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Research article

Utilization of agroindustrial wastes for the production of laccase by *Achromobacter xylosoxidans* HWN16 and *Bordetella bronchiseptica* HSO16

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ABSTRACT

Agroindustrial residual lignocellulosic biomaterial provides an economical and renewable natural bioresource for the large-scale, gainful biofuel production, as well as the production of fine bulk chemicals, which may include industrial biocatalysts. To this end, the laccase-inducing aptitude of some agroindustrial, lignocellulosic residues were appraised in submerged fermentation batch culture of two woodland betaproteobacteria (Hb9c; *Achromobacter xylosoxidans* HWN16, Hb16c; *Bordetella bronchiseptica* HSO16). Significant fermentation factors for laccase production were identified following a one-variable-at-a-time: OVAT screening method, levels of significant factors were optimized using response surface methodology: RSM. Mandarin peelings: MP and wheat bran: WB were suitable substrates for laccase production in Hb9c; 29.4 U/mL and Hb16c; 28.2 U/mL, respectively. However, the numerical optimization of significant factors for laccase production in both isolates presented an overall maximum laccase output encountered throughout the study (Hb9c; 169.39 U/mL, Hb16c; 45.22 U/mL), albeit the simulated conditions of the statistical model were outside the design space of the algorithm such as pH 5, 0.5 g MP, 100 rpm, 0.25 g $NaNO_3$ for Hb9c and pH 3, 2.5 g WB, 50 rpm, 0.05 g yeast extract for Hb16c. Furthermore, a record 17.5 and 15.54 fold increase in laccase turnover depicts the astuteness of the statistical method in the valorization of these lignocellulosic residues for enhanced laccase production, hence, the incorporation of these outcomes at industrial scales might yield tremendous outputs.

1. Introduction

Laccases (*p*-benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are blue copper-containing oxidases that are well represented in the cross-linking of monomers, degradation of polymers, and the ring cleavage of aromatics. Kushwah et al. (2014) however describe them as polyphenol oxidases that could oxidize an interesting array of organic and inorganic substrates, including phenols as well as metal complexes by simultaneous four-electron reduction of available molecular oxygen to water. Given the interesting characteristics of this unique class of enzymes, it is fitting to suppose that they would seemingly exhibit an overlap in multifarious functions in the biotechnology industry, with applications spanning across textile, pulp and paper, bioenergy, agriculture, as well as the food and pharmaceuticals.

The discovery of laccase was pioneered by Yoshida (1883), who isolated it from the sap of a lacquer tree, *Rhus vernicifera*. Subsequently, corroborative studies were carried out by Bertrand (1896) and Laborde (1896) respectively who also characterized fungal extracellular secretions as laccase. Laccases have thereafter been discovered in other plants, insects, and fungi (Wang et al., 2015; Liu et al., 2017; Geng et al., 2018). The first report on bacterial laccase was published by Givaudan et al. (1993), when they studied a plant-growth-promoting non motile strain of *Azospirillum lipoferum* was isolated from rice rhizosphere, whereas confirmation of laccase genes in bacteria was published by Alexandre and Zhulin (2000) using fungal laccase gene sequences as probes. This discovery provided optimism for the further detection of measurable laccase activities in other bacteria, which information had previously been lacking.

Consequently, there is an increasing amount of information concerning the occurrence of laccase producing bacteria from diverse environments, where they are involved in a variety of metabolic processes. Bacterial laccases, which are putatively secreted during the idiophase could either be inducible or constitutive, however, this phenomenon is dependent on the cultural and nutritional factors present in

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the surrounding medium. Their eco-friendliness and environmental innocuousness has made the pursuit of novel and high-profile laccase producing bacterial strains a genuine objective (Odeniyi et al., 2017). Additionally, such interesting groups of enzymes could be synthesized using a wide range of agricultural wastes as cheap feedstock, which serve the purpose of reducing the cost of large largescale production with the concurrent alleviation of environmental toxicity of the residues, making them useful for agricultural purposes or could be engineered to suit other sustainability goals.

Therefore, taking these into consideration, different cultural and nutritional variables were screened with the aim of improving laccase yield, with especial focus on the valorization of selected agroindustrial residues. The traditional method of screening entails the screening of one variable or factor at a time (OVAT/OFAT), which is often associated with major drawbacks such as inaccurate estimation of interaction between independent variables in the experiment, and the unprofitable expenditure of time and energy. However, a further screening of significant independent variables using statistical design of experiments could explain the interaction effect among different variables synchronously. As such, optimization of cultural and nutritional components by response surface methodology has been reported for a modest number of bacterial isolates, however, to our knowledge, there is insufficient information on the use of statistical design of experiments (DOE) for optimization of laccase production from *Achromobacter xylosoxidans* and *Bordetella bronchiseptica* using agro-industrial residues. Therefore, this report served to establish the relationship between the two betaproteobacteria and optimized medium components using the DOE algorithm toward an enhanced laccase production.

2. Materials and methods

2.1. Sample collection and fabrication

Agro-industrial residues were obtained either on site or were stacked up due to purchase of edible parts. Maize stover (MS) and wheat bran (WB) were gathered from the University of Fort Hare farm, post-harvest, while mandarin peelings (MP) and grape stalks (GS) were reserved during the consumption of purchased fruits. All biomasses were washed, oven dried to a constant weight and milled prior to their experimental evaluation.

2.2. Bacterial culture and inoculum preparation

Laccase-producing bacteria coded Hb9c and Hb16c, which were isolated from wood marsh in Hogsback Forest, Eastern Cape were collected from the Biocatalysis repository in AEMREG culture collection. They have been identified molecularly using 16S rRNA sequence analysis as *Achromobacter xylosoxidans* HWN16 and *Bordetella bronchiseptica* HSO16 with accession numbers MF073257 and MF073258 respectively from GenBank, National Centre for Biotechnology Information (NCBI). The axenic cultures from 24 h old streaked plates were twice washed with physiological saline, and then standardized with 0.5 MacFarland Standard to give an optical density (OD 600 nm) comparable to 1.5×10^8 cfu/mL, which were used as the inoculum for subsequent studies.

2.3. Laccase production and extraction

Milled mandarin peelings (5 g/L) and wheat bran (10 g/L) were used as sources of carbon for the ultimate production of laccase, respectively. However, the original medium (20 mL) had the following basal composition (g/L): glucose; 3, $NaNO_3$; 1.24, KH_2PO_4; 0.514, K_2HPO_4; 0.32, $KNaC_4H_4O_6.4H_2O$; 0.32, NaCl; 0.08, $MnSO.H_2O$; 0.032, $MgSO_4.7H_2O$; 0.192, $CaCl_2.2H_2O$; 0.008, $CuSO_4.5H_2O$; 0.0008, $FeCl_3.7H_2O$; 0.0008, $ZnSO_4$; 0.0008, vanillin; 0.012. (Sigma-Aldrich, South Africa) in pH 5, Citrate buffer, and was autoclaved at 121 °C 15 psi prior to inoculation. The respective cultures were thereafter incubated in an orbital shaker at 140 rpm, 30 °C for 96 h, while aliquots were harvested post-incubation and centrifuged at 15000 rpm for 12 min at 4 °C using a benchtop centrifuge (SIGMA-1-14k). The resultant supernatant was used to assay for laccase activity.

2.4. Laccase activity assay

Laccase activity was measured by monitoring the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate according to the method of Unuofin et al. (unpublished). Briefly, a 50 µL aliquot of appropriately diluted crude laccase was reacted with 2 mM ABTS in potassium phosphate buffer (pH 6) at 30 °C, and the reaction was terminated after 10 min with 40 µL 20% TCA. The changes is absorbance due to oxidation of ABTS was monitored spectrophotometrically at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) using a SynergyMX 96-well microtitre plate reader (BioTeK Instruments). One unit of enzyme activity was defined as the amount of enzyme oxidising 1 µmol of ABTS per minute under the aforementioned conditions.

2.5. Medium optimization using traditional methods

Laccase production was optimized in the OVAT mode, which was based on varying the pH, temperature, agitation, carbon sources, nitrogen sources, agroindustrial residues, and aromatic and inorganic inducers, independent of other factors in the experiment. One percent (1% w/v) carbohydrates (glucose, trehalose, xylose, cellobiose, fructose, galactose), and an aliphatic alcohol (glycerol), were varied as carbon sources, while 0.2% w/v nitrogenous sources ($NaNO_3$, KNO_3, NH_4NO_3, L-asparagine, yeast extract, tryptone), and 0.05% inducers ($CoCl_2$, $CuSO_4$, ferulic acid, acetaminophen, 4-nitrophenol, vanillic acid, 2,5-xylidine) were screened for laccase production. Significantly, four agro-industrial residues (MS, WB, GB and MP) were varied.

2.6. Optimization of fermentation conditions using experimental design and response surface methology

Process optimization was achieved with response surface methodology (RSM) to optimize the concentration of selected cultural and nutritional factors from OVAT screening. Four independent variables were selected (pH, agitation, nitrogen and agroindustrial residue) from the traditional screening that significantly contributed to laccase production (Table 1). A central composite design (CCD) was generated by the Design-Expert software (Stat-Ease, Inc., Minneapolis, USA, Trial version 10.0.3) and was applied to interpret the interactions of these independent variables on laccase production. A face-centered experimental design of 30 experiments (3-level-4-factorial) with central points set at zero was formulated, and the experiments were run in 100 mL bottles containing 20 mL of media prepared according to the design and inoculated with 400 µL standardized culture. The flasks were incubator at 30 °C for 96 h, all experiments were conducted in triplicates. The results of the OVAT screening were fit to a second-order quadratic model, Eq. (1) to represent the behaviour of the algorithm. The response plots were designed to understand the interaction of different variables, and were used to assess the optimized components of

Table 1a
Levels of significant variables used in central composite design for Hb9c.

Independent variables	Level			
	Symbol	−1	0	1
pH	$A(X_1)$	3	4	5
Mandarin peelings	$B(X_2)$	2	1.5	2
Agitation speed	$C(X_2)$	50	75	100
$NaNO_3$	$D(X_4)$	0.1	0.15	0.2

Table 1b
Levels of significant variables used in central composite design for Hb16c.

Independent parameters	Level			
	Symbol	−1	0	1
pH	A(X_1)	3	4	5
Wheat Bran	B(X_2)	2	1.5	2
Agitation speed	C(X_2)	50	75	100
Yeast Extract	D(X_4)	0.1	0.15	0.2

the medium, which influences the responses.

$$Y = \beta_o + \sum_{i=1}^{k} \beta i x i + \sum_{i=1}^{k} \beta i i x i i 2 + \sum_{i=1}^{k} \neq \sum_{j=1}^{k} \beta i j x i x j + \varepsilon \quad (1)$$

Y = Responses

K = Total number of independent factors
β_o = Intercept
β_i, β_{ii}, and β_{ij} = Coefficient values for linear, quadratic and interaction effects respectively.
x_i and x_j indicate coded levels for independent variables.

3. Results and discussion

Medium components such as carbon source, nitrogen source, and their respective composition, as well as environmental factors like pH and incubation temperature have been proven to be crucial for laccase production (Asgher et al., 2016; Chenthamarakshan et al., 2017). As such, this study was driven to optimize the different agroindustrial wastes, along with other medium components and environmental factors to the end of an enhanced laccase production from the betaproteobacteria examined so far.

3.1. Determination of significant variables

Optimal nutritional and cultural factors were first screened for, using the one-variable-at-a-time approach, where each of the variables considered were amended, with other factors in the experiment being constant. The isolates, coded Hb9c (*Achromobacter xylosoxidans* HWN16) and Hb16c (*Bordetella bronchiseptica* HSO16) considered in this study both recorded pH 5 (cca. 3.85 and 7.7 U/mL) as optimal for their respective productions of laccase, although a considerably measurable activity was observed for Hb16c at pH 4 (cca. 3.85 U/mL) (data not shown). A similar result was reported by Odeniyi et al. (2017) where the acidic pH regime (3–6) was optimal for laccase production from two bacterial strains. Specifically, Patel and Gupte (2016) observed maximum production of laccase from *Tricholoma giganteum* AGHP per gram of dry substrate at pH 5, however no increase in laccase production was recorded when pH was increased further. Conversely, alkalophilic species of Bacillus were reported to yield maximum laccase titres at an initial pH of 10.5 (Gupta et al., 2015). Although the pH of the medium was only amended with Na_2CO_3 in tap water, which could fluctuate as metabolic activities intensify, the examined strains could be useful in the treatment of effluents from the pulp and paper industry, where pH regimes are mostly alkaline. The influence of pH of any given medium is critical for growth and enzyme production as it strongly affects many enzymatic processes and transport of various components across the cell membrane (Ferreira et al., 2015; Sharma et al., 2017). Therefore, choosing the right buffer is desired for favourable microbial cell proliferation and unabated synthesis of interesting metabolites of particular interest.

Glycerol, an aliphatic alcohol, was preferred as carbon source for Hb9c compared to trehalose which was utilized best by Hb16c, however these outcomes paled in comparison to the copious amounts of

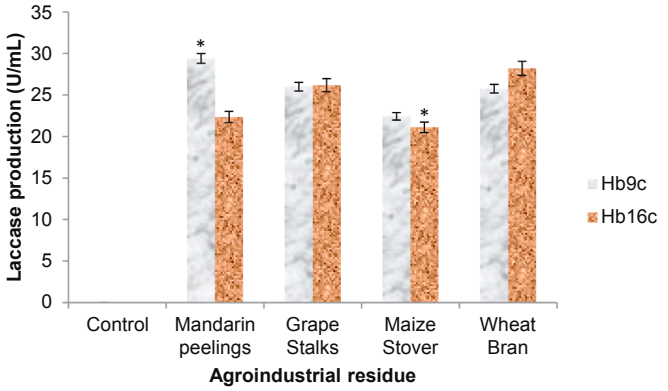


Fig. 1. a: Estimation of bacterial laccase produced from agroindustrial residues. Asterisks indicate significant difference ($p < 0.05$) in laccase production among treatments. **b**: Estimation of bacterial laccases produced from nitrogenous sources. Asterisks indicate significant difference ($p < 0.05$) in laccase production among treatments. **c**: Estimation of bacterial laccases produced from different inducers. Asterisks indicate significant difference ($p < 0.05$) in laccase production among treatments.

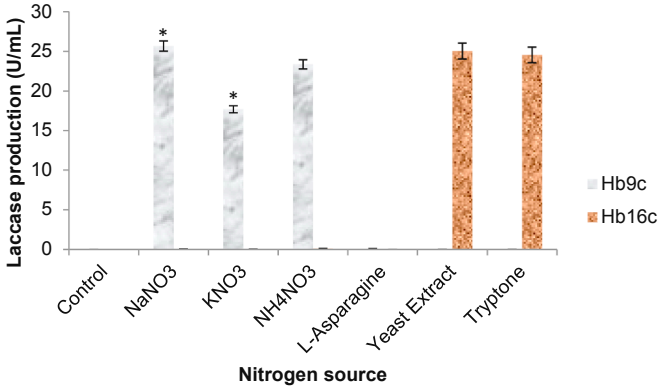


Fig. 1. (*continued*)

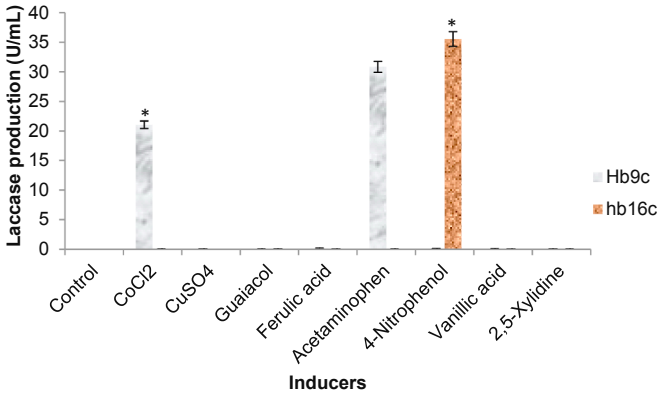


Fig. 1. (*continued*)

laccases produced when agroindustrial residues were employed (Fig. 1). Although both isolates had individual preferences for the residues optimally utilized (Hb9c; MP and Hb16c; WB) with 3.22 and 11.8 fold increase recorded respectively when they replaced the simpler sources of carbon, they collectively recorded GS as second best substrate for laccase production, albeit there was no significant difference ($P < 0.05$) between the influence of WB and GS on laccase production in Hb9c. This may likely imply its potential as an emerging feedstock for bioprocesses, hence the possible prospects of it being a component of a co-substrate is worth visiting in the near future, which is

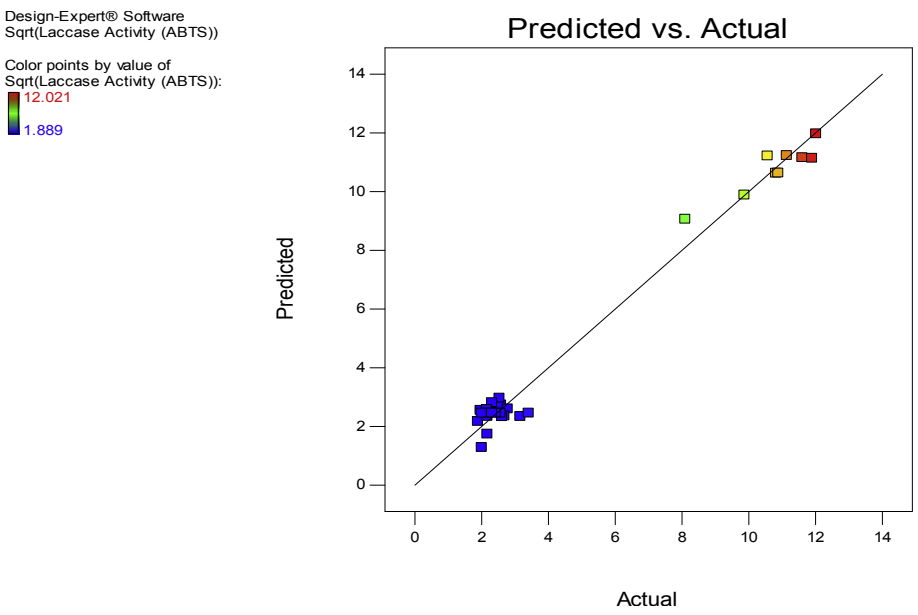
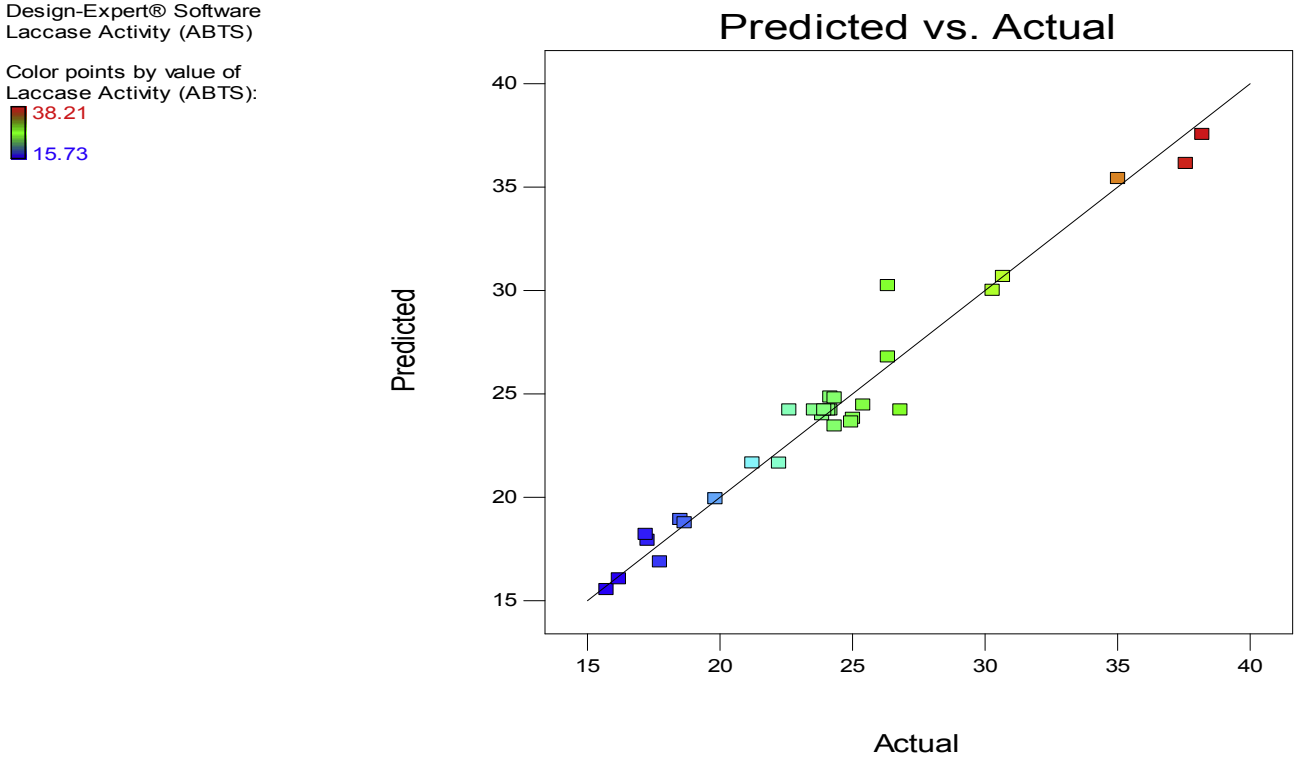


Fig. 2. a: Predicted response versus experimental value for Hb9c laccase. **b**: Predicted response versus experimental value for Hb16c laccase.

anticipated to significantly reduce the production overhead. Kachlishvili et al. (2016) revealed that a MP supplemented medium selectively induced the production of appreciable amounts of lignin-modifying enzymes, whereas WB served as an excellent substrate for a high level production of laccase from *Cerrena unicolor* C-139 (Songulashvili et al., 2015). Other promising lignocellulosic substrates for improved laccase yield are pineapple leaf (Chenthamarakshan et al., 2017) and corn cobs (Noreen et al., 2015). The individual preference of the isolates for the selected agroindustrial residues may not be overtly explained at present, however, the chemical composition of each the substrates and the roles they play could be implicated in future elucidations. The composition and functionality of wheat bran has been reviewed by Onipe et al. (2015), while the functional potentials of mandarin and associated citrus peels has been revealed in a review by Rafiq et al. (in press).

Similarly, both isolates recorded different optimal nitrogen sources (Fig. 2). The inorganic sources of nitrogen were preferred by Hb9c, while Hb16c was more responsive the organic sources; $NaNO_3$ produced the highest measurable activity in Hb9c (cca. 26 U/mL), while yeast extract was eminent for laccase production in Hb16c (cca. 25 U/mL). In another study, KNO_3 demonstrated a significant enhancement in laccase production (Das et al., 2016). The inorganic nitrate optimal for laccase production might have been readily metabolized due to its swift solubility and lower molecular weight, which might have



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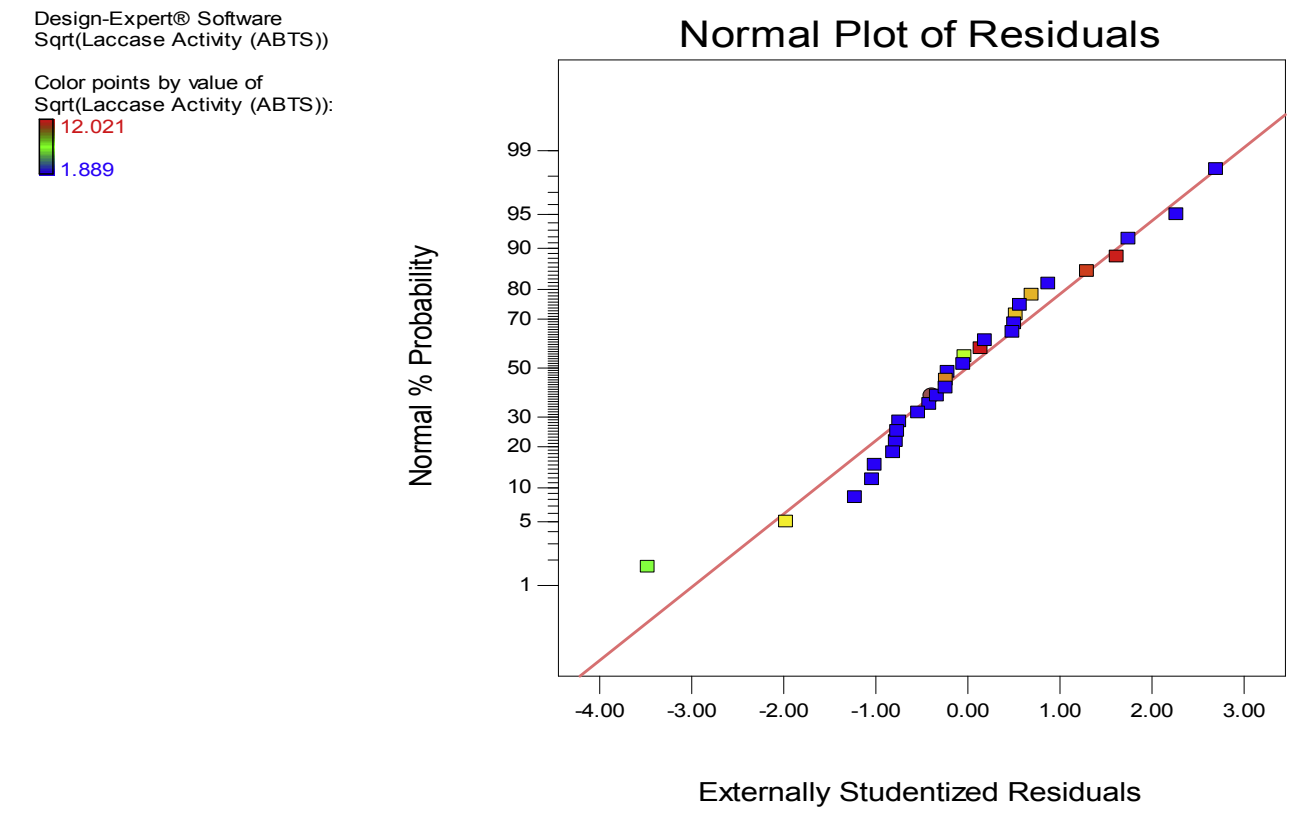


Fig. 3. a: Normal plot of residuals for Hb9c responses. **b**: Normal plot of residuals for Hb16c responses.

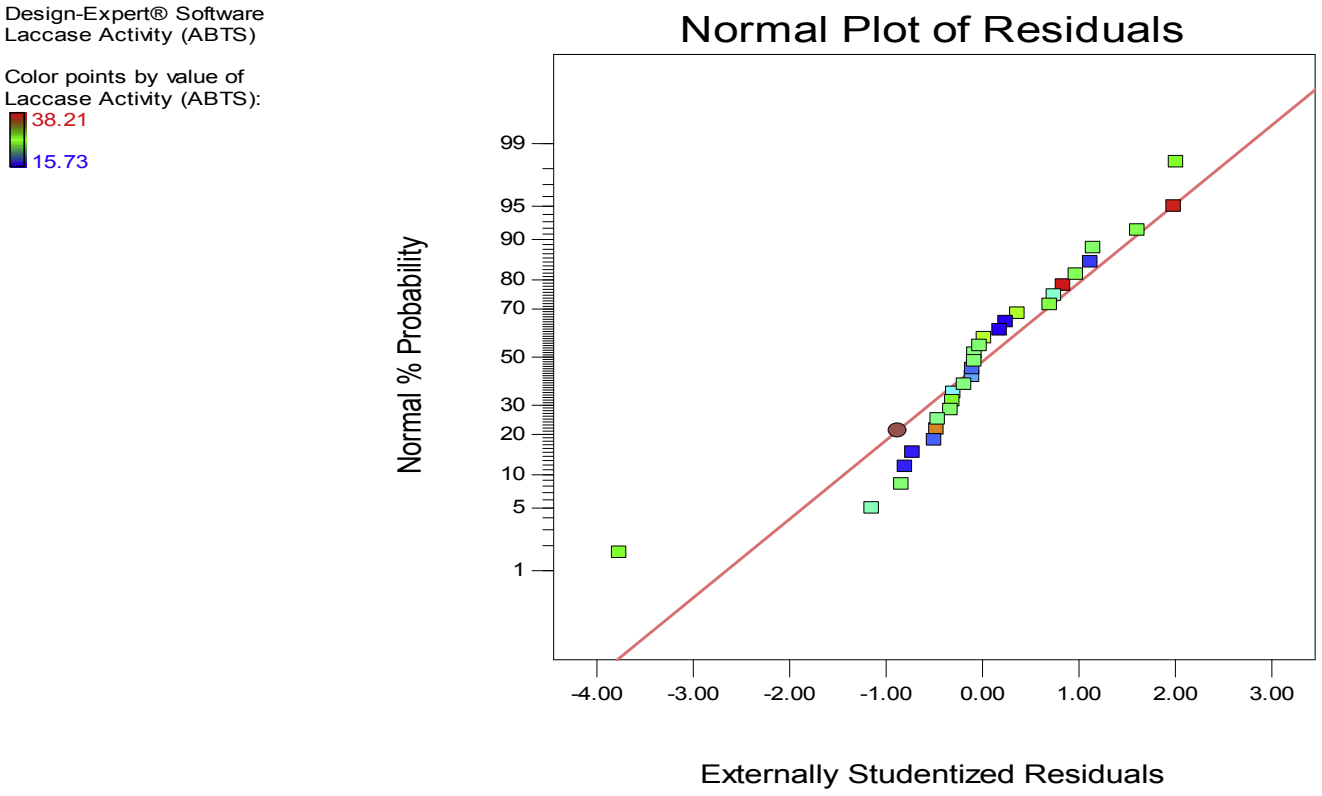


Fig. 3. (*continued*)

facilitated its early depletion, and hence laccase production, since laccase is mostly produced under nitrogen limiting conditions. Contrariwise, Othman et al. (2018) reported maximum secretion of laccase in an organic nitrogen-supplemented medium compared to inorganic forms. The outcome of our study therefore depicts the submission of Collins and Dobson (1997) that the effect of nitrogenous sources on laccase outputs by heterogeneous bacterial strains tend to be considerably controversial.

Table 2a
CCD design matrix for laccase production in Hb9c.

Independent Variables (*X*)					Dependent variables (*Y*) Sqrt. Laccase Activity (U/mL)	
Run Order	pH	Mandarin peels (g/ 200 mL)	Agitation Speed (rpm)	$NaNO_3$ (g/ 200 mL)	Actual Value	Predicted Value
1	4(0)	1.5(0)	100(1)	0.15(0)	2.17	2.34
2	4(0)	1(-1)	75(0)	0.15(0)	1.97	2.55
3	4(0)	1.5(0)	75(0)	0.15(0)	2.14	2.46
4	4(0)	1.5(0)	50(-1)	0.15(0)	2.17	1.74
5	4(0)	1.5(0)	75(0)	0.15(0)	2.43	2.46
6	4(0)	1.5(0)	75(0)	0.15(0)	2.31	2.46
7	3(-1)	2(1)	100(1)	0.1(-1)	2.40	2.48
8	5(1)	1(-1)	50(-1)	0.2(1)	10.88	10.63
9	3(-1)	2(1)	50(-1)	0.2(1)	3.16	2.34
10	4(0)	1.5(0)	75(0)	0.15(0)	2.01	2.46
11	3(-1)	1.5(0)	75(0)	0.15(0)	2.31	2.81
12	3(-1)	1(-1)	50(-1)	0.1(-1)	1.89	2.17
13	4(0)	2(1)	75(0)	0.15(0)	2.69	2.36
14	5(1)	2(1)	50(-1)	0.2(1)	8.10	9.06
15	4(0)	1.5(0)	75(0)	0.1(-1)	2.61	2.33
16	5(1)	1(-1)	100(1)	0.2(1)	12.02	11.97
17	5(1)	1.5(0)	75(0)	0.15(0)	11.90	11.14
18	3(-1)	1(-1)	100(1)	0.2(1)	2.78	2.60
19	4(0)	1.5(0)	75(0)	0.15(0)	2.57	2.46
20	3(-1)	2(1)	100(1)	0.2(1)	2.54	2.96
21	3(-1)	2(1)	50(-1)	0.1(-1)	2.31	2.60
22	5(1)	1(-1)	50(-1)	0.1(-1)	10.82	10.63
23	5(1)	1(-1)	100(1)	0.1(-1)	10.57	11.21
24	3(-1)	1(-1)	50(-1)	0.2(1)	2.58	2.73
25	3(-1)	1(-1)	100(1)	0.1(-1)	2.01	1.28
26	5(1)	2(1)	50(-1)	0.1(-1)	9.87	9.88
27	5(1)	2(1)	100(1)	0.2(1)	11.61	11.16
28	5(1)	2(1)	100(1)	0.1(-1)	11.14	11.23
29	4(0)	1.5(0)	75(0)	0.15(0)	3.41	2.46
30	4(0)	1.5(0)	75(0)	0.2(1)	2.16	2.58

NB: coded values are in parenthesis.

Table 2b
CCD design matrix for laccase production in Hb16c.

Independent Variables (*X*)					Dependent variables (*Y*) Laccase Activity (U/mL)	
Run Order	pH	Wheat bran (g/ 200 mL)	Agitation Speed (rpm)	Yeast Extract (g/ 200 mL)	Actual Value	Predicted Value
1	3(-1)	2(1)	100(1)	0.1(-1)	35.03	35.41
2	4(0)	1.5(0)	75(0)	0.15(0)	23.95	24.22
3	4(0)	1.5(0)	75(0)	0.15(0)	24.18	24.22
4	4(0)	1.5(0)	75(0)	0.15(0)	26.82	24.22
5	4(0)	1(-1)	75(0)	0.15(0)	21.24	21.66
6	5(1)	2(1)	50(-1)	0.1(-1)	17.75	16.87
7	4(0)	1.5(0)	75(0)	0.15(0)	22.63	24.22
8	3(-1)	2(1)	50(-1)	0.2(1)	30.69	30.68
9	5(1)	2(1)	100(1)	0.2(1)	18.68	18.76
10	5(1)	1(-1)	100(1)	0.2(1)	16.20	16.05
11	4(0)	1.5(0)	75(0)	0.15(0)	24.10	24.22
12	4(0)	2(1)	75(0)	0.15(0)	26.35	26.78
13	3(-1)	1(-1)	100(1)	0.1(-1)	24.34	23.44
14	4(0)	1.5(0)	75(0)	0.1(-1)	25.42	24.46
15	3(-1)	1(-1)	50(-1)	0.2(1)	25.03	23.81
16	4(0)	1.5(0)	75(0)	0.15(0)	23.56	24.22
17	4(0)	1.5(0)	50(-1)	0.15(0)	24.34	24.80
18	5(1)	1(-1)	50(-1)	0.1(-1)	17.28	17.92
19	5(1)	1.5(0)	75(0)	0.15(0)	17.21	18.20
20	3(-1)	1(-1)	100(1)	0.2(1)	24.18	24.85
21	5(1)	2(1)	100(1)	0.1(-1)	18.52	18.92
22	3(-1)	2(1)	50(-1)	0.1(-1)	38.21	37.54
23	3(-1)	2(1)	100(1)	0.2(1)	37.59	36.14
24	5(1)	1(-1)	100(1)	0.1(-1)	15.73	15.53
25	3(-1)	1.5(0)	75(0)	0.15(0)	26.35	30.23
26	5(1)	2(1)	50(-1)	0.2(1)	19.84	19.93
27	3(-1)	1(-1)	50(-1)	0.1(-1)	30.30	30.01
28	5(1)	1(-1)	50(-1)	0.2(1)	22.24	21.65
29	4(0)	1.5(0)	75(0)	0.2(1)	23.87	23.98
30	4(0)	1.5(0)	100(1)	0.15(0)	24.96	23.64

NB: coded values are in parenthesis.

Aromatic and some inorganic compounds when supplemented into a chemically defined medium could act as inducers for enhanced laccase production. Notable inducers reported so far are 2,5-xylidine, guaiacol, $CuSO_4$, veratryl alcohol, etc. Hence, in our study, we evaluated eight potential inducers of different classes (Fig. 3). Results showed that Hb9c was best induced by Acetaminophen (cca. 31 U/mL) while a 4-nitrophenol-supplemented medium elicited the highest laccase output in Hb16c (cca. 36 U/mL). However, only traces of measurable laccase activities were discovered for most of the other compounds assayed. A reason for this is not especially decipherable, but an in-depth understanding of the physiology of the bacterial strains tested would be invaluable. Patel and Gupte (2016) recorded a yield index of 2.15 when they supplemented their production medium with 0.3 mM cupric sulphate, a famously reported laccase inducer, any further increase in concentration led to a decrease in enzyme production, although their study was carried out on fungi, while Kuhar and Papinutti (2014) reported ferulic acid to be contributory to enhanced laccase production. Most natural and synthetic organic inducers routinely employed in laccase production so far are aromatic compounds that are structurally analogous to lignin fractions. The importance of inducers in the production of laccase and other remarkable enzymes on industrial scale could be the high titres of enzymes they afford in a relatively short fermentation period, however, the task of identifying their optimal concentration is essential. Although, to our knowledge, there has been no reports concerning the inducement of laccase production by acetaminophen and 4-nitrophenol, their significance in environment can not be annulled, since they could exhibit recalcitrance and hepatoxicity. This suggests that effluents of wastewater treatment plants which customarily contain these aromatic compounds could be adopted as novel supporting medium for the production of novel biomolecules in a submerged fermentation system.

Finally, both isolates recorded maximum activity at a low agitation speed (100 rpm), which implies enzyme secretion was not dependent on

Table 3a
Analysis of variance (ANOVA) for response surface reduced quadratic model for laccase production in Hb9c.

Source	Sum of squares	*df*	Mean square	*F* value	*p* value prob > *F*	
Model	448.39	12	37.37	101.50	< 0.0001	significant
A-pH	311.88	1	311.88	847.16	< 0.0001	significant
B-Mandarin Peel	0.16	1	0.16	0.44	0.5152	
C-Agitation Speed	1.64	1	1.64	4.46	0.0499	significant
D-NaNO3	0.27	1	0.27	0.74	0.4011	
AB	1.39	1	1.39	3.77	0.0689	
AC	2.16	1	2.16	5.88	0.0268	significant
AD	0.32	1	0.32	0.86	0.3679	
BC	0.57	1	0.57	1.56	0.2287	
BD	0.68	1	0.68	1.85	0.1911	
CD	0.56	1	0.56	1.53	0.2327	
A^2	70.29	1	70.29	190.92	< 0.0001	significant
C^2	0.59	1	0.59	1.60	0.2233	
Residual	6.26	17	0.37			
Lack of Fit	5.01	12	0.42	1.68	0.2956	not significant
Pure Error	1.24	5	0.25			
Cor Total	454.65	29				

R^2 = 0.9862, Adj R^2 = 0.9765, Pred R^2 = 0.9284, Adeq Precision = 26.751, C.V. % = 12.34.

Table 3b
Analysis of variance (ANOVA) for response surface cubic model for laccase production in Hb16c.

Source	Sum of squares	*df*	Mean square	*F* value	p value Prob > *F*	
Model	926.15	11	84.20	41.61	< 0.0001	significant
A-pH	651.24	1	651.24	321.82	< 0.0001	significant
B-Wheat Bran	118.17	1	118.17	58.40	< 0.0001	significant
C-Agitation Speed	6.07	1	6.07	3.00	0.1005	
D-Yeast Extract	1.01	1	1.01	0.50	0.4893	
AB	73.66	1	73.66	36.40	< 0.0001	significant
AC	1.49	1	1.49	0.74	0.4014	
AD	20.41	1	20.41	10.08	0.0052	significant
BC	19.65	1	19.65	9.71	0.0060	significant
BD	0.46	1	0.46	0.23	0.6396	
CD	4.81	1	4.81	2.38	0.1406	
ACD	29.19	1	29.19	14.42	0.0013	significant
Residual	36.42	18	2.02			
Lack of Fit	26.61	13	2.05	1.04	0.5227	not significant
Pure Error	9.81	5	1.96			
Cor Total	962.58	29				

R^2 = 0.9622, Adj R^2 = 0.9390, Pred R^2 = 0.8847, Adeq Precision = 24.463, C.V. = 5.87.

their requirement for saturated oxygen. Furthermore, this outcome could be consequent of the physiological response of the isolates to modest supply of oxygen, whereby the particular metabolic pathway responsible for the bioconversion of substrates and the simultaneous secretion of laccase is maintained. Conversely, as oxygen availability increases with agitation rates, there could be a decline in respiration of the cells, due to increasing dissolved oxygen tension and therefore causing a lag in substrate bioconversion and enzyme secretion. A relatively low agitation speed (130 rpm) was recorded as optimum for laccase production in a *Klebsiella pneumoniae* strain (Gaur et al., 2018). Niladevi and Prema (2008) however reported standard agitation (150–175 rpm) to have the best influence on laccase production with *Streptomyces psammoticus*, while Sondhi et al. (2015) reported a similar outcome for *Bacillus tequilensis*. Other ratiocinations concerning the preference for agitation and aeration toward enzyme production and other metabolic activities have been enumerated by Tinoco-Valencia et al. (2014) and Ihssen et al. (2015). Since the selection of suitable cultural and nutritional parameters is the leading strategy for the success of any optimization process, we selected some significant nutritional and cultural factors during the preliminary, OVAT studies which emphasised the need for carbon and nitrogen sources among other cultural parameters.

3.2. Response surface methology

The inability of the just concluded OVAT method to predict the effects of interactions among media components may expose its flaw in scale up experiments, and somehow reduce its reliability. The response surface methodology has proven useful in determining significant parameters and their corresponding optimal levels (Dean et al., 2017). Hence, we explored response surface methodology where we used CCD to study the interaction of the variables extrapolated from the preliminary screening (OVAT). The results obtained from the 30 experimental runs generated by the CCD were fit into a second-order model shown in Table 2a and b, respectively. The results were analyzed by analysis of variance (ANOVA), along with their respective predicted and observed responses. A multiple regression analysis of the experimental data yielded the following noise-free second-order polynomial regression equation to explain laccase production by Hb9c and Hb16c, respectively.

Hb9c Laccase Production (U/mL):

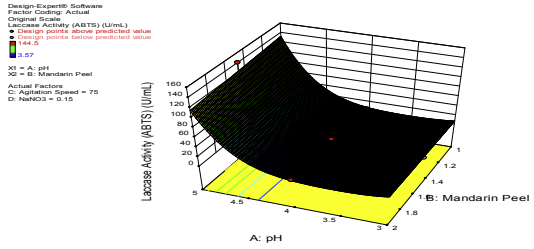
$$Y(Sqrt.) = 2.6 + 4.16\,A - 0.095\,B + 0.30\,C + 0.12\,D - 0.29AB + 0.37AC - 0.14AD + 0.19BC - 0.21BD + 0.19CD + 4.52A^2 - 0.41C^2 \quad (2)$$

Hb16c Laccase Production (U/mL):

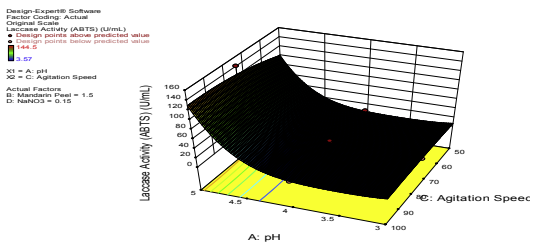
$$Y = 24.22 - 6.02\,A + 2.56\,B - 0.58\,C - 0.24\,D - 2.15AB - 0.31AC + 1.13AD + 1.11BC - 0.17BD + 0.55CD - 1.35ACD. \quad (3)$$

Where Y is the measurable laccase activity (U/mL), A, B, C and D are pH, carbon source, agitation speed and nitrogen source for the isolates, respectively.

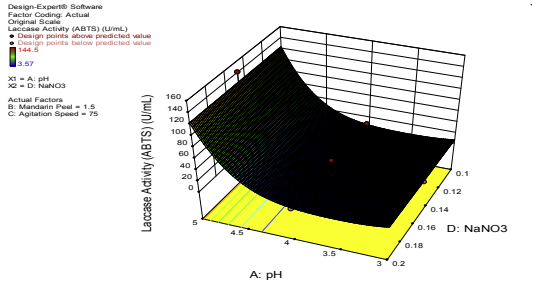
Their respective ANOVA tables are displayed as Table 3a and b, respectively. The experimental data proved a good fit with the second



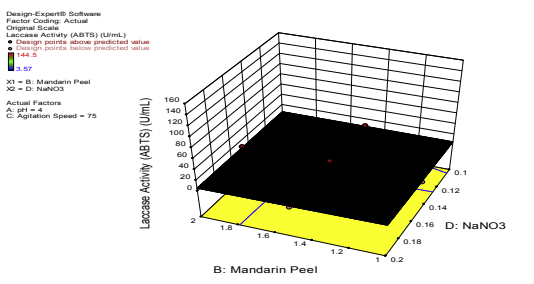
4a: Effect of pH and MP



4b: Effect of pH and agitation speed

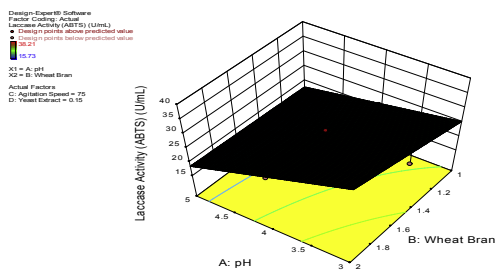


4c: Effect of pH and $NaNO_3$

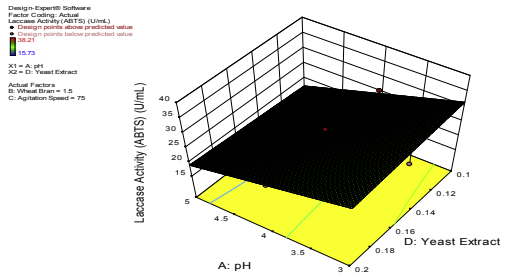


4d: Effect of MP and $NaNO_3$

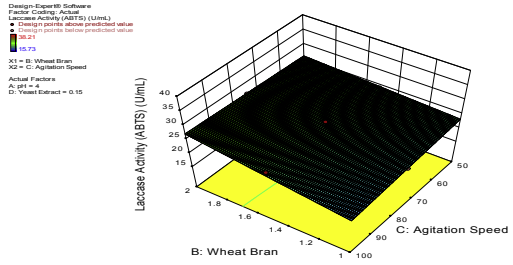
Fig. 4. a – d: Response plots showing different interaction effects on laccase production in Hb9c (HWN16).



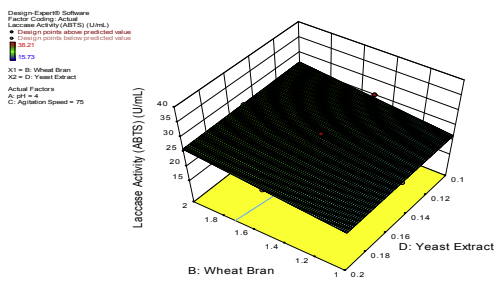
5a: Effect of pH and wheat bran



5b: Effect of pH and yeast extract



5c: Effect of wheat bran and agitation speed



5d: Effect of wheat bran and yeast extract

Fig. 5. Response plots showing different interaction effects on laccase production in Hb16c (HSO16).

order polynomial equations, which were statistically relevant at $P < 0.05$ level, although Hb16c was best suited by a cubic model as opposed to the quadratic model of Hb9c. Also, ANOVA for laccase production in both isolates implied that their respective models were apposite, with *F*-values of 101.5 and 41.61. Furthermore, the rectitude of both models is highlighted by their significant algorithms and statistically nonsignificant lack-of-fit (0.2956 and 0.5227). This is rational because a nonsignificant lack-of-fit is desirable, besides, we want the models to fit. The lack of fit test compares the residual error to the pure error from the replicated design points.

Model terms with values of "Prob > *F*" less than 0.05 were considered significant. Therefore, in our study, A, C, AC and A^2 were significant model terms for Hb9c while A, B, AB, AD, BC and ACD were significant model terms for Hb16c. More specifically, it was observed that the linear effects of pH (A) for both isolates were highly significant ($P < 0.001$), while Hb16c correspondingly recorded a high significance for the quadratic effect of pH, i.e. A^2 ($P < 0.0001$), linear effect of WB (B) and its interaction effect with pH, i.e. AB ($P < 0.0001$), thereby bearing some similarity with the investigation of Bagewadi et al. (2017). The goodness of fit of the model was confirmed by their determination coefficients (R^2), which are displayed in the respective ANOVA tables, and they depict a reasonable agreement between R^2 values highlighted. Thus, 98.62% and 96.22% variabilities of the response could be explained, which means the model fitted well with the experimental data obtained, just as corroborated by Srivastava et al. (2018). An adequate precision ratio value greater than 4 is desirable because it suggests acceptable discrimination of the model. The coefficient of variation and the adequate precision values of Hb9c (12.34; 26.75) and Hb16c (5.87; 24.46) indicate a low disparity between the predicted and experimental values. Also, the adequate signals indicated by the precision values show the models can be used to navigate the design space. This implies, the regression models bestow a brilliant interpretation of the connection between the independent variables and the responses (Sharma et al., 2009). The relationship between predicted and actual responses of laccase activity (Fig. 2a and b), and the normal plot of residuals of both isolates are shown Fig. 3a and b. Here, a good fit of model, and a satisfactory correlation between the actual and predicted values was illustrated by the proximity of the cluster measurements to the diagonal line in the parity plots. From the experimental matrices (Table 2a and b), maximum responses of laccase activity were obtained with a Hb9c inoculated medium of the following conditions; pH 5, 1 g MP, 100 rpm and 0.2 g $NaNO_3$, thereby producing a measurable laccase activity of 144.5 U/mL after 96 h of incubation, while Hb16c at the same incubation period recovered a 38.21 U/mL

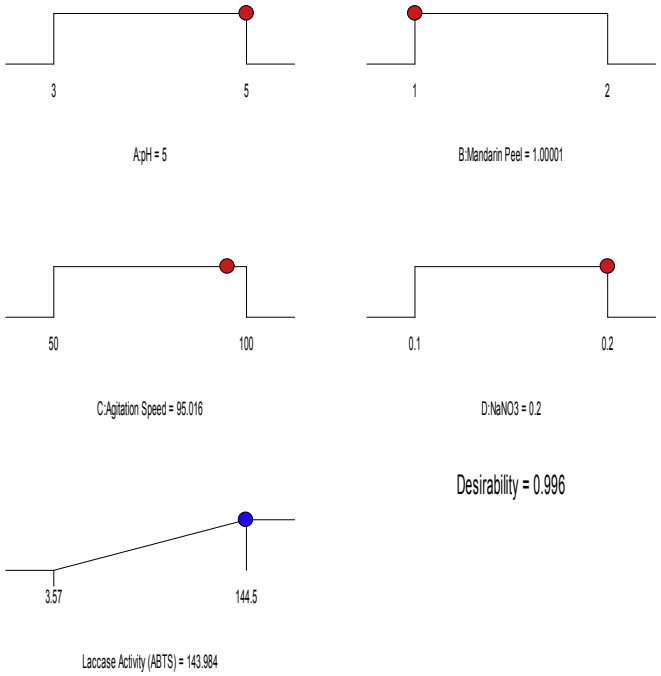


Fig. 6. a: Desirability ramp of laccase production in Hb9c. **b**: Desirability ramp for laccase production in Hb16c.

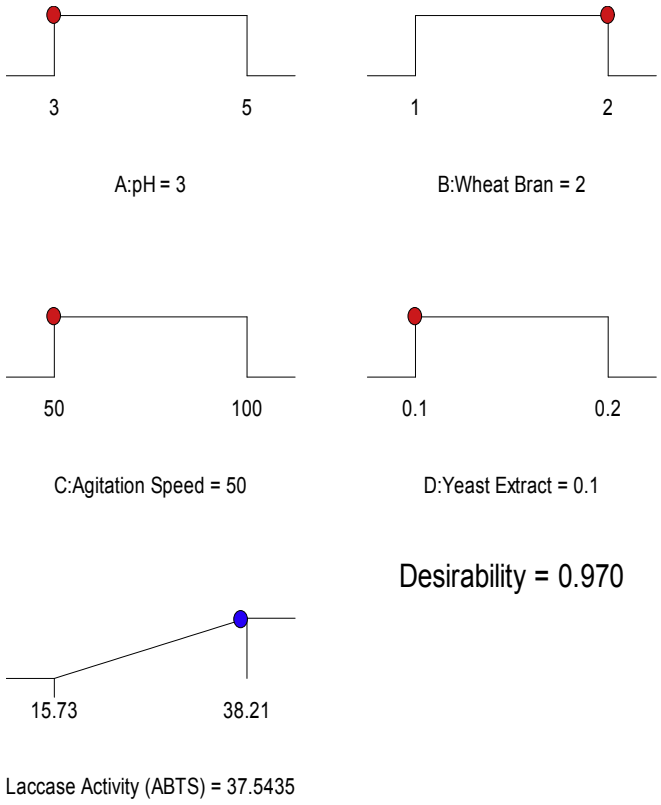


Fig. 6. (*continued*)

equivalent from an unusually different condition (pH 3, 2 g WB, 100 rpm and 0.2 g yeast extract). Minimum detectable activity observed in Hb9c was 3.57 U/mL (pH 3, 1 g MP, 50 rpm, 0.1 g $NaNO_3$), while Hb16c presented a comparably higher minimum response (15.73 U/mL) with pH 5, 1 g WB, 50 rpm, 0.2 yeast extract. A greater insight of the interaction of some variables on laccase production was presented by surface plots of the estimated responses (Figs. 4a–d and 5a – d).

A closer look at the aforementioned figures reveals the levels of two independent factors were varied, while the other two remained constant. Fig. 4a–d emphasizes the dependence of the other variables on the medium pH as corresponding synchronous increases in agitation speed and MP concentrations with increasing pH regimes in Hb9c resulting in improved laccase outputs. However, the effect of changes in $NaNO_3$ concentration at increasing pH regimes was not noticeable, just as the neutrality of the contributions MP and $NaNO_3$ towards laccase production was highlighted. Conversely, the evaluation of selected interactions in Hb16c (Fig. 5a–d) showed that a decline in pH with a simultaneous increase in concentration of WB and yeast extract was required for maximum laccase production, which is similar to the observations of Kuppusamy et al. (2017), where the interaction between starch and yeast extract was significant. Similarly, an increase in agitation speed with the corresponding increase in wheat bran concentration was positively contributory to laccase yield. This outcome was congruent with the interaction of increasing nutritional variables, likewise.

3.3. Numerical optimization and validation of the model

Numerical optimization comprised the choice of a desired goal for each factor and response. These goals were thereafter combined into an overall desirability function, which could range from zero to one, but not outside of the limits. This is usually done to maximize productivity by stretching all the interacting variables to the limit where the highest yield is achievable. Therefore, to obtain a maximum output of 143.98 U/mL from Hb9c, the optimal conditions were pH 5, 1.00 g MP, 95.016 rpm and 0.2 $NaNO_3$ with a desirability function of 0.996 (Fig. 6a). Similarly, for Hb61c, optimal conditions were pH 3, 2 g WB, 50 rpm and 0.1 g yeast extract with a desirability function of 0.97 (Fig. 6b). The closer the desirability value to 1, the more appropriate the selected fermentation conditions are for achieving optimal response, which is laccase production. Agarwal et al. (2016) achieved a desirability of 1 when they attempted the removal of a synthetic textile dye with an adsorbent. In addition, two random levels for the independent variables were assessed, which were not necessarily within the confines of the model algorithm. Altogether, experimental runs were carried out in triplicates to confirm the validity of the respective predictions (Table 4a and b), and results showed them to be close to the predicted values (cca. 99% similarity), hence the model was successfully validated. Furthermore, a striking outcome observed in both experimental runs was the maximum laccase outputs (Hb9c; 169.39 U/mL and Hb16c; 45.22 U/mL, respectively), which were obtained with a combinatorial effect of variable level outside the design space. Similarly, fermentation conditions generated by statistical models have been used to achieve tremendous outputs of laccase from agroindustrial residues (Bagewadi et al., 2017). In our study, the final measurable laccases produced were respectively 17.5 and 15.54 folds greater than outputs of the OVAT approach, thereby suggesting that the response surface model approach is sagacious and highly economically viable for improved yield in the industry.

Table 4a
Validation experiment for laccase production from Hb9c.

Confirmation Report	Independent variables				Response: Laccase Activity (U/mL)					
	pH	Mandarin peelings	Agitation Speed	$NaNO_3$	Predicted mean	Data mean	n	Std Dev.	95% PI low	95% PI high
1	5.00	1.00	95.02	0.20	143.984	144.027	3	14.552	115.742	174.494
2	5.00	0.50	100.00	0.25	168.31	169.39	3	15.7348	115.306	230.444
3	5.00	2.50	100.00	0.25	110.745	111.064	3	12.7598	68.6393	161.981

Table 4b
Validation experiment for laccase production from Hb16c.

Confirmation Report	Independent variables				Response: Laccase Activity (U/mL)						
	pH	Wheat Bran	Agitation Speed	Yeast Extract	Predicted mean	SE Pred.	Data mean	n	Std Dev.	95% PI low	95% PI high
1	3.00	2.00	50.00	0.10	37.5435	1.44	38.51	3	1.422	34.52	40.57
2	3.00	2.50	50.00	0.050	44.9161	2.56	45.22	3	1.422	45.22	50.29
3	3.00	0.50	50.00	0.25	17.458	2.56	17.62	3	1.422	12.08	22.83

4. Conclusions

The optimization of laccase production was carried out with traditional methods to identify significant nutritional and cultural factors, while the selected factors were further optimized statistically using central composite design. Here, the following outcomes were discovered; (1) the pH was very crucial to production of laccase, irrespective of the isolate assessed, (2) the employment of agroindustrial wastes as substrates for production gave an interestingly higher yield when compared to the other carbon and energy sources, however preferences for types of nitrogenous sources differed among the isolates. (3) the respective models were confirmed to be statistically significant with a desirable goodness of fit, hence they could properly explain a good fraction of the experimental data. Furthermore, the models were validated with further predictions outside the design space, which were proven to be of high accuracy by the their corresponding experimental runs that equally gave the highest yields. Bearing this in mind, we conclude, the exploitation of agroindustrial residues with respect to other significant nutritional and cultural media components, through the response surface models would be a good feat for an economically viable process in the biochemical industry.

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