Research article

Functionalization of Bacterial Cellulose With Bacterial Pigments: Optimization Using a Full Factorial Design Approach

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Abstract.
Pigments from natural sources, such as bacterial pigments, have gained increased attention in recent years due to their biodegradability, non-toxicity, and non-carcinogenicity. The intention to replace synthetic and oil-derived compounds is not restricted to synthetic dyes; other applications include the replacement of oil-derived polymers for more environmentally friendly options, such as biopolymers. In this work, the functionalization of a bacterial cellulose (BC) biopolymer with bacterial pigments was explored using a full factorial design methodology to evaluate the best functionalization conditions to produce colored BC. From the factors and interactions evaluated, it was possible to conclude that the variable duration of the functionalization procedure could be reduced to a low level without significantly affecting the functionalization of the BC samples with bacterial pigments. Moreover, BC is a product with high industrial applicability, versatility, and sustainability. Hence, the multifunctional colored BC can be applied in the packaging, paper, and textile industries, among others.

Keywords: bacterial pigments, bacterial cellulose, full-factorial design, optimization

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1. Introduction

The adverse side effects ensued from the extensive use of synthetic dyes in food, pharmaceutical, textile, and cosmetic industries pushes research towards their replacement with safer and more sustainable alternatives [1–3]. Moreover, high concentrations of natural, biodegradable, non-toxic, and non-carcinogenic pigments can be obtained from microorganisms, as an alternative to chemically produced dyes. These pigments exhibit a wide range of colors as well as interesting biological activities [4]. Additionally, microorganisms are also able to produce non-toxic and biodegradable biopolymers, such as Bacterial Cellulose (BC), which exhibits noteworthy intrinsic properties, with high industrial applicability [5,6] Employing microbial resources for BC production simplifies the downstream and upstream processes, due to the capacity of production regardless climatic or environmental conditions, in a controlled environment, and also owing to the

absence of undesirable compounds (such as lignin, pectin, and hemicellulose, contained in vegetable cellulose) [5,6]. Furthermore, factorial designs are commonly used to investigate how one or more responses are affected by the effects of experimental factors (variables evaluated) and the interactions between those factors, using a reduced number of experiments, and at the same time increasing the possibilities to evaluate interactions among the variables, which ultimately may reduce the process evaluation costs [7]. Thus, the aim of the present work is to investigate the functionalization of BC pellicles with the bacterial pigments prodigiosin, violacein and flexirubin-type pigment and improve the functionalization process using full factorial design methodology.

2. Material and methods

Biosynthesis and recovery of bacterial pigments: Prodigiosin was produced by *Serratia plymuthica*, in Peptone Glycerol Phosphate medium, at 20°C, with no light exposure [8]. Violacein production was accomplished using *Chromobacterium violaceum*, in Tryptic Soy Broth, at 30°C [9]. Flexirubin-type pigment was produced by bacteria *Chryseobacterium shigense*, also at 30°C, in nutrient medium [10]. The pigments recovery was achieved using previously established methods with acidified ethanol, ethanol, and acetone, for prodigiosin) [11], violacein [12], and flexirubin-type pigment [10], respectively.

Biosynthesis and recovery of BC : A commercial Kombucha beverage was used as inoculum 10% (v/v) for BC production. The fermentation was carried out for 7 days, at 30°C, using a mixture of 16.5 g/L of black and green tea, and 70 g/L of glucose. The recovered BC pellicles were subjected to an alkaline treatment to remove impurities and possible cellular debris [13].

Functionalization conditions optimization : Experimental runs were designed by Design Expert software, version 7.0.0 (Stat-Ease, Minneapolis), using the full factorial method. The two-level three-factor full factorial experimental design approach was applied for the evaluation of three variables at two levels, for each pigment tested, in order to evaluate the optimal conditions for BC functionalization with violacein, prodigiosin, and flexirubin-type pigment. The variables evaluated in this work were temperature (A), duration of functionalization (B), and pigment concentration (C). Temperature was kept at 40°C (low level) and 90°C (high level), the duration of BC-functionalization was 20 min or 60 min, for low (-1) and high level (+1), respectively, and pigment solutions were prepared at 5 and 30% over the weight of the fiber (owf). In addition, triplicate mid-level of variables, center points, were also included. The Statistical software was also used to establish the validity of the models on the basis

of analysis of variance (ANOVA) and evaluate the effects of process variables (A, B, C) and their interactions on the response variable, BC functionalization, by measuring the color strength (K/S) of the samples produced.

Functionalization procedure and samples' evaluation: A Datacolor AHIBA IR equipment (Datacolor company, USA) was used for BC functionalization with each pigment solution. The pigment solutions were prepared at 30% owf, 17.5% owf, and 5% owf. The functionalization temperatures evaluated were 40, 65, 90°C, and the functionalization duration was 20, 40 and 60 min. A liquor ratio of 1:30, 20 rpm, and a 2°C/min raise velocity were maintained constant during the entire full factorial experimental design study. A Datacolor 110 spectrophotometer (Datacolor company, USA) was used to evaluate the response variable by determining the K/S values of the functionalized samples. The Kubelka–Munk equation was used to determine the K/S values.

3. Results and discussion

Table 1, 2 and 3 summarize the randomized set of the experiments with actual factors and the measured K/S response for BC functionalized with violacein, prodigiosin and flexirubin-type pigment.

ANOVA was performed to statistically evaluate the significance of the factors (data not shown) and the Pareto charts (Figure 1) illustrate the relative importance of the main effects and their interactions. Pareto charts are composed of columns that represent the values obtained from a student's t-test to determine whether the calculated effects (factors and interactions between factors) were significantly different from zero [14]. The charts also show two limits, the Bonferroni and the t-limit, that aid in the evaluation of significant factors and interactions. Significant effects appear above the threshold given by the Bonferroni limit, insignificant factors appear below the t-limit, and in between both limits the effects may be significant [15]. As can be observed in Figure 1, significant factors and interactions, for violacein and prodigiosin pigments (Figure 1 (a) e (b)), were temperature, duration of functionalization, pigment concentration, and the interaction between temperature and concentration. Whereas, for flexirubin-type pigment, the temperature, and the pigment concentration were the only two significant factors (Figure 1 (c)).

TABLE 1: Summary of the randomized set of the experiments with actual factors and the measured K/S response, for the 2^3full factorial design with violacein pigment.

Violacein					
Std	Run	Factor A: Temperature (°C)	Factor B: Duration(Minutes)	Factor C: Concentration	Response: K/S
26	1	65	40	0.175	3.35
16	2	90	20	0.300	6.40
27	3	65	40	0.175	3.36
13	4	40	20	0.300	3.61
22	5	90	60	0.300	6.47
7	6	40	60	0.005	1.63
19	7	40	60	0.300	4.35
25	8	65	40	0.175	3.33
4	9	90	20	0.005	1.36
1	10	40	20	0.005	1.55
10	11	90	60	0.005	2.15
14	12	40	20	0.300	3.59
29	13	65	40	0.175	3.41
20	14	40	60	0.300	4.07
11	15	90	60	0.005	2.05
23	16	90	60	0.300	6.46
30	17	65	40	0.175	3.34
2	18	40	20	0.005	1.22
5	19	90	20	0.005	1.49
8	20	40	60	0.005	1.61
28	21	65	40	0.175	3.67
17	22	90	20	0.300	5.79
32	23	65	40	0.175	3.53
15	24	40	20	0.300	4.05
9	25	40	60	0.005	1.62
12	26	90	60	0.005	2.30
33	27	65	40	0.175	3.96
18	28	90	20	0.300	5.71
3	29	40	20	0.005	1.55
21	30	40	60	0.300	4.42
6	31	90	20	0.005	1.71
24	32	90	60	0.300	6.62
31	33	65	40	0.175	3.60

4. Conclusions

The results indicated that the variable duration was the least significant, for BC functionalized with violacein and prodigiosin pigments, and not even a significant factor,

Table 2: Summary of the randomized set of the experiments with actual factors and the measured K/S response, for the 2^3full factorial design with prodigiosin pigment.

		Prodigiosin			
Std	Run	Factor A: Temperature (ºC)	Factor B: Duration(Minutes)	Factor C: Concentration	Response: K/S
26	1	65	40	0.175	1.97
13	2	40	20	0.300	1.15
19	3	40	60	0.300	1.83
10	4	90	60	0.005	0.67
1	5	40	20	0.005	0.3
25	6	65	40	0.175	1.78
16	7	90	20	0.300	2.74
22	8	90	60	0.300	2.94
7	9	40	60	0.005	0.45
27	10	65	40	0.175	1.57
4	11	90	20	0.005	0.7
23	12	90	60	0.300	2.98
5	13	90	20	0.005	0.78
20	14	40	60	0.300	1.62
29	15	65	40	0.175	1.61
8	16	40	60	0.005	0.49
14	17	40	20	0.300	1.69
30	18	65	40	0.175	1.64
17	19	90	20	0.300	2.61
11	20	90	60	0.005	0.94
2	21	40	20	0.005	0.29
28	22	65	40	0.175	1.24
6	23	90	20	0.005	0.86
15	24	40	20	0.300	1.34
9	25	40	60	0.005	0.44
3	26	40	20	0.005	0.48
24	27	90	60	0.300	2.97
18	28	90	20	0.300	2.76
32	29	65	40	0.175	1.45
21	30	40	60	0.300	1.96
12	31	90	60	0.005	0.87
31	32	65	40	0.175	1.48
33	33	65	40	0.175	1.2

for flexirubin-type pigment-BC functionalization. Moreover, the K/S obtained for the BC functionalization, with the three pigments, under best dyeing conditions would be similar to the K/S achieved if the variable duration was reduced to its low level.

TABLE 3: Summary of the randomized set of the experiments with actual factors and the measured K/S response, for the 2^3full factorial design with flexirubin-type pigment.

Flexirubin-type pigment					
Std	Run	Factor A: Temperature (°C)	Factor B: Duration(Minutes)	Factor C: Concentration	Response: K/S
26	1	65	40	0.175	0.28
4	2	90	20	0.005	0.55
16	3	90	20	0.300	1.50
27	4	65	40	0.175	0.40
10	5	90	60	0.005	0.51
25	6	65	40	0.175	0.44
1	7	40	20	0.005	0.10
7	8	40	60	0.005	0.13
13	9	40	20	0.300	0.24
19	10	40	60	0.300	0.23
22	11	90	60	0.300	1.40
14	12	40	20	0.300	0.21
5	13	90	20	0.005	0.56
28	14	65	40	0.175	0.25
11	15	90	60	0.005	0.45
23	16	90	60	0.300	1.13
2	17	40	20	0.005	0.11
8	18	40	60	0.005	0.12
29	19	65	40	0.175	0.38
20	20	40	60	0.300	0.24
30	21	65	40	0.175	0.39
17	22	90	20	0.300	1.06
21	23	40	60	0.300	0.20
32	24	65	40	0.175	0.27
6	25	90	20	0.005	0.47
24	26	90	60	0.300	1.02
3	27	40	20	0.005	0.11
33	28	65	40	0.175	0.35
31	29	65	40	0.175	0.44
12	30	90	60	0.005	0.60
9	31	40	60	0.005	0.13
15	32	40	20	0.300	0.20
18	33	90	20	0.300	1.43

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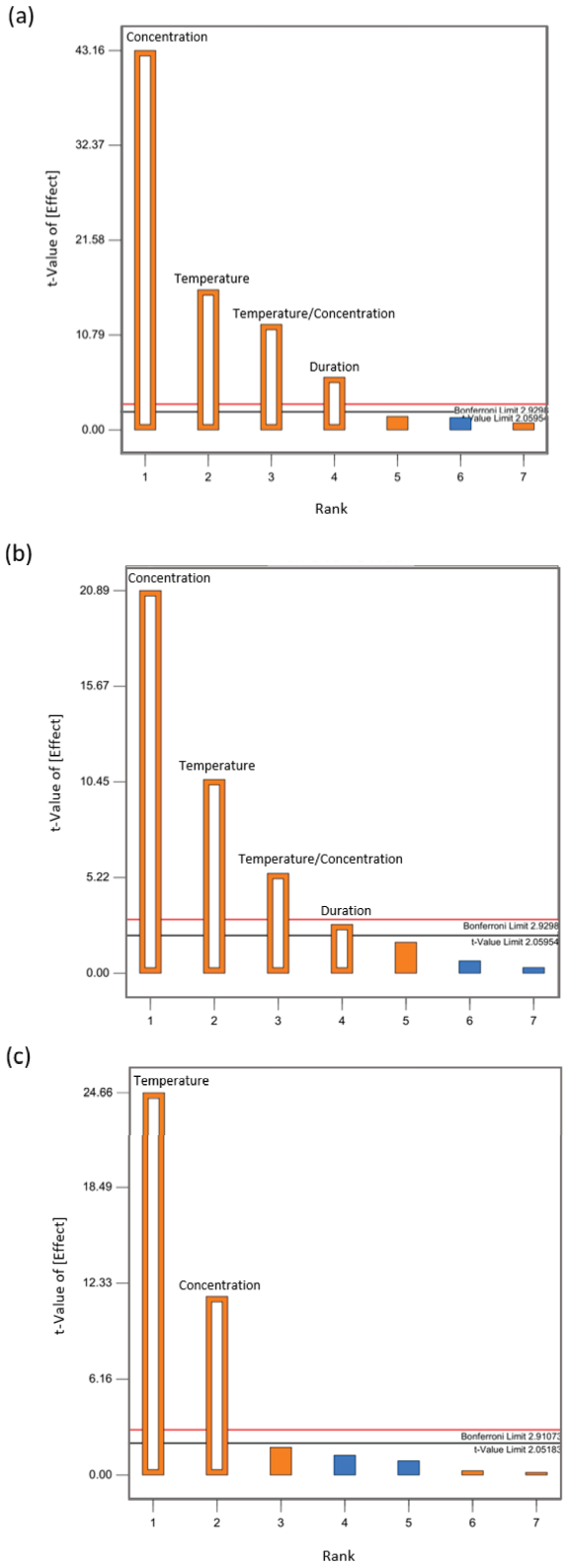

Figure 1: Pareto charts for the models of each pigment, violacein (a), prodigiosin (b), and Flexirubin-type pigment (c), presenting graphically the statistical significance of the factors and interactions.

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