International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 12 No. 4 Sep. 2015, pp. 1022-1028

http://www.ijias.issr-journals.org/

Nutritional improvement process for Blue Cibacron bleaching by Coprinus cinereus strain through statistical mathematical methods

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ABSTRACT: The study of the Blue Cibacron (BC) treatment process through an statistical mathematical methods (SMM) allows us to determine a relation between nutritional variables, which are nitrogen source concentration (N), phosphorus source concentration (P) and vitamin mixture concentration (Vit.) on the decrease of coloration by bio-treatment. A complete factorial design (23) was made in order to determine the factors and their interactions which have a statistically significant influence on the studied response. Nitrogen source and vitamin mixture have a significant effect on the reduction of coloration; by contrast, Phosphorus source concentration does not have a significant effect. On the other hand, the interactions between the three factors have significant effects on the decrease of coloration. The discoloration activity is correlated with laccase activity.

KEYWORDS: Blue Cibacron, *Coprinus cinereus*, Nitrogen, Phosphorus, Vitamins, optimization and laccase.

1 INTRODUCTION

Major improvements in the methods of bioremediation attributed generally to the development of efficient strains are also closely related to parameters such as cultural conditions environment in which the microorganisms are exposed and which affect the performance of treatment [1].

The efforts to improve microbial bio-treatment frequently commence early in the development of a successful fermentation process and continue throughout in support of improved culture introduction and fermentation scale up activities [2]. Development of an economical bio-treatment medium requires the selection of a carbon, nitrogen, phosphorus, sulfur, potassium, and trace element source as well as an energy source that will support not only good microbial growth but also maximize remediation yield [3]. An equally important consideration is the cost of the nutrients, including not only the purchase price but also transportation, handling, and storage costs, any pretreatment costs (such as chemical or enzymatic hydrolysis), and special sterilization costs [4]. As medium development proceeds, other economic considerations, such as performance in stirred vessels, product recovery costs, and quality of the product, become important as well [5].

Medium optimization by the classical method involves changing one variable while fixing the others at a certain level. This mono-dimensional search is difficult and laborious, especially for an enormous number of variables, and often does not promise optimal conditions determination. An alternative method is to introduce a full factorial design exploration that would examine several possible combination of variables at suitable levels [6,7,8]. Media commonly used in environmental microbial process frequently do not become part of the process definition. Undesirable characteristics of culture media include repellant nutrient, support of suboptimal productivity, and support of the synthesis of closely related activity, and support of the synthesis of closely related product components.

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This research has been designed to integrate the perspective of improvement of Cibacron Blue (BC) discoloration and it aims at determining the best values of factors and the best combinations between nutritional factors (Nitrogen concentration (N), Phosphorus concentration (P) & vitamin mixture concentration (Vit.)) susceptible to reach a decrease coloration in order to improve the efficacy BC discoloration. In this context, the present work, initially, aims modeling and optimizing of nutritional parameters of Blue Cibacron discoloration by the strain *Coprinus cinereus* grown on minimal medium.

2 MATERIALS AND METHODS

2.1 MATERIALS

The fungus *Coprinus cinereus* used in this study was isolated from effluents contaminated dyes of an industrial sector of the city of Casablanca (Morocco). It has been identified based on morphological and biochemical characterization of the isolates grown on nutrient agar PDA (potato dextrose agar) [9] and by using taxonomic keys described by [10].

In this study two culture media were used: Potato Dextrose Agar (PDA), used in mycelium transfer and minimal medium (MM) containing [x1 g l-1 K2HPO4, 0.1 g l-1 MgSO4, x2 g l-1 (NH4) 2SO4, 0.5 g l-1 NaCl, 20 mg l-1 CaCl2, 1.1 mg l-1 MnSO4, 0.2 mg l-1 ZnSO4, 0.2 mg l-1 CuSO4 and 0.14 mg l-1 FeSO4 (pH adjusted to 7 with 1M HCl)] used to assess BC discoloration. The MM medium was supplemented by a fraction of vitamin mixture containing 100X of (Biotin 2 µg, Calcium pantothenate 400 µg, Folic acid 2 µg, Inositol 2000 µg, Niacin 400 µg, p-Aminobenzoic acid 200 µg, Pyridoxine hydrochloride 400 µg, Riboflavin 200 µg and Thiamine hydrochloride 400 µg) [11]. The vitamin mixture was sterilized by filtration and aseptically added to the MM medium according to experimental design.

2.2 EVALUATION OF THE DISCOLORATION.

The minimal medium used in each experiment is added, after sterilization for 20 minutes at 121 °C, by Cibacron blue so as to obtain a final concentration of 25mg/liter. Then, each flask was inoculated with 0,1 or 0,2 mg / ml of biomass (dry weight). These are incubated for 24 hours in an incubator at 30 or 37 °C according to the experimental design. Discoloration was evaluated by reading the absorbance at 612nm against the template (untreated medium) [1].

2.3 EVALUATION OF LACCASE ACTIVITY

Laccase activity was determined using guaiacol as the substrate according to the method of Sandhu and Arora [12]. The assay mixture contained 4.80 ml of sodium phosphate buffer pH 6.0 (100 mM), 0.1 ml of Guaiacol (10 mM) and 0.1 ml of enzyme extract. One activity unit (U) was defined as the amount of enzyme oxidized 1µmol Guaiacol per minute. The kinetic reaction was spectrophotometrically recorded at 470 nm (ε = 21,600/M/cm) incubated at 60 °C for 30 min, in an UV-Visible spectrophotometer (VWR Spectrophotometers,UV-VIS,1600PC). The enzyme activity was expressed as EU.

2.4 EXPERIMENTAL DESIGN

Many factors can significantly influence the discoloration of BC. The bibliographical data have advised us to study three factors: Nitrogen concentration (N), pH, Phosphorus concentration (P) and vitamin mixture concentration (Vit.) This study was performed according to factorial experiment design where the calculation of the coefficients of the polynomial model has been accomplished through the method of “least squares” with the use of coded variables. In fact, the act of replacing the natural variables by coded variables allows for the same domain of variation for each factor (between –1 and +1) and hence being able to compare the effect of factors among themselves. The lowest level is coded –1 while the highest level is coded +1 (Table 1).

Table 1. Levels of natural variables

Factors	levels	
	-1	+1
Nitrogen concentration(N) %	0,15	0,3
Phosphorus concentration (P)%	0.075	0,15
vitamin mixture concentration (Vit) (v/v)	1/50	1/10

A complete factorial design with 3 factors and n (number of tries) has been designed which will be equal to 8*3 = (24). In this study, we have conducted 2 replicates for each trial.

A summary of the total tests can be seen in Table 2, which we have called "Table of Experiments".

Table 2. Table of experiments

Variables/trial	Nitrogen (N)	Phosphorus (P)	Vitamin (Vit.)
1	0,15	0,075	1/50
2	0,3	0,075	1/50
3	0,15	0,15	1/50
4	0,3	0,15	1/50
5	0,15	0,075	1/10
6	0,3	0,075	1/10
7	015	0,15	1/10
8	03	0,15	1/10

2.5 STATISTICAL ANALYSIS

The statistical calculations (calculation of coefficients, T-test, analysis of variance, curves were done using the JMP software.

3 RESULTS AND DISCUSSION

On the basis of table 3, the mathematical model is written as follows and has the form:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{123}X_1X_2X_3$$

To be able to conduct the statistical calculations and prevent that n = p, (n = number of tests and p= the number of estimated parameters starting from the model, in other words, the number of the model's coefficients), it is necessary to make replication. This is the case in this study (Y_1, Y_2 and Y_3). a_0, a_1 .... a_3, a_{12} ... ... a_{123}: are the mathematical coefficients of the model. a_{ij}. X_i. X_j correspond to the interactions. n = 24: the number of realized experiments. p: the number of estimated parameters from the model.

Table 3. Factorial design experience of coded variables N(X_1), P(X_2) and Vit.(X_3).

Tests	x_0	x_1	x_2	x_3	X_{12}	X_{13}	X_{23}	X_{123}	Y_1	Y_2	Y_3	Y_{aver}
1	1	-1	-1	-1	1	1	1	-1	12	11	15	12,66
2	1	1	-1	-1	-1	-1	1	1	33	31	35	33
3	1	-1	1	-1	-1	1	-1	1	24	22	26	24
4	1	1	1	-1	1	-1	-1	-1	45	48	52	48,33
5	1	-1	-1	1	1	-1	-1	1	55	58	59	57,33
6	1	1	-1	1	-1	1	-1	-1	99	98	96	97,66
7	1	-1	1	1	-1	-1	1	-1	48	53	54	51,66
8	1	1	1	1	1	1	1	1	67	70	70	69

After the point estimate of effects (table 3), the model is written as:

$$(1)\ Y=49,20+12,79X_1-0,95X_2-19,70X_3-2,37X_1X_2+1,62X_1X_3-7,62X_2X_3-3,37X_1X_2X_3$$

3.1 THE SIGNIFICANCE OF EFFECTS

According to the test of significance of effects (T-test) of the study (table 4), we noticed that at $p<0.0001$, nitrogen and vitamin factors have a significant effect on the decrease of coloration, on the contrary phosphors do not have any significant effect.

Table 4. Parameter estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	49,20	0,48	102,02	<.0001
N	12,79	0,48	26,52	<.0001
P	-0,95	0,48	-1,99	0,0643
Vit.	19,70	0,48	40,86	<.0001
N*P	-2,37	0,48	-4,92	0,0002
N*Vit.	1,62	0,48	3,37	0,0039
P*Vit.	-7,62	0,48	-15,81	<.0001
N*P*Vit.	-3,37	0,48	-7,00	<.0001

We also noted that the interactions between Phosphorus and vitamin (P*Vit), Nitrogen, Phosphorus and Vitamin mixture (N*P*Vit), have significant effects on the discoloration. Phosphorus factor cannot be ignored in the mathematical model because its interaction is high so the mathematical model (1) is retained.

(1) $Y= 49,20+ 12,79X_1 - 0,95X_2 - 19,70X_3 - 2,37X_1X_2 + 1,62X_1X_3 - 7,62X_2X_3 - 3,37X_1X_2X_3$

Variance analysis (Table 5) which aim is to compare the sum of differences squares due solely to regression (therefore to the model) with the sum of squares of the residues with the help of the F test.

Table 5. Variance analysis

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	15138,625	2162,66	387,3422
Error	16	89,333	5,58	Prob> F
C. Total	23	15227,958	-	<.0001

We noticed that F (observed) > F (0.0001) and therefore we accept the hypothesis of the validity of the model.

(1) $Y= 49,20+ 12,79X_1 - 0,95X_2 - 19,70X_3 - 2,37X_1X_2 + 1,62X_1X_3 - 7,62X_2X_3 - 3,37X_1X_2X_3$

Since Y represents the decrease of Bleu Cibacron, and since the aim is to increase its value, it is the Nitrogen source and vitamin mixture which have the most important effect.

Limited to statistically significant words, we can adopt the mathematical model to find the relationships between the factors studied in pairs to draw the isoresponse (Contours plot) curves. This allows to decipher the interactions of the factors in pairs. These allowed us to draw isoresponse curves connecting temperature and pH, temperature and biomass and pH and biomass (Figures 2, 3 and 4). To trace the isoresponse curves, we used the JMP software that facilitates the task.

- **Study of the interaction of Nitrogen (N) and Phosphorus**

To study the fading under the experimental conditions of N and P, the factor X_3, corresponding to the vitamin mixture (Vit.) may take discrete values (-1, +1) corresponding respectively to the values of 1/50 and 1/10 concentration of vitamin. Figure 1 shows the responses obtained in the experimental area bounded by the Nitrogen source (N) and Phosphorus source (P). From this figure, we see that the increase of N factor simultaneously and the decrease of P factor improve discoloration.

This figure (fig.1) explain in a clear the major effect of Nitrogen source in metabolic activities involved in BC treatment process.

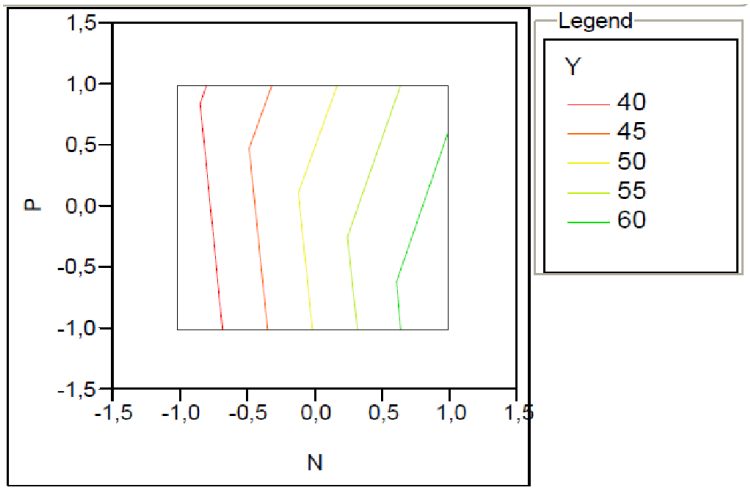

Fig.1 Contours plot of the N-P interaction

- **Study of the interaction between Nitrogen factor (N) and the vitamin mixture factor (Vit.)**

For the study of discoloration under the experimental conditions of Nitrogen factor (N) and the vitamin mixture factor (Vit.), X_2 factor corresponding to the Phosphorus source factor can take discrete values (-1, +1) which are respectively the values 0.07% and 0.15%.

Figure 2 embodies the responses obtained in the experimental area bounded by the Nitrogen (N) and vitamin mixture (Vit). From this figure, it also found that the increase of both factors simultaneously increasing discoloration (green curves) and reduction decreases discoloration. This can be explained by the positive correlation between Nitrogen source and vitamin mixture source.

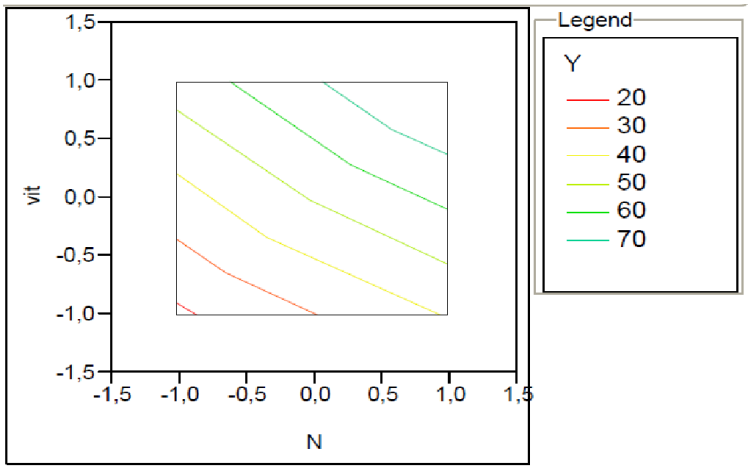

Fig.2 Contours plot of the N-Vitamin mixture interaction

- **Study of the interaction between Phosphorus source and vitamin mixture source**

For the study of discoloration under the experimental conditions of pH and biomass, X_1 factor corresponding to Nitrogen source, can take discrete values (-1 and +1) which are respectively values are 0.15% and 0.30%, figure 3 displays the isoresponses curves in the experimental zone bordered by the Nitrogen and Vitamin mixture. From this figure, we notice the existence BC bleaching in proportionality with the increase of vitamin mixture factor. Thus, the vitamin mixture factor could play an important role in controlling discoloration of Cibacron Blue.

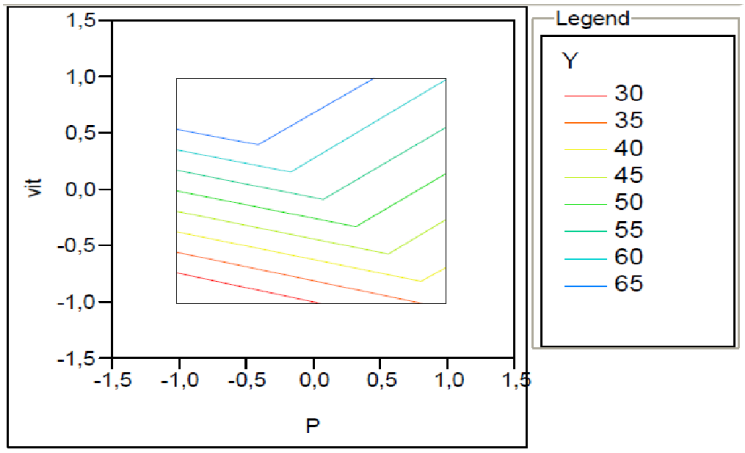

Fig. 3 Contours plot of the P-vitamin mixture interaction

The results shown in Figure 3 demonstrate that vitamin at high concentrations is favorable for maximizing the discoloration of BC. To prove that the discoloration of BC is related to the activities of peroxidases enzymes of the strain *Coprinus cinereus*, we have studied the enzyme laccase as a metabolic indicator of degradation pathways of this industrial dye. The results shown in figure 4 display that there is a high correlation between discoloration and oxidoreductase activity of the enzymes involved in the degradation of xenobiotics such as BC.

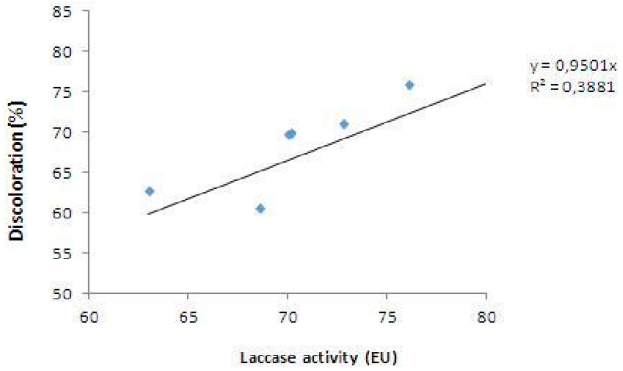

Fig. 4 Correlation between discolorations (%) and Laccase activities (EU)

The coefficient of determination (r2 = 0.3881) means that the correlation coefficient (r) equals 0.62. This value shows the existence of a significant positive correlation between BC discoloration and laccase activity used in this study as a metabolic indicator.

4 CONCLUSION

The overall results of this preliminary study, using the full factorial design shows that the optimal conditions for obtaining the best yields are fading as follows:

- A Nitrogen source at 0.3% concentration,
- A Vitamin mixture at 1/10 dilution.
- The discoloration of BC is due mainly to activities involved in oxidative degradation of refractory compounds in primary metabolism. These results, however preliminary, gives an idea about the methods to optimize the biological treatment of BC.

We intend in this research, further optimize the discoloration of Cibacron Blue studying nutritional factors combined to physicochemical factors in a single plane of experience to master discoloration Cibacron Blue. This multi-dimensional exploration is useful for a small number of variables but impractical for a large number of variables (6 factors : 3

physicochemical and 3 nutritional factors) because of the huge number of experimental run required to complete the search. Therefore, a more practical method (such as the Placqett-Burman design [13]) is recommended when more than five independent variables are to be investigated.

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