

Optimization of Laccase Production from *Penicillium martensii* NRC 345

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Abstract Laccase production by *Penicillium martensii* NRC 345 was optimized. Eight media with different components were screened. The enzyme formed by *P. martensii* NRC 345 was detected mainly exocellularly under shake culture condition. Laccase formation reaches its maximum value with specific activities of about 7.18 U/mg protein at the twenty-sixth day of incubation at pH 5.5 and 30°C. Galactose (5 g/l) and sodium nitrate (0.2 g/l) were found to be the best carbon and nitrogen sources for laccase formation, respectively. Replacement of galactose instead of glucose at the same concentration increased laccase production by about more than ten-fold. Among the various wastes used wheat bran induces the highest laccase production with specific activity of 39.52 U/mg protein. Tween-80 and CuSO₄·5H₂O, were also tested for laccase induction.

Keywords Laccase, *Penicillium martensii*, production, optimization

1. Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC1.10.3.2) are either mono or multimeric copper-containing oxidases that catalyze the one-electron oxidation of large numbers of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and consequently reduced to two molecules of water[1]. Laccase catalyses the four electron reduction of oxygen to water coupled with single-electron oxidation of four hydrogen-donating substrates[2]. This is accomplished by a cluster of four copper atoms (type 1 copper; type 2 copper and two type 3 copper atoms) that form the enzyme active site[3]. Type 1 copper, which confers the typical blue colour to the enzyme due to intense electronic absorption of covalent copper–cysteine bonds, is the site where monoelectronic oxidation of substrate takes place. Type 2 and type 3 copper atoms form a trinuclear cluster, to which the electrons are transferred and where molecular oxygen is reduced to water[4]. Laccases are widely distributed in nature, originating from plants, insects, bacteria and fungi[5]. In fungi, laccases are widely distributed in ascomycetes, deutromycetes and basidiomycetes. These laccase producing fungi include *Trametes (Coriolus) versicolor*, *T.villosa*, *T. hirsute*, *T. gallica*, *T. ochracea*, *Cerrena maxima*, *Phlebia radiata*, *Lentinus tigrinus*, *Corioloropsis polyzona* and *Pleurotus eryngii*. Laccases are also reported in saprophytic ascomycetes of com-

posts (*Myceliophthora thermophila*, *Curvularia*, *Penicillium*, *Aspergillus* and *Chaetomium thermophile*) and in the soil hyphomycete *Mycelia sterilia* INBI 2-26[6, 7].

Many laccase applications were reported some of which have been patented, ranging from effluent decoloration and detoxification to pulp bleaching, removal of phenolics from wines and dye transfer blocking functions in detergents, washing powders and degradation of lignin[1].

The optimization of medium to reduce the cost of laccase is the basic research in the industrial applications[8]. Another approach is to overproduce laccase in a suitable host. Thus, screening studies should be made to select new promising microorganisms producing laccase[9].

The purpose of this work is to study the cultural and nutritional conditions for laccase production by a filamentous fungus which was not previously described in the literature.

2. Materials and Methods

2.1. Microorganism

Penicillium martensii NRC 345 was obtained from Culture Collection of Microbial Chemistry Department, National Research Centre, Egypt.

2.2. Chemicals and Media

Syringaldazine was supplied by Sigma, USA. The other chemicals used in this study were of analytical grade and higher purity. A culture was maintained through periodic transfer on slants of potato-dextrose agar (PDA) medium, and was kept at 4°C. The liquid medium used for laccase production by the fungal culture during its growth in sub-

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merged fermentation was composed as follows; 5g of glucose, 1g of KH_2PO_4 , 0.5g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2g of NaNO_3 , 0.1g of yeast extract, 0.01g of CaCl_2 , 1mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1mg of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ and 1000 ml of distilled water[10]. The liquid medium was then adjusted to pH value 5.0, and sterilized by autoclaving at 1.5 atmospheres and 121°C for 20 min.

2.3. Preparation of Cell Free Filtrates

At the end of the incubation period, the cultures were filtered using Whatman No.1 filter paper. The culture filtrate was used directly as a crude enzyme preparation.

2.4. Preparation of Cell Free Extracts

P. martensii NRC 345 pellets were filtered and homogenized in citrate phosphate buffer (100 mM; pH 5.0) and cold washed sand in a cold mortar (4°C). The crude homogenate was then centrifuged at 5000 rpm for 10 min. The upper layer containing cell free extracts was separated by decantation and used as the crude endocellular enzyme preparation[11].

2.5. Enzyme Assay

Laccase activity was assayed by monitoring the product formation rate of enzymatic oxidation of syringaldazine spectrophotometrically at 525nm ($\epsilon_{525} = 65000 \text{ M}^{-1} \text{ cm}^{-1}$)[12]. The assay mixture (2.0 ml) contained syringaldazine, 0.1 μ mole; citrate-phosphate buffer (pH 5.0), 90 μ moles and appropriate volume of diluted enzyme. One unit of laccase activity was defined as the change in the absorbance of 0.001 per sec[13]. Protein content was estimated by the modified procedure of Lowry *et al.*,[14]. All the data was statistically evaluated according to the method described by Kenney and Keeping[15]. The means and standard deviations of means (Mean \pm S.D.) were calculated for each experiment.

3. Results

3.1. Effect of Media Composition

P. martensii NRC 345 was grown on eight media described by different investigators under both static and shake culture conditions (120 rpm) using New Brunswick scientific Co. Inc. Edison N. J. USA shaker at $28 \pm 2^\circ\text{C}$ for 26 days of incubation and the cell-free filtrate (CFF) and cell-free extract (CFE) were then used as enzyme sources. The eight liquid media were tested for inducing laccase production with their different compositions. Results indicate that, *P. martensii* NRC 345 laccase was mainly formed under shake condition. Results also showed that in the diverse composition of the culture media used for the cultivation of *P. martensii* NRC 345, better enzyme production was obtained maximally in medium N₀ 1 (7.76 U/mg protein) under shake culture condition followed by medium N₀ 2. No laccase activity could be detected in case of using media number 4, 5

and 6 under shake culture condition and media number 3 and 5 under static condition (Table 1). In all successive experiments, medium N₀ 1 under shake condition will be used.

Table 1. Effect of Medium Composition on the Formation of Exocellular Laccase under Static and Shake Culture Conditions by *P. martensii* NRC 345

Medium	Reference	Aeration condition	Final pH	Specific activity (U/mg protein)
1	[10]	static	6.0	6.12 \pm 0.46
		shake	7.0	7.76 \pm 0.71
2	[16]	static	6.5	4.71 \pm 0.36
		shake	7.5	6.09 \pm 0.70
3	[12]	static	8.0	0.00 \pm 0.00
		shake	9.0	0.03 \pm 0.01
4	[17]	static	7.0	0.21 \pm 0.07
		shake	9.0	0.00 \pm 0.00
5	[18]	static	4.5	0.00 \pm 0.00
		shake	6.5	0.00 \pm 0.00
6	[19]	static	8.0	0.14 \pm 0.00
		shake	8.0	0.00 \pm 0.00
7	[20]	static	6.0	0.08 \pm 0.00
		shake	8.0	0.90 \pm 0.02
8	[21]	static	7.0	2.48 \pm 0.05
		shake	7.5	0.21 \pm 0.16

3.2. Localization of Laccase

P. martensii NRC 345 was grown on medium N₀ 1 under static and shake culture conditions. Laccase activity levels in both cell-free filtrate (CFF) and cell-free extract (CFE) were determined.

Results obtained showed that, the enzyme formed by *P. martensii* NRC 345 expressed as specific activities, was detected mainly in the exocellular fraction (CFF) under shake culture condition. Relative specific activities of the corresponding endocellular enzyme values only represent about 22 % and 16 % with static and shake conditions respectively, which indicate that, the enzyme is mainly excreted exocellularly (Table 2).

Table 2. Localization of Laccase by *P. martensii* NRC 345

Enzyme source	static		shake	
	Specific activity (U/mg protein)	Relative specific activity (%)	Specific activity (U/mg protein)	Relative specific activity (%)
Exocellular	6.12 \pm 0.46	100.00	7.76 \pm 0.71	100.00
Endocellular	1.37 \pm 0.01	22.39	1.27 \pm 0.01	16.37

3.3. Effect of Incubation Period

P. martensii NRC 345 was grown on media N₀ 1 under shake culture condition (the best tested media for laccase formation), at pH 5.0 for thirty-one days of incubation. Samples of the fermented medium were collected periodically for the determination of laccase activity. Results indicate that the highest specific activity was obtained at the

twenty-sixth day of incubation with specific activity of 7.18 U/mg protein. After this incubation period, a gradual decrease in enzyme formation level was occurred (Figure 1).

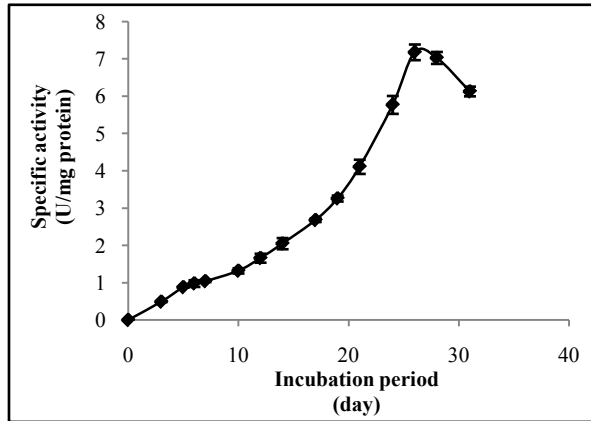


Figure 1. Effect of incubation period on laccase formation by *P. martensii* NRC 345

3.4. Influence of Initial pH Values

The effect of pH on laccase production was carried out by incubating the culture flasks (250 ml Erlenmeyer conical flasks) each containing 50 ml of production medium at different pH values. A series of pH values ranging from 4.0 to 7.0 were studied.

From the obtained results, it is clear that maximal formation of *P. martensii* NRC 345 laccase took place at pH 5.5, and laccase formation occurred at a narrow range of pH values, whereas considerably low levels of enzyme were obtained at pH values below and higher than this value (Figure 2).

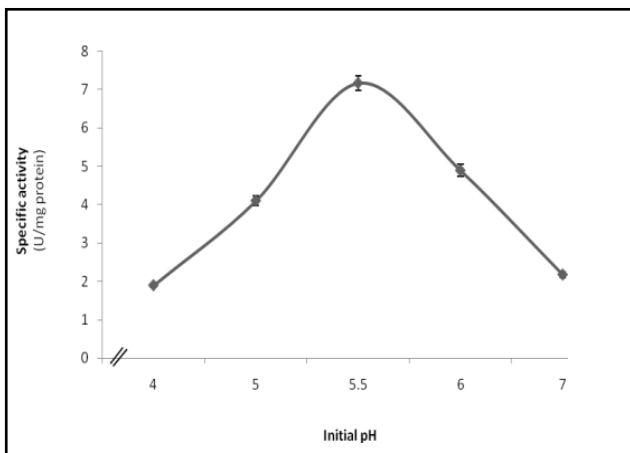


Figure 2. Influence of different initial pH values on the formation of *P. martensii* NRC 345 laccase

3.5. Effect of Incubation Temperature

The present experiment aims to determine the optimal incubation temperature for laccase formation by *P. martensii* NRC 345, which was grown at various degrees of temperatures ranging from 25 to 35°C. Results demonstrated that the optimal temperature for laccase formation was found to be 30°C with specific activity of 7.21 U/mg protein, and below or above this incubation temperature value a considerable

decrease in laccase formation was detected (Figure 3).

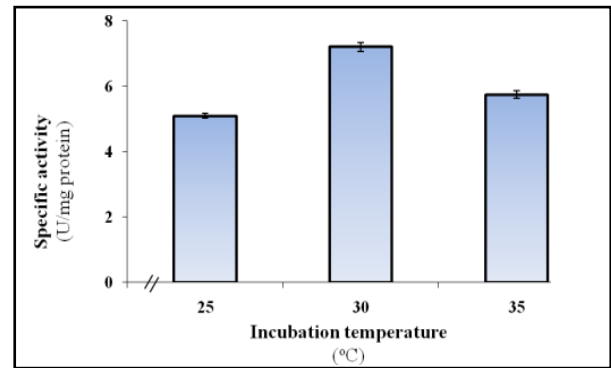


Figure 3. Influence of incubation temperature on the formation of *P. martensii* NRC 345 laccase

3.6. Effect of Inoculums' Size

The present experiment is an attempt to find out the optimal inoculum size for laccase production. Therefore, inocula with size ratios ranging from two to seven agar plugs (14 mm in diameter) were cut from actively growing fungal mycelia and inoculated in the production medium. Results indicated that, as the percentage of the introduced inoculum size was raised from two to five discs, laccase progressively increased. The optimal inoculum size was found to be five discs. Further increase in inoculum size reduced the level of enzyme formation (Figure 4). Consequently, five discs inoculum size, which was the optimal for laccase production from *P. martensii* NRC 345, was used in the subsequent experiments.

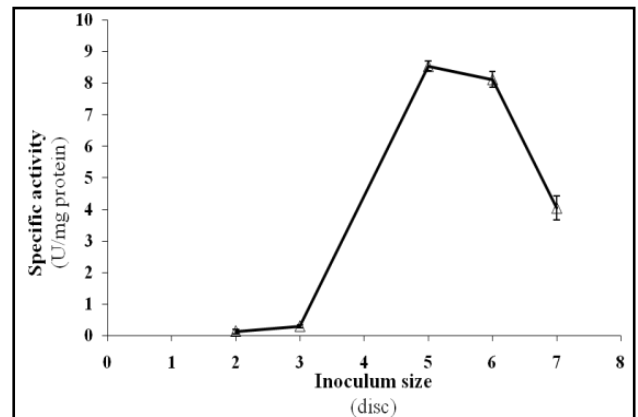


Figure 4. Influence of inoculums' size on the formation of *P. martensii* NRC 345 laccase

3.7. Influence of Lignocellulosic Wastes on Laccase Formation

Different lignocellulosic wastes (wheat bran, wheat straw, saw dust, rice bran, rice straw, corn stalk, corn stover and sugar cane bagasse) were used instead of glucose in the medium for laccase production by *P. martensii* NRC 345. Results cited in Table (3) showed that among the various wastes used, wheat bran followed by corn stover were found to be best substrates for laccase production by *P. martensii* NRC 345 with maximum laccase specific activity (39.52 and

33.33 U/mg protein, respectively). On the other hand, the lowest activity was obtained with wheat straw when used as a substrate. No detectable laccase activity was found when saw dust was used as a substrate instead of glucose.

Table 3. Influence of Lignocellulosic Wastes on the Formation of *P. martensii* NRC 345 laccase

Lignocellulosic waste	Final pH	Specific activity (U/mg protein)
Glucose (control)	6.0	7.55 ± 0.09
Wheat bran	6.6	39.52 ± 0.96
wheat straw	6.5	0.65 ± 0.01
Saw dust	5.5	0.00 ± 0.00
Rice bran	6.5	15.06 ± 0.23
Rice straw	7.0	11.84 ± 0.67
Corn stalk	6.5	1.46 ± 0.02
Corn stover	7.0	33.33 ± 0.68
Sugar cane bagasse	6.0	16.68 ± 0.53

3.8. Effect of Different Carbon Sources

This experiment aims to study the effect of different carbon sources on the formation of laccase by *P. martensii* NRC 345. Each carbon source was added at a concentration of 0.5 % to the medium described by [10] (the best media for laccase formation), as the main only carbon source.

As shown in Figure (5), galactose was found to be the best carbon source for laccase formation by *P. martensii* NRC 345. Alternatively, CMC completely repressed laccase production. On the other hand, all tested carbon sources stimulated laccase production than glucose (the carbon source in the original medium). Galactose reduced the incubation period to be 22 days only instead of 26 days in case of glucose in the original medium. It is worthy to mention that, replacement of galactose in the medium described by [10] instead of glucose at the same concentration increased laccase production by about ten-fold.

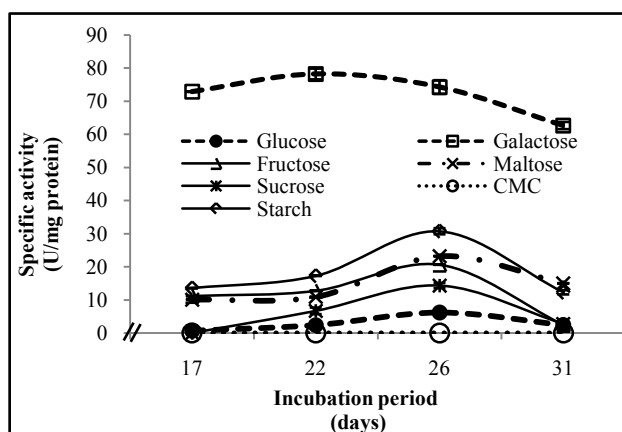


Figure 5. Influence of different carbon sources on the formation of *P. martensii* NRC 345 laccase

3.9. Formation of Laccase as a Function of Galactose Concentration

From the preceding experiment, it has been detected that galactose was the best carbon source for laccase production by *P. martensii* NRC 345. Therefore, in this experiment the

effect of galactose concentration on enzyme formation was tested. Galactose was added to the culture medium instead of glucose at five different concentrations ranging from 2.5 to 20 g/l. It is clear from Figure 6, that the enzyme specific activity was increased with increasing galactose concentration up to 5 g/l, which is the best concentration of galactose for laccase formation. By increasing galactose concentration above this value, a gradual decrease in laccase activity occurred.

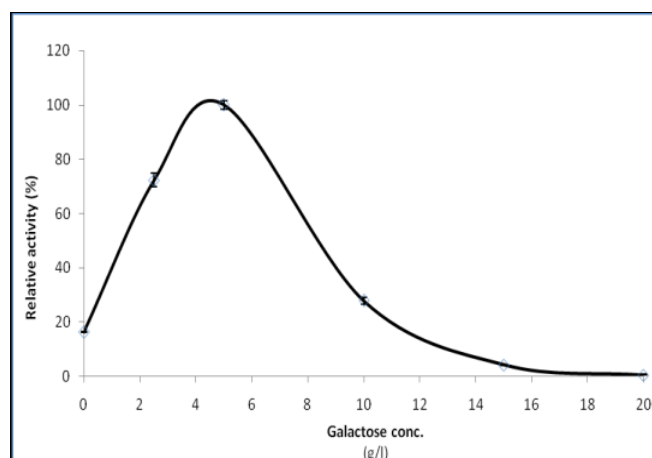


Figure 6. Influence of galactose concentration on the formation of *P. martensii* NRC 345 laccase

3.10. Effect of Nitrogen Sources

Various nitrogen compounds were added separately to the culture medium in amounts equivalent (on nitrogen basis) to the amount of nitrogen in sodium nitrate (0.2 g/l) in the modified medium described by [10] using galactose as a carbon source instead of glucose. Table 4 demonstrated that, the highest level of enzyme formation expressed in terms of specific activity was obtained with sodium nitrate (76.56 U/mg protein) followed by L (+) glutamine (53.92 U/mg protein). On the other hand, no enzyme formation could be detected with ammonium sulfate or ammonium chloride.

Table 4. Influence of Different Nitrogen Sources on the Formation of *P. martensii* NRC 345 Laccase

Nitrogen source	Final pH	Specific activity (U/mg protein)
Sodium Nitrate (Control)	5.5	76.56 ± 1.27
Ammonium sulfate	4.0	0.00 ± 0.00
Ammonium chloride	4.0	0.00 ± 0.00
Urea	4.5	38.38 ± 1.63
L (+) asparagine	4.5	1.92 ± 0.02
L (+) glutamine	5.0	53.92 ± 0.58
L - alanine	4.5	1.88 ± 0.07
DL - alanine	4.5	20.22 ± 1.05
L - arginine	7.0	30.82 ± 0.45
L - tryptophan	4.5	22.93 ± 0.93

3.11. Influence of Addition of Tween-80 and Copper Sulfate

The effect of addition of Tween-80 and copper sulfate at concentrations between 0.1 – 0.75 % (v/v) and 50 - 1500 μM respectively, indicated that both supplements had no induc-

tion effect on laccase production by *P. martensii* NRC 345 (data not shown).

4. Discussion

The objective of this article was to enhance laccase production by *P. martensii* NRC 345, which was not previously described in the literature. The medium components, carbon source, nitrogen source, initial pH and incubation temperatures were studied to investigate their effects on laccase production. Previous studies indicated that these factors were considered to be important for laccase production by white rot fungi[22]. Eight liquid media with different compositions were tested for inducing laccase production by *Penicillium martensii* NRC 345. Results indicated that, within the diverse composition of the culture media used for the cultivation of *P. martensii* NRC 345, better enzyme production (7.76 U/mg protein) was obtained maximally in the medium described by Chawachart *et al.*[10] in the exocellular fraction (CFF) under shake culture condition and this culture medium was found to enhance laccase formation than other tested culture media.

The present research has demonstrated that incubation period is a determining factor in the process of laccase production by *P. martensii* NRC 345 because the production reaches its maximum value in a relatively gradual manner and then falls. The production decline after attaining the maximum might be due to the depletion of macro- and micronutrients in the fermentation medium with the lapse in time, which stressed the fungal physiology resulting in the inactivation of secretory machinery of the enzymes[23]. Zeng *et al.*[24] stated that, the production of ligninolytic enzymes by most white-rot fungi was found to be a 'secondary metabolic' event. Concerning the delay of optimum laccase formation which reached the 26th day of incubation in our study Fortina *et al.*[25] reported that, *Botrytis cinerea* produced appreciable levels of laccase (9.8 U/ml) in a brief period (5-7 days) and they stated that, some fungal species required a longer production time (12-30 days).

Most of fungal cultures prefer a slightly acidic pH in the medium for growth and enzyme biosynthesis[26] in agreement with the results obtained. In this study, maximal formation of *P. martensii* NRC 345 laccase took place at pH 5.5 with a narrow range of pH values. This may be attributed to the fact that change in pH value may alter the three-dimensional structure of the enzymes[27].

In the present study, the optimum production of laccase by this fungus occurred at a temperature of 30°C (7.21 U/mg protein), with a specific activity of 5.10 U/mg protein at 25°C. In this context, Zadrazil *et al.*[28] reported that, temperatures higher than 30°C caused reduction in ligninolytic enzymes activity.

In our study, different lignocellulosic wastes (wheat bran, wheat straw, sawdust, rice bran, rice straw, corn stalk, corn stover and sugar cane bagasse) were used instead of glucose in the medium for laccase production. Srinivasan *et al.*[29]

stated that, the cellulose and lignocellulosic residues were considered to stimulate laccase production. The presence of water-soluble constituents from lignocellulose fractions of the lignocellulosic residues such as p-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol and other aromatic compounds might result in the increased laccase activities in comparison to the growth on glucose[30]. Results in this study showed that, among the various wastes used, wheat bran followed by corn stover were found to be best substrates for laccase production by *P. martensii* NRC 345 with maximum laccase specific activity (39.52 and 33.33 U/mg protein, respectively) when used separately as substrates instead of glucose.

In order to improve laccase production, a wide range of carbon sources added separately in the medium was examined. The production of enzymes is related to the type and concentration of the carbon source in the medium[31], and according to[32], a substance that presents itself as an enzymatic inducer for one species can be an inhibitor of the same activity in another species. Andrade *et al.*[33] reported that, glucose is one of the most used carbon sources for biomass growth because it is easily metabolized but according to our results, galactose was found to be the best carbon source for laccase formation by *P. martensii* NRC 345 which reduced the incubation period to 22 days only instead of 26 days in case of glucose in the original medium. The replacement of galactose instead of glucose at the same concentration increased laccase production by about more than ten-fold.

Dhawan and Kuhad[34] reported that both nature and the concentration of nitrogen source are of considerable importance in laccase production. Results also demonstrated that, the highest level of laccase formation by *P. martensii* NRC 345 expressed in terms of specific activity was obtained with sodium nitrate (78.56 U/mg protein) followed by L (+) glutamine (53.92 U/mg protein).

Zeng *et al.*[35] and Palmieri *et al.*[36] reported to the stimulatory effect of surfactants and copper sulfate on laccase production. Opposite to this facts, in this study, *P. martensii* NRC 345 grew on the medium supplemented separately with different concentrations of Tween-80 and copper sulfate ranging from 0.1 to 0.75 % (v/v) and from 50 to 1500µM respectively, showed no obvious promotion of laccase activity, which indicated that the inducing capability of these compounds might vary among different lignin-degrading fungi.

5. Conclusions

In view of the results obtained, it can be concluded that: media composition, culture conditions, pH values, incubation temperatures, inoculum's size, carbon sources and nitrogen sources have significant influence on laccase production. The optimization of these criteria leads to overproduction of laccase by *P. martensii* NRC 345. It is worthy to mention that, replacement of galactose instead of glucose

increased laccase production by about more than ten-fold. However, such results point out the necessity of a better understanding of production process, mainly regarding to induction and repression processes of the substrates used.

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