Title: SIMULTANEOUS DELIGNIFICATION AND DEAGGREGATION OF BIOMASS

Abstract: Disclosed are methods and systems for treating a lignocellulosic material or lignocellulosic containing biomass to delignify the material or biomass and make it more accessible for enzymatic or chemical modification. The system and methods include treating lignocellulose-containing material or biomass with an alkali in an exemplary alcohol/water co-solvent system. The treatment deaggregates the lignocellulosic material and at the same time delignifies the starting material or substrate. A liquid fraction contains a lignin composition having lignin in a form closer to its native state than any known laboratory procedure is also provided.
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO 2010105048 A1</td>
<td>16-09-2010</td>
<td>NONE</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2699289 A1</td>
<td>26-03-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 101848901 A</td>
<td>29-09-2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2203433 A1</td>
<td>07-07-2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2010539171 A</td>
<td>16-12-2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KR 20100075915 A</td>
<td>05-07-2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NZ 583889 A</td>
<td>12-01-2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RU 2010114727 A</td>
<td>20-10-2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2010222399 A1</td>
<td>02-09-2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2009038681 A1</td>
<td>26-03-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2612765 A1</td>
<td>18-01-2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1907381 A1</td>
<td>09-04-2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 20095000355 A</td>
<td>08-01-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2010168164 A1</td>
<td>01-07-2010</td>
</tr>
<tr>
<td>WO 2004053059 A2</td>
<td>24-06-2004</td>
<td>AU 2003295843 A1</td>
<td>30-06-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BR 0316923 A</td>
<td>18-10-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2508346 A1</td>
<td>24-06-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1585391 A2</td>
<td>19-10-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MX PA05006035 A</td>
<td>18-08-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2005043361 A1</td>
<td>24-02-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2004053059 A2</td>
<td>24-06-2004</td>
</tr>
</tbody>
</table>
hemicellulose within the lignocellulosic feedstock to hydrolytic enzymes; and 2) depolymerizing or hydrolyzing hemicellulose and cellulose polymers to free sugars. To produce alcohols, the sugars are then fermented to an alcohol, e.g., ethanol, and the alcohol is recovered, typically via distillation. Alternatively, the sugars can be converted to hydrocarbons through catalytic reformulation.

Because of the crystalline structure of cellulose, it is not readily convertible to sugars, and represents a major hurdle in the commercialization of processes for biofuels production. Enzymatic conversion to sugars, for example, takes a considerable amount of time and requires large quantities of hydrolytic enzymes, such as cellulases. Likewise for the production of chemically-modified cellulose derivatives, cellulose must be made accessible to reactive chemical agents; this usually requires high temperature, pressures, harsh chemical conditions, and extended periods of time.

As to lignin, it is exceeded only by cellulose as one of the most abundant organic polymers on Earth, employing about 30% of the non-fossil organic carbon, and constituting from a quarter to a third of the dry mass of wood. Lignin is an unusual biopolymer because it is heterogeneous and lacks of a defined primary structure. Lignin is a cross-linked racemic macromolecule with molecular masses in excess of 10,000 to 15,000 u. It is relatively hydrophobic and aromatic in nature. The degree of polymerization in nature is difficult to measure because lignin is fragmented during extraction/isolation. Different types of lignin have been reported depending on the means of isolation. In other words, it is unlikely that a natural unprocessed form has ever been isolated and completely described.

Moreover, most of the processes for the conversion of lignocellulosic biomass into ethanol use high temperature or pressure with acid, caustic or organic solvent. Such separation conditions chemically alter the nature of the recovered lignin, and the suitability of the lignin for high value applications. When more mild conditions are used, e.g., when water is used as the sole agent, the majority of the hemicellulose sugars can be recovered through autohydrolysis, but a very inefficient delignification results.

**BRIEF DESCRIPTION**

In one aspect, a process for the treatment for lignocellulosic material or biomass, e.g., grassy herbaceous biomass, is provided under mild conditions that simultaneously overcomes the two primary barriers to cellulosic biofuels recognized in U.S. Department of Energy publications. The method both delignifies the biomass and enhances accessibility of
cell wall polysaccharides to cell wall hydrolases. As such, the process is economically viable, and it is believed to be an important step toward advancing national goals for cellulosic biofuels.

In an illustrated embodiment, a method of simultaneously delignifying and deaggregating a lignocellulosic material or biomass is provided. The method includes treating a biomass with a concentrated alkali in a co-solvent system to at least partially delignify the lignocellulosic material to form a treated biomass and a liquid fraction, separating the liquid fraction which contains a lignin composition, and washing the treated biomass with co-solvent and then further with water.

In another aspect, a method of solubilizing at least a portion of lignin in a lignocellulose-containing biomass is provided. The method includes treating a lignocellulose-containing biomass with an alkali in a co-solvent to yield a pretreated biomass, and separating a liquid fraction comprising a lignin composition from the pretreated biomass.

In yet a further aspect, an embodiment provides a method of reducing lignin inhibition of glycosidase hydrolysis or saccharification of lignocellulosic material. The method includes contacting the lignocellulosic material or biomass with an alkali in a co-solvent system to at least partially delignify the lignocellulosic material, and separating a liquid fraction containing a lignin composition. The product is then provided as a cellulosic source for a saccharification reaction.

The methods and systems herein also include treating lignocellulosic feedstocks with a solution of an alkali in a co-solvent system, which includes water and a second solvent that is polar and fully water-miscible, to form a deaggregated cellulose, and washing out the alkali from the deaggregated cellulose to stabilize the deaggregated cellulose in an aqueous medium. The washing may be accomplished with a co-solvent system that is the same as in the treating step with the varying ratios of water and second solvent, and finally, with water itself. Among the most effective co-solvents identified are alcohols.

In yet another aspect, the principles of the processes described herein are manifest in a method of enhancing the susceptibility of a lignocellulose-containing biomass to hydrolysis, which includes treating a lignocellulose-containing biomass with an alkali in a co-solvent to yield a pretreated biomass. The pretreated biomass is then provided as a substrate for a hydrolysis or depolymerization reaction.

All methods may be conducted at mild conditions of temperature and pressure, e.g., ambient temperature and pressure. The biomass may suitably be in particulate form.
A lignin composition is also provided that is produced from the methods in accordance with principles described herein. Because of the mild conditions of isolation, it is believed that the lignin is in a relatively natural unprocessed, undegraded state.

According to the principles manifest in embodiments described herein, methods and systems are provided which simultaneously delignify a lignocellulosic material and deaggregate cellulose therein so that the lignin is at least practically solubilized and the cellulose is more accessible for enzymatic or chemical modification, e.g., depolymerization or hydrolysis reactions. The methods and systems, in effect, enhance the conversion of cellulose-based feedstocks for use in production of biofuels and cellulose derivatives.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Embodiments described herein may be better understood and appreciated by reference to the detailed description presented herein in conjunction with the accompanying drawings of which:

FIG. 1 is an x-ray diffractogram of a pulp before and after the pretreatment process in accordance with embodiments described herein;

FIG. 2 is a flowchart illustrating a system in accordance with embodiments described herein including the pretreatment of cellulosic feedstock to increase its accessibility to depolymerization;

FIG. 3 shows comparative x-ray diffractograms of a cellulose isolated at 70°C before and after it is annealed at 150°C;

FIG. 4 is a graph of the width at half-height of cellulose samples annealed at different temperatures;

FIG. 5 is a flow chart describing an embodiment wherein the enzymes are applied without prior pretreatment followed by separation of the residual cellulose, pretreatment according principles of the processes described herein, and then recombination with the supernatant from the separation after the first stage;

FIG. 6 is a flowchart illustrating an alternative embodiment for reducing enzymatic reaction times in accordance with principles of the embodiments described herein, including treating the residual cellulose from a first stage pretreatment with second stages of deaggregation and enzymatic hydrolysis to glucose before fermentation to ethanol;
FIG. 7 is a flowchart illustrating yet another embodiment for reducing enzymatic reaction times in accordance with principles of the processes described herein utilizing a countercurrent system wherein the residual cellulose from the second stages of treatment is recirculated into the first stage of pretreatment.

FIG. 8 is a flowchart illustrating a system in accordance with embodiments described herein including the pretreatment of a biomass feedstock to at least partially delignify and increase the pretreated biomass' accessibility to depolymerization; and

FIG. 9 depicts Raman spectra of untreated corn stover and corn stover treated by the process described in an embodiment herein.

**DETAILED DESCRIPTION**

Methods and systems are provided in which lignocellulosic material or lignocellulose-containing biomass or simply, biomass is at least partially delignified by treatments which include contacting the material or biomass with a concentrated alkali in a co-solvent system that includes water and a water-miscible solvent, e.g., an alcohol or polyol, under mild conditions. The treatment for biomass, e.g., grassy herbaceous biomass, simultaneously delignifies and enhances accessibility of cell wall polysaccharides to cell wall hydrolases. In the method, cellulose is made more accessible for enzymatic and chemical reaction. For example, when applied to corn stover at room temperature and pressure, the method facilitates saccharification at levels equal to or greater than achieved through treatment with acid at elevated temperatures. The methods and systems described herein, thus, increase the efficiency of enzymatic or chemical modification of cellulose for use as biofuels or cellulose derivatives.

Before any embodiments are explained in detail herein, however, it is to be understood that the embodiments are not limited in application to the details of construction and the arrangement of components of processes set forth in the following description, illustrated in the following drawings or exemplified by the Examples. Such description, drawings, and Examples are not intended to limit the scope of the embodiments of the processes described herein as set forth in the appended claims. Other embodiments can be practiced or carried out in various other ways.

Further, no admission is made that any reference, including any patent or patent document, cited in this specification constitutes prior art. In particular, it will be understood that, unless otherwise stated, reference to any document herein does not constitute an admission that
any of these documents form part of the common general knowledge in the prior art in the United States or in any other country. Any discussion of the references states what their authors assert, and the applicant reserves the right to challenge the accuracy and pertinence of any of the documents cited herein.

Throughout this disclosure, various aspects of the methods and systems described herein may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity, and should not be construed as an inflexible limitation on the scope of the processes described herein. Accordingly, as will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof, as well as all integral and fractional numerical values within that range. As only one example, a range of 20% to 40% can be broken down into ranges of 20% to 32.5% and 32.5% to 40%, 20% to 27.5% and 27.5% to 40%, etc. Any listed range is also easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third, and upper third, etc. Further, as will also be understood by one skilled in the art, all language such as "up to," "at least," "greater than," "less than," "more than" and the like include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. In the same manner, all ratios disclosed herein also include all subratios falling within the broader ratio. Further, the phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably. The foregoing are only examples of what is specifically intended.

Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "comprising," "including," "having," and variations thereof herein is meant to encompass the items listed thereafter and equivalents thereof as well as additional items. "Comprising" encompasses the terms "consisting of" and "consisting essentially of." The use of "consisting essentially of" means that the composition or method may include additional ingredients and/or steps, but only if the additional ingredients and/or steps do not materially alter the basic and novel characteristics of the claimed composition or method. Unless specified or limited otherwise, the terms such as "mounted," "connected," "supported," and "coupled" and variations thereof are used broadly and encompass both direct and indirect mountings, connections, supports,
and couplings. Further, "connected" and "coupled" are not restricted to physical or mechanical connections or couplings.

Unless otherwise noted, technical terms are used according to conventional usage. However, as used herein, the following definitions may be useful in aiding the skilled practitioner in understanding the embodiments described herein:

As used herein, the terms "lignocellulosic feedstock", "lignocellulosic substrate", "lignocellulosic material", or "biomass" are meant to refer to any type of biomass that contains cellulose, hemicellulose and lignin. For example, lignocellulosic feedstocks may include grasses such as switch grass, cord grass, rye grass, miscanthus, or a combination thereof; sugar-processing residues such as sugar cane bagasse and sugar beet pulp; agricultural wastes such as soybean stover, corn stover; oat straw, rice straw, rice hulls, barley straw, corn cobs, wheat straw, canola straw, oat hulls, and corn fiber; and forestry wastes, such as recycled wood pulp fiber, sawdust, hardwood, softwood, or any combination thereof. Lignocelluloses feedstock may also include lignocellulosic waste or forestry waste materials such as newsprint, cardboard and the like. Lignocellulosic feedstock may also include one or more species of fiber that originate from different lignocellulosic feedstocks. Wheat straw, barley straw, corn stover, soybean stover, canola straw, switch grass, reed canary grass, sugar cane bagasse, cord grass, oat hulls, sugar beet pulp and miscanthus are particularly advantageous as cellulose feedstocks due to their widespread availability and low cost.

The term "hydrolytic enzyme(s)" is meant to refer to enzymes that catalyze hydrolysis of biological materials such as cellulose. Hydrolytic enzymes include "cellulase enzymes" or "cellulases" (used interchangeably) which are enzymes that catalyze the hydrolysis of cellulose to products such as glucose, cellobiose, cello-oligodextrins, and other cello-oligosaccharides. "Cellulase" is meant to be a generic term denoting a multienzyme complex or family, including exo-cellobiohydrolases (CBH), endoglucanases (EG), and β-glucosidases (β-θ) that can be produced by a number of plants and microorganisms. It is noted that many crude cellulase extracts also include some hemicellulases. Processes described herein may be carried out with any type of cellulase enzyme complex, regardless of their source; however, microbial cellulases are generally available at lower cost than those of plants. Among the most widely studied, characterized, and commercially produced cellulases are, e.g., those obtained from fungi of the genera Aspergillus, Humicola, and Trichoderma, and from the bacteria of the genera Bacillus and Thermobifida. Also, for example, cellulase produced by the filamentous fungi Trichoderma longibrachiatum includes at least two cellobiohydrolase enzymes termed CBHI and CBHII and at least 4 EG enzymes.
"Fermentation enzymes" refer to enzymes that can catalyze the conversion of the cellulosic sugars to alcohols, including ethanol as well as higher chain alcohols such as butanol. Typically, yeast such as Saccharomyces cerevisiae is used to produce the enzymes that catalyze the conversion. Enzymes may also include bacterial enzymes from Clostridium acetobutylicum as well as enzymes produced by engineered microorganisms to produce the higher chain alcohols from the sugars of cellulose.

As used herein, the terms "treatment," "treating," "pretreatment," or "pretreating" in respect of biomass are meant to refer to a process or treatment described herein in which lignin is at least partially removed from the biomass and cellulose is made more accessible for enzymatic or chemical, e.g., chemical catalytic, reaction.

"Modification or degradation" in reference to cellulose is used to refer to the biological, e.g., enzymatic, or chemical-induced alteration of the native structure of cellulose. Such changes and alterations are known to those in the art and include those involved in enzymatic degradation and/or enzymatic or chemical hydrolysis of cellulose, as well as chemical modifications involved in a variety of commercial cellulose-based products, production of alcohols by fermentation of biomass, and generation of hydrogen-rich biofuels.

The term "stable" or "stabilizing" refers to a deaggregated cellulose that does not change materially over a selected period of time and under selected conditions.

In view of the disadvantages inherent in conventional lignocellulose conversion, embodiments described herein provide novel methods for simultaneously delignifying and deaggregating lignocellulose or biomass. The methods include reacting a biomass with a treatment solution, which includes an alkali dissolved in a co-solvent system under mild conditions of temperature and pressure that may be optimized for economic feasibility. Subjecting the biomass to such treatment in accordance with embodiments described herein makes the cellulose therein more accessible for enzymatic or chemical reaction, by opening up, at the nano-scale, the tightly aggregated domains, which are also the source of recalcitrance during hydrolysis. The resulting deaggregated cellulose also allows for much more uniform substitution along the cellulose chains, thus minimizing problems of quality control currently inherent in producing cellulose derivative products.

A system embodying the principles described herein was initially developed to produce nano-deaggregated cellulose, a previously unknown form that differs from all known polymorphs, and that is much more accessible to enzymes than native cellulosates. When applied to commercial microcrystalline cellulose derived from high purity dissolving pulps,
which is frequently the standard in studies of saccharification, the pretreatment results in
reduction of the incubation time necessary for saccharification or, alternatively, in reduction of
the prerequisite dosage of enzymes by an order of magnitude.

As also described in the Examples below, studies were also undertaken to compare
untreated biomass and biomass treated as described in the embodiments herein. A
significant difference in saccharification yields was demonstrated. Studies were further
untaken to compare biomass treated in accordance with the embodiments described herein
and biomass treated by a known pretreatment process. Many pretreatments of biomass
have been developed that include physical treatments such as mechanical comminution,
physico-chemical pretreatment such as steam explosion and ammonia explosion, and
chemical treatments such as ozonolysis, acid or base hydrolysis, and organosolv. In the
Examples below, biomass treated in accordance with the embodiments described herein
and steam exploded biomass were compared with respect to saccharification yields. The
results demonstrated significantly better yields for the biomass treated by the processes
described herein.

The process embodying the principles described herein is a simple one. It requires that the
lignocellulosic substrate be treated with a solution of alkali, e.g., NaOH, in a co-solvent that
is, e.g., 75% ethanol and 25% water. This is followed by removal of the alkali under
conditions that avoid conversion to cellulose II, the mercerized form. The alkali co-solvent is
suitably present in a ratio of about 4:1 to the weight of biomass (volume co-solvent:weight
biomass).

As described above, the treatment solution includes an alkali dissolved in a co-solvent
system. Suitably, the alkali is dissolved in a co-solvent system of water plus a second water-
miscible solvent. In one aspect, the second solvent is suitably an alcohol which may include,
e.g., methanol, ethanol, propanol, isopropanol, butanol, isobutanol, or a polyol. In another
aspect, the second solvent may include other protic solvents as well as aprotic solvents that
are miscible in water, e.g. DMSO. In an illustrated embodiment, the co-solvent system is
ethanol and water.

In some embodiments, the alkali is suitably sodium hydroxide (NaOH) or potassium
hydroxide (KOH), although other alkalis may be used, such as lithium hydroxide (LiOH). The
concentration of, for example, NaOH or KOH needed in the treatment solution depends on
the nature of the lignocellulosic material to be treated, as different lignocelluloses may have
their polymeric forms disrupted at different concentrations of alkali. For example, the
threshold for mercerization of most pulps is approximately 8% NaOH in water; for cotton, it is
about 11 to 12%, depending on prior pretreatment; and for bacterial cellulose, it is about 14%.

It had been found that boiling of cellulosics isolated at room temperature resulted in tight molecular aggregation in cellulose and a decline in accessibility to probe molecules. That work led the inventor to consider the possibility that absent the elevated temperature, lignin in corn stover might be sufficiently loosely bound that its constraint on action of hydrolases may be reduced. The method embodying the principles described herein was applied to biomass that had not been previously processed at elevated temperatures, e.g., a sample of corn stover that had been knife milled to 6 mm. The inventor then discovered that another process was unfolding as well, namely, the removal of a significant fraction of lignin.

While the linkages are known to be quite labile in an alkaline environment, the hydrophobicity of lignins limits their solubility in aqueous media. The effectiveness of the system embodying the principles of the embodiments described herein includes a significant amount of ethanol, which is believed to be effective in at least partially solubilizing the lignin. It is noted that the use of ethanol to facilitate dissolution of lignin monomers has been used in laboratory studies of lignin chemistry.

Evidence for the removal of lignin is presented in Figure 9, which shows the Raman spectra of corn stover before and after it has been treated by the process embodying the principles described herein. Comparison of the spectrum of the treated stover with that of the raw stover indicates significant removal of lignin substructures from the treated stover. The removal of significant amounts of lignins is reflected in the significant decline of the bands at 1600 cm⁻¹, 1630 cm⁻¹ and 1192 cm⁻¹, all of which are signature bands characteristic of lignin monomeric entities (9). There remain some bands at frequencies higher than 1500 cm⁻¹ that are also characteristic of lignin because not all the lignin is likely removed, but clearly a significant amount is removed.

Thus, a liquid fraction from the processes described herein containing a lignin composition is also provided in embodiments described herein. The process described herein extracts lignin from biomass, e.g., grassy herbaceous plants, in a form closer to its native state than any known laboratory procedure; little or no degradation is expected. Simultaneously, the process enhances the accessibility of cell wall polysaccharides to hydrolases in a manner that avoids the formation of inhibitors of enzyme or yeast action. As to the latter, conversion rates of the order of 75% to 80% in 21 hours of enzyme action on corn stover treated in accordance with embodiments described herein in contrast to a conversion of 20% for corn stover that has simply been knife milled to 6 mm.
Establishing the molarity of the alkali, e.g., NaOH or KOH, of the pretreatment solution is an iterative process. As a beginning point, the co-solvent ratio is fixed at a level that was found optimal in the finishing of cotton (4), which is reported to be 75% ethanol and 25% water. The molarity is then varied and the effectiveness of the treatment is assessed until an optimum molarity of the alkali, e.g., NaOH, in the co-solvents is identified.

In Examples below, studies were first carried out on pure cellulose samples, and then applied to lignocellulose or biomass samples. The effect of the solutions on Avicel, a microcrystalline cellulose prepared from northern softwood (American Viscose Company, Marcus Hook, PA) and pulped at 180°C, was compared with earlier observations on other celluloses. It was found that a molarity of NaOH solutions between 1 M and 2.5 M worked well. Avicel was selected for the testing because it has become the standard substrate used in most published studies of bioconversion of cellulose. Avicel is a highly recalcitrant cellulose and representative of the effects of elevated temperature on pulp crystallinity. In additional Examples, kraft pulps derived from a toilet paper were used. The toilet paper was of the type designed for use in septic systems so that it did not contain wet strength additives. The paper was made up of approximately 65% eucalyptus and 35% northern softwood. Use of an organosolv pulp (e.g., see, U.S. Patent No. 4,100,016 to Diebold, et al.) is also included in the Examples below.

In general, the molarity (normality) of hydroxide (e.g., NaOH) in co-solvent is 1 - 2.5 M (N) which may be considered “concentrated alkali”, e.g., compared to alkali concentrations used in prior art processes. On a pH basis, the pH is suitably equal or greater than 14. Also, generally, the mild reaction conditions for the pretreatment of biomass include a temperature from about 0°C to 90°C, ambient pressure, and the hydroxide (OH\(^{-}\)) concentration described above.

As to the co-solvent, a suitable ratio of co-solvents is 75% water-miscible solvent and 25% water. It is anticipated that other ratios may be suitable. In varying the ratio, it is important to avoid levels of ethanol that can result in precipitation of alkali, e.g., NaOH.

Reference is now made to FIG. 2 that illustrates the general treatment process for embodiments described as well as further steps in the processing of cellulosic feedstock to an alcohol, e.g., ethanol. The process begins at step 100 with a cellulosic source. In an illustrated embodiment, Avicel was used as a source of cellulose at step 100.

At step 102, the cellulosic material is subjected to a pretreatment step in accordance with embodiments described herein, e.g., a treatment solution of alkali in a co-solvent system 101
of water and a second solvent, such as an alcohol, e.g., ethanol, or another water-miscible solvent, to deaggregate the cellulose. At step 104, the reaction mixture is separated to yield the decrystallized cellulose 108 and remove the treatment solution 101. At step 106, the treated cellulose is washed with a washing co-solvent solution 107 to remove the alkali. The washing co-solvent is suitably an alcohol/water mixture with final washing with water. At step 112, the treated cellulose in accordance with embodiments described herein is hydrolyzed, for example, by treatment with cellulases 110, to form sugars. At step 114, the sugars, which include glucose and cello-oligodextrins, are suitably fermented, and a cellulosic alcohol 118 is recovered from the fermentation mix via distillation or other separatory method, e.g., membrane separation.

The effectiveness of the treatment solution is suitably measured by the onset of disruption of the Raman spectrum of cellulose, particularly in the low frequency region between 250 cm\(^{-1}\) and 600 cm\(^{-1}\) wherein the band at 378 cm\(^{-1}\) is a very sensitive index of the degree of perturbation of the native lattice.

As to the washing solution 107, if methanol was used as the co-solvent with water, it has been found that the same ratio of methanol to water as in the treatment co-solvent system is suitable for washing NaOH, as the alkali, out of the cellulose. For the ethanol/water system, a suitable ratio was also the same as in the treatment co-solvent.

It was noted earlier that the work with methanol was based on using the same ratio of co-solvents as in the pretreatment and was used as the starting point for ethanol/water co-solvent. The effect of varying the initial co-solvent for the first wash was determined. From a process perspective, it is especially suitable if the co-solvent ratio in the washing mixture is higher in ethanol than that used for the pretreatment as that would reduce the cost of post treatment of the washing solution. However, it is again noted that it is necessary to ensure that the ethanol content of the initial wash is not high enough to cause precipitation of the alkali, e.g., NaOH.

After the first wash is completed, it is necessary to continue washing the cellulose substrate until a neutral pH is achieved. It was found in some cases that it was more effective to transition from the first wash to washes with co-solvents including higher levels of water, before eventually washing with water only.

It has also been found that the degree to which the cellulose is tightly aggregated, and hence, its recalcitrance, is related to the highest temperature to which the cellulose is exposed during isolation (5). See, FIG. 3 and 4 taken from the Atalla et al. reference (5).
FIG. 3 shows the dramatic reduction in the width at half-height of the primary diffraction peak of native celluloses as a result of the annealing at 150°C. The width at half-height for the most prominent reflection in powder diffraction patterns of wood celluloses has always been regarded as one of the most sensitive indices of the degree of coherence of order within the cellulose in the wood cell walls. FIG. 4 shows how the width at half height declines as the temperature of treatment increases. Thus, in essence, the recalcitrance of a cellulosic sample is directly correlated with the temperature of isolation.

Once treated and washed, the degree to which the treated cellulose has become more accessible, i.e., decrystallized, can be assessed. Simple analytical methods, such as the weight loss upon enzymatic hydrolysis, can and were used as the measure of success in decrystallizing cellulose. Methods utilizing accessibility to deuterium oxide (D_2O) of the decrystallized cellulose can also be used. While these methods can rank the treatments, the readiness with which deuterium exchanges with hydrogen suggests that the use of D_2O may result in overstating the degree of accessibility. It has been found that deuterated ethylene glycol (OHCD_2CD_2OH) appropriately assesses the degree of accessibility to enzymatic action.

In utilizing deuterated methods, the most common measures of accessibility have relied on observation of the access to cellulosic hydroxyl groups on the basis of perfusion of samples with D_2O (4). While this is a useful measure, a more reliable measure is based on accessibility to molecules larger than the D^+ ion. Such molecules suitably include perdeutero methanol (CD_3OH), perdeutero ethylene glycol (CD_2OHCD_2OH), and perdeutero glycerol (CD_2OHCDOHCD_2OH), which can be added to the pretreated cellulosic samples in solution in H_2O, and allowed to reach equilibrium. The amount of deuterated molecules within the cellulosic samples is monitored through measurement of the Raman spectra of the samples in the region between 2300 and 2700 cm^-1 where there will be no interference from any other functional groups. The preparation of the perdeuterated samples of the alcohols or polyols can be accomplished by refluxing in D_2O over Raney nickel.

Perdeuterated methanol is available commercially, and perdeuteration of glycol and glycerol can be carried out as noted above. The perdeuterated methanol is used in measurements based on using other cellulosics that are common standards such as Avicel, which is derived from dissolving pulps, and Whatman CF-1 powder, which is derived from cotton linters. These standards are pre-swollen using known protocols.

As most enzymes are much larger in size than the molecules used to assess cellulose accessibility, an assay was developed for the transformations of the celluloses more closely
related to the activity of enzymes. In such assay, the pretreated and washed cellulose are incubated with representative cellulases from *Aspergillus niger* and *Trichoderma reesi* to assess the effect of the transformations on susceptibility to enzyme action. As noted earlier, the increased availability of celluloses to the hydrolytic enzymes should increase the rate of conversion to sugars by at least one order of magnitude or more.

Reference is again made to FIG. 2 wherein it is noted that a portion of the alcohol, e.g., ethanol, produced, i.e., reference numeral 118, can be used in the deaggregation step 102 as the co-solvent. Thus, in accordance with embodiments described herein, the entire cellulose conversion process may suitably have a feedback loop to supply co-solvent for the pretreatment process.

It is noted that another barrier to economic implementation of enzymatic hydrolysis of celluloses is the biphasic nature of the process when the celluloses are subjected to hydrolytic enzymes on a continuous basis in a batch process. The very long residence times required for the second phase result in the need for very large holding tanks to accommodate the time needed for the second phase to be complete. In another embodiment, it is envisioned that the long residence times of enzymatic hydrolysis reactions due to its biphasic nature can be reduced by use of the treatment process in accordance with embodiments described herein. To overcome this barrier, the application of the enzymes can suitably be accomplished in multiple stages, with the cellulosic substrates subjected to the treatment in accordance with embodiments described herein between stages.

At least three such multistage processes are contemplated. As shown in FIG. 5, a first application of enzymatic hydrolysis is carried out in a first stage prior to a pretreatment as described herein in order to take advantage of the relatively rapid early phase in enzymatic hydrolysis. When the rate of hydrolysis has slowed down at the beginning of the second phase, the solid cellulosic residue is separated and pretreated as described herein, and then recombined with the supernatant liquid stream separated from the solids at the end of the first phase. Specifically, a cellulosic material 100 is subjected to enzymatic hydrolysis 112 with cellulose until the first phase of enzymatic hydrolysis begins to slow. At step 120, the reaction mixture is separated into residual cellulose 122 and the remainder 128 of cellulases and glucose. The residual cellulose 122 is subjected to the deaggregation 124 as illustrated in FIG. 2, to yield a deaggregated residual cellulose 126 which is subjected to enzymatic hydrolysis 130 again, utilizing remainder enzymatic solution 128. The sugar products are then fermented at step 114 to produce cellulosic ethanol 118.
An embodiment of a second multistage process is shown in FIG. 6, and is based on repeating the deaggregation process as described herein between hydrolytic stages. At step 132, a deaggregated cellulose 108 as described herein is exposed to the enzymes for a period corresponding to the early phase of rapid hydrolysis. Next, at step 134, the residual cellulose 136 is separated from the enzyme-containing liquid medium 142 by filtration or centrifugation. The residual cellulose 136 is then subjected to a second cycle of deaggregation at step 138, as illustrated in FIG. 6, to yield a deaggregated residual cellulose 140, which, in turn, is exposed again to the enzyme-containing buffered water solution 142 for enzymatic hydrolysis to glucose at step 144 before fermentation to cellulosic ethanol 118 at step 114. It is anticipated that the hydrolysis again proceeds at a rapid rate so that the hydrolysis of the cellulose can be completed in a much shorter period than in the case of a single stage hydrolysis. Thus, one of the major cost factors in processes based on current designs, which need very long holding periods or residence times in the enzyme solution, is overcome and significantly reduced.

An embodiment of a third multistage process is shown in FIG. 7, and includes a countercurrent mixing of the cellulose and the enzyme solutions. At step 146, a deaggregated cellulose 108 as described herein, is exposed to the enzymes. At step 148, the residual cellulose 150 is separated from the enzyme-containing liquid medium 149 by filtration or centrifugation. The residual cellulose 150 is subjected to a second cycle of deaggregation at step 152 to yield a deaggregated residual cellulose 154, which in turn is exposed again to the enzyme-containing buffered water solution at step 156. As shown in FIG. 7, fresh enzyme may be used at step 156 in the second stage of treatment, and after the second stage of treatment, the residual cellulose 160 is separated at step 158, and then introduced into a second cycle of deaggregation 152 to yield a deaggregated residual cellulose 154, and then reintroduced into the enzymatic solution 156. Furthermore, after filtration or complete dissolution at step 158 of the cellulose, the enzyme solution 102 is re-applied to deaggregated cellulose at step 146. The sugar products are then fermented at step 114 to produce cellulosic ethanol 118. Such an approach reduces the amount of enzyme needed for the conversion of the cellulose to glucose. The cost of the enzymes is another major economic barrier for processes based on current designs.

The specific embodiment in this third multistage process will depend on the nature of the cellulosic feedstock. If the cellulose was relatively pure, it is anticipated that the conversion may be complete before the enzyme-containing solution is added to the freshly deaggregated cellulose. However, if the feedstock contains other components of
lignocellulosic matter, a filtration or centrifugation stage would be required prior to using the enzyme solution from the second stage to treat the feedstock in the first stage.

Reference is now made to FIG. 8, illustrating the general treatment process for embodiments described as well as further steps in the processing of biomass. The process begins at step 200 with a lignocellulosic source or biomass. In illustrated embodiments, corn stover or sugarcane bagasse was used as a source of biomass at step 200.

Biomass is suitably in particulate form which may be effected by milling, crushing, grinding, shredding or chopping. Suitable particle sizes may be from 8-200 mesh.

The biomass is subjected to a pretreatment step 202 in accordance with embodiments described herein, e.g., a treatment solution of alkali in a co-solvent system 201 of water and a second solvent, such as an alcohol, e.g., ethanol, or another water-miscible solvent, to deaggregate the cellulose. At step 204, the reaction mixture is separated into a liquid fraction 205 and a deaggregated cellulose 208. Removal of the liquid fraction may suitably be, e.g., by extraction or membrane filtration. At step 206, the treated biomass is washed with a co-solvent solution or mixture 207 to remove the alkali. The washing co-solvent or mixture is suitably an alcohol/water mixture with final wash with water. At step 212, the treated biomass in accordance with embodiments described herein may be hydrolyzed, for example, by treatment with hydrolytic enzymes 210 to form sugars, or by use of other suitable catalysts to yield other products.

An embodiment also contemplated is a kit, the kit including an alkali in an alcohol/water co-solvent, cellulase enzymes, one or more flocculants, and instructions for delignifying and deaggregating the biomass to produce a liquid fraction containing a lignin composition and deaggregated cellulose, and instructions for hydrolyzing the deaggregated cellulose to produce a hydrolysis product.

It is further envisioned that a similar treatment may make the cellulose more accessible homogeneous catalysts that may be used to transform the cellulosic feedstock into other forms. For example, the deaggregated cellulose as described herein could be more easily penetrated by the catalytic systems to reform it into hydrocarbons. Such process could make possible use of the vast amount of cellulosic resources as feedstocks for catalytic reformation to generate biofuels, such as diesel, fuel gases, such as hydrogen, and other high-value chemical types. Thus, in some embodiments, a method of producing cellulosic biofuels is provided. The method includes treating a cellulosic material with an alkali in an alcohol/water co-solvent system to yield a deaggregated cellulose; washing the
deaggregated cellulose to remove the alkali; hydrolyzing the cellulose to glucose and cello-
oligodextrins; and catalytically reforming the glucose and cello-oligodextrins into hydrocarbons.

As described in the Examples below, the methods and systems described herein also include methods of enhancing the susceptibility of a lignocellulose-containing biomass to hydrolysis, of reducing lignin-inhibition of saccharification of a cellulosic material, and of solubilizing at least a portion of lignin in a lignocellulose-containing biomass. The methods include treating the cellulosic or lignocellulosic material or biomass with an alkali in a co-
solvent to yield a treated or pretreated material. This treating step is suitably carried out under mild temperature and pressure conditions. The substrate or material to be treated is suitably dried and in a particulate form, e.g., the material may be milled, e.g., knife-milled.

As noted above, a barrier to much broader use of cellulose as a feedstock in the manufacture of fibers or films is the difficulty in solubilizing the cellulose in an environmentally acceptable system. The systems most often used outside of the United States are based on the century-old cellulose xanthate process, which is environmentally objectionable because the regeneration of the cellulose from solution results in the formation of hydrogen sulfide, and other toxic byproducts. The more recently developed methyl morpholine-N-oxide system relies on a complex and expensive solvent that is prone to explosion if conditions are not carefully controlled. On the other hand, the co-solvent system used herein is environmentally benign. It is envisioned that this system described herein could alter dramatically the economics of rayon and cellophane manufacture as well as biofuels as described herein.

The following Examples, which should not be construed by way of limiting the scope of the processes described herein, further explain embodiments of the principles described herein. Moreover, all experimental processes may be further optimized for efficiency and the process of scale up is expected to achieve greater enhancement of efficiency of conversion of cellulose to sugars.

**EXAMPLES**

Experiments to demonstrate reduction of lignin and the recalcitrance of cellulose were carried out in three phases. The first included the treatment of the native cellulose. The second assessed the consequence of this treatment by exposing the treated cellulose samples to hydrolytic enzymes and measuring its weight loss in comparison to a control consisting of the untreated native cellulose from the same source. The third included the
treatment of biomass or lignocellulosic feedstock to simultaneously delignify and
degraded the feedstock. Treatment of biomass with the processes described herein
was also compared to biomass treated by known methods.

The cellulose chosen as the substrate for the first Example was from a sample of Avicel
PH1, which has been used as a standard in the inventor's laboratory since the 1970s and
was supplied by the American Viscose Company (Marcus Hook, PA). It is a microcrystalline
cellulose usually manufactured by acid hydrolysis of a high purity dissolving grade northern
softwood pulp followed by mechanical disintegration of the pulp fibers and spray drying of
the resulting dispersion of fiber fragments. This type of cellulose was chosen because
Avicel has become a standard substrate in studies of enzymatic hydrolysis of cellulose and
is representative of the most recalcitrant pulp-derived celluloses. In a second set of
Examples, a kraft pulp derived from toilet paper was used. In yet another Example, an
organosolv pulp was used.

The enzymes used in the assessments were a cellulase from the fungus Trichoderma reesii
purchased from Worthington and a glucosidase derived from almonds available from Sigma
Aldrich.

**Example 1: Deaggregation**

A solution prepared for treatment of the Avicel was a 1.5 N solution of sodium hydroxide
(NaOH) in a mixture of ethanol (CH₃CH₂OH) and water that was 75% ethanol by volume. To
prepare the treatment solution, ethanol and water were mixed, and then 6 g of NaOH was
dissolved per 100 mL of the solvent mixture.

The treatment procedure was as follows: 1 g of Avicel was placed in a 300 mL beaker. To
this, 50 mL of the treatment solution were added. The Avicel was allowed to sit in the
treatment solution for 15 minutes. Thereafter, the solution was decanted and replaced with
100 mL of the solvent mixture (75% ethanol, 25% water). This solution was allowed to sit for
a few minutes to allow diffusion of the NaOH out of the cellulose.

The solvent was then decanted and the process repeated two times whereupon the pH was
approximately 8. After decanting the solvent the last time, a solution of 0.05 M ammonium
acetate buffer at a pH of 5 was added; the pH was 5.4 after the rinse in buffer. The buffer
solution was decanted, and 30 mL of buffer added again; the pH was then determined to be
5.0.
The dispersion of cellulose in 30 mL of buffer was transferred to a 50 mL polypropylene centrifuge tube and buffer added to the 40 mL level. Hydrolytic enzymes were added to the tube. These enzymes were 0.2 g cellulase (108 µ/mg) and 0.1 g β-glucosidase (6 µ/mg).

A control sample of 1 g of untreated Avicel was also placed in a 50 mL polypropylene centrifuge tube, and 40 mL of buffer added to it, followed by addition of the same amounts of enzymes as the test sample.

The two centrifuge tubes were then tightly closed with their covers, and inserted in a Vortemp 1550 shaking incubator. The contents of the tubes were incubated at 45°C and agitated at a speed of 900 rpm. It was found necessary to agitate at 900 rpm to keep the cellulose microcrystalline particles adequately dispersed.

For a first experiment, the incubation was for 41 hrs, and for a second, the incubation was for 13 hrs.

After the incubation, the two dispersions were each divided into 8 portions in 15 mL centrifuge tubes. The tubes were inserted in a centrifuge and spun for 2 minutes at 3800 rpm. The buffer-enzyme liquid was decanted from each tube and replaced with 95% ethanol, re-dispersed and spun again; this was done twice for each of the samples. The last ethanol decanted was replaced with acetone followed by dispersion in the acetone.

The acetone dispersions were then, in turn, poured into tared crucibles with sintered glass bottom filters; the crucible filters were mounted on a vacuum flask with full vacuum applied during the filtration. The crucibles were then transferred to a vacuum oven with full vacuum applied, heated to 105°C, and held at that temperature under vacuum overnight.

The samples were then weighed on an analytical balance, and the weight loss taken as a measure of the conversion of cellulose to glucose and soluble oligomers.

It should be noted that the 1.5 M (or 1.5 N) solution of NaOH in the solvent mixture was selected because the Avicel microcrystalline cellulose was derived from a dissolving pulp. Had microcrystalline cellulose made from cotton linters been used, it would have been necessary to use a 2 M (or 2 N) solution of NaOH in the solvent. Conversely, if the cellulose had been isolated from a herbaceous plant at a temperature much closer to ambient temperature, a 1 M (or 1 N) solution may have been adequate. This variability in the normality required for the pretreatment of cellulose reflects the great diversity in the level of aggregation of celluloses from different sources and with different histories into semicrystalline domains.
Results:

As noted above, the initial weights of the test and control samples were 1 g each. The weights after exposure to the enzyme mixture at 45°C are given below in Table 1.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Control</th>
<th>Pretreated</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>13 hrs</td>
<td>0.535 g</td>
<td>0.408 g</td>
<td>0.127 g</td>
</tr>
<tr>
<td>41 hrs</td>
<td>0.251 g</td>
<td>0.189 g</td>
<td>0.062 g</td>
</tr>
</tbody>
</table>

where Δ represents the difference in weight loss between the control and pretreated samples. Thus, in both instances the loss in weight of the sample treated as described herein was significantly greater than that of the control sample.

The results demonstrated that the loss in weight for both samples during the first 13 hr exposure was significantly higher than the loss during the further exposure for an additional 28 hrs. This is typical of the biphasic nature of enzyme action on celluloses where the rate of conversion to glucose or soluble oligomers proceeds rapidly at first but then levels off to a much slower rate. The results of these experiments demonstrate that the decrystallization treatment described herein increases the disorder in cellulose substrates, and makes them more susceptible to enzymatic hydrolysis by cellulases.

Example 2: A Two Stage Process

A solution prepared for treatment of the Avicel was a 1.5 N solution of sodium hydroxide (NaOH) in a mixture of ethanol (CH₃CH₂OH) and water that was 75% ethanol by volume. To prepare the treatment solution, one mixes the ethanol and water, and then dissolves 6 g of NaOH per 100 mL of the solvent mixture.

The treatment procedure was as follows: Two samples of 1 g each of Avicel were placed in 50 mL centrifuge tubes, one experimental sample, and one control. To each, 45 mL of 0.05 N ammonium acetate buffer with a pH of 5.01 was added. Both tubes received 0.15 g of cellulase, which was assayed at 136 μ/mg DW, with no supplemental β-glucosidase.

Both samples were placed in a Vortemp 1550 shaking incubator. They were incubated at 50°C and agitated at a speed of 900 rpm. Initial incubation was for 5.5 hours.
After the initial incubation period, the experimental sample was removed from the incubator and chilled in an ice bath to halt the enzyme action. The experimental sample was then placed in a centrifuge and spun at 4500 rpm to extract the supernatant. The supernatant was decanted and set aside for later return to the sample tube.

The sample tube then had 50 mL of the NaOH treatment solution added, and was shaken for 5 minutes, after which it was placed back in the centrifuge to extract the treatment solution.

Thereafter, the solution was decanted and replaced with 50 mL of the solvent mixture (75% ethanol, 25% water). It was shaken for 5 minutes to allow diffusion of the NaOH out of the cellulose. It was then centrifuged at 4500 rpm.

The solvent was then decanted and the process repeated two times. After the last decanting of solvent, a solution of 0.05 M ammonium acetate buffer at a pH of 5.01 was added; the pH was 8.4 after dispersing the sample in buffer. The buffer solution was centrifuged and decanted and 40 mL of buffer added again; the pH was then determined to be 5.15. This cycle was repeated one more time, after which the pH of the sample in buffer was 5.04. The buffer was then removed.

The supernatant enzyme solution extracted previously was returned to the sample tube, and incubation was resumed at 50°C and 900 rpm. The second phase of incubation lasted 2.5 hours.

After the incubation, both experimental and control sample tubes were inserted in a centrifuge and spun for 2 minutes at 4500 rpm. The buffer-enzyme liquid was decanted from each tube, and the remaining solids poured onto tared fiberglass paper for drying in a microwave oven with a built-in analytical balance, with the weight loss taken as a measure of the conversion of cellulose to glucose and soluble oligomers.

**Results:**

As noted above, the initial weights of the test and control sample were 1 g each. The weight after exposure to the enzyme mixture at 50°C is given below in Table 2.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Control</th>
<th>Pretreated</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 hrs</td>
<td>0.529 g</td>
<td>0.269 g</td>
<td>0.26 g</td>
</tr>
</tbody>
</table>

Table 2
where $\Delta$ represents the difference in weight loss between the control and pretreated samples. Thus, the loss in weight of the sample treated as described herein was significantly greater than that of the control sample.

**Example 3: A Two Stage Treatment Using a Kraft Pulp Paper**

A solution prepared for treatment of toilet paper (Cottonelle™ brand) was a 1.5 N solution of sodium hydroxide (NaOH) in a mixture of ethanol (CH$_3$CH$_2$OH) and water that was 75% ethanol by volume. Preparation of the treatment solution was the same as described in previous examples.

The treatment procedure was as follows: 2 samples (one control and one experimental sample) of toilet paper were weighed and then cut into small pieces and placed in 50 mL centrifuge tubes. The tubes were filled with water and put in a Vortemp 1550 shaking incubator at room temperature at 900 rpm and left to disperse overnight.

Each tube was filled to the 50 mL mark with 0.05 N ammonium acetate buffer with a pH of 5.01. Both tubes received 0.125 g of cellulase, which was assayed at 136 $\mu$/mg DW, with no supplemental $\beta$-glucosidase. Both samples were placed in the Vortemp incubator. They were incubated at 50°C and agitated at a speed of 900 rpm. Initial incubation was for 4.25 hours.

After the initial incubation period, both samples were removed from the incubator and chilled in an ice bath to halt the enzyme action. The experimental sample was then placed in a centrifuge and spun at 4500 rpm to extract the supernatant. The supernatant was decanted and set aside for later return to the sample tube.

The sample tube then had 50 mL of the NaOH treatment solution added, and was shaken for 2 minutes, after which it was placed back in the centrifuge to extract the treatment solution.

Thereafter, the solution was decanted and replaced with 50 mL of the solvent mixture (75% ethanol, 25% water). It was shaken for 2 minutes to allow diffusion of the NaOH out of the cellulose.

The solvent was then decanted and the process repeated two times. After decanting the solvent the last time, a solution of 0.05 M ammonium acetate buffer at a pH of 5.01 was added; the pH was 6.4 after dispersing the sample in buffer. The buffer solution was centrifuged and decanted and 40 mL of buffer added again; the pH was then determined to be 5.23. The supernatant enzyme solution extracted previously was returned to the sample.
tube, and incubation was resumed at 50°C and 900 rpm. The second phase of incubation lasted approximately 9.5 hours.

After the incubation, both experimental and control sample tubes were inserted in a centrifuge and spun for 2 minutes at 4500 rpm. The buffer-enzyme liquid was decanted from each tube, and the remaining solids poured onto tared fiberglass paper for drying in a microwave oven with a built-in analytical balance, with the weight loss taken as a measure of the conversion of cellulose to glucose and soluble oligomers.

**Results:**

The initial weights of the test and control samples, along with the weights after exposure to the enzyme mixture at 50°C are given below in Table 3.

<table>
<thead>
<tr>
<th></th>
<th>Initial wt</th>
<th>Final wt</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.021 g</td>
<td>0.314 g</td>
<td>69.25%</td>
</tr>
<tr>
<td>Pretreated</td>
<td>1.026 g</td>
<td>0.226 g</td>
<td>77.97%</td>
</tr>
</tbody>
</table>

The difference in the percentage of sample weight remaining demonstrates that conversion of the sample treated as described herein was greater than that of the control sample.

**Example 4: A Single Stage Treatment Using a Kraft Pulp Paper**

A solution prepared for treatment of toilet paper (Cottonelle™ brand) was a 1.5 N solution of sodium hydroxide (NaOH) in a mixture of ethanol (CH₃CH₂OH) and water that was 75% ethanol by volume. Preparation of the treatment solution was the same as described in previous examples.

The treatment procedure was as follows: 2 samples (one control and one experimental sample) of toilet paper were weighed and then cut into small pieces and placed in 50 ml centrifuge tubes. The tubes were filled with water and put in a Vortemp 1550 shaking incubator at room temperature at 900 rpm and left to disperse overnight.

The experimental sample was put in a centrifuge for 2 minutes at 4500 rpm and the extracted water decanted. The tube was refilled with 200 proof ethanol, and shaken for 5 minutes at 900 rpm, after which the tube was centrifuged again, the ethanol decanted, and then the tube was refilled with a mix of 75% ethanol and 25% water, shaken for 5 minutes, centrifuged and decanted again.
The sample tube then had 50 mL of the NaOH treatment solution added, and was shaken for 5 minutes, after which it was placed back in the centrifuge to extract the treatment solution.

Thereafter, the solution was decanted and replaced with 50 mL of the solvent mixture (75% ethanol, 25% water). It was shaken for 5 minutes to allow diffusion of the NaOH out of the cellulose.

The solvent was then decanted and the process repeated two times. After decanting the solvent the last time, a solution of 0.05 M ammonium acetate buffer at a pH of 5.01 was added; the pH was 12.63 after dispersing the sample in buffer. The buffer solution was centrifuged and decanted and 40 mL of buffer added again; the pH was then determined to be 9.37. This cycle was repeated 4 more times, with the pH determined at 6.02, 5.29, 5.14, and then 5.05 in the last cycle.

The control tube was filled to the 50 mL mark with the same ammonium acetate buffer solution. Both tubes received 0.125 g of cellulase, which was assayed at 136 µ/mg DW, with no supplemental β-glucosidase. Both samples were placed in a Vortemp 1550 shaking incubator. They were incubated at 50°C and agitated at a speed of 900 rpm for a total incubation of 16 hours and 25 minutes.

After the incubation, both experimental and control sample tubes were inserted in a centrifuge and spun for 2 minutes at 4500 rpm. The buffer-enzyme liquid was decanted from each tube, and the remaining solids poured onto tared fiberglass paper for drying in a microwave oven with a built-in analytical balance, with the weight loss taken as a measure of the conversion of cellulose to glucose and soluble oligomers.

Results:

The initial weights of the test and control samples, along with the weights after exposure to the enzyme mixture for 16 hours and 25 minutes at 50°C are given below in Table 4.

<table>
<thead>
<tr>
<th></th>
<th>Initial wt</th>
<th>Final wt</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.020 g</td>
<td>0.367 g</td>
<td>64.02%</td>
</tr>
<tr>
<td>Pretreated</td>
<td>1.017 g</td>
<td>0.225 g</td>
<td>77.88%</td>
</tr>
</tbody>
</table>

The difference in the percentage of sample weight remaining demonstrates that conversion of the sample treated as described herein was greater than that of the control sample.
Example 5: A Two Stage Treatment Using an Organosolv Pulp

An organosolv pulp (e.g., U.S. Patent No. 4,100,016) was initially treated with sodium chlorite to delignify it, after which it was allowed to air dry. The sodium chlorite treatment is a well-established, mild bleaching technique. Two samples (one control and one experimental sample) were weighed out from the resulting delignified, dried pulp.

A solution prepared for treatment of the delignified organosolv pulp was a 1.5 N solution of sodium hydroxide (NaOH) in a mixture of ethanol (CH$_3$CH$_2$OH) and water that was 75% ethanol by volume. Preparation of the treatment solution was the same as described in previous examples.

The treatment procedure was as follows: 2 samples (one control and one experimental sample) of pulp were weighed and then placed in 50 mL centrifuge tubes. The tubes were filled with water and put in a Vortemp 1550 shaking incubator at room temperature at 900 rpm and left to disperse for two days. After dispersion, both tubes were put in a centrifuge and spun at 4500 rpm for about 3 minutes, after which the water was decanted.

Each tube was then filled to the 50 mL mark with 0.05 N ammonium acetate buffer with a pH of 5.01. Both tubes received 0.2 g of cellulase, which was assayed at 136 µ/mg DW, with no supplemental β-glucosidase. Both samples were placed in the Vortemp incubator. They were incubated at 50°C and agitated at a speed of 900 rpm. Initial incubation was for 5.5 hours.

After the initial incubation period, both samples were removed from the incubator. The experimental sample was then placed in a centrifuge and spun for 7 minutes at 4700 rpm to extract the supernatant. The supernatant was decanted and set aside for later return to the sample tube.

The sample tube then had 50 mL of the NaOH treatment solution added, and was shaken for 2 minutes, after which it was placed back in the centrifuge to extract the treatment solution.

Thereafter, the solution was decanted and replaced with 50 mL of the solvent mixture (75% ethanol, 25% water). It was shaken for 2 minutes to allow diffusion of the NaOH out of the cellulose.

The solvent was then decanted and the process repeated two times. After decanting the solvent the last time, a solution of 0.05 M ammonium acetate buffer at a pH of 5.01 was added; the pH was 7.32 after dispersing the sample in buffer. The buffer solution was centrifuged and decanted and 40 mL of buffer added again; the pH was then determined to
be 5.18. The supernatant enzyme solution extracted previously was returned to the sample tube, and incubation was resumed at 50°C and 900 rpm. The second phase of incubation lasted approximately 3.5 hours.

After the incubation, both experimental and control sample tubes were inserted in a centrifuge and spun for 2 minutes at 4500 rpm. The buffer-enzyme liquid was decanted from each tube, and the remaining solids poured onto tared fiberglass paper for drying in a microwave oven with a built-in analytical balance, with the weight loss taken as a measure of the conversion of cellulose to glucose and soluble oligomers.

**Results:**

The initial weights of the test and control samples, along with the weights after exposure to the enzyme mixture at 50°C are given below in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>Initial wt</th>
<th>Final wt</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.003 g</td>
<td>0.539 g</td>
<td>46.26%</td>
</tr>
<tr>
<td>Pretreated</td>
<td>1.005 g</td>
<td>0.409 g</td>
<td>59.3%</td>
</tr>
</tbody>
</table>

The difference in the percentage of sample weight remaining demonstrates that conversion of the sample treated as described herein was greater than that of the control sample.

**Example 6: Comparison of Treated and Untreated Dried, Knife-Milled Corn Stover**

In this experiment, milled corn stover, which was treated with the alkali/co-solvent described herein, was compared with an untreated control in both pretreatment and saccharification. The alkali/co-solvent used was potassium hydroxide (KOH) in an ethanol/water solvent.

The corn stover samples were used in particulate form. Dried corn stover was knife-milled with a 6 mm screen size. The milled corn stover had a moisture content (determined by microwave balance) of approximately 4.35%. Two samples (one control and one experimental sample) of the dried, milled corn stover were selected, weighed out, and labeled.

A solution prepared for treatment of the corn stover consisted of a 1.5 N solution of potassium hydroxide (KOH) in a co-solvent mixture of ethanol and water that was 75% ethanol by volume. The treatment solution was prepared by mixing the ethanol and water together and then dissolving 8.42 g of KOH per 100 ml of the ethanol/water solvent mixture.
The treatment procedure was as follows. Both samples of corn stover were placed in 50 ml centrifuge tubes. The tube containing the control sample was filled with 0.05 N ammonium acetate buffer (pH 5.01) and set aside.

The tube containing the experimental sample received 45 ml of KOH treatment solution, followed by shaking for 5 minutes. After shaking, the tube was centrifuged and decanted over filter paper, then refilled with the co-solvent mixture (75% ethanol, 25% water). The tube was again shaken for 5 minutes to allow diffusion of the KOH out of the cellulose. The tube was once again centrifuged and decanted over filter paper, and the process was repeated two times. After decanting the solvent the third time, the sample was washed with water, decanted, and refilled with water. Using a pH meter, 85% phosphoric acid was added gradually to the sample until the pH reached 5.23. The treatment was under ambient temperature and pressure.

Next, each tube (control and experimental) received 0.12 ml of Cellic C-Tec 1 enzyme solution (Novozymes A/S) and were incubated at 50°C shaking at 600 rpm for 21 hours, 40 minutes. After incubation, each sample was centrifuged and decanted over filter paper for drying in a microwave oven with a built-in analytical balance to determine the weight loss in each sample.

Results:

The initial weights of the test and control samples, along with their weights after exposure to the enzyme mixture at 50°C are given below in Table 6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial Weight</th>
<th>Estimated Dry Weight</th>
<th>Final Weight</th>
<th>Change</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>2.014 g</td>
<td>1.926 g</td>
<td>0.322 g</td>
<td>1.088 g</td>
<td>77.1%</td>
</tr>
<tr>
<td>Control</td>
<td>2.022 g</td>
<td>1.944 g</td>
<td>1.549 g</td>
<td>0.395 g</td>
<td>20.3%</td>
</tr>
</tbody>
</table>

The change in weight of the experimental sample, representing the amount of solid converted during incubation, was significantly greater than that of the control sample.

These results indicate the inhibition of the saccharification of the corn stover by lignin had been significantly reduced, and that the method in accordance with the embodiments described herein is advantageous in overcoming the recalcitrance of cellulose to hydrolysis.
When the co-solvent wash and the first water wash were neutralized with acetic acid, a precipitation of solubilized material resulted, indicating that a portion of the constituents of corn stover were solubilized during the alkali/co-solvent treatment. Raman spectroscopy showed the precipitated material to contain a lignin composition as shown in FIG. 8.

Example 7: Pretreatment and Hydrolysis of Corn Stover compared to Known Pretreatment

In the following experiment, pretreatment and hydrolysis of corn stover treated in accordance with the processes described herein were compared to corn stover pretreated with a known process, steam explosion, as the control. The corn stover and steam exploded corn stover were single sourced.

Samples of corn stover were used to determine weight loss resulting from the treatment process in accordance with embodiments described herein.

Samples were weighed out as follows:
- A - 2.000g - raw stover, used to determine weight loss from pretreatment
- B - 2.000g - raw stover, used to determine weight loss from pretreatment
- C - 2.000g - raw stover, subjected to pretreatment followed by enzymatic hydrolysis
- D - 2.000g - raw stover, subjected to pretreatment followed by enzymatic hydrolysis
- E - 2.292g - (est. dry weight 1.375g) steam exploded stover, control
- F - 2.292g - (est. dry weight 1.375g) steam exploded stover, control

The raw stover was determined to have a moisture content of approximately 6.5%, and the steam exploded stover was determined to have a moisture content of approximately 40%.

Samples A, B, C, and D were placed in 50 ml centrifuge tubes, which were then filled with a solution of 1.5 molar sodium hydroxide in a co-solvent mixture of 75% ethanol and 25% water. These samples were then placed in a Vortemp 1550 incubator and shaken for 5 minutes at room temperature (22° C), after which they were centrifuged and the treatment solution was decanted.

The tubes containing A, B, C and D were then refilled with a co-solvent mixture of 75% ethanol and 25% water and shaken in the incubator for 5 minutes. The tubes were then centrifuged and decanted. Co-solvent washing was repeated 2 more times for a total of 3 co-solvent washes after the treatment solution.
The tubes containing A, B, C and D were then filled with water, shaken, centrifuged and decanted a total of 3 times. After this, samples C and D had acetic acid dropped in until the pH reached 5.05 and 5.15 respectively.

Samples A and B were centrifuged and decanted. The solids were dried in a microwave balance to determine how much solid material had been removed from each sample as a result of applying the alkali/co-solvent treatment protocol. The results are shown below in Table 7A:

**Table 7A (raw corn stover samples)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial weight</th>
<th>Est. dry weight</th>
<th>Final weight</th>
<th>Δ (dry basis)</th>
<th>% lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.000g</td>
<td>1.870g</td>
<td>1.390g</td>
<td>0.480g</td>
<td>25.67%</td>
</tr>
<tr>
<td>B</td>
<td>2.000g</td>
<td>1.870g</td>
<td>1.362g</td>
<td>0.508g</td>
<td>27.17%</td>
</tr>
</tbody>
</table>

The results demonstrate that a portion of the constituents of corn stover were solubilized during the alkali/co-solvent treatment.

The average loss on a weight basis for raw corn stover samples A and B was 26.42%. This value was used to adjust the starting dry weight of treated samples C and D in the results given below when calculating percentage enzyme loading and conversion percentage.

Control samples E and F were placed in a 50ml centrifuge tube, which was then filled with a 0.05M ammonium acetate buffer with a pH of 5.03. Samples C, D, E, and F each received 0.04 ml doses (3% of the starting dry weight of the biomass) of Novozymes Cellic C-Tec 2 enzyme, and placed in the Vortemp incubator. These four samples were then incubated for 19 hours at 50 C @ 600 rpm.

At the end of the incubation period, C, D, E, and F were centrifuged and the supernatant decanted and set aside. The remaining solids were placed in a microwave balance and dried to determine dry weight of the remaining solids from each sample. The results for the experimental samples are shown in Table 7B, and for the control samples in Table 7C.

**Table 7B (experimental samples).**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial weight</th>
<th>Est. dry weight</th>
<th>Post-Treatment</th>
<th>Final weight</th>
<th>pH</th>
<th>Δ</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.000g</td>
<td>1.870g</td>
<td>1.376g</td>
<td>0.493g</td>
<td>5.15</td>
<td>-0.883g</td>
<td>64.17%</td>
</tr>
</tbody>
</table>
Table 7C (control samples).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial weight</th>
<th>Est. dry weight</th>
<th>Final weight</th>
<th>pH</th>
<th>Δ</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>2.292g</td>
<td>1.375g</td>
<td>0.896g</td>
<td>5.03</td>
<td>-0.479g</td>
<td>34.84%</td>
</tr>
<tr>
<td>F</td>
<td>2.292g</td>
<td>1.375g</td>
<td>0.881g</td>
<td>5.03</td>
<td>-0.494g</td>
<td>35.93%</td>
</tr>
</tbody>
</table>

The change in weight represents the amount of solid converted during incubation. The results demonstrate that the change in weight of experimental samples was significantly greater than that for the control sample, and that the inhibition of the saccharification of the corn stover had been significantly reduced by the method in accordance with the embodiments described herein.

**Example 8: Treatment of Sugarcane Bagasse**

In this experiment, sugarcane bagasse, which was treated with the alkali/co-solvent described herein, underwent pretreatment and saccharification compared with untreated control.

Samples of sugar cane bagasse were used to determine weight loss resulting from the alkali/co-solvent pretreatment process described herein.

Samples were weighed out as follows:

- **A** - 2.211g - used to determine weight loss from pretreatment
- **B** - 2.239g - used to determine weight loss from pretreatment
- **C** - 2.222g - subjected to pretreatment followed by enzymatic hydrolysis
- **D** - 2.230g - subjected to pretreatment followed by enzymatic hydrolysis
- **E** - 1.779g - 1.601g est. dry wt - untreated bagasse, control
- **F** - 1.770g - 1.6g est. dry wt - untreated bagasse, control

The raw bagasse was determined to have a moisture content of approximately 10%

Samples A, B, C, and D were placed in 50 ml centrifuge tubes, which were then filled with a solution of 1.5 molar sodium hydroxide in a co-solvent mixture of 75% ethanol and 25% water. These samples were then placed in a Vortemp 1550 incubator and shaken for 5 minutes at room temperature (22°C), after which they were centrifuged and the treatment solution was decanted.
The tubes containing A, B, C and D were then refilled with a co-solvent mixture of 75% ethanol and 25% water and shaken in the incubator for 5 minutes. The tubes were then centrifuged and decanted. Co-solvent washing was repeated 2 more times for a total of 3 co-solvent washes after the treatment solution.

The tubes containing A, B, C and D were then filled with water and shaken, centrifuged and decanted a total of 3 times. After this, samples C and D had acetic acid dropped in until the pH reached 5.22 and 5.15 respectively.

Samples A and B were centrifuged and decanted. The solids were dried in a microwave balance to determine how much solid material had been removed from each sample as a result of applying the treatment protocol described herein. The results are shown below in Table 8A:

**Table 8A (raw bagasse samples)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial weight</th>
<th>Est. dry weight</th>
<th>Final weight</th>
<th>Δ (dry basis)</th>
<th>% lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.21 g</td>
<td>1.989</td>
<td>1.579 g</td>
<td>0.41 g</td>
<td>20.61%</td>
</tr>
<tr>
<td>B</td>
<td>2.239g</td>
<td>2.015</td>
<td>1.625</td>
<td>0.39 g</td>
<td>19.35%</td>
</tr>
</tbody>
</table>

The results demonstrate that a portion of the constituents of bagasse were solubilized during the alkali/co-solvent treatment.

The average weight loss for samples A and B was 19.98%. This value was used to adjust the starting dry weight of alkali/co-solvent-treated samples C and D in the tables given below when calculating percentage enzyme loading and conversion percentage.

Samples E and F were placed in a 50ml centrifuge tube, which was then filled with a 0.05M ammonium acetate buffer with a pH of 5.08. Samples C, D, E, and F each received 0.048ml doses (3% of the starting dry weight of the biomass) of Novozymes Cellic C-Tec 2 enzyme, and placed in the Vortemp incubator. These four samples were then incubated for 20 hours at 50°C @ 800rpm.

At the end of the incubation period, C, D, E, and F were centrifuged and the supernatant decanted and set aside. The remaining solids were placed in a microwave balance and dried to determine dry weight of the remaining solids from each sample. The results for the experimental samples are shown in Table 8B, and for the control samples in Table 8C:
Table 8B (experimental samples)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial weight</th>
<th>Est. dry weight</th>
<th>Post-Treatment</th>
<th>Final weight</th>
<th>pH</th>
<th>Δ</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.222g</td>
<td>2.000g</td>
<td>1.600g</td>
<td>0.771g</td>
<td>5.22</td>
<td>-0.829g</td>
<td>5.181%</td>
</tr>
<tr>
<td>D</td>
<td>2.230g</td>
<td>2.007g</td>
<td>1.606g</td>
<td>0.780g</td>
<td>5.13</td>
<td>-0.826g</td>
<td>5.143%</td>
</tr>
</tbody>
</table>

Table 8C (control samples)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial weight</th>
<th>Est. dry weight</th>
<th>Final weight</th>
<th>pH</th>
<th>Δ</th>
<th>% conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>1.779g</td>
<td>1.601g</td>
<td>1.486g</td>
<td>5.08</td>
<td>-0.115g</td>
<td>7.18%</td>
</tr>
<tr>
<td>F</td>
<td>1.770g</td>
<td>1.6g</td>
<td>1.468g</td>
<td>5.08</td>
<td>-0.132g</td>
<td>8.25%</td>
</tr>
</tbody>
</table>

The change in weight represents the amount of solid converted during incubation. The results demonstrate that the change in weight of experimental samples was significantly greater than that for the control sample, and that the inhibition of the saccharification of the bagasse had been significantly reduced by the method in accordance with the embodiments described herein.

The foregoing description is considered as illustrative only of the principles of the embodiments described herein. Further, since numerous modifications and changes may readily occur to those skilled in the art, it is not desired to limit the embodiments to the exact construction and operation shown and described, and accordingly, all suitable modifications and equivalents are considered to fall within the scope of the embodiments. Various features and advantages of the embodiments and processes described herein are set forth in the following claims.

All publications, patents and patent applications referenced in this specification are indicative of the level of ordinary skill in the art to which this application pertains. All publications, patents and patent applications are herein expressly incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference. In case of conflict between the present disclosure and the incorporated patents, publications and references, the present disclosure should control.
REFERENCES


CLAIMS

1. A method of simultaneously delignifying and deaggregating a lignocellulosic biomass, comprising:
   a) partially solubilizing the biomass with a concentrated alkali/co-solvent system to yield a liquid fraction and a treated biomass under mild conditions; 
   b) removing the liquid fraction; 
   c) washing the treated biomass with the co-solvent system; and 
   d) further washing of the treated biomass with water to provide a cellulosic source for saccharification reaction.

2. The method of claim 1, wherein step (a) deaggregates the cellulose of the biomass.

3. The method of claim 1, wherein the liquid fraction includes at least a portion of the lignin in the biomass.

4. The method of claim 1, further comprising treating the product of step (d) with saccharification enzymes capable of hydrolyzing the biomass to saccharification products.

5. The method of claim 4, wherein partial solubilization of the lignin of the biomass reduces lignin inhibition and improves reaction rates of hydrolysis of the product of step (d) and hydrolysis yields relative to untreated biomass.

6. The method of anyone of claims 1-5, wherein the co-solvent comprises water and a second water-miscible solvent.

7. The method of claim 6, wherein the water-miscible solvent is (i) a primary, secondary or tertiary alcohol or a polyol, or (ii) an organic polar solvent.

8. The method of claim 6, wherein the alcohol is ethanol or methanol.

9. The method of any one of claims 1-8, wherein the alkali is sodium hydroxide (NaOH) or potassium hydroxide (KOH).

10. The method of claim 4, further comprising treating the saccharification products with chemical or biochemical reagents to yield conversion products.
11. The method of claim 10, wherein the biochemical reagents are fermentation enzymes or microorganisms.

12. The method of claim 1, wherein the alkali co-solvent is present at ratio of about 4:1 (volume co-solvent:weight biomass) to weight biomass.

13. The method of claim 9, wherein the alkali concentration in the co-solvent is from 1 N to 2.5 N.

14. The method of claim 6, wherein the co-solvent is 75% (v/v) ethanol and 25% (v/v) water.

15. The method of any one of claims 1-9, wherein the mild conditions comprise:
   e) a temperature from about 0°C to about 90°C;
   f) ambient pressure; and
   g) a hydroxide concentration greater or equal to about 1 N in co-solvent.

16. The method of any one of claims 1-14, where the biomass is in particulate form.

17. The method of claim 16, wherein the particulate form of the biomass is effected by milling, crushing, grinding, shredding or chopping.

18. A method of enhancing the susceptibility of a biomass to hydrolysis, comprising treating the biomass with an alkali in a co-solvent to yield a treated biomass.


20. A method of solubilizing at least a portion of lignin in a biomass, comprising treating the biomass with an alkali in a co-solvent to yield a liquid fraction and a treated biomass, the liquid fraction comprising a lignin composition.
X-ray diffractograms of bleached kraft pulp before and after decrystallization treatment.

FIG. 1
CELLULOSIC FEEDSTOCK

NaOH in Cosolvents

DECRYSTALLIZATION TREATMENT

SEPARATION
Centrifugation Or Filtration

Cosolvents Wash (107)

DECRYSTALLIZED CELLULOSE

CELLULASES In BUFFER SOLUTION

ENZYMATIC HYDROLYSIS To GLUCOSE

FERMENTATION To ETHANOL

Cosolvents + NaOH

PRIMAR Y CELLULOSIC ETHANOL OUTPUT

FIG. 2

SUBSTITUTE SHEET (RULE 26)
X-ray diffractograms of cellulose isolated from southern pine. (C) control pulped at 60°C, (A) Annealed heated up to 150°C in water.

FIG. 3
Width at half-height of samples annealed at different temperatures

FIG. 4
NaOH in Co-Solvent

Pretreatment

Separation

Liquid Fraction

Co-Solvent and Water Wash

Deaggregated Cellulose

Hydrolytic Enzymes or Other Catalysts

Hydrolysis

See FIG. 6 for use of enzymatic hydrolysis to sugars

FIG. 8
### INTERNATIONAL SEARCH REPORT

---

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C08H8/00  C08H7/00  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

---

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C08H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

---

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>wo 2012/027767 AI (ANNI KKI GMBH [AT]); FACKLER KARIN [AT]; TERS THOMAS [AT]; ERTL ORTWIN [ ] 8 March 2012 (2012-03-08) page 11, line 3 - page 15, line 9; claims 1-6; examples 1, 2</td>
<td>1-20</td>
</tr>
<tr>
<td>E</td>
<td>wo 2012/037250 A2 (CELLULOSE SCIENCES INTERNATIONAL INC [US]; ATALLA RAJAI H [US]) 22 March 2012 (2012-03-22) examples 1-5</td>
<td>1-20</td>
</tr>
<tr>
<td>X</td>
<td>US 4 395 543 A (WANG DANI EL I C ET AL) 26 July 1983 (1983-07-26) column 1, line 57 - column 2, line 17; claims 1, 4-10; example 1</td>
<td>1-20</td>
</tr>
</tbody>
</table>

---

**Date of the actual completion of the international search**

25 July 2012

**Date of mailing of the international search report**

02/08/2012

---

Name and mailing address of the ISA

European Patent Office, P.B. 5018 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040
Fax: (+31-70) 340-3016

Authorized officer

Gerber, Myriam
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>WO 2012027767 A1</td>
<td>08-03-2012</td>
<td>AT 510346 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2012027767 A1</td>
</tr>
<tr>
<td>WO 2012037250 A2</td>
<td>22-03-2012</td>
<td>NONE</td>
</tr>
<tr>
<td>US 4395543 A</td>
<td>26-07-1983</td>
<td>NONE</td>
</tr>
<tr>
<td>WO 2009124240 A1</td>
<td>08-10-2009</td>
<td>CA 2720177 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2274433 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RU 2010144861 A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2011091940 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2009124240 A1</td>
</tr>
</tbody>
</table>