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FUNGAL LACCASES PRODUCTION USING TOMATO-BASED MEDIUM: A FACTORIAL DESIGN APPROACH

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Abstract

This study was aimed to stimulate laccase production by *Trametes pubescens* MUT 2400 by using a tomato-based medium. Two sequential 2x2 factorial designs were used to determine the effect of carbon (C) and nitrogen (N) source concentration on laccase activity and biomass concentration; copper was used as the sole inducer (0.75 mM). Analysis of the first factorial design showed that N had a strong positive effect on the maximum laccase activity, which occurred at day 12 of cultivation and final biomass concentration. When both C and N concentration (25% v/v, 28.7 g/L) were set at the high level, laccase activity and biomass growth were maximal (9.5 U/mL and 7.15 mg/mL). A second factorial experiment with C and N concentrations in the ranges of 25 - 50 % v/v and 28.7 - 48.7 g/L, respectively, established based on the results of the first one, showed that laccase activity could be further increased by either increasing C or N to their high levels. The enzymatic peak occurred at the 17th day in this second design with a maximum laccase activity of 28.6-32.8 U/mL. Laccase peak occurred when reducing sugars were completely depleted from the medium. The results of this study indicated that laccase activity could be enhanced by acting on nutrient content, and tomato-based medium is a good fermentation substrate.

Key words: factorial design, fungi, laccases, tomato-based medium

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1. Introduction

Agriculture and food industrial chains produce large amount of wastes which represent a constant issue in term of disposal and handling. Common processing strategies foresee landfill storage, animal feeding, composting, burning for energy production, etc. Although some of them are able to manage the huge scale wastes produced, low valuable products are generated. The valorization of wastes as barley bran, sugarcane bagasse, grape, rice and corn wheat straw, vegetables derivates, etc. has indeed become a major research topic. Their organic components may be fruitful for greener technologies, extracting valuable chemicals (polymers, ethanol etc.) or producing fine compounds as enzymes (Arancon et al., 2013; D'Annibale et al., 2014; Gonzalez et al., 2013).

Tomato is one of the most consumed crops worldwide, producing tomato pomace as the major waste. It contains peel, seeds and also a minor fraction of pulp (Del Valle et al., 2006). Tomatoes are rich in sugars (glucose, fructose and sucrose), organic acids (malic and citric acid), phenolic compounds (flavonoids), unsaturated hydrocarbons (carotenoids) and minerals (mainly P, K, Ca and Cu) (Acosta-Quezada et al., 2015). Considering this various chemical composition, tomato derivatives can be used as cultivation medium; proper nourishment content is associated to high economic and environmental sustainability. Laccases (EC 1.10.3.2) are multicopper oxidases, able to catalyze

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the electron transfer from a substrate to a molecule of oxygen, which is thereby reduced to water. They occur in bacteria, insects and plants, but in fungi the highest production yields have been monitored (Rivera-Hoyos et al., 2013). Thanks to the high redox potential, laccases of Basidiomycetes are one of the most promising industrial biocatalysts that can be employed in textile and paper industry, wastewater treatment, chemicals synthesis, beverage clarification etc. (Rivera-Hoyos et al., 2013). Enzymatic treatments are indeed eco-friendly methodologies, displaying low energy requirements and easy process control (Torres et al., 2003), but the availability of large amount of laccases is an essential prerequisite.

Laccases are usually secreted in small amount during secondary metabolisms, but their production can be enhanced by several exogenous stimuli (Desai and Nityanand, 2011; Elisashvili and Kachlishvili, 2009). Metal ions (i.e. Cu), aromatic compounds and nutrient sources are predominant factors, sometimes directly acting at gene transcription level (Piscitelli et al., 2011). Since interactions effects among these factors occur, conventional experimental approaches in which one factor at a time is changed are not only inefficient, but lead to erroneous conclusions and sub-optimal results. Designs of experiment methods are instead able to evaluate the effects of several factors, highlighting combinatorial interactions and identifying optimum range. This approach has been successfully applied for the optimization of fungal laccases production, taking into consideration medium composition and inducers addition (Aghaie-Khouzani et al., 2012; Junghanns et al., 2008; Tinoco et al., 2011).

Obtaining low cost enzymes first passes through a reduction of production costs. To date, most of the researches have used synthetic costly media based on glucose and model inducers as veratryl alcohol, ABTS, dyes etc. (Galhaup et al., 2002; Kanwal and Reddy, 2011) but selected agro-food wastes offer alternative nourishment substrates (Elisashvili and Kachlishvili, 2009). In addition, the high phenolic content as well as the natural occurrence of minerals as Cu makes them (i.e. olive mill wastewaters, coffee husk, soybean pod, apple pomace, etc.) efficient inducers of laccase production (Cambria et al., 2011; Gonzalez et al., 2013: Park et al., 2014). For example, the presence of caffeine and tannins in coffee husk has been univocally associated to laccase production stimulation of *Trametes pubescens* strain (Gonzalez et al., 2013).

In the present study, laccase production by *Trametes pubescens* MUT 2400 was stimulated by medium composition variations. The strain was selected because of its demonstrated capability to transform organic compounds (Anastasi et al., 2012; Spina et al., 2013) making the expressed laccases good candidates for biotechnological uses. To enhance the economic sustainability of the biocatalysts production, complex not-synthetic media were used: previous evidences already demonstrated the capability of this fungal strain to grow and produce the enzyme of interest in presence of agro-industrial wastes (Gonzalez et al., 2013). A tomato-based medium was set up and the concentration of C and N source was varied to define the range where fungal productivity was maximized.

Assuming that these factors may both influence laccase production, the identification of the optimal culture conditions needed to consider single and combinatorial effects. Replicated factorial designs were used to evaluate the behavior of the fungus in response to the medium variations: the production stimulation of relevant enzymes and the biomass growth were both considered as responses.

2. Experimental

2.1. Fungal strain

Trametes pubescens MUT 2400 was selected for its capability to decolorize and detoxify wastewaters producing high concentration of laccases (Anastasi et al., 2011; Spina et al., 2013). The strain is preserved at the *Mycotheca Universitatis Taurinensis* (MUT, University of Turin, Department of Systems Biology and Life Sciences, Torino, Italy).

2.2. Chemicals

All the chemicals were purchased from Sigma Aldrich. The tomato sauce was used as a commercially available formulate (Cirio, San Lazzaro di Savena, Bologna, Italy). The partial chemical composition includes (mass fractions) 5.3 % carbohydrates (including 4.2 % sugars), 1.7 % fibers, 0.1 % fatty acid and 1.2 % proteins. Reducing sugars were analyzed following the method described in 2.4. paragraph: 37 g/L were detected in commercial tomato sauce.

2.3. Production of laccases

The experiments were carried out in 100 mL Erlenmeyer flasks with 40 mL of liquid culture as final volume. Each flask was inoculated with a 0.5 mL fungal suspension, prepared by homogenizing agar squares (1 cm^2) derived from the margins of a grown colony together with sterile water.

A tomato-based medium (TM) was used, using tomato sauce as C source and bactopeptone as N source. After two days from inoculation, laccase production was stimulated by copper addition ($CuSO_4$ 0.75 mM final concentration). A control without Cu was set for a unique cultural composition: 12.5% v/v tomato sauce and 18.7 g/L bactopeptone (corresponding to TM5-center point of *Design I*). Flasks were incubated at 25 °C and 120 rpm for 20 days. Every two days, a sample was collected; enzymatic activity, pH and reducing sugars concentration were measured. At the end of the experiment, biomass growth was also evaluated. The fungal dry weight (mg/L) was calculated after incubation at 60 °C for 24 h.

2.3.1. Description of experimental set up

To evaluate the effects of several parameters on laccase production by *T. pubescens* MUT 2400, a replicated 2^2 factorial design was carried out. Tomato sauce (X_1) and bactopeptone (X_2) were chosen as the independent variables. Tomato sauce was properly diluted with sterile water, and the concentration expressed as volume tomato sauce / total volume (% v/v). Bactopeptone concentration range was fixed considering previous evidences about laccase production by *T. pubescens* MUT 2400 in a synthetic medium (data not shown). The laccase activity and the biomass concentration were taken as response variables. The experimentation was carried out in a sequential fashion by setting up two factorial experiments.

Design I

Table 1 reports the factor levels in both natural and coded values for *Design I*. The low (-1) and high (+1) levels for tomato sauce concentration or bactopeptone concentration were 6.25 and 25% v/v or 8.7 and 28.7 g/L, respectively. An additional medium (TM5) was added with 12.5% v/v tomato sauce and 18.7 g/L bactopeptone and used to estimate response surface curvature. All the experiments were carried out in triplicate with the exception of TM5 medium that was replicated five times.

Design II

A second design was evolved according to the data of *Design I*. One of the experimental points was repeated in the two designs (labeled as TM4 in *Design I* and TM1 in *Design II*). The design factors are tomato sauce concentration (25-50% v/v) and bactopeptone concentration (28.7-48.7 g/L).

As described in Table 2, each parameter was assessed at two levels (-1, +1) and the center point was fixed at 37.5% v/v tomato sauce and 38.7 g/L bactopeptone. All the experiments were carried out in triplicate with the exception of the center point that was replicated five times. Fig. 1 reports the two designs carried out on the X_1-X_2 plane.

2.4. Enzymatic activity assay and glucose consumption analysis

Both enzymatic activity and reducing sugar content were evaluated by colorimetric analysis. A multimode reader spectrophotometer (TECAN Infinite M200, Austria) was used and the protocols were validated to be run in 96-wells microplates.

Table 1. Experimental conditions of the Design I. X_1: tomato sauce (% v/v); X_2: bactopeptone (g/L)

	coded levels		*actual levels*	
	X_1	X_2	X_1	X_2
TM1	-1	-1	6.25	8.70
TM2	+1	-1	25.00	8.70
TM3	-1	+1	6.25	28.70
TM4	+1	+1	25.00	28.70
TM5	-0.333	0	12.50	18.70

Table 2. Experimental conditions of the Design II. X_1: tomato sauce (% v/v); X_2: bactopeptone (g/L)

	coded levels		*actual levels*	
	X_1	X_2	X_1	X_2
TM1	-1	-1	25.0	28.7
TM2	+1	-1	50.0	28.7
TM3	-1	+1	25.0	48.7
TM4	+1	+1	50.0	48.7
TM5	0	0	37.5	38.7

Laccase activities were determined by following the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) in sodium-citrate buffer (pH 3.0) at 420 nm (ε_{420} = 36 mM^{-1} cm^{-1}) (Niku-Paavola, 1988). The enzymatic activity was expressed as international units (U), where one unit is the amount of enzyme that oxidizes one µmol of substrate per minute.

Reducing sugars concentration in the culture was determined using the 2,4-dinitrosalicyclic acid (DNS) (λ 540 nm) assay, according to the method of Miller (Miller, 1959). Data were expressed as residual amount (%) in comparison with the initial content before the fungal inoculum.

3. Results and discussion

Enzymes technologies feasibility passes mainly by the maintenance of low costs of fermentation for the production of industrial enzymes.

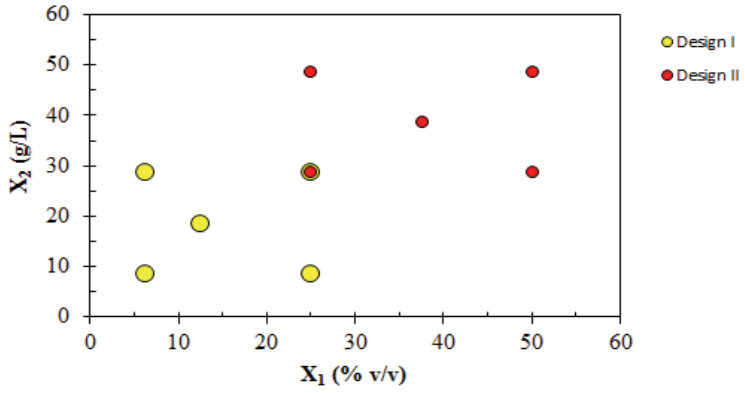

Fig. 1. Design points of *Design I* and *Design II*

Since carbon source in the fermentation medium is of major relevance on the economic balance of the process, pure synthetic sugars can be substituted by cheaper materials as by-products of agro-food industries.

The possibility to use fruits and vegetables derivatives has been recently investigated, taking into consideration for example banana and mandarin peel (Sathishkumar et al., 2010), cedar sawdust (Gonzalez et al., 2013), apricot and pomegranate wastes (Akpinar and Urek, 2014), apple pomace (Park et al., 2014), etc. Among these substrates, tomato based medium have been efficiently used for fungal fermentation by several authors (Carabajal et al., 2014; Freixo et al., 2008; Liers et al., 2007; Junghanns et al., 2008; Ramirez-Cavados et al., 2014).

The addition of inducers as syringaldazide, anthraquinonic dyes, etc. has been often required to achieve high production yields among which copper has often triggered laccase overexpression. In the present study, Cu was the sole added elicitor, taken the assumption that tomato-based medium (TM) already contains a consistent amount of uncharacterized aromatic compounds which could potentially stimulate enzymatic production (Ullrich et al., 2005). In accordance with other reports (Galhaup et al., 2002; Galhaup and Hatrich, 2001; Gonzalez et al., 2013; Hess et al., 2002), in this study *T. pubescens* enzymatic productivity was remarkably influenced by Cu.

The sole addition of 0.75 mM $CuSO_4$ in a culture medium (12.5% v/v tomato sauce and 18.7 g/L bactopeptone) increased laccase production up to 2-fold than the control without Cu (from 3.22 U/mL to 6.43 U/mL). It is indeed essential for the proper folding of laccase active site; it may play as transcriptional inducer of laccase genes due to the present of metal response elements in the promoter (Piscitelli et al., 2011). Moreover, Cu interferes with laccase denaturation catalyzed by proteases (Baldrian and Gabriel, 2002; Palmieri et al., 2001).

3.1. Laccase production by TM: Design I

Laccase production by *T. pubescens* MUT 2400 was stimulated by 5 media (TM1-5) whose composition varied for the tomato sauce and bactopeptone concentration ($CuSO_4$ 0.75 mM). Data concerning the major parameters measured during the experiment are listed in Table 3. In all the media, the maximal laccase activity was reached at the 12th day. The highest laccase activities (8.4 and 8.3 U/mL) and final biomass concentrations (7.1 and 6.1 g/L) were obtained when N source was at its maximum tested concentration (TM4 and TM3, respectively, with 28.7 g/L bactopeptone). Table 4 lists the results obtained from the analysis of variance (ANOVA). Since the p-value of each term was below 0.05 except for the curvature term of biomass, the tested factors significantly influenced laccase productivity and biomass development.

Data on laccase activity show a statistically significant curvature ($p < 0.05$), suggesting that a model with only main effects and interaction is not adequate to describe the system. Since the mean response for the center point (6.43 U/mL) is lower than the mean response at the corners of the experimental design when N is at the high level (8.3-8.4 U/mL) (Fig 2), it could be assumed that the optimum does not belong to the experimental region. A significant interaction between C and N was detected: the effect of each factor depends on the levels of the other factor. Despite this, N played a major role ($p = 0.000$). As shown in Fig 2, when bactopeptone concentration increased from -1 to +1, laccase activity increased irrespective of the low and high level of tomato sauce.

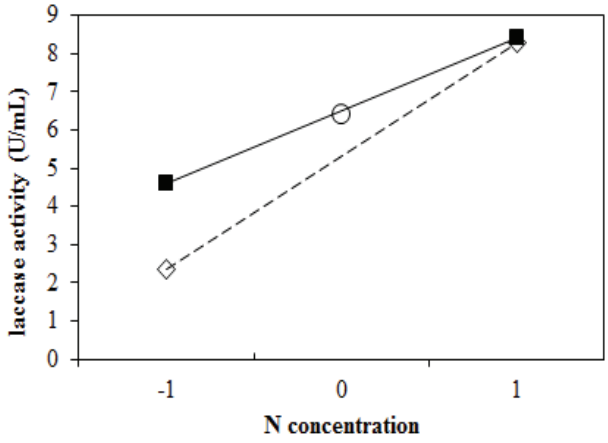

Fig. 2. Interaction plot of C and N for laccase activity: ── high level of C (+1); - - - low level of C (-1)-; ○ internal point

Table 3. Laccase activity (U/mL) at 12th day, dry biomass (g/L), pH and residual reducing sugars content (%) at the end of the experiment for Design I

	laccase activity	*biomass*	*pH*	*residual sugar %*
TM1	2.4 ± 0.1	1.8±0.0	7.6 ± 0.0	3.8
TM2	4.6 ± 1.1	4.5±0.4	8.4 ± 0.9	2.6
TM3	8.3 ± 0.1	6.1±0.4	6.7 ± 0.0	8.4
TM4	8.4 ± 0.5	7.1±1.0	8.3 ± 1.1	2.7
TM5	6.4 ± 1.1	5.0±0.2	7.5 ± 0.0	5.8

Table 4. ANOVA table of Design I data using laccase activity at 12th day (U/mL) and final biomass (g/L). SS: adjusted sum of squares; df: degrees of freedom; MS: adjusted mean sum of squares; F-value: ratio of MS and mean sum of squares for pure error. Data on lack of fit tests (curvature) are not reported

	Laccase activity			***Biomass growth***		
	C	**N**	**C*N**	**C**	**N**	**C*N**
SS	3.10	56.15	2.59	8.25	28.50	1.65
df	1	1	1	1	1	1
MS	3.10	56.15	2.59	8.25	28.50	1.65
F-value	11.16	202.29	9.34	31.15	107.59	6.23
p-value	0.016	0.000	0.022	0.001	0.000	0.047

Biomass development was similarly influenced by nutrients concentration (Table 3), with a strong interaction effect between C and N but no curvature in the system ($p > 0.05$). Within the tested conditions, the analysis of the data suggested that an increase of both N and C levels results in an increase in both laccase activity and final biomass concentration, taking into account the effect of interaction (increase of C at the high level of N does not produce any increase in laccase activity). In fact, maximum laccase activity and final biomass concentration appeared positively correlated (r^2=0.92); greater laccase productions corresponded to greater biomass growth.

pH is an important parameter to take into consideration because its variations have been often correlated with specific fermentation stages, associating this information with laccase production profile. Despite an initial acidification and a further basification of the medium has been often observed (Akpinar and Urek, 2010; Du et al., 2012; Galhaup et al., 2002), the medium composition has a predominant role on pH values fluctuations. When tomato-based media were used for fungal fermentation, the acid initial pH (pH 4-4.5) increased until alkaline values of 7-8.5 (Michniewicz et al., 2006; Ullrich et al., 2005).

Confirming these evidences, in the present study, the pH of TM was barely acidic (pH 5-6) and linearly increased during the following two weeks up to pH 7.6-8.4 (Table 3). The consumption of the C source by *T. pubescens* MUT 2400 was assessed by determining the reducing sugars concentration (Table 3). Despite their initial concentration was not high (2.40 and 7.62 g/L for the -1 and +1 level of tomato sauce concentration), it has to be considered that tomato sauce is a heterogeneous substrate in which both simple and complex carbohydrates are present (Acosta-Quezada et al., 2015), while reducing sugars concentration includes just a fraction of its overall C content.

The fungus was indeed able to develop properly (up to 286 mg dry weight) by using the nutrients provided by TM, consuming most of them. The reducing sugar content at the end of the experiment was almost absent (0.12-0.43 g/L).

3.2. TM development: Design II

Culture conditions assessed in *Design I* allowed to enhance laccase production but the region of the optimum was still far. A second factorial design (referred to as *Design II*) was then set increasing both N and C concentration. Results are listed in Table 5.

The highest laccase activity was obtained at 17th day of cultivation in TM2 (design point +1, -1). As can be seen in Fig. 3, enzymatic production started increasing a lot after 1 week from the induction and the maximal laccase productivity was reached at the 17th day for all the culture media tested.

Biological replicates slightly differed but the error percentage among them was not above 27%. These data (~ 33 U/mL) are among the best ones reported in literature for laccase production in presence of tomato-based medium. The use of media based on agro-industrial sources is indeed becoming more and more actual, as confirmed by the few evidences on complex tomato juice medium.

Table 5. Laccase activity (U/mL) at 17th day, dry biomass concentration (g/L), pH and residual reducing sugars content (%) at the end of the experiment for Design II

	lac activity	***biomass***	***pH***	***residual sugar %***
TM1	21.2 ± 3.0	89 ± 6	7.7 ± 0.1	5.3
TM2	32.8 ± 5.8	156 ± 20	8.1 ± 0.1	7.8
TM3	28.6 ± 7.9	137 ± 10	8.2 ± 0.0	9.8
TM4	19.3 ± 0.7	192 ± 4	8.1 ± 0.0	10.6
TM5	14.6 ± 1.2	174 ± 15	8.3 ± 0.0	9.5

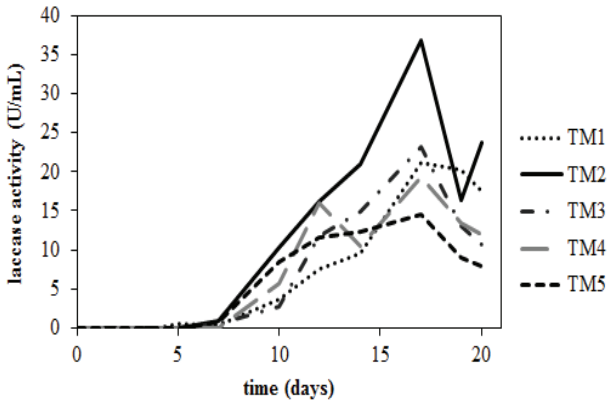

Fig. 3. Time course of laccase activity (U/mL) for the media of *Design II*

However using similar fermentation medium, lower enzymatic concentrations were found in cultures of *Cerrena unicolor* (18.7 U/mL)

(Michniewicz et al., 2006), *Trametes versicolor* (7.9 U/mL) (Carabajal et al., 2014), *Agaricus blazei* (5.0 U/mL) (Ullrich et al., 2005). Higher laccase activity (52.5 U/mL) was instead obtained by *Pycnoporus sanguineus* but, for comparative purpose, it should be mentioned that tomato juice was supplemented with high Cu concentration (3 mM) and soybean oil (1% v/v) as inducers (Ramirez-Cavados et al., 2014).

Table 6 lists the results obtained from the analysis of variance (ANOVA). As regards laccase activity, main effects were not significant upon the response variables ($p > 0.05$). The system was instead dominated by the two-way interaction and the presence of curvature. The effect of C was positive at the low N level and negative at the high N level. Thus increasing tomato sauce concentration from 30 to 50% v/v resulted in an increase in the laccase activity when bactopeptone was at 28.7 g/L (from 21.4 U/mL to 32.8 U/mL), but in a decrease when it was at 48.7 g/L (from 28.6 U/mL to 19.3 U/mL) (Fig. 4). Thus, laccase activity obtained in *Design I* could be increased by either increasing the C level or the N level, while increasing both levels at the same time results in a decrease of the laccase activity. Similarly, the laccase activity determined from the center point did not result in any improvement compared to the (-1, -1) condition (corresponding to the best medium of *Design I*).

Thus, while it was possible to improve laccase activity compared to the results of *Design I*, further experimentation would be necessary to reach optimal conditions for laccase stimulation.

Concerning biomass growth, main effects of C and N appeared statistically significant, and both tomato paste and nitrogen concentration exerted a positive effect on final biomass development. The interaction between the two parameters showed a high p-value ($p > 0.05$), resulting indeed not significant. Furthermore the system exhibited some curvature. As regard the pH and the reducing sugars consumption, *Design I* data were confirmed in *Design II* (Table 6). The pH linearly increased during the fermentation, till alkaline values closed to 8. Besides, most of the available reducing sugars were already consumed after 7-10 days, detecting a final residual amount lower than 10%. Noteworthy, only when the concentration of reducing sugars in the medium decreased to low levels, the laccase activity started to increase (around day 6-7, Fig. 5).

The strict correlation between enzymatic production and depletion of nutrients was already observed by other authors (Du et al., 2002; Hess et al., 2002; Gaitan et al., 2011; Galhaup et al., 2002), and it has been associated to the activation of energy-saving response (Piscitelli et al., 2011).

4. Conclusions

Previous reports have posed the basis for sustainable fermentations: tomato-based medium was here investigated positively stimulating *Trametes pubescens*. Carbon and N concentration exerted an interaction effect on laccase production making a factorial approach essential to screen different media.

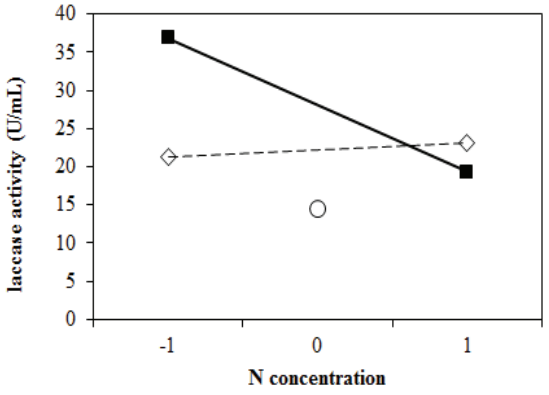

Fig. 4. Interaction plot of C and N for laccase activity: ── high level of C (+1); - - - low level of C (-1); ○ central point

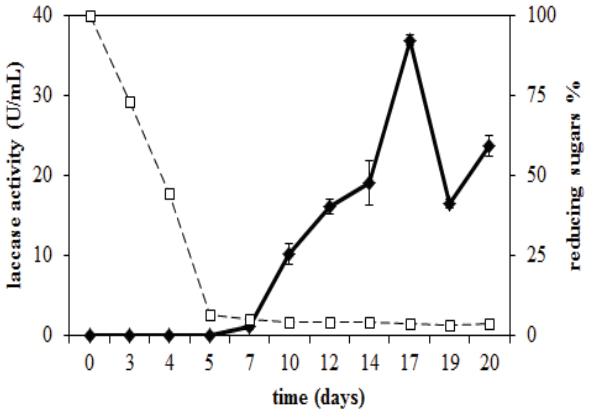

Fig. 5. Time course of *T. pubescens* grown on TM2 medium: ── laccase activity (U/mL); - - - residual reducing sugars %

Table 6. ANOVA table of Design II data using laccase activity at 17th day (U/mL) and final biomass (g/L). SS: adjusted sum of squares; df: degrees of freedom; MS: adjusted mean sum of squares; F-value: ratio of MS and mean sum of squares for pure error

	Laccase activity				*Biomass growth*			
	C	***N***	***C*N***	***curvature***	***C***	***N***	***C*N***	***curvature***
SS	3.80	28.58	324.06	283.87	11342.10	5303.60	99.00	2151.90
df	1	1	1	1	1	1	1	1
MS	3.80	28.58	324.06	283.87	11342.10	5303.60	99.00	2151.90
F-value	0.12	0.90	10.15	8.89	46.75	21.86	0.41	8.87
p-value	0.737	0.366	0.010	0.014	0.000	0.001	0.537	0.014

This method is not yet a common practice in medium engineering but it demonstrated its huge potential to define the optimal growth condition: the final laccase activity (32.8 U/mL) was one the highest reached by similar media. This result can be considered sub-optimal since laccase activity data obtained in *Design II* did not show a peak inside the experimental region investigated.

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