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Surfactant-Aided Phosphoric Acid Pretreatment to Enable Efficient Bioethanol Production from *Glycyrrhiza Glabra* Residue

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ABSTRACT

Glycyrrhiza glabra residue (GGR) was efficiently subjected to concentrated phosphoric acid (PA) pretreatment with/without surfactant assistance, and promising results were obtained following separate enzymatic hydrolysis and fermentation (SHF) of the biomass. Pretreatment was carried out using 85 % PA either at 50 or 85 °C with 12.5 % solid loading for 30 min. In parallel experiments, the intact GGR was impregnated in 2 % (w/w) surfactant (Polyethylene glycol) aqueous solution prior to the PA pretreatment. Consequently, the pretreated materials were subjected to enzymatic hydrolysis (50 °C, 72 h) using 25 FPU/g cellulase, and the most digestible biomass was nominated for conversion to bioethanol. Substantial improvement in digestibility of GGR (~92 % hydrolysis yield) was observed following surfactant-assisted PA pretreatment, whereas digestibility yield from the untreated biomass was only 16.1 %. Consequently, the ethanol production form GGR was significantly enhanced by 19.7-fold through separate hydrolysis and fermentation of biomass. Different analytical approaches including water retention value, Simons' staining, and crystallinity together with FESEM imaging revealed that the improved surface hydrophilicity, increased substrate accessibility to enzyme, and decreased crystallinity could be the major effects of PA pretreatment, leading to higher susceptibility of GGR to enzymatic hydrolysis and subsequent ethanol production.

1. INTRODUCTION

Bioethanol is the most important liquid biofuel for the transportation sector. Production of second-generation ethanol from non-edible lignocellulosic wastes has key advantages over the conventional processes utilizing sugar- and starch-based resources. Lignocellulosic wastes are considered a sustainable source of biomass for transformation into biofuels; however, they are highly resistant to physical and biological attacks. Therefore, an efficient pretreatment is vital prior to hydrolysis and subsequent biological conversion of lignocelluloses. To date, several pretreatments have been developed which use energy and/or chemicals [1-5]. Chemical-based pretreatments were shown to be effective to render downstream processing of biomass, whereas physical and biological techniques typically need high energy input and a long time, respectively. Moreover, some operation such as dilute sulfuric acid pretreatment generates toxic degradation products which make the process less environmentally-friendly and adversely influence the success of bioprocessing. Despite substantial study, engaging a cost-effective pretreatment for economical production of biomass-derived fuel is still the focus of intense research and continues to be challenging [6,7]. Cellulose dissolution pretreatment has been recognized as a promising approach for fractionation of lignocellulosic materials [8]. It could be applied at relatively milder conditions than many thermal operations. Concentrated phosphoric acid (PA) is able to dissolve cellulose in the presence of water under mild reaction conditions (e.g., 50 °C). It has been demonstrated that the PA (85 %) could change the inner crystalline structure of cellulose molecules and cause a phase transition from cellulose swelling to cellulose dissolution [9,10]. Compared to other mineral acids, PA is advantageous as it is non-corrosive, non-toxic, inexpensive, and safe to be used. Moreover, pretreatment with PA poses no inhibitory effect on the subsequent hydrolysis and fermentation processes [11-14,10]. Recent studies have investigated the consolidated use of surfactants and cellulose solvents for efficient pretreatment of lignocellulosic biomass. It has been reported that non-ionic surfactants, which combines both hydrophobic and hydrophilic properties, could emulsify hydrophobic substances (e.g., hemicellulose and lignin) and enhance the water solubility of them by decreasing surface tension at the interface. A few studies have developed a biomass impregnation approach using surfactants polyethylene glycol (PEG), Tween 20, and Tween 80, prior to the ionic liquid pretreatments, and noticeable improvements in the yield of enzymatic hydrolysis were observed [15-18]. Despite promising results, no previous study has involved the synergy between surfactant impregnation and PA pretreatment prior to the hydrolysis and fermentation of the biomass. Pretreatment should efficiently reduce biomass recalcitrance to saccharification and facilitate the release of energy locked within the lignocellulosic structure. Inherent resistance of lignocellulosic biomass is mainly attributed to various physicochemical factors such as cellulose crystallinity, accessible surface area, lignin and hemicellulose protection, cellulose degree of polymerization, degree of hemicelluloses acetylation, and enzyme adsorption and desorption behavior. Enzymatic hydrolysis is a heterogeneous reaction; thus, an efficient saccharification yield should be reached when the biomass pores are large enough to provide high accessibility to enzyme molecules [19-23]. For the first time, The present study dealt with the PEG assisted-PA pretreatment, and

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separate enzymatic hydrolysis and fermentation (SHF) of *Glycyrrhiza glabra* residue to ethanol. Moreover, a novel pretreatment approach, relying on the impregnation of GGR prior to the IL pretreatment, was developed for enhancement of ethanol production. Consequently, in parallel experiments, the biomass was presoaked in PEG solution (as an impregnation agent) and the synergistic effects of surfactant impregnation with the PA pretreatment were also tracked using rapid semi-quantitative methods.

2. MATERIALS AND METHOD

2.1. Raw materials

The intact *Glycyrrhiza glabra* residue was originally obtained from Atran Daru Co. (Esfahan, Iran). The waste materials were washed with tap water for surface dust removal and then dried at room temperature. The dried biomass was then milled (Retsch, Germany) to achieve mesh size between 20 and 50 (Fan Azma Gostar, Iran). The biomass moisture content was subsequently measured following 4 h oven-drying at 105 °C (Behdad, Iran). The composition of the GGR was analyzed for cellulose, hemicellulose, and lignin contents according to the previously established procedure with some modifications [24]. Except when noted, all the chemicals were of analytical-reagent grade and were purchased from Sigma and Merck.

2.2. Phosphoric acid pretreatment

One gram of the native GGR was well mixed with PA (85 %) in a 50 mL plastic centrifuge tube (12.5 % solid loading) either at 50 or 85 °C and manually stirred every 3 min. After 30 min, the reaction was rapidly quenched by the addition of 20 mL cold acetone (Commercial solvent-grade aceton, 99 %) to the medium. Subsequently, the pretreated GGR was separated from the mixture following 20 min centrifugation at 4000×g (PIT320, Pole Ideal Tajhiz, Iran). The filter cake was initially washed with 40 mL acetone and centrifuged (three times) then repeatedly washed with distilled water and centrifuged until pH 7 was reached (Metrohm 827, Switzerland). In parallel experiments, the intact GGR was impregnated using polyethylene glycol 4000 surfactant (PEG) prior to the PA pretreatment. Consequently, the GGR was soaked in 2 % (w/w) PEG aqueous solution overnight. The impregnated biomass was then isolated by centrifugation and subjected to PA pretreatment. The PA pretreated materials were oven-dried at 45 °C for 48 h, and stored at 4 °C until use [25,10].

2.3. Simons' stain

The change in available surface area after pretreatment was evaluated based on the competitive adsorption of two direct dyes, according to the Simons' stain technique with minor modifications [26]. The dyes Direct Blue 1 (DB) and Direct Orange 15 (DO) have different molecular sizes and binding affinity for cellulose. They were obtained from Pylam Products Co. Inc. (Garden City, NY) under the commercial names of Pontamine Fast Sky Blue 6BX (DB) and Pontamine Fast Orange 6RN (DO). The high molecular weight fraction of the orange dye was separated using ultrafiltration through a 100 KDa MWCO membrane (Millipore, United States). Approximately, 100 mg of GGR was placed in 15 mL test tubes and supplemented with 1.0 mL of phosphate-buffered saline solution (pH 6, 0.3 M PO_4, 1.4 mM NaCl). The tubes were supplemented with identical volumes of 10 mg/mL dyes stock solutions (0.25-2 mL) and then diluted to 10 mL with distilled water. All the samples were incubated at 50 °C and 150 rpm for 15 h. The stained materials were separated by centrifugation at 4000×g for 5 min, and the final concentrations of free DB and DO in the supernatant were determined spectrophotometrically at 455 and 624 nm, respectively [27,16].

2.4. Water retention value

The GGR porosity and its surface hydrophilicity were qualitatively examined by measuring its ability to hold water molecules based on the Water retention value (WRV) technique. The method is based on centrifuging a water-swollen fibrous sample and determining the remained water molecules inside the porous biomass. Approximately, 1 g of the biomass was soaked in extra amounts of deionized water for 60 min. The wet biomass was then collected in a nonwoven bag and soaked again in 10 mL deionized water at room temperature for 2 h. The swollen sample was finally centrifuged (3000×g, 15 min) and oven-dried (105 °C) for 24 h. The WRV was calculated according to the following formula [28,29]:

$$WRV = \left(\frac{M_1}{M_2} - 1 \right) \quad (1)$$

where M_1 and M_2 are the wet and dry mass of the GGR, respectively [30].

2.5. Crystallinity measurement

An X-ray diffractometry (XRD) analysis was conducted (D8 ADVANCE, Bruker, Germany) to track the crystallinity change following PA pretreatment. The XRD patterns were recorded in the 2θ range of 5 to 90° at a rate of 0.02°/s. Accordingly, crystallinity index (CrI) of the untreated and selected pretreated GGR was calculated based on the following equation [31,32]:

$$CrI = \left(\frac{I_{002} - I_{AM}}{I_{002}} \right) \times 100 \quad (2)$$

where I_{002} and I_{AM} are intensities of the first and second highest peaks occurred at 2θ = 22.5° and 16.6°, corresponding to the crystalline and amorphous regions, respectively.

2.6. Field emission scanning electron microscopy (FE-SEM)

The untreated and selected pretreated GGR were coated with a thin layer of gold and then subjected to FE-SEM imaging (TScan-Czech, Czech Republic).

2.7. Enzymatic hydrolysis

The untreated and different pretreated GGR were subjected to enzymatic hydrolysis in 50 mM sodium citrate buffer (pH 4.8) with 2 % (w/v) solid loading. The experiments were conducted at 50 °C and 120 rpm for 72 h. The enzymes' loading was adjusted 25 FPU activity per gram dry weight of either untreated or PA pretreated GGR and the medium was supplemented with 0.5 g/L sodium azide to prevent possible

bacterial contamination. The samples were withdrawn at 72 h enzymatic hydrolysis, immersed in boiling water (5 min) for enzyme deactivation. The hydrolyzates were cooled to room temperature and then subjected to glucose analysis using a glucose kit (Pars Azmoon, Tehran). The substrate enzymatic digestibility (SED) was calculated as follows [33,24]:

$$SED\ (\%) = \frac{[G]_{72h}}{1.111 \times F \times [S]} \times 100 \quad (3)$$

where [G] and [S] are the final glucose and initial substrate concentrations (g/L), respectively and F stands for the cellulose fraction in the substrate (As shown in Table 2).

2.8. Fermentation

The untreated GGR together with the most digestible pretreated biomass were similarly hydrolyzed using the previously-mentioned procedure except that the samples were sterilized at 121 °C for 20 min. Subsequently, necessary nutrients for microbial cultivation were supplemented, the pH was adjusted 5, and the media were fermented to ethanol (32 °C for 48 h) with 5 g/L Saccharomyces cerevisiae (PTCC 5052). The final ethanol concentration was determined using an Agilent 6890 gas chromatograph equipped with an HP-Innowax column. The ethanol production yield was subsequently calculated as follows [15,24]:

$$Ethanol\ yield\,(\%) = \frac{[E]_{48h} \times 100}{1.111 \times F \times [S] \times 0.51} \quad (4)$$

where $[E]_{48h}$ is the ethanol concentration (g/L) after 48 h fermentation and 0.51 in the dominator stands for the maximum theoretical ethanol production yield.

All the laboratory experiments were performed at least in duplicate and the average values have been reported.

3. RESULTS AND DISCUSSION

3.1. Enzymatic digestibility

All the untreated and pretreated materials were enzymatically hydrolyzed for 72 h and the enzymatic digestibility is represented in Table 1. The biomass digestibility (without any pretreatment) was only 16.1 %, whereas the PA pretreatment at 50 °C significantly improved the hydrolysis yield by over 5.0-fold. A slight decrease (11.0 %) in the hydrolysis yield of GGR was found after increasing PA pretreatment temperature from 50 to 85 °C. The adverse effect of pretreatment temperature on hydrolysis efficiency could be attributed to the greater chance of cellulose degradation at higher temperatures. It has been reported that the cellulose decrystallization rate at 70 °C was much less than that at 50 °C, indicating that higher temperatures may considerably attenuate the decrystallization reaction [34]. The results also revealed the surfactant considerably assisted hydrolysis yield of biomass. Impregnation of GGR prior to the PA pretreatment at the mild temperature of 50 °C could significantly promote the hydrolysis yield to the maximum value of 92.3 %. However, the effect of surfactant impregnation was less pronounced at elevated temperature, whereas it slightly improved (7.4 %) enzymatic digestibility of GGR at 85 °C. It was also remarkable that the pretreatment under mild temperature (50 °C) was much more effective than that at 85 °C even with surfactant assistance.

Table 1. Yield of enzymatic hydrolysis (%) of untreated and different pretreated GGR.

Pretreatment	Enzymatic digestibility (%)
PA50	80.5
PA85	72.5
SAPA50	92.3
SAPA85	77.9
Untreated	16.1

3.2. Composition

The carbohydrate (cellulose and hemicellulose) and Klason lignin fractions in the GGR (before and after pretreatments) were determined (Table 2). The initial biomass had a considerable amount of cellulose (27.5 %), while hemicelluloses accounted for almost 20 % of biomass composition. Besides, lignin was the major non-carbohydrate constituent in the GGR with nearly 42 % of the total biomass weight. The results were well supported by the recent studies which reported GGR composition; however, negligible differences between the samples may arise from differences in the growth, collection, and storage conditions [15,24]. Noticeably, PA pretreatment at mild condition (50 °C) efficiently improved composition of the biomass, whereas the cellulose content of biomass was increased to ~31 %. Furthermore, presoaking in PEG solution prior to the mild PA pretreatment could substantially enrich the cellulose fraction up to ~37 % and decrease the lignin content to the minimum value of 35.7 %. The remarkable effect of PEG surfactant most likely contributed with formation of lignin-surfactant hydrophobic interactions, which may increase lignin solubilization and prevent its deposition on biomass surface. The lignin removal was increased by 19.0 % and 9.1 % upon SAPA pretreatment at 50 and 85 °C, respectively, suggesting that the surfactant assistance was more effective at mild temperature.

Table 2. Chemical composition (%) of untreated and selected pretreated GGR.

Pretreatment	Cellulose (%)	Hemicellulose (%)	Lignin (%)
PA50	30.9	12.6	44.1
PA85	18.5	8.1	61.3
SAPA50	36.8	15.1	35.7
SAPA85	22.4	8.6	55.7
Untreated	27.5	20.3	41.9

However, noticeable degradation of carbohydrates (cellulose and hemicellulose) was occurred following PA pretreatment at elevated temperature of 85 °C. Consequently, the cellulose and hemicellulose fractions were decreased from 27.5 and 20.3 % to minimum values of 18.5 and 8.1 %, respectively. The cellulose and hemicellulose degradation products, which are usually in the liquid phase, would be discharged during the severe post-washing process. Therefore, it should be expected that after pretreatment the proportion of cellulose and hemicellulose in the residual solids decreases, whereas the lignin fraction increases. Although the exact mechanism of the surfactant action has not been established, it is believed that the presence of PEG could facilitate lignin removal during the PA pretreatment by decreasing the surface tension and

entrapment of lignin in the liquid phase. The aforementioned effect could lead to the enhanced lignin wash-out during the post-washing process [18,16].

3.3. Surface hydrophilicity and cellulose accessibility

Water retention value (WRV) of the materials was measured as a general indicator of biomass swelling capacity. As shown in Table 3, WRV of the intact biomass was only 0.61, which was slightly increased to 0.77 upon 30 min pretreatment with PA at 50 °C. It should be noted that cellulose hydroxyl group tends to bond with water molecules via hydrophilic interactions; thus, biomass surface hydrophobicity can limit its water swelling capacity. The ability of the materials pretreated at mild temperature of 50 °C to hold water molecules was at least 1.6-fold higher than that at high temperature of 85 °C. WRV of the biomass was unexpectedly decreased to a minimum value of 0.49 at high temperature of 85 °C, suggesting that a more hydrophobic surface was formed following PA pretreatment.

Table 3. WRV and DAR for the untreated and pretreated GGR.

Pretreatment	WRV (g/g DM*)	DAR+
PA50	0.77	1.21
PA85	0.49	0.89
SAPA50	1.37	1.26
SAPA85	0.75	0.97
Untreated	0.61	0.55

*Dry material
+(DO15/DB1) adsorption ratio

This phenomenon could be attributed to either cellulose degradation or lignin deposition on the biomass surface following PA pretreatment at high temperature. However, impregnation with a surfactant solution could substantially increase surface hydrophilicity of the PA pretreated GGR, whereas the WRV was increased by 1.5- to 1.8-fold. The materials were also subjected to Simons' stain experiments and the dye adsorption ratio (DAR) was estimated. The observations revealed that the PA pretreatment successfully increased DAR of the materials by 1.62- to 2.29-fold depending on the treatment conditions. As shown, PA pretreatment at 50 °C was much more successful to increase DAR of the materials (compared to that at 85 °C); however, the ratio was almost unchanged upon impregnation of the biomass prior to the treatment. It is well acknowledged that the DO15 dye composed of large molecules which are not able to penetrate small pores. However, DO15 molecules could push off DB1 molecules from the surface in the large pores. Since DO15 molecular diameter is in the order of the catalytic domain of cellulase, the DO15/DB1 adsorption ratio (DAR) could be interpreted as the cellulose accessibility to enzyme. According to the results of composition, WRV, and Simons' stain analyses, the major effect of PEG should contribute to the increased surface hydrophilicity of the biomass by hindering lignin redeposition during the pretreatment, most likely leading to subsequent attenuation of the non-specific enzyme adsorption [16].

3.4. Crystallinity and surface morphology

The crystallinity of biomass before and after pretreatment was determined by XRD analysis, and the results are summarized in Table 4. As shown, the SAPA pretreated biomass at 50 °C showed a substantially lower proportion of crystalline cellulose when compared to the intact GGR. Accordingly, the CrI of the untreated biomass was 0.72 which dropped by ~31 % after pretreatment. The activation energy for dissolving cellulose in PA and NaOH/urea system have been estimated to be 42 and 101 kJ/mol, suggesting that PA could pose powerful solubilization towards cellulose [35].

Table 4. Crystallinity index of untreated and selected pretreated GGR.

Pretreatment	Crystallinity index
SAPA50	0.55
Untreated	0.72

Therefore, PA, as a cellulosic solvent, could efficiently open up the highly ordered cellulose structure. However, the disordered network cannot return to its original form and a less crystalline structure would be generated during the subsequent short-time quenching process. The morphology of the lignocellulosic biomass was also explored by SEM imaging. As shown in Figure 1a, the surface morphology of the native biomass underwent a significant change after pretreatment (Figure 1b). Evidently, the SAPA pretreatment could extensively expand the fibrillar pattern of biomass, while regular texture can be observed for untreated GGR.

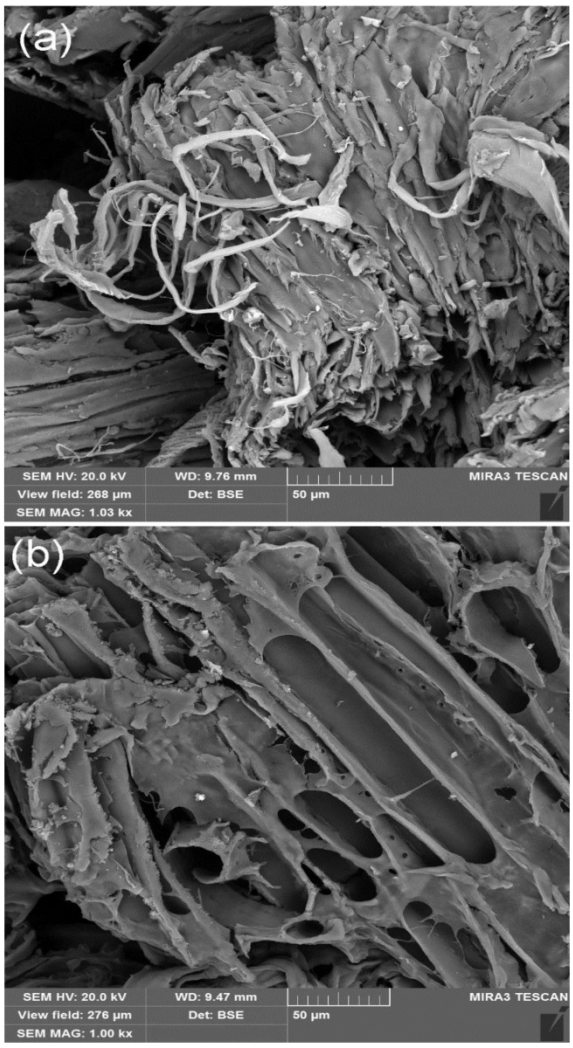

Figure 1. SEM images of (a) untreated and (b) selected pretreated GGR.

Moreover, compared to the compact structure of intact biomass, SAPA pretreated GGR exhibited a highly disordered and rough surface with more sponge-like structures. The above-mentioned improvement could provide a higher accessible surface area for enzymatic attack as previously evidenced by Simons' staining experiments.

3.5. Bioethanol production

Fermentability of the GGR was evaluated through an SHF process using the yeast S. cerevisiae and the main results are illustrated in Figure 2. The yeast could produce high ethanol yield (84.7 % of theoretical ethanol yield) from the SAPA pretreated biomass following 72 h hydrolysis and 48 h fermentation. It should be compared with the ethanol production yield obtained from untreated GGR (21.3 %).

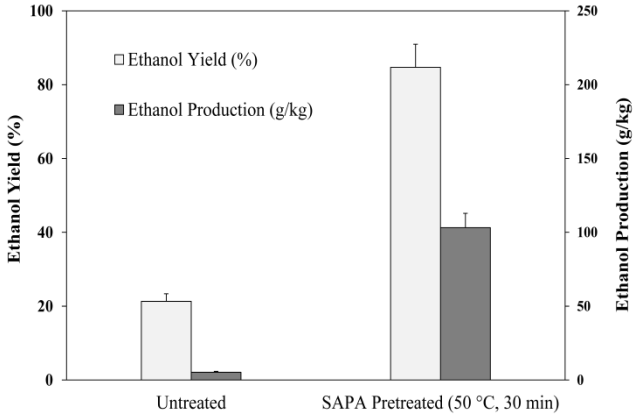

Figure 2. Bioethanol production from untreated and selected pretreated GGR.

Considering the biomass recovery yield after pretreatment, the SAPA pretreatment at mild temperature could significantly improve the ethanol production up to ~103 g per kg of pretreated GGR, while it was only 5.2 g per kg of raw biomass. It should be also noted that the selected SHF process allows both hydrolysis and fermentation processes to be performed at their corresponding optimum conditions. The improved yield of hydrolysis and consequent bioethanol production following PEG assistance could be due to enhanced GGR accessibility to cellulase enzyme. It has been previoulsy found that the hydrolysis yield could be improved either by loading high enzyme activity and/or applying lignin-blocking additives (e.g., surfactants). It should be noted that the presence of lignin could inhibit the hydrolysis reaction by covering the surface layer and preventing direct contact of the enzyme with cellulose. It has been proposed that the surfactant could inhibit the non-productive lignin binding to the enzyme, promote enzyme stability, and modify the biomass structure. The present study also found that the surfactant was mainly responsible for the enhanced delignification during PA pretreatment and preventing lignin accumulation during the hydrolysis reactions [36-38].

4. CONCLUSIONS

Glycyrrhiza glabra residue was efficiently subjected to surfactant-assisted PA pretreatment and promising results were obtained following separate hydrolysis and fermentation of the pretreated biomass. The pretreatment at mild temperature led to the highest hydrolysis yield (~92 %) and subsequent bioconversion of GGR to ethanol (~85 %). Moreover, in-depth composition, hydrophilicity, enzyme accessibility, crystallinity, and morphological analyses revealed that the pretreatment could successfully render GGR recalcitrance to enzymatic and biological processing.

5. ACKNOWLEDGEMENT

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NOMENCLATURE

GGR	*Glycyrrhiza glabra* residue
PA	Phosphoric acid
PA50	Phosphoric acid at 50 °C
PA85	Phosphoric acid at 85 °C
SAPA50	Surfactant-aided phosphoric acid at 50 °C
SAPA85	Surfactant-aided phosphoric acid at 85 °C
SHF	Separate hydrolysis and fermentation
DAR	Dye adsorption ratio

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