Understanding the effects of process parameters in the bioscouring of cotton and their interactions on pectate lyase activity by factorial design analysis

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ABSTRACT
Pectinases can be used to remove impurities and hydrophobic material from cotton fibers by the degeneration of pectic substances. The biochemical characterization and the evaluation of process parameters that influence the enzyme’s activity are mandatory to optimize the industrial application. In the present study, a factorial experimental design was conducted to evaluate the activity of a commercial pectate lyase at different reaction conditions of temperature, pH, and nonionic surfactant concentration, aiming its application in bioscouring of textiles. The main effects of all three factors were found to be statistically significant. The second-order interactions between temperature and pH, and pH and surfactant concentration, were also important. Maximum activity on polygalacturonic acid was achieved at 60 °C, pH 9, and without surfactant. These results provided support for the subsequent bioscouring assays, resulting in a high level of cleanliness and good wettability of knitted cotton fabric at the lowest enzyme concentration (1 g/l), and could make textile processing more sustainable.

1. Introduction
Cotton is an important natural fiber used in the textile industry (Abdulrachman et al., 2017; Demir et al., 2018). Cotton fiber is composed of mostly cellulose, 88.0-96.5% (Gordon & Hsieh, 2007), and it has four elementary layers in its morphological structure, which are illustrated in Figure 1 with emphasis on the cuticle and the primary cell wall. The cuticle is a slim outer layer composed of wax, proteins, and pectins, protecting the cotton from environmental attacks and water penetration (Li & Hardin, 1997). The primary cell wall is about 0.1 microns thick, which comprises only 1% of the total fiber thickness and consists of 52% cellulose with a mixture of pectins, waxes, proteins, ashes, and other organic compounds comprising the remainder (Etters, 1999). Natural hydrophobic non-cellulosic impurities are located mainly in the cuticle and primary cell wall and are responsible for the low wettability and water absorption of raw cotton (Gordon & Hsieh, 2007). Therefore, raw cotton fabrics must be prepared, cleaned, and converted into hydrophilic before the subsequent dyeing, printing, and finishing processes (Besegatto et al., 2018).

The conventional cleaning and preparation procedure for cotton involves alkaline scouring with sodium hydroxide (NaOH) and other chemicals, at boiling temperature, followed by multiple rinsing operations (Varadarajan & Venkatachal, 2016). The result is a fiber with a cellulose content of over 99% and excellent water absorbency (Wakelyn et al., 2006). However, scouring represents one of the most aggressive stages inside the textile processing industry (Losonczi et al., 2004), with corrosive actions on equipment parts, toxic fumes, long residence time, high energy consumption, loss in fabric strength, and environmental pollution (Ramadan, 2008).

A gentle and environmentally friendly scouring can be achieved using enzymes, biodegradable proteins that function as efficient and highly specific biological catalysts (Demir et al., 2018). Enzymes display high substrate selectivity and act exclusively on the compounds that must be eliminated, permitting better control to achieve the desired fiber properties (Andreaus et al., 2019). Enzymatic scouring combines several advantages compared to conventional chemical processing, such as reduced usage of water, chemicals, and energy and milder temperature and pH conditions (Varadarajan & Venkatachal, 2016). Pectinases, for example, are capable of hydrolyzing pectins (a polymer of α-1,4 linked galacturonic acid residues, Figure 2), which act as an impermeable biological glue binding the non-cellulosic components of the primary wall of cotton within the cellulose matrix (Etters, 1999), without causing significant fiber damage. Pectinase enzymes act on pectic compounds such as the polygalacturonic acid backbone of pectin without attacking cellulose, which is an undesirable side activity of alkaline scouring (Abdulrachman et al., 2017).
Pectate lyases (EC 4.2.2.2), which have been found to be the most interesting pectinases for bioscouring, cleave α 1-4-glycosidic linkages in the pectic acid and form 4,5-unsaturated products from the nonreducing end, through trans-elimination reaction (Figure 3) (Andreaus et al., 2019; Linhardt et al., 1986). The digestion of pectins on the surface of cotton fibers by pectate lyase destabilizes the structure, loosens the cellulosic matrix, and releases other non-cellulosic products for subsequent removal by emulsification (Niaz et al., 2011). Here, the inclusion of surfactants in the bioscouring bath is essential to ensure adequate hydrophilicity of the textile article (Tzanov et al., 2001).

The surfactant is a complementary but necessary additive in the composition of the enzymatic scouring bath, which has a high impact on the removal of waxes and fats to achieve good wettability of cotton fabrics (Tzanov et al., 2001). Surfactants can complex with waxes and fats, making them water-soluble and amenable to rapid elimination from the fabrics (Hebeish et al., 2009). Also, surfactants reduce surface tension and allow easier penetration of the enzymes into fibers micropores and cracks, as well as they may help
to maintain the enzymes in the liquid phase, making them available for further catalytic action (Tzanov et al., 2001). However, surfactant type and concentration should be chosen carefully to avoid negative effects on enzyme activity and conserve the desired wetting and detergency capacity of the scouring bath. According to Holmberg (2018), the nonionic surfactants are more compatible with enzymes than ionic surfactants.

The rising academic interest in this area could be shown by a search in the Web of Science on March 28, 2020, that rendered more than 1400 research papers for the term pectate lyase (and this number is continuously rising, Figure 4), but only 92 publications were found for the combination of “pectate lyase” with “textile”.

Given the need to amplify the studies on enzymatic treatments with pectate lyase in the textile industry, the objective of this study was to characterize the performance of the commercial enzyme Bioprep® 3000 L under typical activity assay conditions on polygalacturonic acid (Experimental part 1, using polygalacturonic acid as the model compound for pectic substances in cotton fibers) and establish therefrom a roadmap for bioscouring under optimized conditions (Experimental part 2). A factorial experimental design was used to identify the more significant variables (temperature, pH, and surfactant concentration), considering the possibility of increasing the enzymatic activity and bioprocess efficiency. It is important to know these enzyme characteristics and understand the catalytic mechanism to evaluate the best operating range and its sensitivity to the surfactant, due to the possibility of the enzyme becoming unstable or inactive during industrial processes.

2. Material and methods

2.1. Material

The commercial enzyme BioPrep® 3000 L (an alkaline pectinase with pectate lyase activity from Bacillus licheniformis) was kindly supplied by Novozymes®. The nonionic surfactant Berol® 175 is based on natural primary alcohol (alcohol ethoxylate, 90%) and was kindly provided by Macler Produtos Químicos Ltda. Polygalacturonic acid was purchased from Sigma-Aldrich. The 100% cotton greige knitted fabric (Jersey) (160 g/m²), made from combed yarns of Ne 30.1, was purchased from the local market. All chemical reagents used were of analytical grade.

2.2. Experimental part 1 – enzyme activity on polygalacturonic acid

2.2.1. Enzyme assay

Pectate lyase activity was determined according to Collmer et al. (1988). Polygalacturonic acid was used as the substrate (substrate stock solution: 50 mM Tris-HCl, 0.6 mM calcium chloride, and 0.24% (w/v) polygalacturonic acid) and the increasing absorbance at 232 nm of 4,5-unsaturated reaction products was monitored with a Shimadzu 1601 spectrophotometer. To initiate the assay, 2.5 ml of the substrate stock solution with surfactant and 0.5 ml of enzyme plus water (1 ml/l), both equilibrated at the desired temperature, were rapidly mixed in a cuvette with a 1 cm light path. The increase in absorbance was monitored as a function of time so that the enzyme produced a linear rate of reaction for 30 s.

One unit of the enzyme (U) releases 1 μmol of 4,5-unsaturated pectic fragments per minute under the conditions of the assay. The molar extinction coefficient for the unsaturated products at 232 nm is 4600 M⁻¹ cm⁻¹ (Collmer et al., 1988). The formula for enzyme activity is given in equation (1), where: ΔAbs/Δt is the increase in absorbance of enzyme solution per time of incubation (min), ε is the molar extinction coefficient, V_total is the total volume of the mixture (3.0 ml), V_enzyme is the volume of the enzyme (0.5 ml), and

![Figure 3](image1.png) Mode of enzymatic action of pectate lyases in a pectin molecule according to Linhardt et al. (1986).

![Figure 4](image2.png) Papers reported by Web of Science for pectate lyase.
d is the dilution factor of the enzyme before addition to the reaction mixture (1000 times).

\[
\frac{U}{ml} = \frac{\Delta \text{Abs}}{\Delta t} \times \frac{1}{\varepsilon} \times \frac{V_{\text{total}}}{V_{\text{enzyme}}} \times d
\]  

(1)

2.2.2. Experimental design

The experimental design is an efficient statistical tool used for process optimization and to evaluate the effects of many parameters, individually and their interactions. The “change-one-factor-at-a-time” method used for optimization is very time consuming and could lead to less accurate conclusions since it does not consider the interactions between factors, so multivariate techniques have been used increasingly in analytical applications, in the industrial world, and in bioprocesses (Kalantzi et al., 2019).

In this case, the pectate lyase activity, dependent factor, was determined from three independents factors (Table 1): temperature (40, 50, and 60 °C), pH (7, 8, and 9), and nonionic surfactant concentration (0.0, 0.5, and 1.0 g/l), which potentially affect the catalytic behavior. A 2³ full factorial experimental design with center point (0) was used, where the factors were evaluated at the minimum (-1) and maximum (+1) levels, totaling 9 test runs (Table 2) carried out in triplicate. The upper and lower limits of each variable were selected according to pectate lyase characteristics and preliminary investigations. The experiments were conducted randomly, and enzyme activity (U) was measured as described in item 2.2.1.

2.2.3. Statistical analysis

The enzyme behavior concerning the studied factors (pH, temperature, and surfactant concentration) was compared to a first-order prediction model, according to equation (2), and response surface plots were generated.

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j
\]  

(2)

Where: Y is the predicted response, \( \beta_0 \) is an offset term, \( \beta_i \) is the individual effect of each factor, \( \beta_{ij} \) is the interactive coefficient between factors, \( x_i \) and \( x_j \) are the levels of the independent variables (factors), and k is the number of independent variables (factors studied). All data were analyzed using Analysis of Variance (ANOVA) considering a significance level of 1%. Statistical analysis was performed by STATISTICA® software (version 13).

2.3. Experimental part 2 – bioscouring of cotton with pectate lyase

2.3.1. Enzymatic treatment of cotton fabrics

Bioscouring experiments were carried out in 500 ml stainless steel recipients, in a textile laboratory washing machine (wash-tester, Kimak, Brusque, Brazil) with vertical agitation of 40 rpm. Swatches (5.0 g) of knitted cotton jersey fabrics were treated with different enzyme concentrations (1.0, 5.0, and 10.0 g/l) at a fabric to liquor ratio of 1:20 (g/ml) without or with nonionic surfactant (1 g/l, Berol® 175). Treatment with pectinate BioPrep® 3000 L was carried out at pH 8.5 (0.05 M Tris-HCl buffer) at 55 °C for 30 min. Then the solution was heated to 90 °C and agitated for 10 min to deactivate the enzyme. Then fabrics were washed with distilled water at 55 °C for 10 min, extensively rinsed with tap water at room temperature, and air-dried. Control treatments were carried out similarly without enzyme addition. All experiments were performed in triplicate.

2.3.2. Fabric testing

Fabric weight loss was calculated according to equation (3), where \( W_1 \) and \( W_2 \) correspond to the weights of the fabric before and after the cleaning process, respectively. Before each weighing, the samples were conditioned for at least 24 h in a controlled atmosphere above saturated copper chloride solution at a relative humidity of 65 ± 4%.

\[
\text{Weight loss (\%)} = \left( \frac{W_1 - W_2}{W_1} \right) \times 100
\]  

(3)

The whiteness index (Berger) of the fabrics was evaluated with a reflectance spectrophotometer (CM-3610D, Konica Minolta), using white ceramic as the calibration standard.

The hydrophilicity of the fabric was determined by the water drop test using NBR 13000 (ABNT (Associação Brasileira de Normas Técnicas), 1993). According to

### Table 1. Actual and coded levels of the independent variables.

| Independent variables | Levels |
|-----------------------|--------|
| \( x_1 \) (temperature, °C) | -1 (40) 0 (50) 1 (60) |
| \( x_2 \) (pH) | 7 8 9 |
| \( x_3 \) (surfactant concentration, g/l) | 0.0 0.5 1.0 |

### Table 2. Matrix of the 2³ full factorial experimental design with one center point, and enzymatic activity of pectate lyase at different temperatures \( (x_1) \), pH \( (x_2) \), and surfactant concentration \( (x_3) \).

| Run | \( x_1 \) (°C) | \( x_2 \) | \( x_3 \) (g/l) | Experimental activity* (U/ml) | Predicted activity (U/ml) | Difference (%) |
|-----|---------------|----------|----------------|-----------------------------|----------------------------|----------------|
| 1   | -1 (40)       | -1 (7)   | -1 (0.0)       | 114.00 ± 3.65               | 111.19                     | 2.5            |
| 2   | -1 (40)       | -1 (7)   | +1 (1.0)       | 110.61 ± 1.71               | 115.45                     | 4.4            |
| 3   | -1 (40)       | +1 (9)   | -1 (0.0)       | 421.57 ± 9.84               | 414.67                     | 1.6            |
| 4   | -1 (40)       | +1 (9)   | +1 (1.0)       | 244.78 ± 16.28              | 253.72                     | 3.7            |
| 5   | +1 (60)       | -1 (7)   | -1 (0.0)       | 147.91 ± 7.21               | 152.76                     | 3.3            |
| 6   | +1 (60)       | -1 (7)   | +1 (1.0)       | 159.83 ± 13.10              | 157.02                     | 1.8            |
| 7   | +1 (60)       | +1 (9)   | -1 (0.0)       | 618.17 ± 70.67              | 627.10                     | 1.4            |
| 8   | +1 (60)       | +1 (9)   | +1 (1.0)       | 473.04 ± 32.09              | 466.15                     | 1.5            |
| 9   | 0 (50)        | 0 (8)    | 0 (0.5)        | 295.39 ± 50.34              | 287.26                     | 2.8            |

* Values are the average from three replicates ± standard deviation.
Values are statistically significant with a 99% confidence interval. Holmberg (2018).

Changes may eventually lead to the loss of enzymatic activity when the surfactant interacts with the enzyme, conformational changes affecting the wettability of fabrics (Raza et al., 2014). However, if detergents for immiscible fatty substances from the fiber are used, enzymatic activity increases. Surfactants act as emulsifiers and allow subsequent dyeing, printing, and finishing processes (Andreaus et al., 2019; Besegatto et al., 2018).

Along with the enzyme, surfactants are used in many bioscouring formulations. Surfactants act as emulsifiers and detergents for immiscible fatty substances from the fiber surface and make saponification of these hydrophobic impurities (oils and waxes) in the aqueous medium, increasing the wettability of fabrics (Raza et al., 2014). However, if the surfactant interacts with the enzyme, conformational changes may eventually lead to the loss of enzymatic activity (Holmberg, 2018).

From a practical point of view, understanding the magnitude of interactions between factors pH, temperature, and surfactant concentration, and their effects, on enzyme activity is necessary to improve bioscouring. Table 2 displays the experimental and predicted results (using equation 4) carried out by factorial experimental design. The effects of the factors can be seen in Table 3.

According to Table 2, the enzyme showed the highest activity (618.17 U/ml) at 60°C, pH 9, and in the absence of surfactant (run 7). The standard deviation for this same treatment was also the largest. This may be due to the enzyme being very close to their extreme temperature and pH values at a temperature of 60°C and pH 9.0 (Foulk et al., 2008), leading to possible instabilities in the protein structure of the pectate lyase and in their ability to serve as a catalyst. The temperature at which the enzyme exhibited its best activity was 60°C, however, at 70°C and 80°C the enzyme retained only 55% and 15% of its maximum activity, respectively (unpublished data), indicating that higher temperatures cause significant conformational changes or even lead to denaturation of the enzyme and loss of its catalytic activity. At pH 10, also, most commercial pectinases lose significant enzyme activity (Daniell et al., 2019).

The independent variable with the most pronounced effect on enzyme activity was pH (Table 3). In fact, enzymes have ionic groups in their active site which must be in a stable form (Zohdi & Amid, 2013). As Linhardt et al. (1986) report, the protein chain of pectate lyases is predominantly cationic. Variation in pH of the medium results in changes in the ionic form of the active site which affects the reaction rate and decreases enzyme activity (Zohdi & Amid, 2013). The high positive effect of 306.3 indicates that enzyme activity increases under the studied conditions with increasing pH from 7 to 9, as in the work of Foulk et al. (2008), and can be attributed to the high pKa of the basic residue (arginine or lysine) in the catalytic site of pectate lyase (Abbott et al., 2010). Comparing runs 5 and 7, in which only the pH was different, enzyme activity augmented four times when pH was increased from 7 to 9. These results are corroborated by other studies that have also shown that some pectinases are most active at alkaline pH (Khan & Barate, 2016). From the perspective of bioscouring, a higher enzyme activity at alkaline pH is beneficial, because under these conditions a greater cleaning action and interaction with dirt (better repulsion of dirt particles from the cotton surface) is achieved (Vineyard & Freitas, 2015) since alkaline pH increases the zeta potential and thus the stability of dispersions and micelles (fat droplets trapped by surfactant molecules).

It is perceived that the other two factors (temperature and surfactant) are also significant for the activity of the pectate lyase (Table 3). Temperature as an isolated factor showed the expected behavior: the catalytic capacity of the enzyme increases with temperature following the Arrhenius equation. The surfactant, despite its unfavorable impact on enzyme activity (negative sign), had the least effect on enzyme activity at alkaline pH is beneficial, because under these conditions a greater cleaning action and interaction with dirt (better repulsion of dirt particles from the cotton surface) is achieved (Vineyard & Freitas, 2015) since alkaline pH increases the zeta potential and thus the stability of dispersions and micelles (fat droplets trapped by surfactant molecules).

Nerurkar et al. (2015), water drop absorption in five seconds or less represents adequate absorbency of the cotton fabric. To determine the hydrophobicity of the greige cotton fabric, contact angle measurement was carried out with a goniometer (Ramé-Hart Goniometer/Tensiometer, model 250).

Micrographs for morphological analyses of cotton fabrics were obtained with a conventional JEOL JSM-6390LV SEM instrument (Scanning Electron Microscope) with a tungsten filament. Samples were previously coated with a thin layer of gold.

| Independent variables | Effect |
|-----------------------|--------|
| Temperature           | 127.0* |
| pH                    | 306.3* |
| Surfactant concentration | –78.3* |
| Temperature X pH       | 85.4*  |
| Temperature X surfactant concentration | 11.7 |
| pH X surfactant concentration | –82.6* |

* Values are statistically significant with a 99% confidence interval.

**Table 3.** Effect of the studied variables on the enzymatic activity of pectate lyase.

2.3.3. Statistical analysis

Bioscouring data were analyzed using the Tukey test to determine significant differences with a significance level of 5%, by STATISTICA® software (version 13).

3. Results and discussion

3.1. Multivariate effect of pH, temperature, and surfactant on the activity of pectate lyase enzyme

Pectate lyase enzyme is widely employed in bioscouring of natural cellulosic fibers, especially cotton, for the degradation of the pectin layer on the fiber to grant hydrophilicity and allowing subsequent dyeing, printing, and finishing processes (Andreaus et al., 2019; Besegatto et al., 2018).

Pectate lyase requires Ca²⁺ for activity, has an optimum pH for the action in the alkaline region (7.5-10.0), and has an optimum temperature between 40 and 50°C (Uenojo & Pastore, 2007). Bioprep® 3000 L enzyme, specifically, acts efficiently in specific conditions of the pH between 7 and 9 and temperature between 50 and 60°C (Foulk et al., 2008). Along with the enzyme, surfactants are used in many bioscouring formulations. Surfactants act as emulsifiers and detergents for immiscible fatty substances from the fiber surface and make saponification of these hydrophobic impurities (oils and waxes) in the aqueous medium, increasing the wettability of fabrics (Raza et al., 2014). However, if the surfactant interacts with the enzyme, conformational changes may eventually lead to the loss of enzymatic activity (Holmberg, 2018).

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In this work, we observed some loss of enzyme activity with the increase in nonionic surfactant concentration (Tables 2 and 3). The surfactant can influence enzyme activity in two different principal ways: (1) by binding to the enzyme and modifying intrinsic enzymatic properties such as structure or flexibility; and (2) by changing the environment in which the enzyme functions (Rubingh, 1996). It seems that the surfactant diminishes the interaction between enzyme and pectin, or by adsorption to the enzyme or by adsorption to the textile substrate. According to Holmberg (2018), the nonionic surfactant only binds to the enzyme through hydrophobic interactions, leading to conformational changes that can lead to denaturation of the protein. That is, the lipophilic part (or hydrophobic part) of the surfactant molecule may interact with the hydrophobic regions inside the enzyme proteins, causing the breakdown of its secondary and tertiary structure. The surfactant may influence the conformational dynamics of the enzyme, and these changes will cause a loss in enzyme activity. Specific enzyme-surfactant interactions, such as competitive binding to the active site or by adsorption to the interface where the substrate is bound, can also dramatically affect activity (Rubingh, 1996). Enzymes that act on carbohydrates are well known to have binding Modules, that help to increase the enzyme concentration on the polymeric substrate (Tzanov et al., 2003). The surfactant can adsorb to the substrate and difficult enzyme adsorption to the substrate, which is an essential step in heterogeneous biocatalysis. So, the nonionic surfactant may be slowing down the reaction catalyzed by the Bioprep® 3000 L by adsorption of the amphiphile in hydrophobic regions of the polygalacturonic acid, preventing adequate contact between the pectinase and its substrate and/or decreasing the ability of pectinase to access the substrate.

The interaction between pH and temperature as well as the interaction between pH and surfactant concentration are also important in the studied range (Figure 5a and c), indicating that the response of one factor depends on the other. Comparing the pH change at different temperatures (Figure 5a), it is noted that at 60°C a much higher increase in activity results from increasing the pH from 7 to 9, than at 40°C. The same occurs with respect to surfactant concentration (Figure 5c), wherein the absence of surfactant a more pronounced increase in enzymatic activity was observed by raising the pH to 9.

The interaction between temperature and surfactant concentration was not significant, which can be seen from the parallel lines in Figure 5b. It means that these two factors are independent of each other, which is the enzyme activity change due to one of the parameters is not influenced by the other parameter.

Figures 6 and 7, respectively, display through the slope of the response surface curves, the positive correlation between pH and temperature (synergism) and the negative interaction between pH and surfactant concentration (antagonism). At pH 7 the enzymatic activity was low with and without nonionic surfactant concentration, but at pH 9 the concentration of 1 g/L of surfactant reduced the activity by up to 40%. Even so, maximum activities are obtained at pH 9 and 60°C, regardless of the presence (473.04 U/ml) or absence (618.17 U/ml) of nonionic surfactant.

The analysis of variance (Table 4) confirms these main and interaction effects that significantly interfere with enzyme activity, according to the 99% confidence level. An even more stringent confidence level could be assumed because the p-values are smaller than 10^-4, except for the interaction between temperature and surfactant concentration which is not significant. The analysis of variance (Table 4) also establishes the values of the determination and adjustment coefficients, by comparing the predicted and observed values of the model. The coefficient of determination (R²) was 0.9767, explaining that 97.67% of the variation presented in pectate lyase activity resulted from the variation of the proposed factors (temperature, pH, and surfactant concentration) with low external interference and with good data reproducibility. The regression equation obtained with coded variables is presented as equation (4), after the removal of insignificant coefficients. The values obtained in the analysis of the curvature of the linear predictive model demonstrated a good fit to the observed data, with no change in linearity with the tests performed at the center point. Therefore, it can be considered that the model allows predicting values of enzymatic activity (Y = dependent variable) for various intermediate values of the studied factors, under the conditions imposed by the independent variables (x1, x2, and x3). The average difference between the experimental values and predicted values using equation 4 was 2.5%, confirming the quality fit represented by the high R² value.

$$Y = 287.3 + 63.5 x_1 + 153.2 x_2 - 39.2 x_3 + 42.7 x_1 x_2 - 41.3 x_2 x_3$$

(4)

3.2. Enzymatic scouring of cotton using pectate lyase

All processes developed by fundamental research must pass through an optimization step, based on reliable statistical operations, in which the obtained results are analyzed and the best conditions for industrial application are established (Pustianu et al., 2018). Therefore, the investigation of the effect of Bioprep® 3000 L in bioscouring of knitted cotton fabrics (Figure 8), should be based on the results of the experimental design with this enzyme on polygalacturonic acid (see item 3.1). Based on the pre-established experimental boundaries, the obtained data suggest that the most effective reaction conditions for maximum enzymatic activity are a temperature of 60°C, pH 9.0, and no use of surfactant. However, evaluating previous studies with an alkaline pectinase enzyme (Hebeish et al., 2009), a temperature of 55°C and pH 8.5, both slightly below the optimum operating condition found by the experimental design in this
work, were preferred. It was considered that less severe conditions would be beneficial, especially concerning the eventual recycling of the enzyme bath. Also, the addition of surfactant can aid in the removal of hydrophobic substances and this effect may compensate or even overcompensate for a loss in enzyme activity.

The error bars represent ± standard deviation. Equal characters in the same color columns are not statistically different at 5%. Control is the treatment without enzyme (treatment using surfactant only) at the same condition of the enzymatic scouring. The value of the greige fabric for the whiteness index was 16.34 ± 1.11 Berger degree.
Table 4. ANOVA table for the $2^3$ full factorial experimental design with one center point.

| Source of variation | SS     | DF  | MS      | F-ratio | p-value |
|---------------------|--------|-----|---------|---------|---------|
| Temperature         | 96772.7| 1   | 96772.7 | 95.09   | 0       |
| pH                  | 562933.5| 1   | 562933.5| 553.15  | 0       |
| Surfactant concentration | 36829.9| 1   | 36830.0 | 36.19   | $1.1 \times 10^{-5}$ |
| Temperature X pH    | 43793.9| 1   | 43794.0 | 43.03   | $4 \times 10^{-5}$ |
| Temperature X surfactant concentration | 827.3 | 1   | 827.3   | 8.81    | $0.3791$ |
| pH X surfactant concentration | 40942.3| 1   | 40942.3 | 40.23   | $6 \times 10^{-5}$ |
| Curvature           | 223.3  | 1   | 223.3   | 0.22    | 0.6451  |
| Lack-of-fit         | 323.5  | 2   | 161.8   | 0.16    | 0.8542  |
| Error               | 18318.2| 18  | 1017.7  |         |         |
| Total               | 800741.6| 15  |         |         |         |

SS = sum of squares; DF = degrees of freedom; MS = mean square.

Figure 8. Physicochemical properties of scoured cotton fabrics treated without (control) and with pectate lyase: (a) weight loss, (b) whiteness index, and (c) hydrophilicity.
Enzymatic cleaning of cotton is generally characterized by weight loss and this parameter depends on the used enzyme, fabric type, reaction time, agitation, etc. (Kalantzi et al., 2008), which is confirmed by data plotted in Figure 8a. All treatments with pectate lyase caused a constant weight loss and increasing enzyme concentration did not result in further weight loss. The highest weight loss (3.45% ± 0.04) was already obtained with the lowest enzyme concentration (1.0 g/l) with a surfactant, suggesting in advance to be sufficient for the digestion of the available pectin impurities. In contrast, Hebeish et al. (2009) and Kalantzi et al. (2008) found that an increase in the alkaline pectinase concentration resulted in an additional increase in the weight loss of the cotton fabric. It is important to note that the magnitude of the losses was less than 4%, as found in several other studies with pectinases, which seems to be the maximum amount of pectic and waxy impurities that can be removed (Hebeish et al., 2009; Kalantzi et al., 2008; Li & Hardin, 1997; Niaz et al., 2011) but is also in agreement with the amount of pectic and waxy substances in the cotton fiber, which is not superior than 2.4% (Andreaus et al., 2019). Weight loss is a measure of the removal of fabric’s impurities and, on an industrial level, a weight loss of 3-6% is considered acceptable; since excessive loss results in reduced fabric strength and durability (Kalantzi et al., 2019).

In the control treatment without enzyme, an average weight loss of 1.6% was found, which can be associated with the elimination of waxes and fats and other impurities, due to the use of surfactant (1 g/l) and/or subsequent hot washing (90 °C, 10 min). Choe et al. (2004) showed that a short treatment of the fabric at temperatures higher than 80 °C was essential for the removal of waxes from the cotton. And generally, a surfactant is added to enhance the solubilization of hydrophobic compounds and/or prevent their redeposition back on the fabric (Glad et al., 2006).

Bioscouring also increased whiteness (Figure 8b) above that of the crude fabric (16.34 ± 1.11 Berger degree). Although the removal of natural pigments is typically achieved during the bleaching step (Kaur et al., 2020; Mojsov, 2019; Singh et al., 2020), some of them can be removed in the scouring step, since these pigments are adsorbed in pectic, lipidic, and proteic substances in cotton fiber (Karapinar & Sariisk, 2004). No significant difference was observed for the whiteness values obtained with the three different enzyme concentrations, which were in the range of 33 to 34 Berger degree (in the presence of 1 g/l nonionic surfactant) and 28 to 29 Berger degree (in the absence of 1 g/l nonionic surfactant). Hebeish et al. (2009) also found that the enhancement in the whiteness index by increasing alkaline pectinase concentration from 2-4 g/l, was practically negligible. Fabric whiteness from control treatments without enzyme (≈ 33 Berger degree) and of the treatments with enzyme plus surfactant showed no statistically significant differences, so it can be argued that the increase in whiteness may be attributed mainly to the washing process itself.

The greige cotton fabric did not absorb water and a contact angle of 134.24 ± 6.16 between a drop of distilled water and the substrate surface (Figure 9) was measured, so this can be classified as hydrophobic. The cotton fiber is coated with 4-12% natural non-cellulosic impurities and the raw cotton fabric has a highly hydrophobic character (Li & Hardin, 1997). Before wet dyeing, greige cotton fabrics must be cleaned and converted into hydrophilic to achieve excellent water penetration and color uniformity (Besegatto et al., 2018). The scouring step is done to provide good wettability for cotton fabric and the values found for hydrophilicity are shown in Figure 8c.

As expected, treatment with BioPrep® 3000 L plus surfactant improved the cotton fabric’s wetting ability and gave high hydrophilicity for all tested concentrations (5 s of wetting time). As already stated above with respect to fabric weight loss, the lowest enzyme concentration used in this study (1.0 g/l) was sufficient to remove all accessible hydrophobic impurities, so that an increase to 5.0 or even 10.0 g/l did not cause any further improvement in hydrophilicity of the cotton fabric. Considering the catalytic action of the enzyme, the maximum achievable efficiency of the process (amount of removed impurities divided by time or weight loss/time) depends not only on the catalyst concentration but also on process time and the accessible substrate, which further depends on fabric structure, yarn diameter, and fabric and fiber porosity. The lowest used enzyme concentration (1.0 g/l) is probably very close to the saturation levels for the enzymes on the fibers, so a further concentration increase did not change the results under the reaction conditions (55 °C, 30 min, liquor ratio 1:20). Wang et al. (2007) also found when optimizing bioscouring of 100% cotton knitted fabrics with an alkaline pectinase, that an enzyme concentration of 1.0 g/l in the presence of 0.1% nonionic surfactant (pH 9.1, temperature 57 °C, and incubation time 1.25 h) was the optimum parameter for the desired pectin solubilization and adequate fabric wettability.

The control treatment with surfactant only resulted in a much lower wettability (hydrophilicity of 18.47 ± 5.83 s), than the pectate lyase plus surfactant treatment, indicating that only little of the hydrophobic compounds was removed in comparison to the untreated raw cotton fabric (nonwetting) and that the breakdown of the pectin matrix from the fibers by pectate lyase is necessary.

The results of hydrophilicity from enzymatic treatment without surfactant were also not satisfactory. Enzyme activity of Bioprep® 3000L was studied firstly with the soluble substrate of polygalacturonic acid, so the enzyme-catalyzed
reaction occurred under homogenous conditions (see item 3.1), while the cotton fabric used in bioscouring is insoluble, the reaction heterogenous and the enzyme must function at solid/liquid interfaces. Here, surfactants lower the surface tension of the aqueous scouring bath, which ultimately improved the penetration of the enzymes into the fibers (Agrawal et al., 2008; Singh et al., 2018). Surfactants can also have a stimulatory effect by improving the turnover number of pectinase and increasing the contact frequency between the active site of the enzyme and the cotton substrate (Oumer & Abate, 2017) as well as aiding in adsorption and desorption of the enzyme to and from the fabric. Like this: (1) the pectin substances will be effectively removed by pectinase in the available areas, because of the specificity of the enzyme (Xiaokang et al., 2019); (2) the surfactant can promote permeability and enlarge the area accessible to the pectinase, facilitating the contact between enzymes and pectin and improving the catalytic reaction rate; and (3) the surfactant can ensure that the decomposition products of pectin and other impurities are promptly removed from the surface of the fiber. While enzymes catalyze the decomposition of a specific substrate (Erdem & Ibrahim Bahtiyari, 2018), surfactants remove material from surfaces in a non-specific manner (Holmberg, 2018). In bioscouring, the surfactant removes the oily layer from the cotton fibers by either emulsification or solubilization or by a combination of both (Holmberg, 2018). The mechanism of pectinase scouring starts with the degradation and elimination of pectins that loosen waxes and makes them more easily removable with the help of surfactants and mechanical agitation (Cui et al., 2009). This allows cotton to achieve superior hydrophilicity without fiber deterioration.

Figure 10 shows the surface morphology of raw and bioscourged cotton by SEM images. The enzymatic treatment removed the hydrophobic impurities, cleaned the fabrics, and turned the surface more uneven; however, the fibers seem to undergo mild changes in their architecture, due to the milder conditions of the process. According to Demir et al. (2018), with the use of enzymes for the treatment of raw cotton, conditions are much milder and do not create any damages to the surface of cotton fabrics.

4. Conclusion

In this study, a commercial pectate lyase was tested in several different reaction conditions on polygalacturonic acid, in an attempt to maximize its catalytic activity and to find its ideal operating conditions for the scouring of raw cotton fabrics. Through statistical and mathematical methods, the influence of the three independent variables pH, temperature, and nonionic surfactant in the medium was confirmed. Enzyme activity was most favored by an increase in pH from 7 to 9, followed by the temperature increase from 40 to 60 °C. Despite its negative effect, the presence of surfactant proved to be the factor that less influenced pectate lyase
activity on polygalacturonic acid. It can be further expected that the beneficial cleansing action of the nonionic surfactant during scouring at least compensates for an eventually reduced enzyme activity. As there was an interaction between factors, it is understood that it is not appropriate to evaluate the enzymatic activity of each factor separately, in the case of using the enzyme in industrial processes where the system is even more complex.

After careful investigations and the establishment of the best combination between the parameters, the enzyme showed great potential for application in the textile processing of bioscouring, with a significant increase in hydrophilicity of cotton fiber. The characterized pectate lyase allowed enzymatic scouring with effective performance, which represents an alternative to conventional chemical (mainly alkaline) scouring, towards the establishment of eco-friendly textile industry.

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