ACTA SCIENTIFIC MICROBIOLOGY (ISSN: 2581-3226)

Volume 2 Issue 11 November 2019

Research Article

Application of Central Composite Designs Utilized for Scale-up Strategies in the Optimization Process for Development Laccase Production and for the Bioremediation of Various Industrial Pollutants

Soad A Abdelgalil[1], Ahmed M Attia[2], Reyed M Reyed[1]*and Nadia A Soliman[1]

[1]Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technological Applications, Alexandria, Egypt

[2]Institute of Graduate Studies and Research, Environmental Studies Department, Alexandria University, Egypt

***Corresponding Author:** Reyed M Reyed, Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technological Applications, Alexandria, Egypt.

Received: September 05, 2019; **Published:** October 11, 2019

DOI: 10.31080/ASMI.2019.02.0393

Abstract

The additives (activators or inhibitors) influencing the Alcaligenes faecalis NYSO (ac: KP859538) laccase productivity were studied in submerged fermentation as well as the exploitation the ultimate benefit from lingo-cellulosic waste by employing Central Composite Design (CCD) under Response Surface Methodology (RSM) was investigated in this study. Among the various amino acids and vitamins used as a growth factor and or/ nitrogen source, L-glutamine, L-cysteine, L-arginine, and biotin were found to be the most suitable for laccase production. Ethanol addition caused about 40% increment followed by petroleum ether, then acetonitrile and methanol whilst, isopropanol caused a slight decrease in production. Promotion of laccase production was achieved in media supplemented with fast blue, ethidium bromide and azure B by 37 - 35%. The humic acid, 2,2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS) and alkali lignin proved to be the best synthetic inducers for laccase. Among the various wastes used, sugarcane bagasse followed by black liquor and prickly pears peel were found to be the best natural substrates could be used for laccase production with maximum activity (1408 and 1365 U $ml^{-1}min^{-1}$), respectively. Considering this trend, pre-formulation media was designed using yeast extract, copper sulfate, and mixture of black liquor and sugarcane bagasse, thereafter, experimental modeling of Central composite design of RSM was adopted to acquire the best process conditions among the selected variables where a five-level Central Composite Design (CCD) was employed to create a polynomial quadratic model creating the relationship between these variables and laccase activity.

Keywords: Microbial Biotechnology Application; Laccase; Central Composite Design (CCD); Response Surface Methodology (RSM); Central Composite Response Surface Statistical Matrix; Analysis Of Variance "ANOVA Test" STATISTICA 5.0; Software *Alcaligenes faecalis* NYSO; Phenolic Compounds; Submerged Fermentation; Bacterial Bioremediation

Introduction

With industrialization, the pollution of the environment with hazardous compounds has become a dangerous problem. Recalcitrant and phenolic compounds are associated with wastes from several industrial processes [1]. Pulp and paper industry is considered as one of the most polluter industry around the world [2], large amounts of toxic and intensely colored effluents waste which causing severe water pollution are produced annually. The black liquor and sugarcane bagasse which characterized by a high level of chemical oxygen demand (COD) and high lignin content, are considered as the most important effluents from pulp and paper industry. The primary contributors to the color and toxicity of these effluents are lignin and its derivative. As a result, lignin is high molecular weight compound and resistance to degradation either chemically or biologically [3]. One of the main concerns for the reasonable and sustainable development of planet Earth in the 21st century finds a way to elimination of widely dispersed, anthropogenic, recalcitrant pollutants. Bioremediation is one of the green chemistry strategies which considered the safest, least disruptive and most cost-effective treatment in comparison with traditional physicochemical treatments [4]. Laccases (p-benzenediol: oxygen oxidoreductase, 1.10.3.2) belong to the family of blue multicopper oxidases, which catalyze the one-electron oxidation of four reducing-substrate molecules concomitant with the four-electron reduction of molecular oxygen to water [5]. They have a unique molecular structure of glycoprotein with copper atoms distribution [6]. Researchers in last decades paid more attention toward the study of laccases due to they have a broad range of substrate specificity, which can facilitate industrial purposes and bioremediation processes [7]. As a result of potential capability of laccases for degradation of synthetic azo dyes and dyes of diverse chemical structure, this opens a new era for the biological removal of these azo dyes from nature [8]. As a result of these diversity laccase applications in the industrial and biotechnological field, studies on laccase producing organisms and the optimization of its production are being carried out by many investigators. The ever-increasing demand for this enzyme requires the production process to be economical. Exploitation of inexpensive raw materials for laccase production could be viewed as a solution to make the entire process cost-effective and utilization of natural occurring inducers for further enhancement may add to the benefit was reported by many investigators [9].

The present study was focused on the utilization of *Alcaligenes faecalis* NYSO (KP859538) laccase for bioremediation of some environmental pollutants to exploit the ultimate benefit from lignocellulosic waste. Efforts were taken to enhance the laccase production by using a wide variety of chemical and natural inducers. The potential of this bacterial strain to utilize various synthetic dyes belonging to different categories was also evaluated. Conservative and traditional approaches of learning method by using "one at a time" difference of action variables do not depict the joint effect of all the factors involved. These methods are also time overwhelming and want large number of investigates to determine optimum levels, which are unpredictable. All these limitations can be eliminated by optimizing all the operating parameters collectively, "all at the same time" using statistical experimental design such as central composite design (CCD) coupled with response surface methodology (RSM) knowing by central composite response surface statistical matrix.

To the best of our knowledge, By intensely exploring the review articles, it was perceived that secreted out in the medium extracellulary by bacteria-source laccases are precious little hitherto.

Materials and Methods

Microorganism and its maintenance

Alcaligenes faecalis NYSO (KP89538) used in the present study was isolated from a discharged effluent of Tanning and leather industry, Alexandria, Egypt and identified by 16S rDNA sequence analysis as described previously [10]. The culture was grown and maintained on buffered LB agar slants.

Medium and Cultural conditions for submerged fermentation

In order to study the influence of different inducers on laccase productivity, the inducers were added to buffered optimized media (w/v%): yeast extract; 0.896; $(NH_4)2SO_4$, 0.035; $CuSO_4.5H_2O$, 0.02; $FeSO_4.7H_2O$, 0.000133; glucose, 0.0943, pH (11), at 30°C and 200 rpm as described by Abdelgalil., *et al*. (2018) [10]. Aliquots (5.0 ml) were withdrawn and cell pellets were used for determination of laccase activity after sonication.

Laccase activity assay

Laccase activity has been estimated calorimetrically using 2,2-azino-di-[3-ethylbenzo-thiazolin-sulphonate] (ABTS) a substrate with an extinction coefficient (ε) 436 = 29,300 $M^{-1}cm^{-1}$ at 436 nm [11]. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidization 1.0 µmol of ABTS per minute under above standard assay conditions; the activities were expressed in U ml^{-1} min^{-1}.

Effects of different amino acids and vitamins on laccase production

The effect of some of amino acids and vitamins such as isoleucine, tyrosine, L-arginine, L-glutamine, lysine, phenylalanine, valine, cysteine, glycine, nicotinic acid, thiamin HCl, biotin and histidine-HCl on laccase production was studied at concentration 0.1%. All amino acids and vitamin were sterilized by filtration.

Effects of different solvents on laccase production

1.0% of various solvents such as ethanol, acetone, petroleum ether, isopropanol, methanol, and acetonitrile were used for the study of their influence on laccase production.

Effects of different synthetic dyes on laccase production

Different synthetic dyes were examined in order to characterize their influence on laccase production. Methyl orange, bromophenol blue, Eriochrome blue-black T, phenol red, safranine, fast green, fast blue, Dinitro salicylic acid, basic fuchsin, azure B, methylene blue, bromocresol purple, bromothymol blue, congo red, malachite green, ethidium bromide, crystal violet, acridine orange, Coamisse blue R250, methyl red and Nile red were used at concentration of 10 µM except ethidium bromide at concentration of 2.5 µM. All dyes were dissolved in 10% DMSO and sterilized by autoclaving except ethidium bromide sterilized by syringe filter.

Effects of different synthetic substrates and inducers on laccase production

In order to study the effect of putative phenolic and aromatic inducers on enzyme production, various aromatic and phenolic compounds such as guaiacol (2-methoxy phenol), veratryl alcohol (3,4-dimethoxy benzyl alcohol), catechol (benzene-1,2-diol), pyrogallol (benzene-1,2,3-triol), para-anisidine (p-methoxy aniline), vanillic acid (4-hydroxy,3-methoxy benzoic acid), o-toluidine, syringaldehyde, p-dimethylamine-benzaldehyde, 4-aminophenol, 4-aminobenzoic acid, p-phenylenediamine, benzophenol, 4 nitrophenol, alkali lignin, tannic acid, benzoic acid, kojic acid, hydroquinone, humic acids, benzaldehyde, Tween 80, phenol, inuline, aniline, mercaptoacetic acid, ascorbic acid, sodium azide, dimethyl sulfoxide (DMSO) and ABTS were exploited at concentration 1.0 mM at the time of inoculation under sterile conditions. Catechol, vanillic acid, and alkali lignin were dissolved in sterile water while all the other inducers were dissolved in 10% DMSO.

Effects of different lignocellulosic residues on laccase production

To investigate the effects of natural inducers on *Alcaligenes faecalis* NYSO (KP859538) laccase production, a range of agricultural wastes such as wheat bran, wheat straw, rice straw, rice bran, sugarcane bagasse, corn cobs, banana stalk, grape seed, grape skin, green tea, yellow corn, pear peel, oat, prickly pears peel, pomegranate peel, hazelnuts peel, black liquor, sugarcane pith and orange peel were exploited at concentration of 1% (w/v) [12]. Banana stalks, prickly pears peel, sugarcane bagasse, green tea, pear peel, and pomegranate peel, which collected from the fruit opened market were washed and chopped into small chips. The chips were sun-dried, oven dried (50°C) to constant weight, ground to 40 mm mesh size, and stored in plastic jars to keep the material moisture-free [13]. While orange peel the chips was socked in 18.17 mM KOH for 1.0 hour then, sun-dried/ oven dried (50°C) to constant weight, ground to 40 mm mesh size and stored in plastic jars. The wheat bran, wheat straw, rice straw, rice bran, yellow corn, corn cobs, hazelnuts peel and oat are obtained from Agriculture Insti-

tute Research-Alexandria were air-dried and cut into10–20 mm lengths. Black liquor was obtained from pulp and paper factory, Alexandria, Egypt.

Utilization of agro-waste materials for evaluation of culture conditions

In order to obtain the ultimate benefit from lignocellulosic wastes, pre-formulation media was designed. Sugarcane bassage and black liquor were selected for evaluation of culture conditions. This study carried out in four trials experiment compared with control; the first trial consists of same ratio 1:1 of 1.0% sugarcane bagasse and black liquor, 0.8 mM copper sulfate and 0.8% yeast extract dissolved in 0.1 M glycine-NaOH buffer pH 11.0. The second trial consists of the same component as the first trial except yeast extract was omitted, whilst, the third trial had the same media composition as the first trial but cupper sulfate was deleted. In the fourth trial, only consist of 1.0% sugarcane bagasse and black liquor. Finally, the control trial is the previous optimized media (previous submitted). The inoculated flasks were incubated at 30°C in a shaker incubator (200 rpm) for 24 hrs. The culture was filtered through cheese cloths for remove of agro-waste materials residual from cultural media and laccase activity was estimated in bacterial cell pellets.

Central composite response surface statistical matrix

In biostatistics, a Central Composite Design- is an mathematical design, valuable in Response Surface Methodology, for building a second order (quadratic) model for the response variable without needing to use a complete three-level factorial experiment was adopted to find the optimum levels of the significant variables (Copper sulfate, yeast extract, and selected agro-waste material) and the effects of their mutual interactions on enzyme production. A total of 20 trials were carried out. Each independent variable was studied at five different levels coded as (-2, –1, 0, +1 and +2; respectively) [14]. The center point of the design was replicated six times for the estimation of error. The CCD and the coded levels of each factor were shown in table 1. The experimental design matrix used for the study is shown in table 2. The JMP program was used for experimental design, data analysis, and quadratic model building. Laccase yield obtained was taken as the experimental values of the dependent variable or response (Y), while predicted values of the response were obtained from quadratic model fitting techniques. The optimal values of the three parameters were achieved by solving the obtained polynomial equation. In addition to it, three-dimensional plots were constructed for visual observation of the trend of maximum response and the interactive effects of the significant variables on the response by STATISTICA 5.0 software. The response was fitted by a second order model to be correlated with the independent parameters. The correlation between the three parameters and the response (laccase activity) was described by the following predictive quadratic polynomial equation:

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta12(X_1X_2)+\beta_{13}(X_1X_3)+\beta_{23}(X_2X_3)+\beta_{11}X_{12}+\beta_{22}X_{22}+\beta_{33}X_{32}$$

Variable	Variable code	Low level (-2) g/l	(-1) g/l	middle level (0) g/l	(+1) g/l	high level (+2) g/l
Yeast extract	X1	3	6	9	12	15
$CuSO_4.2H_2O$	X2	0.075	0.15	0.225	0.3	0.375
Mixture of agro-wastes	X3	6	12	18	24	30

Table 1: The levels of variables chosen for the by Central Composite Response Surface Statistical Matrix. “Optimization Condition”.

Trials	X1 (Y.E.)	X2 ($CuSO_4$)	X3 (waste)	Activity ($Uml^{-1}min^{-1}$)
1	0	2	0	503.7543
2	1	1	-1	829.3515
3	1	-1	-1	712.628
4	0	0	-2	509.8976
5	-1	-1	1	411.6041
6	0	0	0	511.9454
7	0	0	2	530.3754
8	-2	0	0	303.0717
9	0	-2	0	573.3788
10	-1	1	1	337.884
11	-1	-1	-1	243.686
12	0	0	0	550.8532
13	1	-1	1	620.4778
14	0	0	0	532.4232
15	0	0	0	526.2799
16	1	1	1	628.6689
17	-1	1	-1	346.0751
18	0	0	0	466.8942
19	2	0	0	1044.369
20	0	0	0	522.1843

Table 2: Central Composite Response Surface Statistical Matrix designed for Alcaligenes faecalis NYSO (KP859538)

where Y is the predicted response (laccase activity U ml-1 min-1); β_0 is the model intercept; X_1, X_2, and X_3 are the independent variables, β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are cross product coefficients; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by a coefficient of determination, R^2. The enzyme activity data were subjected to multiple linear regressions using the JMP program to estimate the t-values, P-values, and confidence levels expressing the P-values as a percentage.

Validation of the central composite response surface statistical matrix

The Central Composite Response Surface Statistical Matrix was validated further for predicted versus actual responses. Each experiment was carried out in triplicate, and the results were compared with the predicted responses by the mathematical model.

Determination of lignin residual

Residual of lignin (Klason lignin) from biodegradation of sugarcane bagasse according to the previous experiment was estimated by the method described by Templeton and Ehrman (1995) [15]. 1.0 oven dried gram of sample was mixed with 20 ml of sulfuric acids (72%) and the mixture swirled at room temperature overnight. 540 ml distilled water was added and the sample was heated by autoclave at 100°C for 4.0 hrs. After cooling to room temperature, samples were separated into liquid and solid fractions by filtration (Whatman filter paper #1). The filter paper was washed with hot water for 1.0 hour. Filter paper that contained lignin residues was muffled at 450°C for 30 min, and then at 800°C for 45 min for determining the ash content.

Lignin% = weight of lignin – the weight of its ash/weight of the dry raw material.

Results and Discussion

Effects of different amino acids and vitamins on laccase production

Among the various amino acids and their analogs studied in this work, the maximum laccase production was stimulated in the presence of L-glutamine, L-cysteine and L-arginine by 1.84, 1.49 and 1.34 - fold, respectively (Table 3). A slight stimulation was observed in the presence of thiamin HCl, tyrosine and isoleucine by 10, 9.3 and 8.6%; respectively greater than control cultures. It has been found that short-chain amino acids with up to four carbons, like L-glycine, caused a slight reduction in laccase production, (0.92%) compared to the control culture. In contrast, the presence of histidine-HCl in bacterial culture did not stimulate the laccase production but caused a sharp decline in laccase titer (93.2%). Laccase titer was gone down approximately to four-fifths, three fifths and one-fifth time less than control culture when the cultures supplemented with nicotinic acid, phenylalanine, and valine; respectively. Also, it was noticed that the laccase production was promoted in presence of biotin and lysine in culture media by about 1608 and 1466 U $ml^{-1}min^{-1}$; respectively, more than that obtained from culture control (1198 U $ml^{-1}min^{-1}$). This find may be in accordance with others citation that reported the enhancement of laccase production in presence of L-glutamine, tyrosine, L-cystine, lysine, and isoleucine [16]. Contrary to other finding recorded that the L-valine, L-arginine, L-cysteine, and biotin caused repression of laccase titer [17,18] and phenylalanine, nicotinic acid and L-histidine HCl were recorded as enhancers for laccase production.

Amino-acids (0.1%)	Laccase activity (U ml^{-1} min^{-1})	Laccase activity (%)
aControl	1198.635	100
L-Glutamine	2214.334	184.738
L-Cysteine	1788.396	149.2027
L-Arginine	1608.191	134.1686
Biotin	1583.618	132.1185
Lysine	1466.212	122.3235
Thiamin HCl	1318.771	110.0228
Tyrosine	1310.58	109.3394
Isoleucine	1302.389	108.656
Glycine	1187.713	99.08884
Nicotinic acid	1067.577	89.06606
Phenylalanine	764.5051	63.78132
Valine	354.9488	29.61276
Histidine-HCl	81.91126	6.833713

Table 3: Effect of different amino-acids on laccase production by *Alcaligenes faecalis* NYSO (KP859538).

Effects of different solvents on laccase production

Addition of organic solvents, with respect to their concentration, showed varied effects on laccase production. The cultures with ethanol expressed more laccase activity than the other treatments exhibiting an increase in activity and reaching a peak of 1679 U $ml^{-1}min^{-1}$ in compared with culture control without any solvent (1198 U $ml^{-1}min^{-1}$). The causes of increase laccase production in the presence of ethanol were previously explained by Chhaya., *et al*. (2013) [16] as a result of its dual role, i.e. gene expression and inhibition of protease activity. Moreover, the results (Table 4) show that petroleum ether, acetonitrile, and methanol enhanced the laccase activity by 1.307, 1.23 and 1.18-fold than that found by control trial. On the other hand, isopropanol caused a slight decline in laccase titer about 4.4% reduction was noticed, while a sharp reduction in laccase production was achieved by acetone, more than one-half of enzyme activity was lost. The present results are consistent with that obtained by other investigators who recorded that laccase production was increased in presence of ethanol and methanol, and suppressed in presence of acetone [16,19], but in contrast to result obtained by Malhotra, Sharma., *et al*. (2004) [20] and Singh., *et al*. (2009) [21] who recorded that the ethanol and methanol caused reduction in laccase production, while, acetone and isopropanol caused no change in laccase production.

Solvents (1.0%)	Laccase activity (U $ml^{-1}min^{-1}$)	Laccase activity (%)
Control	1198.635	100
Ethanol	1679.181	140.0911
Petroleum ether	1567.235	130.7517
Acetonitrile	1474.403	123.0068
Methanol	1419.795	118.451
Isopropanol	1146.758	95.67198
Acetone	693.5154	57.85877

Table 4: Effect of different solvents on laccase production by *Alcaligenes faecalis* NYSO (KP859538).

Effects of different synthetic dyes on laccase production

The dye decolorization studies showed great variation in the ability of *Alcaligenes faecalis* NYSO (KP859538) to degrade dyes belonging to diverse categories. As well as laccase production varied greatly with a different dye-containing media (Table 5). The laccase titer had induced by 37.5, 37.5, 35.3, 34.8, 26.19, 25.2, 24.1, 21.6, 19.5 and 14.1% more than that control culture when ethidium bromide (phenanthridine) and fast blue (benzene diazonium), Azure B (N, N,N′-trimethylation), bromophenol blue (triphenylmethane), congo red (azo), Dinitro salicylic acid, bromocresol purple, methyl orange (azo), Eriochrome black T (azo), basic fuchsin and phenol red (triphenylmethane); respectively was added to culture media. However, bromothymol blue (triphenylmethane) was not shown a significant effect on laccase production only 6.0% more than control culture. Laccase production went down when fast green, safranine (heterocyclic), Coamisse blue R250, methyl red, acridine orange, and crystal violet were used by 8.5%, 15.5, 28.3 and 31.5%, 48.1% and 75%; respectively. In addition to this, a sharp decline was noticed by using the Nile red, methylene blue (heterocyclic) and malachite green (triphenyl-methane), only 51, 43 and 19 U $ml^{-1}min^{-1}$; respectively of enzyme titer had retained in comparison with control culture (1198 U $ml^{-1}min^{-1}$). Comparing the obtained results with those cited by the other investigators, it was found that the ethidium bromide was the only dye reported to increase laccase activity in fungi and bacteria [20]. Bromophenol blue, congo red, basic fuchsin, Dinitro salicylic acid, phenol red, and methyl orange were recorded as promotors for laccase production [18,22] which matched the results of the present study. On the other hand, Eriochrome black T (azo), methyl orange (azo), bromophenol blue (triphenylmethane), congo red (azo) and bromothymol blue (triphenylmethane) caused repressed to laccase production [20], unlike the findings of the results of the present study. It was found that through the study of other investigators the malachite green, and the crystal violet were used for induction of laccase production [23].

Effects of different synthetic substrates and inducers on laccase production

The different inducers were added in an equal concentration (1.0 mM) to the medium to investigate their effect on laccase production by *Alcaligenes faecalis* NYSO (KP859538) (Table 6). The results indicated that the humic acid (1752 U $ml^{-1}min^{-1}$), provided a 1.46-fold increase in laccase activity in relation to control (1198 U $ml^{-1}min^{-1}$). Also, the laccase titer was increased in presence of ABTS, alkali lignin and Tween 80 by approximately 42.8, 36.9 and 35.9%; respectively more than that obtained from control trial which free from any inducer. The phenolic compound such as phenol (1556 U $ml^{-1}min^{-1}$) also proved to be a promising inducer for laccase production by this strain. Furthermore, vanillic acid, mercaptoacetic acid, inuline, DMSO, ascorbic acid and guaiacol stimulated with a moderate increase in laccase production to a certain extent. However, inducing effect was not observed clearly when the other aromatic compounds were used such as benzoic acid, catechol, tannic acid, and syringaldazine; whereas, an increase in laccase induction by 7.5, 5.9, 5.2 and 4.5% were obtained using these compounds; respectively. Sodium azide had not shown any significant effect on laccase production. It can be clearly seen that laccase level was affected slightly by 4-aminobenzoic acid, pyrogallol, veratryl alcohol, kojic acid and o-toluidine where laccase titer was little bit re-

Synthetic Dyes (10 µM)	Laccase activity (U ml^{-1} min^{-1})	Laccase activity (%)
Control	1198.635	100
Ethidium bromide (2.5µM)	1649.147	137.5854
Fast blue	1649.147	137.5854
Azure B	1621.843	135.3075
Bromophenol blue	1616.382	134.8519
Congo red	1512.628	126.1959
Dinitrosalicylic acid	1512.628	126.1959
Bromocresol purple	1501.706	125.2847
Methyl orange	1488.055	124.1458
Eriochrome blue black T	1458.02	121.6401
Basic fuchsin	1433.447	119.59
Phenol red	1367.918	114.123
Bromothymol blue	1272.355	106.1503
Fast green	1097.611	91.57175
Safranine	1012.969	84.51025
Coamisse blue R250	860.0683	71.75399
Methyl red	821.843	68.56492
Acridine orange	622.5256	51.93622
Crystal violet	300.3413	25.05695
Nile red	51.87713	4.328018
Methylene blue	43.68601	3.644647
Malchite green	19.11263	1.594533

Table 5: Effect of different synethetic dyes on laccase production by *Alcaligenes faecalis* NYSO (KP859538).

turn back to 1.6, 3.2, 7.1, 8.7 and 8.9%; respectively, which is less than that obtained from control trial. In the same way, p-dimethy lamino benzaldehyde, hydroquinone, aniline, and 4- nitrophenol exhibited a moderate repression of laccase production by four and two-fifth, four-fifth, three and seven-fifth, and three and five-fifth; respectively, less time than the control trial. On the contrary, a strong repressed for laccase activity was observed in presence of p-anisidine, benzaldehyde, benzophenol, and 4-aminophenol in cultivation media, where laccase was retained only about 245, 185, 38 and 10.0 U $ml^{-1}min^{-}$; respectively, from its activity obtained from the control trial (1198 U $ml^{-1}min^{-1}$). While the bacterium failed to grow in the p. Phenylenediamine supplemented medium, consequently, the laccase production was fully inhibited. Results obtained in the present study are in a good agreement with that obtained by other investigators [18, 24] but inconsistent with that obtained by Malhotra, Sharma., *et al.* (2004) [20].

Synthetic substrates (1.0 mM)	Laccase activity (U ml^{-1} min^{-1})	Laccase activity (%)
Control	1198.635	100
Humic acids	1752.901	146.2415
ABTS	1711.945	142.8246
Alkali lignin	1640.956	136.9021
Tween 80	1630.034	135.9909
Phenol	1556.314	129.8405
Vanillic acid	1534.471	128.0182
Mercaptoacetic acid	1474.403	123.0068
Inuline	1449.829	120.9567
DMSO	1351.536	112.7563
Ascorbic acid	1337.884	111.6173
Guaiacol	1318.771	110.0228
Benzoic acid	1288.737	107.5171
Catechol	1269.625	105.9226
Tannic acid	1261.433	105.2392
Syringaldazine	1253.242	104.5558
Sodium azide	1215.017	101.3667
4-aminobenzoic acid	1179.522	98.40547
Pyrogallol	1160.41	96.81093
Veratryl alcohol	1113.993	92.9385
Kojic acid	1094.881	91.34396
O-tolidine	1092.15	91.11617
p-dimethylaminobenz-aldehyde	999.3174	83.3713
Hydroquinone	969.2833	80.8656
Aniline	881.9113	73.57631
4- nitrophenol	865.529	72.20957
p-anisidine	245.7338	20.50114
Benzaldhyde	185.6655	15.48975
Benzophenol	38.22526	3.189066
4-aminophenol	10.9215	0.911162
p.phylenediamine	0	0

Table 6: Effect of different synthetic inducers on laccase production by *Alcaligenes faecalis* NYSO (KP859538).

Effects of different lignocellulosic residues on laccase production

Results cited in table 7 showed that among the various agricultural wastes screened for laccase production, sugarcane bagasse was found to be the most suitable substrate as it supported maximum laccase production (1408 U $ml^{-1}min^{-1}$) in comparison with control trial (1198 U $ml^{-1}min^{-1}$). Similarly, black liquor, prickly pears peel, corn cob, and sugarcane pith exerted a stimulatory effect on Alcaligenes faecalis NYSO (KP859538) laccase production, compared to the control media. Black liquor and prickly pears peel were found to be effective next to sugarcane bagasse; they had nearly the same induction effect on laccase titer (1365 U $ml^{-1}min^{-1}$). This result was considered acceptable, since black liquor, which contains lignin from pulp and paper bleaching, induce laccase activity as reported by Mongkolthanaruk*., et al*. (2012) [24].

The media supplement with corn cob and sugarcane pith characterized by tiny induction effect, where laccase level slightly climbed up by 7.9 and 6.6% more than control trial. However, the rice straw and rice bran had not significant effect on laccase production; about 1.9 and 0.9% more increase than the control trial was obtained. Other worth mentioned before, the orange peel, oat, wheat straw caused little bite reduction in laccase production to about 12.1, 15.1 and 16.9% less than control trial. Whereas the laccase productions by wheat bran, corn yellow, pear, apple and pea nut did not show any increment in the laccase production but they were retained only about 69, 66.7, 66, 55 and 51%; respectively of its activity when compared to the control trial. Laccase activity reduction reached 50, 38 and 14% was obtained in media supplement with banana stalk, grape skin, and hazelnuts peel; respectively. On the other hand, the lowest activity was obtained with pomegranate peel when used as a substrate. No detectable increase in laccase activity was found when grape seed and green tea were used as a substrate. The present study results are in disagreement with observations reported by other investigators [9,17,25] who found that rice straw, wheat bran, peanut shell, banana peel, and black grapes were identified as promising substrates for laccase production, while the other substrates like corn cops, rice straw, and sugarcane bagasse caused low laccase production. However, the observation documented by other investigators [26,27] are compatible with the results of this study in term of apple and wheat bran caused decline in laccase production.

Utilization of sugarcane bagasse and black liquor for media formulation

The exploitation of the ultimate benefit from lignocellulosic waste was investigated by using the most important and the cheapest agro-waste as a substrate for the formulation of suitable and simple media for bacterial growth and laccase production. The recorded results (Figure 1) revealed that the media composition of trial one, which consisted of (yeast extract, cupper sulfate, and mixture sugarcane bagasse and black liquor), was considered the

Natural substrates (1.0%)	Lignin content (%) [36,80,90]	Laccase activity (U ml^{-1} min^{-1})	Laccase activity (%)
Control	0.0	1198.635	100
Sugarcane bagasse	25	1408.874	117.5399
Black liquor	17	1365.188	113.8952
Prickly pears peel	2.4	1365.188	113.8952
Corn cob	4.5-6.6	1294.198	107.9727
Sugarcane pith	16.1	1277.816	106.6059
Rice straw	9.9	1220.478	101.8223
Rice bran	5	1209.556	100.9112
Orange peel	12	1053.925	87.92711
Oat	13.7	1018.43	84.96583
Wheat straw	14.5	996.587	83.14351
Wheat bran	11.4	832.7645	69.47608
Corn yellow	18	800	66.7426
Pear	29.8	791.8089	66.05923
Apple	32	660.7509	55.12528
Peanut shell	41.2	622.5256	51.93622
Bannana stalk	8	603.413	50.34169
Grape skin	5	455.9727	38.041
Hazelnuts peel	51.3	174.744	14.57859
Pomegranate peel	4.5	8.191126	0.683371
Grape seed	44	0.0	0.0
Green tea	5-6	0.0	0.0

Table 7: Effect of different natural inducers on laccase production by *Alcaligenes faecalis* NYSO (KP859538).

most suitable media for bacterial growth and production of laccase (1138 U $ml^{-1}min^{-1}$) in comparison with control trial (1471 U $ml^{-1}min^{-1}$). However, enzyme titer was reduced to about 22.7% from initial activity but the ultimate goal of this study is looking for a strategy to formulate simple media with maximizing utilization available agro-waste, to avoid their accumulation in the environment. About 96, 97.5 and 98% reduction in initial laccase activity was obtained from trial two, three and four; respectively, this result reflected components of media trial number one are complemented each other and necessary for both bacterial growth and enzyme production. The present study proved the utility of sugarcane bagasse and black liquor as an inexpensive and easily available raw material for laccase production, and convenient with combinations study carried to explore the interaction between the two agricultural residues [28]. Additionally, the employed lignocellulosic residues are abundant and renewable, which are suitable for both growth and laccase enzyme induction [29].

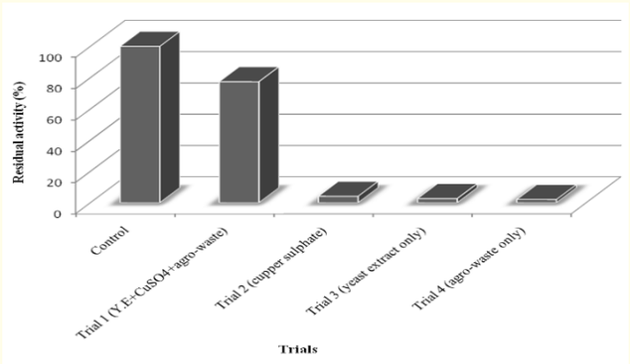

Figure 1: Pre-formulation of media for laccase production by Alcaligenes faecalis NYSO (KP859538) using sugarcane bagasse and black liquor.

Central composite design (CCD) coupled with response surface methodology (RSM) "Central Composite Response Surface Statistical Matrix"

The central composite design (CCD) technique in conjunction with the response surface methodology (RSM) were both applied to study the interactions between independent variables with each other along with the possible maximal levels of the process outcome at five different levels viz; -2, -1, 0, +1, +2 as presented in Table 1. Table 2 depicts the design matrix of the coded variables together with the experimental results of the enzyme activity. The main results of this study are presented in figure 2, which represents the expected laccase response and the correlation between variables in three-dimensional plots. It can be clearly observed from Figure 2a, 2b that, the effects of pairs of factors were additive since there are low interactions between the yeast extract-cupper sulfate and yeast extract – a mixture of agro-wastes. Figure

2c showed non-additive effects of cupper sulfate and a mixture of agro-wastes due to the significant interaction between them. By additive of the two-factor effects, it is meant that the effect of one factor on the response does not depend on the level of the other factor. In Figure 2b it is obvious that maximum laccase activity was attained at higher levels of yeast extract (15g/L) and lower levels of cupper sulfate (0.075 g/L). Figure 2b illustrates that increasing yeast extract concentration in the medium independent on a mixture of agro-wastes concentration led to increases in laccase activity. The optimum point deduced from Figure 2 is in accordance with the mathematically calculated optimum point.

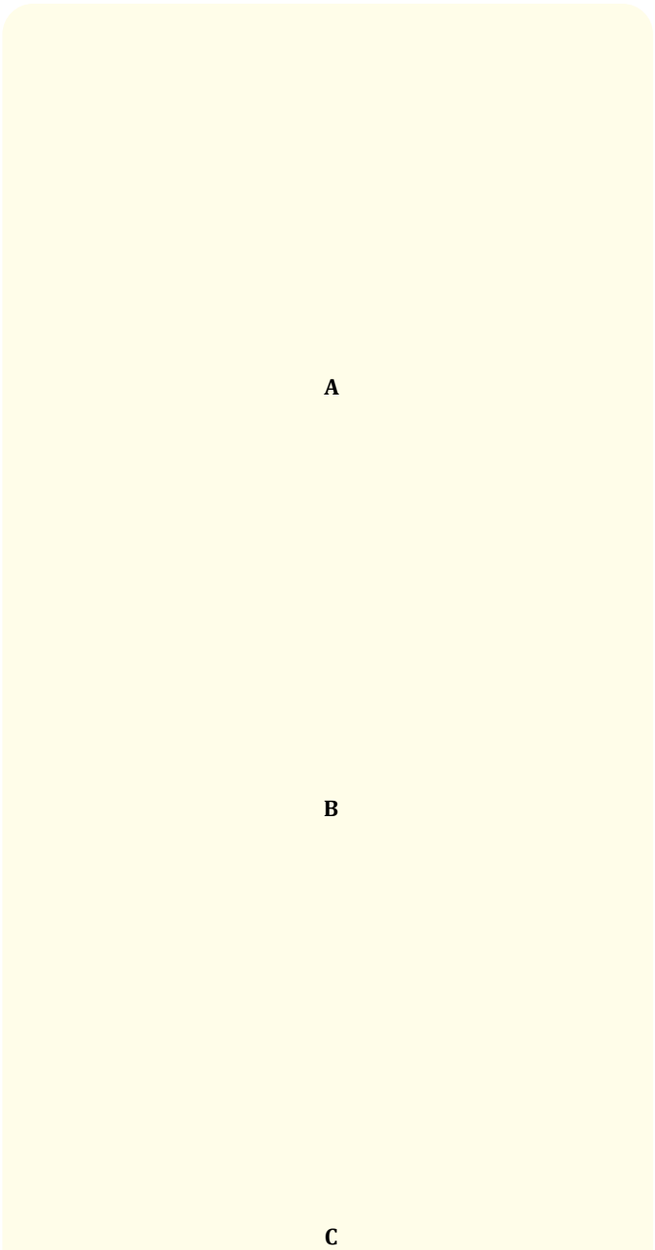

Figure 2: Three dimensional Central Composite Response Surface Statistical Matrix representing laccase activity yield (U ml^{-1} min^{-1}) from Alcaligenes faecalis NYSO (KP859538) as affected by culture conditions.

$Y = 509.99 + 183.40X_1 + 0.895X_2 - 5.759X_3 + 12.03X_1X_2 - 56.57X_1X_3 - 35.58X_2X_3 + 4.60X_{12} + 0.814X_{22} - .79X_{32}$.

where, X_1, X_2, and X_3 are yeast extract, $CuSO_4.5H_2O$, and a mixture of agro-wastes; respectively. At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation coefficients R and the determination coefficient R^2. The closer the value of R is to 1.0, the better is the correlation between the measured and the predicted values. The value of the determination coefficient in this experiment $R^2 = 0.966$ for laccase activity, is a measure of fit of the model, indicates that about 3.4% of the total variations created by variables are not explaining laccase activity. The analysis of variance using the ANOVA test in the central composite experiment was generated which gives p = 0.0001. Since the p-value indicated in the ANOVA table is less than 0.05, it is concluded that there is a statistically significant relationship among the studied variables at 95% confidence level (p = 0.05) (Table 8).

The optimal levels of the five studied variables as obtained from the maximum point of polynomial model were estimated using the JMP programme and Solver function of Microsoft Excel tools, and found to be: yeast extract, 15 g/l; $CuSO_4.5H_2O$, 0.375 g/l and mixture of agro-wastes, 6 g/l with prediction calculated enzyme activity equal to 1433.33U $ml^{-1}min^{-1}$ (Figure 3). Results obtained in this study are in accordance with others findings [30,31] recorded that the bagasse fibers were dignified in an eco-friendly method using the laccase enzyme and the delignification process was optimized using surface response methods.

Verification central composite response surface statistical matrix

In order to determine the accuracy of the quadratic polynomial, a verification experiment was carried out under predicted optimal conditions monitoring growth and enzyme activity in the optimized medium. The bench-scale experiments show that experimental laccase activity was 1386.31U $ml^{-1}min^{-1}$. The calculated model accuracy was 96.74% and this high degree of accuracy is evidence of the model validation under the following optimal conditions: 15 g/l; $CuSO_4.5H_2O$, 0.375 g/l and mixture of agro-wastes, 6 g/l, and buffered condition pH 11.0 under cultivation temperature of 30°C and incubation time of 24 hrs. According to method described by Templeton and Ehrman (1995) [15] for determination of residual of lignin (Klason lignin) from biodegradation of sugarcane bagasse, the result referred that the laccase enzyme from *Alcaligenes faecalis* NYSO (KP859538) had the capacity to degrade the sugarcane bagasse to about 3.2% according to previously described growth condition, where lignin content for raw sugarcane bagasse before cultivation of bacterial strain was approximately 27.8%, while, the lignin content after cultivation of bacteria strain for 24 hrs incubation time was 24.6%.

Term	Estimate	Std Error	t Ratio	Prob>|t|	Confidence level (%)
Intercept	509.9907	18.4104	27.70123	<.0001	
X1&RS	183.4044	11.53933	15.89386	<.0001	99.9
X2&RS	0.895904	11.53933	0.077639	0.939647	7
X3&RS	-5.75939	11.53933	-0.49911	0.628499	37.2
X1*X2	12.03072	16.31907	0.737218	0.47793	52.3
X1*X3	-56.57	16.31907	-3.46649	0.006057	99.395
X2*X3	-35.5802	16.31907	-2.18028	0.05422	94.6
X1*X1	34.60285	9.205199	3.759056	0.003728	99.63
X2*X2	0.814459	9.205199	0.088478	0.931244	6.9
X3*X3	-3.79305	9.205199	-0.41206	0.688993	31.2

Table 8: Statistical analysis of Central Composite Experimental Design Matrix showing coefficient values, t- and p-values for each variable on laccase activity.

Figure 3: JMP profile showing the predicted optimal levels of studied three variables, X1, yeast extract; X2, copper sulphate; X3, mixture agro-wastes along with the predicted laccase activity.

Conclusion

The present study focused on the utilization of *Alcaligenes faecalis* NYSO (KP859538) laccase for bioremediation of some environmental pollutants to exploit the ultimate benefit from lignocellulosic waste. A five-level statistical experimental design such as central composite design (CCD) was employed to create a polynomial quadratic model correlating the relationship between the three variables and laccase activity. It was found that the following optimal conditions: 15 g/l; CuSO4.5H2O, 0.375 g/l and a mixture of agro-wastes, 6 g/l, and buffered condition pH 11.0 under cultivation temperature of 30oC and incubation time of 24 hrs for utilization of agro waste with maximum laccase production around 1386.31U ml-1min-1. Efforts were taken to enhance the laccase production by using a wide variety of chemical and natural inducers. Among the various inducers used as a growth factor L-glutamine, ethanol and humic acid were found to be the most suitable for laccase production. The potential of this bacterial strain to utilize various synthetic dyes belonging to different categories was also evaluated. It was found that the promotion of laccase production was achieved in media supplemented with fast blue, ethidium bromide and azure B by 37 - 35%.

Financial support

This work is financially supported by ULIXES project.

Acknowledgment

Authors would like to acknowledge PROF. Dr. Yasser R. Abdel-Fattah for funding and scientific supporting.

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Volume 2 Issue 11 November 2019

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