Alkali pretreatment of cotton stalk for bioethanol

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Abstract

Cotton stalk biomass was treated with NaOH and NaOH-steam pretreatments to get maximum cellulose content. Three factors with three levels such as biomass concentration (5, 10 and 15%), NaOH concentration (1, 3 and 5%) and residence time (4, 6 and 8 h) was performed through Box-Bhenken Design of response surface methodology. The treatment was performed with and without heating at 121°C for 15 min and 15 psi in an autoclave. Among these two types of treatment, maximum yield of cellulose content 87.80% was observed with 5% w/v NaOH concentration, 10 g substrate loading and 4 h residence time. The substrate having high cellulose content under optimized pretreatment conditions were analysed through FTIR revealing efficiency of pretreatment. The proposed model for this study was found significant in terms of lower p<0.05 values and findings of this study could be utilized for further processes like saccharification and fermentation to bioethanol.

Keywords: NaOH; Pretreatment; Cotton stalk; Cellulose; RSM

Introduction

Lignocellulose is regarded as chief renewable biomass source for biofuels production (Sun et al., 2013). Lignocellulosic biomass generated ethanol has diverted attention as an energy source that reduces greenhouse gas emissions up to 85% (Garcia-Aparicio et al., 2011). Almost half of world biomass comes from dry lignocellulosic biomass, 10–50 billion tons per year (Galbe and Zacchi, 2002). In this study cotton stalk was used as lignocellulosic biomass. Cotton stalk (CS) a renewable lignocellulosic biomass is by-product of cotton crop. Pakistan is the fourth largest cotton producer all around the world. Cotton products and cotton contribute 10% to its GDP (Muhammad et al., 2009). In Pakistan, 13, 210 thousand tons of cotton stalk residues produce annually (Hanif et al., 2004). Cotton stalks have high cellulose content and, are 3 times more in quantity than per acre generated cotton fibre which are frequently discarded through burning due to limited use (Reddy and Yanga, 2009). Cotton stalk biomass comprises of 58.50% cellulose, 14.40% hemicellulose and 21.50% lignin content % on dry basis (Nigam et al., 2009).

First step in biomass to biofuel conversion is hydrolysis which is ineffective without pretreatment due to resistance against bacterial and enzymatic attacks (Taherzadeh and Karimi, 2008). A pretreatment is therefore needed to make cellulose prone to enzymatic attack before enzymatic hydrolysis (Mosier et al., 2005). The chemical pretreatment methods are fast and more effective as compared to physical and biological pretreatment methods (Mosier et al., 2005). Physiochemical characteristics of various lignocellulosic biomass are different, appropriate pretreatment techniques must be adopted based on properties of particular raw material. Alkaline reagents like sodium hydroxide are widely used for lignocellulosic biomass pretreatment (Alvira et al., 2010).

Intermolecular ester bonds (crosslinking hemicellulose, xylan and lignin) saponification describes alkaline treatment mode of action (Sun and Cheng, 2002). Cotton stalks pretreated with 2% w/v sodium hydroxide at 120 for 90 min decrease lignin content up to 65% (Silverstein, 2004). Objective of this study was to find optimum pretreatment conditions, for NaOH and NaOH-steam pretreatment, for maximum cellulose content through response surface methodology and characterization of high cellulose containing substrate through Fourier Transform Infra-Red spectroscopy.

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Materials and methods

Materials

All chemicals used in this study were of analytical grade.

Biomass preparation

Cotton stalk (CS) was obtained from field of Shahkot, District Nankana Punjab, Pakistan. These cotton stalks were chopped, washed to remove redundant matters and oven-dried at 70°C till constant weight. Dried CS biomass was milled to fine powder form and stored in sealed plastic bags at room temperature for further processing.

Pre-treatment of substrate

CS was pretreated according to method described by Iqbal et al. (2017). This grounded CS biomass was soaked in different concentrations of NaOH for various time periods as per experimental design at room temperature. After this the material was filtered and residue was saved for further analysis. In thermo-chemical pretreatment, the material after specific soaking time was subjected to steaming at 121°C for 15min under 15psi. The pretreated samples were recovered through filtration and washed thoroughly with water until neutralized pH. These were then oven-dried for 24h at 105°C and stored in sealed plastic bags at room temperature for further processing.

Cellulose estimation

Cellulose contents of pretreated CS samples were measured through a method suggested by Gopal and Ranjhan (1980). Sample weighing 0.5g (w1) was taken in round bottom flask and added 15ml of 80% acetic acid and 1.5ml of Conc. HNO3, and refluxed for 20 min. The material was filtered through Whatman filter paper #1 and washed with hot water. Digested material was oven dried at 105°C overnight and weighed (w2) of digested residue. Dried matter was incinerated at 550°C for 5h in muffle furnace and weighed again (w3) of ash. The percentage of cellulose of pretreated CS on dry matter basis was calculated using the formula.

\[
\%\text{Cellulose} = \frac{\text{Weight of digested material (w2) - Weight of ash (w3)}}{\text{Weight of material on dry basis (w1)}} \times 100
\]

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Chemical changes in cellulose structure of pretreated biomass were studied through Fourier Transform Infra-Red spectroscopy FTIR. Pretreated CS biomass samples with maximum cellulose content for NaOH pretreatment and NaOH-Steam pretreatment were analysed by Agilent technologies Cary 630 FTIR and absorption spectrum was recorded in the range of 650-4000cm\(^{-1}\) with resolution of 4.

Experimental design

Experimental designs were made using response surface methodology (RSM), employing Box-Bhenken Design (BBD) to study the combined effect of three independent variables and their response. Conditions were optimized using BBD to find the optimum NaOH and NaOH-Steam pretreatment condition for maximum cellulose content of cotton stalk. Three independent variables for BBD, NaOH concentration (1, 3, 5%), substrate loading (5, 10, 15%) and time (6, 8, 10h) were used. The BBD is appropriate for examination of quadratic response surfaces and creates a second degree polynomial model, which is used in improving a process, using a few experimental runs. Number of experiments N for BBD are designed as N = 2k (k+1) + C\(_0\). Here k represents number of factors, C\(_0\) is denotes number of central points. BBD with three factors k=3 and one central point C\(_0\) = 1 total 13 experimental runs were required N = 13. According to BBD total 13 experiments were performed for each NaOH pretreatment, and NaOH-Steam pretreatment.

In Table I, \(x_1\), \(x_2\) and \(x_3\) are coded independent variables for NaOH concentration, substrate concentration and reaction time respectively, at three levels 1, 0 and 1. Three levels for NaOH concentration are 1, 3 and 5%w/v, substrate concentration 5, 10 and 15% and 4, 6 and 8h reaction time. Thirteen experimental runs were randomized separately for both NaOH & NaOH-Steam pretreatment of samples to exploit the effects of unsolved variability in the observed responses due to extraneous factors. The relation between observed and coded response is as follows:

\[
x_t = \frac{x_t - x_0}{\Delta x_t} (2)
\]

Here \(x_t\) is coded independent variable, \(x_t\) actual value for independent variable \(x_0\), actual value at central point and \(\Delta x_t\) is step change for actual value.

Table I. Code and actual level of the three independent variables for the design of pretreatment experiment used in the BBD

| Independent Variable | Code | Code and actual factor level |
|----------------------|------|-----------------------------|
| NaOH concentration (%w/v) | \(x_1\) | 1 3 5 |
| Substrate concentration (g) | \(x_2\) | 5 10 15 |
| Reaction time (h) | \(x_3\) | 4 6 8 |
For dependent variable response $Y$ and its optimal point, a second degree polynomial was fitted to observed data using the statistical package software Minitab v. 17.0. The second degree polynomial expressed as follows:

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

Here $Y$ is the %cellulose response; $\beta_0$ is the constant coefficient; $\beta_i$, $\beta_{ii}$, $\beta_{ij}$ are coefficients for the linear, quadratic and interaction effects, respectively; and $x_i$ is the independent factor variable, representing either NaOH concentration (g/l), temperature (°C), or reaction time (min).

**Statistical analysis**

Minitab v. 17.0 Trial Version of Statistical software package was used to plot the response surfaces and regression analysis of experimental data. Significance of NaOH concentration, substrate concentration and reaction time were examined through Analysis of variance ANOVA. And values differences were showed in terms of probability $p < 0.05$ values.

**Results and discussion**

**Experimental design**

Box-Bhenken design of response surface methodology (RSM) was used to analyze the effects and interaction of NaOH concentration, substrate (CS biomass) concentration and reaction time over actual response % cellulose concentration. The experimental design runs with their respective predicted values and actual responses for % cellulose of NaOH pretreated and NaOH-steam pretreated CS samples are presented in Table II. Cellulose content of untreated CS was assessed as 37.8% which increased in pretreated CS biomass. This resembles with 39.85% cellulose reported by Wang *et al.* (2016), 40.1% cellulose of dried raw cotton stalk stated by Keshav *et al.*(2016), and varied from 30% cellulose determined by Binod *et al.* (2012). Reason for dissimilarity in lignocellulosic material composition is attributable to season, geographical location, lignocellulosic biomass heterogeneity, processing techniques and analytical techniques used for composition analysis (Binod *et al.*, 2012; Silverstein *et al.*, 2007).

Cellulose content in experimental response ranged from 40.8% to 87.80% in NaOH pretreated CS biomass samples. Table II shows run number 1 with minimum cellulose yield of 40.8% and run number 4 yielded maximum cellulose content of 87.80%. The cellulose percentage of 87.80% during NaOH pretreated CS was higher as compared to 64.20% in HPAP pretreatment reported by Wang *et al.* (2016), and 60.6% in NaOH pretreated CS estimated by Asghar *et al.* (2016). NaOH is acknowledged as an efficient chemical reagent for lignocellulosic biomass delignification (Wang *et al.*, 2016).

Steam explosion of NaOH pretreated CS biomass at 121, for 15min, and 15psi results in minimum of 51.66% to maximum of 61.2% cellulose. Table II shows that run number 5 having maximum cellulose content of 61.2% and run number10 with minimum cellulose content of 51.66%. This is comparable with 64.20% cellulose of HPAP pretreatment (2g CS, 3% NaOH, at 5% S/L ratio, at 121, for 40min and 13kpa) investigated by Wang *et al.* (2016). Asghar *et al.* (2016) reported maximum 73.19% cellulose and 77.7% delignification at 2.5% NaOH, 121and 60min reaction time in physiochemical pretreatment of *Gossypiumherbaceum* (cotton stalks). Main purpose of combined alkali and steam pretreatment is lignin and hemicellulose removal which are main obstacles in efficient enzymatic hydrolysis (Ohgren *et al.*, 2007). An increase in NaOH concentration results in significantly higher p<0.05 lignin removal at room temperature for all reaction times (Keshav *et al.*, 2016). They estimated cellulose content of alkaline extracted steam exploded cotton stalk (SECOH) (NaOH 3% treated for 6h) as 81.641.06%. Improved cellulose content from 40.101.55 to 81.641.06% could be due to NaOH and steam collectively degradation of lignin and hemicellulose.

Increased cellulose content of pretreated CS biomass will produce enhanced reducing sugars on hydrolysis and consequently increased ethanol yields on fermentation. In this study cellulose content increased from 37.8% to 87.8% in chemical pretreatment and 61.2% in thermochemical pretreatment of CS biomass. Similar results were reported by Wang *et al.* (2016) 39.85% cellulose of raw cotton stalk increased to 64.20% in HPAP pretreated material. Upon hydrolysis with commercial cellulases this results in increased sugar yield from 55.90mg/g of raw cotton stalk to 271.70 mg/g sugars which is 4.8 folds from untreated material.
Table II. BBD with the predicted and actual values of the experimental response for cellulose content of CS biomass under different pretreatment conditions

| Run # | X_1 | X_2 | X_3 | NaOH | NaOH-steam |
|-------|-----|-----|-----|------|------------|
|       | Observed | Predicted | Residual | Observed | Predicted | Residual |
| 1     | 40.80 | 40.80 | 0.00 | 53.30 | 53.30 | 0.00 |
| 2     | 47.40 | 47.60 | 0.20 | 57.00 | 56.90 | 0.09 |
| 3     | 43.20 | 42.57 | 0.62 | 54.36 | 53.64 | 0.71 |
| 4     | 87.80 | 87.45 | 0.35 | 54.30 | 54.77 | 0.47 |
| 5     | 50.40 | 51.17 | 0.77 | 61.20 | 61.54 | 0.34 |
| 6     | 44.20 | 43.42 | 0.77 | 54.04 | 53.69 | 0.34 |
| 7     | 61.80 | 61.37 | 0.42 | 57.08 | 56.26 | 0.81 |
| 8     | 78.00 | 78.35 | 0.35 | 53.90 | 53.43 | 0.47 |
| 9     | 48.40 | 48.82 | 0.42 | 52.76 | 53.57 | 0.81 |
| 10    | 52.60 | 52.40 | 0.20 | 51.66 | 51.75 | 0.09 |
| 11    | 45.40 | 46.02 | 0.62 | 54.28 | 54.99 | 0.71 |
| 12    | 50.80 | 49.82 | 0.97 | 59.48 | 59.23 | 0.24 |
| 13    | 50.20 | 51.17 | 0.97 | 52.48 | 52.72 | 0.24 |

The response obtained through experiments were calculated through second order polynomial regression equations (eq 4,5). These were in pragmatic relationship among test variables and % cellulose yield.

\[
\text{Cellulose} = 141.09 + 12.72 x_1 + 1.970 x_2 - 40.68 x_3 + 2.331 x_1^2 - 0.1730 x_2^2 + 4.081 x_3^2 - 0.1500 x_1 x_2 - 4.113 x_1 x_3 + 0.2300 x_2 x_3 (p-value = 0.000, Adj. R^2 value = 0.9952) (4)
\]

\[
\text{Cellulose} = 56.20 + 1.220 x_1 - 1.206 x_2 + 0.32 x_3 + 0.179 x_1^2 + 0.0780 x_2^2 + 0.050 x_3^2 - 0.1655 x_1 x_2 + 0.0288 x_1 x_3 - 0.0530 x_2 x_3 (p-value = 0.003, Adj. R^2 value = 0.9690) (5)
\]

Fig. 1. Observed values versus mathematical model prediction of cellulosic content for NaOH pretreated CS biomass samples.
The response obtained through experiments were calculated through second order polynomial regression equations (eq 4,5). These were in pragmatic relationship among test variables and % cellulose yield.

Analysis of variance

Models significance was checked by analysis of variance (ANOVA) coefficient determination $R^2$. Higher $R^2$ values of 0.9983 and 0.9690 indicates that model explained the reaction well. The higher of Adj. $R^2$ values of 0.9952 and 0.9131 also support model’s significance. Higher determination coefficient $R^2$ values and their corresponding adjusted $R^2$ illustrate that actual and predicted values are in agreement as shown in Figure 1 A and B. The model have F values of 321.97 and 17.35, and probability values of 0.000 and 0.003 for NaOH and NaOH-steam pretreated CS biomass samples respectively (Table III and IV). A p-value of less than 0.05 shows significance of the model. Model terms $x_1$, $x_2$, $x_3$ and quadratic term interactions $x_1x_2$, $x_2x_3$, $x_1x_3$, $x_1^2$, $x_2^2$ and $x_3^2$ were also found significant. The model was found accurate as it can explain variation up to 99.83% in NaOH pretreated CS biomass and 96.90% in NaOH-steam pretreated CS biomass.

Fig. 2 and 3 depicts 3-D views for cellulose content after NaOH pretreatment and NaOH-steam pretreatments respectively. These plots indicated that each factor had significant effect on cellulose content. Increased concentration of NaOH resulted in increased cellulose content. Sukri et al. (2014) exhibited an increase in NaOH conc. 0.50-5.25% and pretreatment time 15-52.50min improved cellulose content to 42.78% of oil palm fronds (OPF) biomass.

Figure 4 A and B illustrated desirability for percent cellulose content of CS biomass. This chart shows that if NaOH concentration of 3%, substrate concentration of 10% and reaction time of 6h, it results in 81.939% maximum cellulose content in NaOH pretreatment of CS biomass (Fig. 4A). Fig. 4. (B) illustrates that at 3% NaOH concentration, 10% substrate concentration and 6h reaction time it yields 60.714% maximum cellulose content in NaOH-Steam pretreatment of CS biomass.

### Table III. ANOVA for the regression equation of cellulosic production for NaOH pretreated CS biomass samples

| Source               | DF | Adj. MS | Adj. SS | F Value | p-value |
|----------------------|----|---------|---------|---------|---------|
| Model                | 9  | 294.28  | 2648.54 | 321.97  | 0.000   |
| Linear               | 3  | 56.19   | 168.57  | 61.48   | 0.000   |
| NaOH conc. $x_1$    | 1  | 9.25    | 9.25    | 10.11   | 0.025   |
| Substrate conc. $x_2$| 1  | 62.72   | 52.72   | 68.62   | 0.000   |
| Time. $x_3$         | 1  | 96.61   | 96.61   | 105.69  | 0.000   |
| Square              | 3  | 455.80  | 1367.40 | 498.69  | 0.000   |
| $x_1^2$             | 1  | 321.07  | 321.07  | 351.28  | 0.000   |
| $x_2^2$             | 1  | 69.07   | 69.07   | 75.57   | 0.000   |
| $x_3^2$             | 1  | 984.02  | 984.02  | 1076.61 | 0.000   |
| 2-Way Interaction   | 3  | 370.86  | 1112.57 | 405.75  | 0.000   |
| $x_1x_2$            | 1  | 9.00    | 9.00    | 9.85    | 0.026   |
| $x_1x_3$            | 1  | 1082.41 | 1082.41 | 1184.26 | 0.000   |
| $x_2x_3$            | 1  | 21.16   | 21.16   | 23.15   | 0.005   |
| Error               | 5  | 0.91    | 4.57    |         |         |
| Lack-of-Fit         | 3  | 1.52    | 4.57    |         |         |
| Pure Error          | 2  | 0.00    | 0.00    |         |         |
| Total               | 14 | 2653.11 |         |         |         |
Table IV. ANOVA for the regression equation of cellulose production of NaOH-steam pretreated CS biomass samples

| Source               | DF | Adj. MS  | Adj. SS  | F Value | p-value |
|----------------------|----|----------|----------|---------|---------|
| Model                | 9  | 10.89    | 98.02    | 17.35   | 0.003   |
| Linear               | 3  | 23.55    | 70.66    | 37.53   | 0.001   |
| NaOH conc. $x_1$     | 1  | 20.99    | 20.99    | 33.45   | 0.002   |
| Substrate conc. $x_2$| 1  | 42.41    | 42.41    | 67.57   | 0.000   |
| Time $x_3$           | 1  | 7.25     | 7.25     | 11.56   | 0.019   |
| Square               | 3  | 5.07     | 15.22    | 8.09    | 0.023   |
| $x_1^2$              | 1  | 1.88     | 1.88     | 3.01    | 0.143   |
| $x_2^2$              | 1  | 14.04    | 14.04    | 22.37   | 0.005   |
| $x_3^2$              | 1  | 0.14     | 0.14     | 0.24    | 0.648   |
| 2-Way Interaction    | 3  | 4.04     | 12.13    | 6.44    | 0.036   |
| $x_1x_2$             | 1  | 10.95    | 10.95    | 17.46   | 0.009   |
| $x_1x_3$             | 1  | 0.05     | 0.05     | 0.08    | 0.0783  |
| $x_2x_3$             | 1  | 1.12     | 1.12     | 1.79    | 0.0239  |
| Error                | 5  | 0.62     | 3.13     |         |         |
| Lack-of-Fit          | 3  | 1.04     | 3.13     |         |         |
| Pure Error           | 2  | 0.00     | 0.00     |         |         |
| Total                | 14 | 101.16   |          |         |         |

Fig. 2. Surface plot for cellulose content after NaOH pretreatment of CS biomass
confirmed the efficiency of chemical treatment. These results suggested that it could be potentially used in saccharification.

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Fig. 3. Surface plot for cellulose content after NaOH-steam pretreatment of CS biomass
Degradation of β-D-cellulose makes cellulose accessible to enzymatic hydrolysis through cellulases. Strong peak for polysaccharides (cellulose, hemicellulose) was found at 1026.9 cm⁻¹ in untreated CS material. Increase in intensity of peaks 1025 cm⁻¹, 1049.2 cm⁻¹, 1030.6 cm⁻¹, and 1053 cm⁻¹ in pretreated samples established C-O, C=C, and C-C-O stretching for polysaccharide. Increase in intensity of peaks is referred as xylan (hemicellulose) decomposition in pretreated CS samples, as decreased in transmittance signal at 1037 cm⁻¹ stated by Wang et al. (2016). NaOH-Steam pretreatment attained upsurge in peak at 1053 cm⁻¹ displaying increased xylan decomposition. C=O stretch for ketone aldehyde showed decreased absorbance at 1718.3 cm⁻¹ in NaOH pretreated samples than 1730 cm⁻¹ of untreated CS. Decreased C=O stretching peak describes hemicellulose solubilisation in NaOH pretreatment, and maximum hemicellulose degradation in NaOH-steam pretreatment of CS biomass. Decrease in intensity of peculiar hemicellulose band was fund at 1733 cm⁻¹ in H₂O₂ pretreated wheat straw signifying partial hemicellulose removal after pretreatment. Strong O-H stretching peaks at 3324.1 and 3363.9 cm⁻¹ presented higher lignin content in untreated CS samples. Increase in the intensity of O-H stretching at 3326.6, and

Fig. 4. Desirability chart of cellulose content for NaOH pretreatment (A) and NaOH-steam pretreatment (B) of CS biomass

**FTIR Analysis**

To study the physiochemical and conformational properties of pretreated CS biomass samples, these were analysed through Fourier Transform Infra-Red spectroscopy (FTIR). FTIR spectra for untreated, NaOH pretreated and NaOH-Steam pretreated CS are shown in Fig. 5. Cellulose peaks absorbance at 674.6 cm⁻¹, 896.4 cm⁻¹ and 1026.9 cm⁻¹ increased in the pretreated samples. Peaks for C-O-C asymmetrical stretching appears in pretreated CS samples at 1101.4 cm⁻¹ and 1105.2 cm⁻¹ in chemical and thermochemical pretreatment respectively. Placido and Caparedo (2014) also reported rise in peaks at 898, 1030, 1050, 1090, 1150, and 1170 cm⁻¹ in alkali pretreated cotton gin trash (CGT). They found stronger peaks with U-NaOH 15% + HW + E pretreatment combination for CGT. Increased absorbance of cellulose peaks can be attributed to combination of various pretreatment conditions at cellulose structure. The cellulose peak found at 896 cm⁻¹ in untreated CS biomass represented glycosidic linkage of β-D cellulose. Sharp peaks in NaOH treated CS for cellulose at 896 cm⁻¹ wavelength, and increased intensity to 898 cm⁻¹ were found in NaOH-Steam pretreated CS sample. This is in accordance with the 897 cm⁻¹ band depicted by Wang et al. (2016).
confirmed the efficiency of chemical treatment. These results of acetylated CS. It shows decreased hydroxyl group content as reported by Bello et al. -1

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BBD total 13 experiments were performed for NaOH concentration, substrate concentration and reaction time were examined through Analysis of variance and run number10 with minimum cellulose content of 87.80%. The cellulose percentage of 87.80% was also found significant. The model was found to be 0.9983 and 0.9690 indicates that model explained the variation in the responses for % cellulose of NaOH pretreated and NaOH concentration, substrate (CS biomass) concentration of 3%, substrate concentration of 10% and 60.714% maximum cellulose content in NaOH-Steam pretreatment of CS biomass (Fig. 4A). Fig. 5. FTIR spectra of CS biomass

3334.4cm\(^{-1}\) shows delignification in pretreated CS biomass. As reported by Bello et al. (2016), O-H absorption band intensity decreased 3434.37 to 3409.34cm\(^{-1}\) for FTIR spectra of acetylated CS. It shows decreased hydroxyl group content in pretreated CS.

Conclusion

Results concluded that among two types of treatment, chemical treatment was found best (maximum yield of cellulose content (87.80%)at 5% w/v NaOH concentration, 10g substrate loading and 4h residence time). FTIR confirmed the efficiency of chemical treatment. These results suggested that it could be potentially used in saccharification process for liberation of sugars for the production of beneficial products.

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