



Organosolv process for deconstruction of highly recalcitrant cotton stalks

Santosh N Sankh & Vidhya Rangaswamy*

High Value Chemicals, Reliance Technology Group, Reliance Industries Limited, Mumbai, Maharashtra, India

Received 24 January 2020; revised 12 August 2020

Burning crop residues in a cotton fields adds to environmental pollution and it demands a sustainable solution. As an alternative to burning, cotton biomass can be fractionated into sugars which would not only mitigate the pollution concern but also bring value addition. Here, we propose a process for deconstruction of the highly recalcitrant cotton stalk using a combination of organic and mineral acid fractionation. It includes treatment with formic acid (67 wt. %) at a solid to liquid ratio of 1:4 for 30 min at 130°C. Subsequent treatment with 2% (w/w) nitric acid and de-esterification with 0.5% (w/v) sodium carbonate resulted in delignification of about 89.01%. The enzymatic hydrolysis using 25 FPU of Cellic Ctec3 at a biomass load of 5% (w/v) gave a glucan yield of 68% in 48 h. This is one of the few studies on deconstruction of a highly recalcitrant biomass with high lignin content.

Keywords: Agricultural residue, Air Pollution, Enzymatic hydrolysis, Formic acid, *Gossypium hirsutum*, Nitric acid

The global cotton (*Gossypium hirsutum* L.) production has continuously increased¹ up to current estimates of about 26.15 million tons and India ranks as the one of the largest producer with 6.42 million tons per annum production. After harvesting, cotton residues such as cotton stalks, cotton leaves and cotton shells have limited use and mostly incinerated directly on the field. This leads to release of considerable amount of solid grains and smoke which pollute the environment. Therefore, a desirable and practical solution would be to convert the lignocellulosic cotton stalks into value added products such as biofuels viz. bioethanol, biobutanol and chemicals¹. This would also provide an environmentally sound method of disposal and simultaneous destruction of feeding and fruiting sites of boll weevils and other insects.

Conversion of lignocellulosic biomass to biofuel is thus far the most challenging process due to the complex and recalcitrant structure of the plant cell wall. Several different pretreatment methods have been explored with variable success rate as the process needs to be efficient, simple and cost-effective. Adequate pretreatment is required to alter the structural and chemical composition of biomass to facilitate rapid and efficient enzymatic hydrolysis. A variety of physical (comminution, hydrothermolysis),

chemical (acid, alkali, solvents, ozone), physicochemical (steam explosion, ammonia fiber explosion), and biological pretreatment techniques have been developed to improve the accessibility of enzymes to cellulosic fibers. Improved alkaline pretreatment process for waste biomass have been reported² and value-added chemicals such as poly 3-hydroxy butyrate, have been produced using alkali pretreated biomass hydrolysate³. Mild alkali such as sodium bicarbonate have also been used as green liquor chemical for biomass pretreatment in 2-3 butanediol production⁴. Mechanocatalytical conversion is another pretreatment process applied for conversion of barley straw into fermentable sugars⁵. A comprehensive review covering all pretreatment methods used in biorefinery process development has recently been published⁶. Using suitable method of pretreatment, economically viable process can be developed⁷.

Organosolv pretreatment has provided a promising route for clean fractionation of biomass feedstock to produce multiple products in a concept of lignocellulose biorefinery^{8,9}. The process involves pretreatment of lignocellulosic biomass with organic solvents or their aqueous solution systems with or without added catalysts at a temperature range of 100-250°C⁹. Organosolv pretreatment yields three separate fractions such as lignin fraction, hemicellulosic syrup and cellulose fraction with potential of conversion to various high value products. The organic solvents used in the organosolv process usually include

*Correspondence:

Phone: +91 2279678831

E-Mail: vidhya.rangaswamy@ril.com

methanol, ethanol, acetone, ethylene glycol, triethylene glycol, tetrahydrofurfuryl alcohol, glycerol, aqueous phenol or aqueous n-butanol, etc.¹⁰. Due to the high cost of classical solvents and the difficulty in their recovery, recent research is focused on cheaper organic solvents such as acetic acid and formic acid. The advantages of organosolv method lies in easy separation and recovery of solvent by thermal operations and environment friendly treatment of effluents.

The solvents like formic and acetic acid are commonly used for biomass delignification with significant lignin solvency making it a sought after process in organosolv pulping for pulp and paper production¹¹. However, potential of use of formic acid or acetic acid for pretreatment of lignocellulose to increase its bioconvertibility is still inadequately explored¹²⁻¹⁵, primarily due to formylation or acetylation of cellulose in the presence of formic acid¹² or acetic acid¹⁶, respectively. These modifications can decrease the cellulose digestibility and inhibit the growth of microorganism during the ensuing process of saccharification and fermentation. Nonetheless, use of formic acid in pretreatment does merit some notice, as the formylation that occurs can be reversed by mild alkali treatment. Importantly, formic acid is a low-cost chemical, recoverable by distillation, enabling recycling with ensuing process cost reduction.

Pretreatment methods for feedstocks such as corn stover, wheat straw and rice straw have been extensively investigated in the literature¹⁰. However, there is barely any detailed report with complete mass balance on the deconstruction of recalcitrant cotton stalks, a topic of extreme importance in the Indian context due to the environmental pollution. In the current study, we made an attempt to develop an optimized deconstruction process for cotton stalks that can yield maximum amount of sugar.

Materials and Methods

Materials

Cotton stalks were sourced from Jetpur, Rajkot, Gujarat, India and were subjected to size reduction in a shredder to obtain 2 mm size particles. Compositional analysis of the cotton biomass was performed using the methods of Laboratory Analytical Procedure (LAP) provided by the National Renewable Energy Laboratory (NREL)¹⁷. Moisture content of crushed cotton biomass was determined by moisture analyzer¹⁸. Standard chemicals used for

HPLC calibration like glucose, xylose, arabinose and cellobiose were procured from Sigma-Aldrich (Shanghai). Laboratory grade formic acid and nitric acid were procured from Merck. The cellulase enzyme Cellic Ctec3 was supplied by Novozymes.

Process details

Detailed description of the process employed for deconstruction of cotton stalk is elaborated in this section. All steps and assays have been repeated thrice to ensure reproducibility.

Formic acid pretreatment

The crushed dry biomass was subjected to mild organic acid pre-treatment in a reactor. The pre-treatment involved treating the biomass (16.6% w/v) with formic acid (67% w/v) at a solid to solvent ratio of 1:4 in a total volume of 200 mL.

Reactor was maintained at 130°C for 30 min at 200 rpm agitation speed. After pre-treatment, the slurry from the reactor was cooled to 50-60°C and filtered through 297 µm mesh followed by 37 µm mesh. The liquor passing through the mesh was collected and kept at 4°C for further analysis on HPLC. The pulp was subjected to RO water wash to remove adhering formic acid, xylose and other components. The washed pulp was dried and weighed to check the mass loss after pre-treatment. Moisture content of pulp was checked by moisture analyzer. The pulp was subjected to compositional analysis for determination of hexosan, pentosan and acid insoluble lignin content using the methods of LAP provided by the NREL¹⁷. The sugars in the pulp and liquor fraction, such as glucose, xylose, arabinose and inhibitory products such as hydroxymethylfurfural, furfural and levulinic acid were analyzed on HPLC¹⁹.

Nitration of formic acid pretreated pulp

The formic acid pre-treated pulp (4 g dry wt.) was added to 100 mL RO water containing 2% (w/w) nitric acid (67 wt % stock) and heated at 120°C for 30 min at 200 rpm. The slurry was cooled to 20°C and pulp was washed under RO water using 37µm mesh filter until pH became neutral. After drying, the moisture content of the pulp was checked by moisture analyzer. Compositional analysis of the dried pulp was done using the methods of LAP provided by the NREL¹⁷.

De-esterification of pretreated cotton pulp

It has been reported that de-esterification of acetate groups from cellulose can be accomplished using alkali²⁰. We attempted a similar approach in the current study for de-esterification of the formylated

cellulose. The pre-treated and nitrated dried pulp was subjected to de-esterification using sodium carbonate. The pre-treated pulp (4 g dry wt.) was added to 100 mL RO water and pH of the slurry was raised to 11 using 1% w/v sodium carbonate. Subsequently, the slurry was subjected to heating on hot plate with stirring at 200 rpm for 10 min and washed under RO water until the pH became neutral. The de-esterified pulp was dried and the moisture content was determined by moisture analyzer. The de-esterified and dried pulp was subjected to compositional analysis using the methods of LAP provided by the NREL¹⁷.

Enzymatic hydrolysis conditions

The pulp obtained after pre-treatment (formic acid pretreatment followed by nitration) and de-esterification was subjected to enzymatic hydrolysis (pulp load 5 % w/v, sodium citrate buffer pH 4.8 (50 mM), incubation temperature 50°C and agitation 200 rpm) using Cellic Ctec3. Enzyme loading was 100 FPU/g of biomass and the sugar released was analyzed after 120 h of incubation.

Effect of formic acid concentration

Pre-treatment of dry cotton biomass involved treating the biomass (16.6% w/v) with varying concentrations of formic acid (50, 67 and 84% w/v) in a total volume of 200 mL in a reactor maintained at 130°C for 30 min with 200 rpm agitation speed. All other steps were similar to that described previously. The pre-treated pulp was subjected to de-esterification using sodium carbonate as described in methods.

Effect of pretreatment temperature on cellulose recovery and enzymatic hydrolysis

Dry biomass (16.6% w/v) was mixed with formic acid (67% w/v) in a total volume of 200 mL and the reactor was maintained at various temperatures such as 115, 130 and 145°C for 30 min at 200 rpm. The washed and dried pulp was subjected to de-esterification using 1% w/v sodium carbonate. The pulp prepared after pretreatment at various temperatures and de-esterification was subjected to compositional analysis and enzymatic hydrolysis as explained in methods.

Effect of cold and hot filtration

Two pretreatment batches of dry cotton biomass (16.6% w/v) with formic acid (67% w/v) at a solid to solvent ratio of 1:4 was taken in a total volume of 200 mL. Reactor was maintained at 130°C for 30 min with 200 rpm agitation speed. After pre-treatment, one batch of slurry from the reactor was cooled to

50-60°C and filtered through 297 µm mesh and 37 µm mesh. Another batch of slurry was filtered immediately under hot condition at 130°C using hot filtration assembly. Pulp prepared under cold filtration and hot filtration were washed, dried and subjected to de-esterification using 1% w/v sodium carbonate. The pulps were subjected to compositional analysis and enzymatic hydrolysis as explained in methods.

Effect of alkali as de-esterification agent

Effect of various alkali as de-esterification agent on cellulose recovery and enzymatic hydrolysis was explored. The formic acid treated, washed and dried pulp was subjected to de-esterification using either 1% w/v of either sodium carbonate, NaOH, KOH, Ca(OH)₂ or 1% v/v liquid ammonia (35 wt % stock). The pulp prepared after pretreatment and de-esterification with various alkali was subjected to compositional analysis and enzymatic hydrolysis.

Optimization of nitric acid concentration

The formic acid pre-treated pulp (4 g dry wt.) was added to 100 mL RO water containing various concentrations of nitric acid (67 wt % stock) such as 1, 2 and 3% w/w and heated at 120°C for 30 min at 200 rpm. The slurry was cooled at 20°C and pulp was washed under RO water using 37 µm mesh filter until the pH became neutral. The pulp was subjected to de-esterification with sodium carbonate. The pulp prepared after pretreatment, nitration and de-esterification was subjected to compositional analysis and enzymatic hydrolysis.

Optimization of sodium carbonate concentration

For optimization of concentration of sodium carbonate as de-esterification agent, the formic acid pre-treated pulp (4 g dry wt.) was added to 100 mL RO water having either 0.5% w/v or 1% w/v sodium carbonate. The slurry was subjected to heating at 100°C, 200 rpm for 10 min, cooled to 20°C. The pulp was washed under RO water using 37 µm mesh filter until the pH became neutral. The de-esterified pulp was dried and subjected to the moisture content determination, compositional analysis and enzymatic hydrolysis.

Analytical methods

The main components of cotton stalk, pretreated pulp and biomass liquor were determined using the methods of LAP provided by the NREL¹⁷. Cellulose (glucan) and hemicellulose (xylan, galactan, and arabinan) content was determined based on monomer content measured after a two-step acid hydrolysis procedure to fractionate the fiber. A first step of

treatment with 72% H_2SO_4 at 30°C for 60 min was followed by a second step wherein the reaction mixture was diluted to 4% H_2SO_4 and autoclaved at 121°C for 1 h. This liquid hydrolysate was then analyzed for sugar content by HPLC with refractive index detector. An AMINEX HPX-87H carbohydrate analysis column operating at 85°C with 5 mM H_2SO_4 as mobile-phase (0.6 mL/min) was used. The solid residue remaining after this acid hydrolysis is considered as acid insoluble lignin.

Results and Discussion

Compositional analysis indicated that the dry cotton stalk biomass used in this study consisted of 32.2% glucan, 14.11% xylan, 20.55% lignin, 1.83% acid soluble lignin and 2.3% ash (on dry wt. basis).

Pretreatment of cotton stalk

Compositional analysis confirmed the relatively high lignin content of cotton stalks²¹. A systematic optimization regime was designed to enable maximal release of the C5 and C6 sugars.

Several pretreatment processes reported in literature involve reaction of biomass with alkali which helps in removal of lignin²². In the current study, we attempted two methods viz. alkali treatment and formic acid treatment and evaluated their efficacy for high cellulose recovery in pulp (data not shown). Formic acid pretreatment was found to be a better process, giving 33% more cellulose recovery than alkaline pretreatment process. Hence, all further experiments were carried out using formic acid pretreatment.

Lignin can absorb enzymes and is known to adversely affect the action of cellulases. Formic acid pretreatment is regarded as an efficient method for removing lignin from lignocellulosic biomass¹¹. Formic acid pretreatment also aids in hemicellulose removal thereby increasing porosity and enzymatic digestibility²³. At the solvent:solid ratio of 3:1, formic acid treatment resulted in 20% delignification with 83% cellulose recovery. To improve the delignification, higher solvent:solid ratio was evaluated. However, it was observed that increasing formic acid concentration enhanced degree of delignification only up to a point beyond which there was an adverse effect on recovery of cellulose (Fig. 1). The solvent:solid ratio of 5:1 showed better delignification albeit with reduced cellulose recovery by 13 % as compared to that of 3:1 ratio. This could be due to the loss of cellulose into the biomass liquor

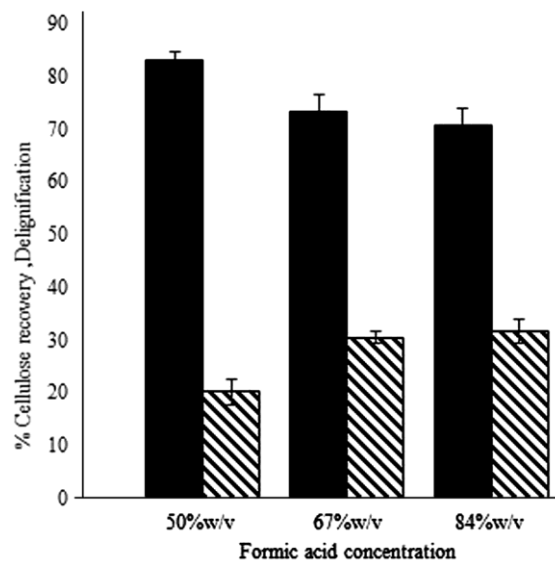


Fig. 1 — Effect of formic acid concentration on cellulose recovery and delignification of cotton pulp. [Solid bar, % Cellulose recovery; Diagonal Striped bar, % Delignification]

at higher concentration of formic acid. The intermediate ratio of 4:1 gave a reasonable delignification of 30.4% with 10% loss in cellulose. As all subsequent steps of deconstruction relies on easy access to cellulose, which is hindered by lignin, all further optimizations were carried out at the solvent:solid ratio of 4:1. One of the key determining factors for an economical pretreatment step is the ability to recycle the solvent used. This would lower the operating cost and prevent any inhibitory effect on the subsequent procedures²⁴. In the current process, 80% (v/v) of the formic acid used in pretreatment could be recovered by the distillation process.

Formic acid treatment of cellulose leads to formylation of cellulose, which can inhibit the enzymatic hydrolysis of cellulose. Therefore, a de-esterification step where the formyl group is removed with mild alkali such as sodium carbonate, thereby aiding in cellulose hydrolysis was introduced. Enzymatic treatment of the de-esterified pulp with Cellic Ctec3 resulted in 78.78% hydrolysis (Fig. 2). It was significantly higher compared to cellulose conversion with only formic acid pretreated pulp (70%). This 8% increase in cellulose hydrolysis of the de-esterified pulp was probably aided by the improved delignification (31.61%), which was 2-fold higher as compared to only formic acid treated pulp.

Effect of temperature

The temperature at which the formic acid pretreatment step is carried out also plays an essential

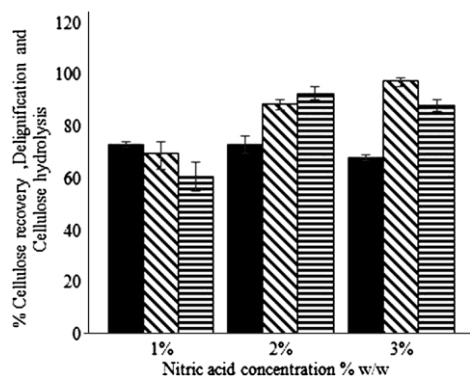


Fig. 2 — Effect of cotton pulp de-esterification on cellulose recovery, delignification and enzymatic hydrolysis. [Solid bar, After formic acid pretreatment; Striped bar, After de-esterification. Process conditions were pretreatment with 67% w/v formic acid, de-esterification with 1% w/v sodium bicarbonate, enzymatic hydrolysis using Cellic Ctec3 at 100 FPU/g]

role in the cellulose recovery and lignin removal. Mild pretreatment conditions preserved the sugars as compared to severe conditions²⁵. As expected, increasing pretreatment temperature from 115°C to 130°C increased delignification by 2.7-fold, which in turn improved cellulose hydrolysis by 2-fold (Fig. 3). No further improvement was seen either in lignin removal or cellulose hydrolysis at higher temperature of 145°C.

Effect of cold and hot filtration

Lignin is a major hurdle in enzymatic hydrolysis of pulp. For maximum removal of lignin the formic acid treated biomass was filtered under hot conditions. However, this resulted in delignification increase by only 2% with increased cellulose loss (17.03%) compared to cold filtration (Fig. 4). This could probably be due to the highly recalcitrant nature of the cotton stalk biomass²⁶. A 7 % increase in cellulose hydrolysis was observed with hot filtered pulp.

Comparison of various alkali as de-esterification agent

As described earlier, pretreatment with organic acids such as formic or acetic acid results in esterification of the cellulose moiety making it unavailable for enzymatic hydrolysis^{12,16}. The mechanism of interference of formyl group with enzymatic hydrolysis of cellulose in case of formic acid treated pulp has not been elucidated in great detail¹⁶. Formyl group might inhibit the formation of productive binding (hydrogen bonds) between cellulose and the catalytic domain of cellulases. It has been found that enzymatic digestibility can be recovered after formyl groups are removed by saponification¹⁶. De-esterification by alkali helps to

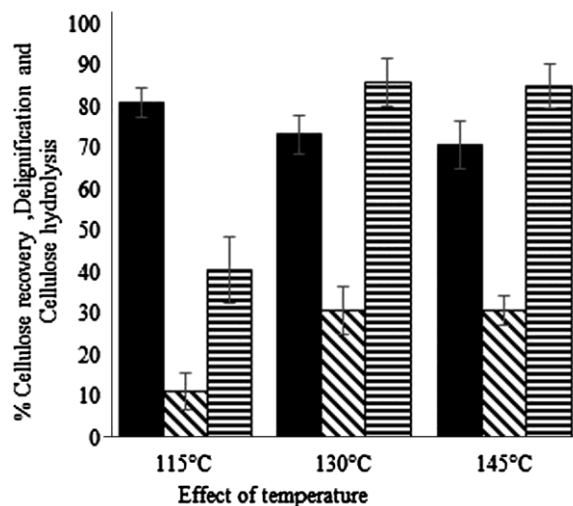


Fig. 3 — Effect of temperature on pretreatment of cotton stalk. [Solid bar, % Cellulose recovery after de-esterification; Diagonal Striped bar, % Delignification; Horizontal striped bar, % Cellulose hydrolysis. Process conditions were pretreatment with 67% w/v formic acid, de-esterification with 1% w/v sodium bicarbonate, enzymatic hydrolysis using Cellic Ctec3 at 100 FPU/g]

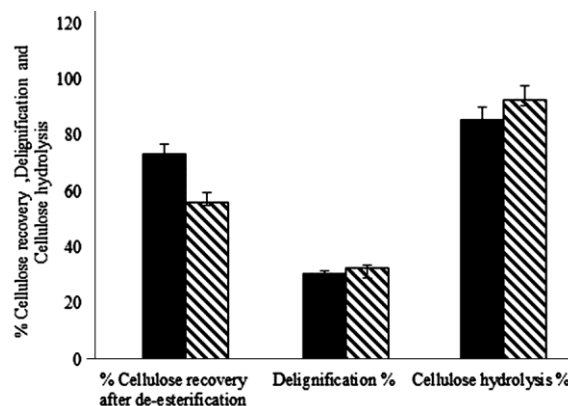


Fig. 4 — Effect of cold and hot filtration of cotton pulp after pretreatment reaction. [Solid bar, Cold filtration; Diagonal Striped bar, Hot filtration. Process conditions were pretreatment with 67% w/v formic acid, de-esterification with 1% w/v sodium bicarbonate, enzymatic hydrolysis using Cellic Ctec3 at 100 FPU/g]

overcome this hurdle and also removes lignin thereby efficiently increasing the accessibility of cellulose. Besides, it also gives numerous beneficial traits that helps in the deconstruction process. These include cellulose swelling, decrease in polymerization degree, increase of internal surface area, disruption of the lignin structure and separation of structural linkages between lignin and carbohydrates.

Since de-esterification using sodium carbonate improved the cellulose hydrolysis in formic acid pretreated pulp, efficacy of few other alkali viz.,

NaOH, KOH, $\text{Ca}(\text{OH})_2$ and liquid ammonia were explored to determine their influence on cellulose hydrolysis. KOH showed highest cellulose hydrolysis (87.51%) compared to other alkali agents albeit with low cellulose recovery (Fig. 5). Sodium carbonate, a mild alkali proved to be a better reagent as it gave the highest cellulose recovery of 80.80% with reasonably good hydrolysis efficiency. Besides, strong alkali treatment is less preferred in commercial processes due to ensuing high neutralization costs and their impact on the effluent stream.

Optimization of enzymatic hydrolysis time and enzyme load

Other than the pretreatment steps, the factor that has profound effect on the economics of any biomass deconstruction process is the cost of the enzyme. This, in turn, is dictated by the amount of enzyme used and the incubation time for hydrolysis. Other factors that influence enzymatic cellulose digestibility include cellulose crystallinity which affects its susceptibility for cellulase action and the presence of residual hemicellulose and lignin²⁷. It was reported that adsorption of cellulase onto lignin restricted the movement of cellulase and hindered the cellulase recycling in bioconversion of lignocellulose²⁸.

Table 1 represents optimization of enzymatic hydrolysis time and enzyme load for hydrolysis of formic acid pretreated and de-esterified pulp using Cellic CTec3. The results indicated that after 120 h of hydrolysis time at 100 FPU enzyme load, maximum cellulose hydrolysis of 88.22% was observed and the total glucose released was 22.63 g /100 g of biomass. Considering the recalcitrant nature of cotton stalks, this 68% recovery of the glucan is significant. However,

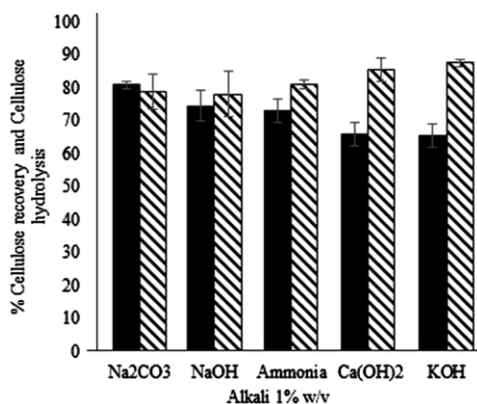


Fig. 5 — Screening of various alkali for de-esterification. [Solid bar, % Cellulose recovery; Diagonal Striped bar, % Cellulose hydrolysis. Process conditions were pretreatment with 67% w/v formic acid, enzymatic hydrolysis using Cellic Ctec3 at 100 FPU/g]

the enzyme loading of 100 FPU and the long incubation time of 120 h is still a matter of concern. Importantly, the pulp still contains 75% of its original lignin content giving scope for further improvement in efficiency of the enzymatic hydrolysis.

Optimization of nitric acid concentration

It has been reported that complete release of lignin from sugarcane biomass was successful by treating with nitric acid at variable concentration of 2-6% for 300 min at temperature of 100-128°C²⁹. Nitric acid reacts with lignin at higher temperature and releases the monomers of lignin into solution leaving white colored cellulose pulp as precipitate. The treatment removes most of the hemicellulose and lignin from the sample, rendering the cellulose fibres accessible for enzymes. A nitration step was therefore introduced between formic acid pretreatment and de-esterification in the current study. As expected, nitration of pulp with 2% w/w nitric acid (67 wt. % stock) gave 89% delignification and 92.89% cellulose hydrolysis with 25 FPU Cellic Ctec 3 in 48 h (Fig. 6).

Table 1 — Optimization of enzymatic hydrolysis time and enzyme load after formic acid pretreatment and de-esterification grams of glucose released/100 g of cotton stalk (% of hydrolysis)

Hr	E-25FPU/g	E-50FPU/g	E-75FPU/g	E-100FPU/g
24	11.12±2.29 (43.37)	14.45±2.31 (56.34)	15.51±4.62 (60.48)	16.36±3.46 (63.80)
48	12.78±3.56 (49.83)	16.89±2.34 (65.87)	17.68±1.15 (68.94)	19.51±2.40 (76.06)
120	13.43±3.50 (52.37)	18.61±3.48 (72.56)	19.73±3.56 (76.94)	22.63±3.52 (88.22)

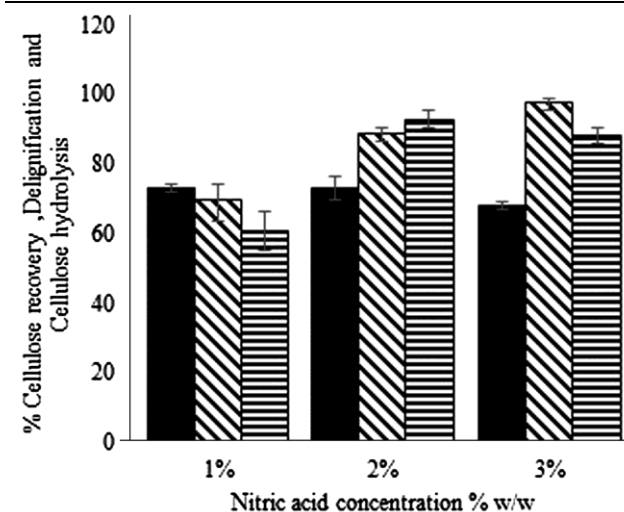


Fig. 6 — Optimization of nitric acid concentration. [Solid bar, % Cellulose recovery after de-esterification; Diagonal Striped bar, % Delignification; Horizontal striped bar, % Cellulose hydrolysis. Process conditions were pretreatment with 67% w/v formic acid, de-esterification with 1% w/v sodium bicarbonate, enzymatic hydrolysis using Cellic Ctec3 at 100 FPU/g]

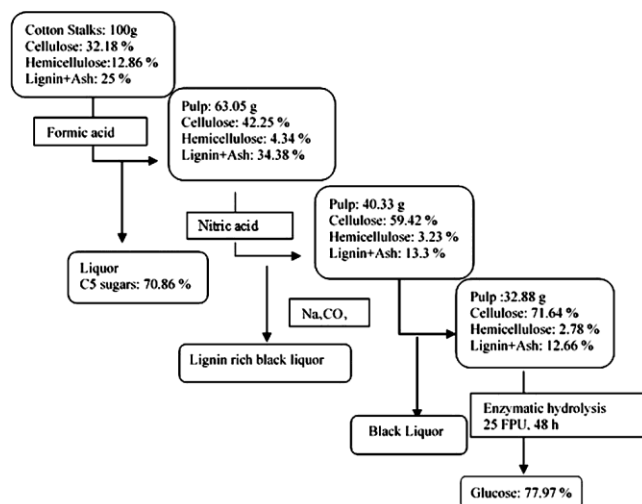


Fig. 7 — Process flow sheet for deconstruction of cotton stalks

Table 2 — Glucan yield with optimized conditions

Pre-treatment steps	% Cellulose Recovery	% Delignification	g glucose/100 g cotton stalk (% Cellulose hydrolysis)
Formic acid pre-treated	82.78 ±3.56	14.19 ±2.41	6.36±1.15 (23.87)
Formic acid pre-treated + 2% w/w Nitric acid	74.49 ±4.62	60 ±4.56	17.41±2.32 (72.65)
Formic acid + 2% w/w Nitric acid + 1% w/v Na carbonate	73.21 ±3.47	89.01 ±3.46	22.88±1.24 (92.89)

This step helped in reducing the enzyme load from 100 FPU to 25 FPU and assisted in the hydrolysis step by decreasing the time from 120 h to 48 h thereby clearly suggesting its potential as one of pretreatment steps.

A flow sheet summarizing the process for deconstruction of cotton stalks in the current study is represented in Fig. 7. The process gave 71% recovery of the glucan content as glucose (22.88% glucose from 32.2% glucan) while achieving 89% delignification (Table 2). The glucose thus obtained can be used in variety of applications including production of biofuels and biochemicals. The process developed proved to be reasonably efficient for high lignin containing recalcitrant biomass such as cotton stalk and can find application for fractionation of such types of biomass.

Conclusion

The current study describes an efficient defractionation process for recalcitrant cotton biomass that yields maximum amount of sugar. A sequence of steps consisting of treatments with formic acid followed by nitric acid and de-esterification by

sodium carbonate rendered the cotton stalk amenable to enzymatic hydrolysis. Optimized conditions for deconstruction of cotton stalk includes treatment with formic acid (67 wt. %) with subsequent treatment with 2% (w/w) nitric acid and de-esterification with 0.5% (w/v) sodium carbonate which resulted in delignification by about 89.01% and glucan recovery of about 68%. Optimized enzymatic load gave net glucan yield of 22.88 g per 100 g of cotton stalk in 48 h with a hydrolysis efficiency of 92.89%. About 80% of the formic acid used was recovered by distillation and reused in the process thereby bringing down the process cost. This is the first report for deconstruction of high lignin content biomass such as cotton stalks and has a significant bearing on the environmentally safe disposal of this surplus agricultural biomass, especially in Indian context.

Conflict of interest

The authors declare no conflict of interest

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