DMSO                 | 5                      | 101.85 ± 2.05 a        |
| -                      | 2.5                | 112.15 ± 1.7 c         | -                    | 10                     | 101.98 ± 1.78 a        |
| -                      | 5                  | 105.23 ± 2.46 a        | -                    | 20                     | 101.04 ± 1.46 a        |
| -                      | 7                  | 115.82 ± 1.24 d        | -                    | 40                     | 100.93 ± 1.18 a        |
| Cu²⁺                  | 1                  | 113.80 ± 3.14 d        | Tween 20             | 5                      | 101.90 ± 2.04 b        |
| -                      | 2.5                | 122.71 ± 3.64 f        | -                    | 10                     | 100.71 ± 1.76 a        |
| -                      | 5                  | 107.65 ± 1.12 c        | -                    | 20                     | 100.41 ± 1.36 a        |
| -                      | 7                  | 97.78 ± 1.56 a         | -                    | 40                     | 102.48 ± 2.14 b        |
| EDTA                   | 1                  | 121.64 ± 1.42 a        | -                    | -                      | -                     |
| -                      | 2.5                | 122.19 ± 1.2 a         | -                    | -                      | -                     |
| -                      | 5                  | 114.09 ± 1.38 a        | -                    | -                      | -                     |
| -                      | 7                  | 118.07 ± 2.46 b        | -                    | -                      | -                     |
| Benzoic Acid           | 1                  | 114.38 ± 3.16 c        | -                    | -                      | -                     |
| -                      | 2.5                | 108.03 ± 2.0 a         | -                    | -                      | -                     |
| -                      | 5                  | 110.05 ± 1.4 b         | -                    | -                      | -                     |
| -                      | 7                  | 110.93 ± 1.65 b        | -                    | -                      | -                     |
| SDS                    | 1                  | 111.32 ± 1.52 b        | -                    | -                      | -                     |
| -                      | 2.5                | 107.82 ± 2.06 a        | -                    | -                      | -                     |
| -                      | 5                  | 116.18 ± 2.32 c        | -                    | -                      | -                     |
| -                      | 7                  | 121.84 ± 3.04 d        | -                    | -                      | -                     |

Values represent mean ± SD of three replicates. Values with the same superscript alphabet along the same column segment are not significantly different (p > 0.05).

Figure 4. Molecular snapshot of amplified fragments of laccase gene from (a) Bordetella parapertusis JWO16. Lane 1: ladder mix, lane 2: CueOP gene, lane 3: MCOStm gene, lane 4: CueOCit gene.
2.4. Dye Decolorization and Denim Bleaching

Synthetic dyes of the heterocyclic cationic, triarylmethane, azo and anthraquinone groups, which form the majority of pollutants in textile effluents, were degraded by Jb1b at novel high concentrations (0.2% w v\(^{-1}\)), without redox mediators (Figure 5a). AB, MO, CR and BB were satisfactorily degraded between 56 h and 80 h incubation period, if the initial concentrations of the mixtures were considered. However, the recalcitrance of MG and RB was observed, and this might be due to the dyes’ inability to access the active site of laccase, as a result of non-congruent atomic orientation.

![Figures](image1.png)

**Figure 5.** Cont.
Figure 5. (a): Decolorization of different synthetic dyes (0.2%) by Jb1b laccase: azure B (AB), malachite green (MG), reactive blue (RB), methyl orange (MO), congo red (CR), brilliant blue (BB). Phosphate buffer was used, with no mediator. Asterisks indicate significant difference ($p > 0.05$) among treatments; (b): Experiential denim bleaching using Jb1b laccase. The control (middle), containing the mediator and TCA, is flanked by Enzyme + Mediator (E+M) treatment on the left, and Enzyme only on the right; (c): Dissection microscope $\times 30$ magnification of Jb1b laccase-bioscouring fabric. Comparisons were made between untreated fabric (control) and the treated variants; laccase only (middle), and laccase + mediator; ABTS (right); (d): Spectrum plot of Jb1b laccase treated denim. JWO16E1 represents enzyme only treatment, whereas JWO16E+M1 represents enzyme + mediation treatment. Absorbance of samples was read from 200 to 900 nm and the log inverse ($10^{-A}$) was plotted as reflectance.

However, these dyes are increasingly susceptible to laccase catalysis through different strategies [24–26], which could be adopted and further developed to enhance the biodegradation capacities of these malignant class of pollutants.

Although, to our knowledge, there have been no preceding studies regarding decolorization of the phenomenally high-dye concentrations, as was employed in this investigation, the studies of selected authors can be referred to in [27,28]. An amalgam of Jb1b laccase and a synthetic mediator (ABTS) was sufficient for the experiential bleaching of indigo from the denim into the surrounding solvent, although the addition of TCA—a component of laccase assay, which is iconic in the laccase oxidation of ABTS, was added. A characteristic greenish solution of ABTS oxidation was briefly formed, which was then transformed to that of the denim’s pigment, suggesting the practical leaching of denim indigo into the surrounding medium (Figure 5b).
The non-interference of TCA, a potential bleaching agent, in the laccase denim bleaching, was ascertained in the control, which had all other components of the reaction mixture, except laccase. It was observed that the bioscouring of the indigo was visible only when the laccase-mediator amalgam was oxidized; however, only Solis-Oba et al. [29] drew particular attention to this phenomenon. Furthermore, the presence of the mediator elicited the brightest hue on the denim (Figure 5c). This outcome has been corroborated through colorimetric assessment [29], and it implies that the investigated laccase only required an exogenous supply of oxidized mediators for effective indigo wash-away. Other effective mediators in bioscouring include violuric acid [30] and 1-hydroxybenzotriazol [31]. Conversely, a study has revealed the indigo wash-away was stimulated by a purified manganese peroxidase from *Cerrena unicolor*, without an exogenous supply of mediators [32]. However, the possible intervention of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), a bleaching agent, was not evaluated in their study, though it was applied as a cosubstrate during treatment. Although a fungal laccase had been reported to wash-away indigo from fabric, without an exogenous supply of mediators [33], other oxygenases might possess the denim bioscouring ability, as inferred from a recent study [32]. Reflectance readings (Figure 5d) revealed that the contrast between the untreated and the treated is comparable to what was reported by Iracheta-Cárdenas et al. [30], where the treated fabric recorded a higher reflectance reading.

Ultimately, a step-by-step decolorization of the dye bath, hours after the initiation of scouring was observed, just as recorded by Unuofin [34]. A precise rationalization for this interesting phenomenon has not been presented to date, but understanding of the mechanism of this synchronized reaction (denim bleaching and dye decolorization) is much anticipated in the not-too-distant future, which will afford process manipulations that can holistically tackle challenges arising in the textile industry. Moreover, we propose that intermediates which might emanate from step-by-step decolorization should be evaluated for other interesting biochemical and biotechnical potentials, as they might serve as valuable unconventional startup chemicals for other industrial and environmental applications.

### 2.5. Sustainability Assessment

In order to understand the environmental implications of the just concluded process in this study, an upstream and downstream perspective was assigned (Figure 6), where the chronicle of waste minimization was observed during the enzyme production (upstream) and application process (downstream). Furthermore, a surgical representation of the feedstock and consumables adopted for the production of a 42.1 U/mL Jb1b laccase equivalent and its subsequent application is portrayed in Table 4. Here, it was inferred that, overall, the environmental footprint associated with the production and application of Jb1b laccase in denim bioscouring was minimal. This can be ascribed to the relative quantities of the reagents used, as well as the feedstock: the minute quantities of the mineral salts, trace elements and inducers make them readily degradable, therefore reducing their bioavailability in the environment, whereas the use of a considerable amount of biomass (wheat bran), implies the reduction in agroindustrial wastes through effective solid waste management. Likewise, during application, inconsequential volumes of reagents were used, which were assumed to be detoxified by laccase catalysis (Table 4). An immensely important metric during the overall process is the environmental perception of the byproducts of each process: hence, it was assumed that enriched sludge would contribute to sustainable agriculture, horticulture or forestry, as would the decolorized batch effluent. This would reduce the untold devastating impacts of chemical fertilizers on soil microflora, microfauna and microbiome [35], especially when they are substituted by the enriched sludge as a “mulch mix”, which is presumed to be an embodiment of basic elements for soil fertility. Conversely, reusing the decolorized batch effluent will reduce its water footprint, thus leading to the conservation or effective management of freshwater sources. This would favor the thesis of wastewater being a resource, as earlier remarked [36]. Although this study only affords a snapshot of the lifecycle assessment, it is comparable to the all-
inclusive study of Nielsen et al. [5], where industrially produced enzymes were appraised. However, the irrepressible contribution of fermentation and electricity driven processes to global warming was highlighted in their study, which makes the overall inclination of production and packaging toward environmental sustainability somewhat cynical. In this study, however, the microaerophilic environment provided during fermentation effected a cut-back in direct emission of greenhouse gases to the atmosphere. Moreover, condensed steam was observed in the fermentation bottles, which added to the fermentation broth volume. Although an economic appraisal could not be conducted, due to the inaccessibility of the data needed for comparison, it was presumed that the overhead of production and application was feasible, given that enzyme purification and formulation was disregarded. Ultimately, a critical assessment of the finished commercialized products corroborates the Jb1b laccase produced in this study as economically beneficial. This product is a 1 g *Agaricus bisporus* laccase concentrate (≥4 U/mg) (CAS Number 80498-15-3), which is sold at USD 974.94 (Sigma-Aldrich), and must have incurred costs of purification, formulation and biofinishing. Even so, it is assumed that Jb1b crude laccase will derive significance biotechnological applications, similar to the aforementioned concentrate.

**Figure 6.** Main process involved in Jb1b laccase production and its application on a laboratory scale. There was minimum consumption of water and electricity during the experiments.

**Table 4.** Ingredients used in the production and application of Jb1b laccase.

| Process                        | Substance                              | Product                          | Derivative          | Environmental Footprint                                                                 |
|--------------------------------|----------------------------------------|----------------------------------|---------------------|----------------------------------------------------------------------------------------|
| Fermentation and separation    | Citrate buffer (pH 3, 100 mM, 200 mL)  | Ca. 150 mL laccase (42.1 U/mL)    | Enriched Sludge     | Minimum: this is because the resultant sludge is presumed to be a conglomerate of mineralized elements, nutrients and microorganisms. Furthermore, the sludge can be portrayed as a symbiosis model, as it could fulfil either bioaugmentation or biostimulation needs, or both. |
|                                | Wheat Bran (2.5 g)                      |                                  |                     |                                                                                        |
|                                | NH₄NO₃ (0.25 g)                         |                                  |                     |                                                                                        |
|                                | Mineral salts (≤ 0.1 g)                 |                                  |                     |                                                                                        |
|                                | Trace elements (negligible) and inducer (≤ 0.0025 g) |                                  |                     |                                                                                        |
Table 4. Cont.

| Process                     | Substance                          | Product               | Derivative          | Environmental Footprint |
|-----------------------------|------------------------------------|-----------------------|---------------------|-------------------------|
| Application (Denim bioscoring) | Phosphate buffer (pH 6, 100 mM, 40 mL) | Biofinished fabric    | Decolorized Batch effluent | Minimum: this is because the process water could be reused for other batches, or can be used in other sectors, such as horticulture or forestry. It is assumed that the laccase-catalysed degradation of the aromatic compounds would reduce their toxicity. |
|                            | Crude laccase (3 mL)               |                       |                     |                         |
|                            | ABTS 2 mM (500 µL)                 |                       |                     |                         |
|                            | Trichloroacetic acid 20% (40 µL)   |                       |                     |                         |
|                            | Denim fabric (10 g)                |                       |                     |                         |

3. Materials and Methods

3.1. The inoculum and Its Protein Estimate

Jb1b was isolated from decaying trunk, and identified as *Bordetella* sp. JWO16, with accession numbers MF073262 from GenBank, National Center for Biotechnology Information (NCBI). A standardized culture (50 µL) of Jb1b was inoculated in a basal medium, which contained (g/L): KH₂PO₄; 0.514, K₂HPO₄; 0.32, NaC₆H₅O₇; 0.08, MnSO₄·H₂O; 0.032, MgSO₄·7H₂O; 0.192, CaCl₂·2H₂O; 0.008, CuSO₄·5H₂O; 0.0008, FeCl₃·7H₂O; 0.0008, ZnSO₄; 0.0008, vanillin; 0.012. (Sigma-Aldrich, Modderfontein, South Africa) pH 5, citrate buffer. The medium was assessed for the optimal source of carbon (1 g/L) using agroindustrial residues like maize stover (MS), mandarin peelings (MP), wheat bran (WB) and grape stalks (GS). Nitrogenous sources (0.2% w/v) such as NaNO₃, KNO₃, NH₄NO₃, l-asparagine, yeast extract, tryptone pH (3–8), and putative inducers (0.05%) CoCl₂, CuSO₄, ferulic acid, acetaminophen, 4-nitrophenol, vanillic acid, 2,5-xyldine. The respective cultures were then incubated in an orbital shaker at 140 rpm, 30 °C for 96 h, while aliquots were harvested post-incubation and centrifuged at 15,000 rpm for 12 min at 4 °C using a benchtop centrifuge (SIGMA-1-14k). The resultant supernatant was used to assay for laccase activity. The Folin-phenol reagent was used to express the protein concentration, according to Lowry et al. [11]. Laccase activity was estimated using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate [37], where 2 mM ABTS in pH 6 potassium phosphate buffer were incubated at room temperature with 50 µL of appropriately diluted crude laccase at 30 °C, allowed to react for 10 min, and stopped with 40 µL 20% TCA. The control contained all reaction conditions, except that the crude laccase was replaced with a thermally deactivated enzyme. One unit of enzyme activity referred to the amount of enzyme oxidizing 1 µmol of substrates per minute under assay conditions.

3.2. Optimization of Production Conditions by Response Surface Methodology

Optimal nutritional and cultural parameters from preliminary screening (OVAT) conducted earlier were selected for response surface methodology (RSM) (Table 5). Here, 30 experimental runs were formulated using Design Expert® version 10 (Stat-Ease, Minneapolis, Min. United States), which were performed in triple iterations. The outcomes of these experiments were fit to a quadratic model, Equation (2), which was used to forecast the accuracy of the algorithm and the astuteness of probabilistic outcomes. Ultimately, the results were computed as mean and standard deviation, using Microsoft Excel.

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i}^{k} \beta_{ij} x_i x_j + \epsilon
\]  

(2)

\(Y = \) Responses.  
\(K = \) Total number of independent factors  
\(\beta_0 = \) Intercept
$\beta_{ii}$, $\beta_{iii}$, and $\beta_{ij}$ = Coefficient values for linear, quadratic and interaction effects respectively.

$x_i$ and $x_j$ indicate coded levels for independent variables.

Table 5. Levels of significant variables used in central composite design for Jb1b.

| Independent Parameters | Symbol | Level |
|------------------------|--------|-------|
| pH                     | A ($X_1$) | -1  3  4  5|
| Wheat bran             | B ($X_2$) | 2  1.5  2|
| Agitation speed        | C ($X_2$) | 50  75  100|
| $\text{NH}_4\text{NO}_3$ | D ($X_4$) | 0.1  0.15  0.2|

3.3. Assay for Biochemical Novelty of the Laccases and Associated Gene

Jb1b laccase was assessed for its biochemical properties in buffered solutions (citrate-phosphate buffer). Notable parameters include temperature optima (0–90 °C) and stability (40–90 °C), pH optima and stability (pH 3–11), effects of some metal ions ($\text{Mn}^{2+}$, $\text{Cu}^{2+}$, $\text{Fe}^{2+}$, $\text{Fe}^{3+}$, $\text{Ba}^{2+}$, $\text{Zn}^{2+}$, $\text{Mg}^{2+}$, $\text{Co}^{2+}$) and surfactants (EDTA, benzoic acid, SDS, urea, DMSO, Tween 20), potent halides (NaCl, NaF) etc. Correspondingly, substrate specificity studies using ABTS, guaiacol, 1-naphthol, 2,6-Dimethoxyphenol (DMP), pyrogallol, syringaldazine and potassium ferrocyanoferrate (PFC) were conducted in replicates. All reagents used were purchased from Sigma-Aldrich, Modderfontein, South Africa.

The total nucleic acid of the Jb1b was extracted by a modified boiling method [16], where axenic culture pellets were washed in microbiological saline, twice, before heating in an AccuBlock Digital dry bath (TECHNE, Lasec SA (Pty) Ltd, Cape Town, South Africa). Thereafter, the PCR conditions for DNA amplification, with the used primers, and the procedures for gel electrophoresis and visualization of target band, were described previously [16].

3.4. Biodecolorization Potentials

The decolorization of the synthetic dyes (Sigma-Aldrich, Modderfontein, South Africa): Azure B, Malachite Green, Reactive Blue, Methyl Orange, Congo Red, and Brilliant Blue was attempted, following a recently reported procedure. Aliquots of crude laccase (50 µL) were incubated in 1950 µL homogenized (0.2% in 50 mM phosphate buffer pH 6) dyes at 30 °C. Absorbance readings were taken in termittently for over 80 h with the aid of Microplate reader (Synergy MX, BioTekTM, Winooski, VT, USA) at respective wavelengths [17]. Thereafter, their decolorization quotients were computed as follows

$$\% \text{ decolorization} = \left( \frac{A_{\text{initial}} - A_{\text{observed}}}{A_{\text{initial}}} \right) \times 100$$  \hspace{1cm} (3)

where $A_{\text{initial}}$ is the initial absorbance, and $A_{\text{observed}}$ is the observed absorbance.

Correspondingly, the bioscouring potential of Jb1b laccase was evaluated according to Unuofin [17], where absorbance lectures at wavelengths ranging from 200 nm to 900 nm were translated to reflectance through the derived equation

$$R = 10^{-A}$$  \hspace{1cm} (4)

where $R$ = reflectance, and $A$ = absorbance value.

3.5. Data Analysis

The results of replicates were computed as mean ± standard deviation (SD) using Microsoft Excel Spreadsheet. Data were subsequently subjected to one-way analysis of variance (ANOVA) and the least significant difference was carried out. Significance was identified at $p \leq 0.05$.  

4. Conclusions

The environmental sustainability of production and application of Jb1b laccase in denim bioscouring was assessed in this present study. It was observed that the statistical optimization of the fermentation process afforded the production of a secretion with phenomenal biochemical and catalytic properties. Furthermore, Jb1b was considered to be appropriate for the decolorization of novel high concentrations of synthetic dyes, and the simultaneous denim bioscouring and batch-effluent decolorization, which further improves environmental and water footprints. This suggests that more studies should be encouraged to elucidate the metatranscriptomics of Jb1b laccase production. Moreover, the perceived economic feasibility of the processes appraised warrants further investigation in a comprehensive cost and environmental feasibility analysis.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/catal11060677/s1, Figure S1: Effect of pH on Jb1b laccase production, Figure S2: Effect of nitrogenous sources on Jb1b laccase production, Figure S3: Effect of agitation speed on Jb1b laccase production, Figure S4: Effect of pH regimes on Jb1b laccase activity, Figure S5: Effect of temperature profile on Jb1b laccase activity.

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