



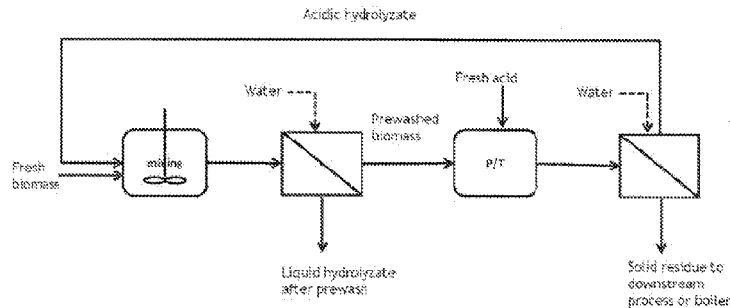
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(54) Title: PREPARATION OF BIOMASS

FIGURE 1



(57) Abstract: Methods and systems are provided for preparing biomass for downstream pretreatment operations to release sugar molecules. Biomass is prewashed with acid hydrolyzate prior to pretreatment. In so doing, the amount of chemicals that are required to efficiently process biomass for downstream uses is greatly reduced.

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PREPARATION OF BIOMASS

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application claims the benefit of U.S. Provisional Application No. 61/955,138, filed on March 18, 2014, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[02] The invention relates to reacting and/or removing compounds from biomass that may be deleterious to downstream pretreatment and/or fermentation processes by prewashing the biomass with acid hydrolysate.

BACKGROUND

[03] Cellulosic biomass provides a readily available source of sugar molecules, which may be used as a carbon source in microbial fermentations to produce bioproducts of interest. Polymeric carbohydrate components of the biomass can be hydrolyzed to release soluble sugar molecules. In acidic hydrolysis (*e.g.*, dilute acid pretreatment) of biomass, acid represents a significant fraction of the cost of sugar production, so reducing the amount of acid required is very important to the process economics. It is well known that compounds such as ash in biomass can neutralize acid and reduce the effectiveness of the pretreatment. The negative impact of these biomass components has been referred to as a neutralizing or buffering effect. Removal or inactivation of the neutralizing effect of such compounds prior to acid hydrolysis could reduce the amount of acid required for biomass pretreatment, which would in turn reduce the alkali usage in downstream processing and improve overall process economics. The reduction in acid and alkali usage also facilitates process recycle and makes the wastewater treatment easier by reducing the total dissolved solids (TDS). There is a need for a more efficient and cost effective method for producing fermentable sugar molecules from cellulosic feedstock.

BRIEF SUMMARY OF THE INVENTION

[04] Methods and systems are provided herein for reacting and/or removing compounds from biomass feedstock. Such reacting and/or removal of the compounds may advantageously reduce the amount of acid that is needed for downstream pretreatment processes such as hydrolysis to release a sufficient amount of soluble sugar molecules and/or may reduce the amount of base that is needed to bring the pH of the hydrolysate back

up to a suitable level for further processing (e.g., microbial fermentation, catalysis) after acid hydrolysis of the biomass, thus lowering chemical usage in an overall biomass conversion process. Not reacting and/or removing the compounds can be deleterious to downstream processes such as microbial fermentation (for example, by inhibiting microbial growth and/or bioproduct formation and/or forming secondary products that are inhibitory to microbial growth and/or bioproduct formation). Further, salts or dirt that are associated with the biomass can enter process streams and foul, contaminate, wear, and/or disable equipment such as hydrolysis or fermentation equipment, if not removed prior to pretreatment of the biomass or fermentation of hydrolysate prepared from the biomass.

[05] In one aspect, a method is provided for reacting and/or removing non-carbohydrate compounds from biomass, including (a) washing a second biomass with a first acid hydrolysate that is produced by acidic hydrolysis of a first biomass, thereby producing an acid hydrolysate washed second biomass; and (b) separating the acid hydrolysate washed second biomass from at least a portion of the first acid hydrolysate that was used to wash the second biomass. In some embodiments, at least one compound (e.g., at least one non-carbohydrate compound) in the first biomass is reacted to produce another compound. In some embodiments, at least a portion of at least one non-carbohydrate compound is removed from the second biomass. In some embodiments in which at least one non-carbohydrate compound is removed from the second biomass, the first acid hydrolysate that is separated from the acid hydrolysate washed second biomass in (b) contains the at least one non-carbohydrate compound. In some embodiments, the separated first acid hydrolysate of (b) is used as a primary substrate for fermentation. In various embodiments, the acid in the first acid hydrolysate used for washing the second biomass in (a) includes at least one acid selected from nitric acid, sulfuric acid, sulfurous acid, SO₂, hydrochloric acid, phosphoric acid, formic acid, and acetic acid. In one embodiment, the acid is nitric acid.

[06] In some embodiments, non-carbohydrate compounds that are removed from the second biomass include inorganic salts, mineral oxides, and/or organic acids. In some embodiments, at least a portion of the non-carbohydrate compounds that are reacted and/or removed from the second biomass are capable of buffering and/or neutralizing acid. In some embodiments, the reaction and/or removal of the non-carbohydrate compounds in a method as described herein reduces or eliminates the buffering and/or neutralizing capacity of such compounds in the second biomass in a downstream pretreatment process such as acid (e.g., dilute acid) hydrolysis. In some embodiments, the reaction and/or removal of the non-carbohydrate compounds in a method as described herein improves downstream

enzymatic hydrolysis and/or microbial fermentation performances. In some embodiments, removal of soluble (e.g., non-structural) sugar molecules reduces or prevents degradation of the sugar molecules from occurring in downstream pretreatment processes, such as acid hydrolysis, avoiding or reducing formation of inhibitors of microbial fermentation and/or bioproduct production.

[07] In some embodiments, the method further includes: (c) contacting the acid hydrolysate washed second biomass with acid and treating under conditions sufficient to depolymerize at least one polymeric carbohydrate component of the second biomass, thereby producing: (i) a second acid hydrolysate that includes soluble sugar molecules; and (ii) residual solids. Typically, less acid is required for said depolymerization from acid hydrolysate washed second biomass than from the same biomass that has not been prewashed as in (a), when treated with acid under identical conditions (e.g., temperature, time, pH). In some embodiments, less base is required to raise the pH of the acid hydrolysate to a level that is suitable for enzymatic hydrolysis and/or microbial fermentation than a hydrolysate produced from the same biomass that has not been prewashed as in (a), when treated with acid under identical conditions (e.g., temperature, time, pH). In various embodiments, the acid used for production of acid hydrolysate in (c) includes at least one acid selected from nitric acid, sulfuric acid, sulfurous acid, SO₂, hydrochloric acid, phosphoric acid, formic acid, and acetic acid. In one embodiment, the acid is nitric acid. In some embodiments, the acid in the first acid hydrolysate used for washing second biomass in (a) and the acid used for production of second acid hydrolysate in (c) are the same acid, for example, selected from nitric acid, sulfuric acid, sulfurous acid, SO₂, hydrochloric acid, phosphoric acid, formic acid, and acetic acid. In one embodiment, the acid is nitric acid. In some embodiments, the acid in the first acid hydrolysate used for washing second biomass in (a) and the acid used for production of second acid hydrolysate in (c) are different acids, for example, selected from nitric acid, sulfuric acid, sulfurous acid, SO₂, hydrochloric acid, phosphoric acid, formic acid, and acetic acid.

[08] In some embodiments, the method further includes: (d) separating the second acid hydrolysate produced in (c) from the residual solids produced in (c); and (e) using at least a portion of the second acid hydrolysate separated in (d) to wash a third biomass, as in (a). In some embodiments, acid hydrolysis of acid hydrolysate washed biomass, separation of the resulting acid hydrolysate from residual solids, and use of the acid hydrolysate for washing of additional unhydrolyzed biomass (e.g., steps (c), (d), and (e)) are conducted in a continuous process.

[09] In some embodiments, the amount of acid in (c) that is required for about 60% to about 95% depolymerization of the at least one polymeric carbohydrate component (*e.g.*, hemicellulose) from the acid hydrolysate washed second biomass is reduced by about 20% to about 60% in comparison with the same biomass that has not been pretreated as in (a). In some embodiments, the amount of acid in (c) that is required for about 75% to about 85% depolymerization of the at least one polymeric carbohydrate component (*e.g.*, hemicellulose) from the acid hydrolysate washed second biomass is reduced by about 30% to about 45% in comparison with the same biomass that has been washed with water instead of pretreated as in (a).

[10] In some embodiments, the second biomass includes lignocellulosic biomass, for example, including but not limited to, rice straw, rice husks, wheat straw, barley straw, corn stover, switchgrass, sugar cane bagasse, sugar cane straw or trash, palm empty fruit bunches, and/or Kenaf.

[11] In some embodiments, the at least one polymeric component that is depolymerized from the acid hydrolysate washed second biomass in (c) includes hemicellulose. In some embodiments, the at least one polymeric component further includes cellulose.

[12] In some embodiments, the lignocellulosic biomass includes a high silica lignocellulosic biomass, for example, including but not limited to, rice straw, wheat straw, rice husks, and/or corn stover. In some embodiments, at least a portion of the silica is removed by alkali extraction prior to step (a), and non-carbohydrate compounds that are removed include residual alkali. In some embodiments, acid in the first acid hydrolysate neutralizes at least a portion of residual alkali in the second biomass, and the first acid hydrolysate that is separated from acid hydrolysate washed second biomass contains neutralization product(s) of acid and residual alkali.

[13] In some embodiments, residual solids that are separated in (d) are subjected to a further hydrolysis process, for example, such as but not limited to, acid hydrolysis, enzymatic hydrolysis, or hydrolysis with a supercritical fluid. In one embodiment, the residual solids are hydrolyzed with one or more enzyme(s) (for example, including at least one cellulase) to produce additional soluble sugar molecules in an enzymatic hydrolysate. In some embodiments, soluble sugar molecules in the enzymatic hydrolysate are fermented by a microorganism to produce a bioproduct of interest.

[14] In some embodiments, residual solids in (d) are subjected to one or more process steps selected from catalytic processing, preparation for other purposes such as paper production, or use as a fuel.

[15] In some embodiments of the methods disclosed herein, the second biomass contains non-structural sugar molecules (for example, but not limited to, sucrose), and at least a portion of the non-structural sugar molecules are washed into the first acid hydrolysate. For example, the first acid hydrolysate that is separated from acid hydrolysate washed second biomass may contain about 50% to about 95% of the non-structural sugar molecules that were associated with the second biomass. In some embodiments, the acid hydrolysate washed second biomass retains a portion of the non-structural sugar molecules and the second acid hydrolysate produced in (c) includes soluble non-structural sugar molecules from the second biomass.

[16] In some embodiments, soluble sugar molecules in the first acid hydrolysate that is separated in (b) (after washing of biomass) and/or acid hydrolysate that is produced by acid hydrolysis of biomass that has been prewashed with acid hydrolysate as described herein are fermented by a microorganism to produce one or more bioproduct(s) of interest, for example, but not limited to, one or more solvent(s), organic acid(s), and/or alcohol(s).

[17] In some embodiments, the acid hydrolysate that contains the soluble sugar molecules is conditioned to remove at least a portion of at least one substance that inhibits microbial growth and/or bioproduct production, prior to fermentation. For example, conditioning may include, but is not limited to, at least one process selected from evaporation, steam stripping, charcoal adsorption, electrodialysis, and reverse osmosis.

[18] In some embodiments of fermentation processes described herein, one or more solvent(s) may be produced. For example, the solvent(s) may include ethanol, acetone, and/or butanol (e.g., n-butanol).

[19] In another aspect, biomass is provided from which at least a portion of one or more non-carbohydrate compound(s) has been reacted and/or removed, according to a process described herein. Hydrolysates that are prepared from such biomass are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[20] **Figure 1** schematically shows pretreatment of biomass to produce acid hydrolysate, and washing of fresh biomass with a stream of the acid hydrolysate to produce acid hydrolysate washed biomass

[21] **Figure 2** schematically shows prewashing of sugar cane bagasse with acid hydrolysate that is generated in a downstream acid hydrolysis operation and used for prewashing fresh bagasse.

[22] **Figure 3** schematically shows alkaline desilication and prewashing of rice straw with acid hydrolysate that is generated in a downstream acid hydrolysis operation and used for prewashing fresh rice straw.

[23] **Figure 4** schematically depicts an embodiment of a biomass washing procedure, exemplified in Example 1.

[24] **Figure 5** shows the concentration of free hydrogen ion in liquid phase of bagasse-water slurries (2.5 wt% solids) as a function of acid loading, as described in Example 1. The solid line represents pure aqueous solution (no solids).

[25] **Figure 6** schematically depicts an embodiment of a biomass washing procedure, exemplified in Example 2.

[26] **Figure 7** shows the concentration of free hydrogen ion in liquid phase of rice straw-water slurries (2.5 wt% solids) as a function of acid loading, as described in Example 2. The solid line represents pure aqueous solution (no solids).

[27] **Figure 8** schematically depicts an embodiment of a biomass sugar recovery procedure, exemplified in Example 3. This figure shows a flow diagram depicting an experiment that tested the effect of carry-over sugar on net sugar production and fermentability.

[28] **Figure 9** depicts results net sugar production (glucose + XMG + arabinose) and butanol titers from fermentation of enzymatic hydrolysates, as described in Example 3.

DETAILED DESCRIPTION

[29] Methods and systems are provided herein for preparing biomass for pretreatment (*e.g.*, acid hydrolysis, enzymatic hydrolysis). In the methods and systems herein, unwanted materials (*e.g.*, non-carbohydrate components) are reacted and/or removed from biomass. For example, compounds such as ash (*e.g.*, mineral oxides), organic acids, extractives, and/or other soluble components, may be removed, and/or compounds such as salts (*e.g.*, calcium salts) and/or oxides may be reacted to form insoluble products in the biomass. Compounds that are reacted and/or removed from biomass as described herein may otherwise affect the amount of one or more chemical(s) (*e.g.*, acid(s)) required for pretreatment. If the unwanted materials are not reacted and/or removed, they may become deleterious to one or more downstream process. In the methods and systems disclosed herein an acid hydrolysate is used to prewash biomass prior to downstream pretreatment processes. In one embodiment, the acid hydrolysate may be a stream from downstream acid hydrolysis of biomass. For example, production of acid hydrolysate from prewashed

biomass and prewashing of further biomass with a stream of the acid hydrolysate may be conducted in a continuous process.

Definitions

[30] “A,” “an” and “the” include plural references unless the context clearly dictates otherwise.

[31] “Bioproduct” refers to any substance of interest produced biologically, *i.e.*, via a metabolic pathway, by a microorganism, *e.g.*, in a microbial fermentation process.

Bioproducts include, but are not limited to fuel molecules (*e.g.*, *n*-butanol, acetone, ethanol, isobutanol, farnesene, etc.), solvents, biomolecules (*e.g.*, proteins (*e.g.*, enzymes), polysaccharides), organic acids (*e.g.*, formate, acetate, butyrate, propionate, succinate), alcohols (*e.g.*, methanol, propanol, isopropanol, hexanol, 2-butanol, isobutanol), diols (*e.g.*, 1,3-propanediol), fatty acids, aldehydes, lipids, long chain organic molecules (for example, for use in surfactant production), vitamins, and sugar alcohols (*e.g.*, xylitol). As nonlimiting examples, bioproducts may be used for catalysis, as a solvent, as a chemical intermediate, as a co-monomer, as a fuel (biofuel), or as a lubricant.

[32] “Byproduct” refers to a substance that is produced and/or purified and/or isolated during any of the processes described herein, which may have economic or environmental value, but that is not the primary process objective. Nonlimiting examples of byproducts of the processes described herein include lignin compounds and derivatives, carbohydrates and carbohydrate degradation products (*e.g.*, furfural, hydroxymethyl furfural, formic acid), and extractives (described *infra*).

[33] “Feedstock” refers to a substance that can serve as a source of sugar molecules to support microbial growth in a fermentation process.

[34] “Deconstruction” refers to mechanical, chemical, and/or biological degradation of biomass to render individual components (*e.g.*, cellulose, hemicellulose) more accessible to further pretreatment processes, for example, a process to release monomeric and oligomeric sugar molecules, such as acid hydrolysis.

[35] “Conditioning” refers to removal of inhibitors of microbial growth and/or bioproduct production from a hydrolysate produced by hydrolysis of a cellulosic feedstock or adjustment of a physical parameter of the hydrolysate to render it more amenable to inclusion in a microbial culture medium, for example, adjustment of the pH to a pH that is suitable for growth of the microorganism when added to a microbial growth medium.

[36] “Titer” refers to amount of a substance produced by a microorganism per unit volume in a microbial fermentation process. For example, titer of butanol in a microbial fermentation may be expressed as grams of butanol produced per liter of solution.

[37] “Yield” refers to amount of a product produced from a feed material (for example, sugar, relative to the total amount that of the substance that would be produced if all of the feed substance were converted to product. For example, yield of butanol in a microbial fermentation may be expressed as % of butanol produced relative to a theoretical yield if 100% of the feed substance (for example, soluble, *e.g.*, non-structural, sugar molecules) were converted to butanol.

[38] “Productivity” refers to the amount of a substance produced by a microorganism per unit volume per unit time in a microbial fermentation process. For example, productivity of butanol in a microbial fermentation may be expressed as grams of butanol produced per liter of solution per hour.

[39] “Sugar conversion” refers to grams of sugar consumed by a microorganism (*e.g.*, in a microbial fermentation process) per grams of sugar provided to the microorganism (*e.g.*, grams of sugar provided in a microbial growth medium).

[40] “Wild-type” refers to a microorganism as it occurs in nature.

[41] “ABE fermentation” refers to production of acetone, butanol, and/or ethanol by a fermenting microorganism.

[42] “Lignocellulosic” biomass refers to plant biomass that contains cellulose, hemicellulose, and lignin. The carbohydrate polymers (cellulose and hemicellulose) are tightly bound to lignin.

[43] “Lignins” are macromolecular components of lignocellulosic biomass that contain phenolic propylbenzene skeletal units linked at various sites.

[44] “Solvent” refers to a liquid or gas that is capable of dissolving a solid or another liquid or gas. A solvent may be produced as a bioproduct by a microorganism as described herein. Nonlimiting examples of solvents produced by microorganisms include *n*-butanol, acetone, ethanol, acetic acid, isopropanol, *n*-propanol, methanol, formic acid, 1,4-dioxane, tetrahydrofuran, acetonitrile, dimethylformamide, and dimethyl sulfoxide.

[45] *n*-Butanol is also referred to as “butanol” herein.

[46] “Vinasse” or “backset” or “stillage” refers to a fermentation broth from which one or more bioproduct has been removed. For example, fermentation broth of a microorganism that produces ethanol and from which ethanol has been removed is termed “ethanol vinasse.” As a further example, fermentation broth of a microorganism that produces

butanol and from which butanol has been removed is termed “butanol vinasse.” In some embodiments, vinasse is the bottom fraction of distillation of a solvent-containing fermentation medium, and solvent and other volatile compounds are separated from the fermentation broth while the rest of the constituents (e.g., residual sugar, organic acids, glycerol, biomass) are slightly concentrated in the vinasse.

[47] “Plant” and “facility” are used interchangeably herein to describe a location and equipment in which a disclosed process (e.g., sugar cane processing, ethanol production, bioproduct production) occurs.

[48] “Base” or “alkali” are used interchangeably herein to refer to a molecule or compound that is characteristically basic (pH greater than 7) at room temperature. Non-limiting examples of basic or alkaline compounds include oxides, carbonates, and hydroxides of alkali metals.

[49] “Acid” refers herein to a molecule or compound that can donate a proton or that can accept an electron pair in reactions.

[50] “Hydrolysis” refers to the chemical breakdown of a compound due to reaction with water. For example, hydrolysis of cellulosic biomass refers to the breakdown of glycosidic bonds in sugar polymers that are contained within the biomass. A “hydrolysate” is the liquid product of a hydrolysis reaction, e.g., liquid product of biomass hydrolysis containing soluble sugar molecules.

[51] “Pretreatment” refers herein to use of a method (e.g., mechanical, thermal, chemical, and/or biological) to modify the characteristics of a biomass material. For example, pretreatment may modify biomass such that hydrolytic enzymes and/or microorganisms may access and/or hydrolyze or utilize carbohydrate molecules in the biomass. Nonlimiting examples of pretreatment operations include acid or enzymatic hydrolysis to release soluble sugar molecules as a liquid hydrolysate.

[52] “CSF” or “combined severity factor” represents a combined effect of pretreatment temperature (°C), time (min), and pH (end of pretreatment). The equation used for the calculation is: $CSF = \log(t * ((T - 100) / 14.75)) - pH$

[53] “XMG” refers to xylose, mannose, galactose.

Acid hydrolysate prewashing

[54] In the methods and systems provided herein, unwanted non-carbohydrate compounds are reacted and/or removed from biomass feedstock prior to downstream processes such as dilute acid pretreatment and/or enzymatic hydrolysis, by prewashing the biomass with an acid

hydrolysate of biomass. Unwanted non-carbohydrate compounds may include compounds that would be deleterious to one or more downstream process(es) if not removed, such as hydrolysis of the biomass (*e.g.*, acid and/or enzymatic hydrolysis) and/or fermentation of a hydrolysate prepared from the biomass. The acid hydrolysate that is used for biomass feedstock prewashing is generated by acid hydrolysis of biomass, either fresh biomass that has not been prewashed with acid hydrolysate or biomass that has been prewashed as described herein. After prewashing with the acid hydrolysate, the washed biomass solid material is separated from the liquid hydrolysate (separated from all, substantially all, or at least a portion of the liquid hydrolysate) that was used for prewashing, thereby producing acid hydrolysate washed biomass from which at least a portion of at least one non-carbohydrate compound has been removed and/or reacted. In embodiments in which at least one non-carbohydrate compound is removed from the biomass in the prewashing process, the liquid hydrolysate that is separated from acid hydrolysate washed biomass solid includes at least one non-carbohydrate compound from the biomass that was washed with the hydrolysate.

[55] In one embodiment, the method includes: reacting and/or removing non-carbohydrate compounds from biomass, including: (a) washing biomass with an acid hydrolysate that is produced by acidic hydrolysis of another portion of biomass (*e.g.*, acid hydrolysate from either the same or different biomass as the biomass to be washed); and (b) separating the solid washed biomass from at least a portion of the liquid acid hydrolysate that was used for washing, thereby producing: (i) acid hydrolysate washed biomass from which at least a portion of at least one non-carbohydrate compound has been reacted and/or removed; and (ii) acid hydrolysate, wherein in embodiments in which at least one compound is removed from the biomass, the acid hydrolysate contains at least one non-carbohydrate compound from the biomass that was washed with the hydrolysate.

[56] Compounds removed from biomass by prewashing with acid hydrolysate may include one or more compound(s) (*e.g.*, non-carbohydrate compounds) that neutralize acid, compound(s) that buffer acid, and/or compound(s) that would be deleterious to one or more downstream process(es), such as acid hydrolysis, enzymatic hydrolysis and/or microbial fermentation of a hydrolysate prepared from the biomass if not removed. Nonlimiting examples of compounds (*e.g.*, extractives) that may be removed in the prewashing procedures described herein include ash (mineral oxides), inorganic salts (*e.g.*, oxides and/or salts of potassium, calcium, iron, and/or other cations), and/or organic acids (*e.g.*, lactic acid, acetic acid, formic acid, and/or carbonic acid). In some embodiments, at least a portion of the compound(s) that are removed from the biomass are capable of buffering and/or neutralizing

acid. By removing such compounds, the amount of acid that is required for downstream acid hydrolysis of the solid washed biomass material may be reduced, and/or the amount of base that is required to neutralize and/or bring the pH of the hydrolysate produced from the washed biomass material to a suitable level for a downstream process such as fermentation may be reduced.

[57] Compounds in the biomass that are reacted by prewashing with acid hydrolysate may include salts and/or oxides. "Reacting" includes formation of an insoluble product in the biomass by reaction of a biomass component with one or more component(s) of the acid hydrolysate that is used for prewashing, such as, for example, the acid that is used to produce the hydrolysate. In one non-limiting example, dilute sulfuric acid in an acid hydrolysate may react with a calcium salt or oxide in the biomass, forming calcium sulfate, which is insoluble and can form deposits within the biomass. Other minerals and salts may also form insoluble products which are not readily separated from the biomass in the liquid hydrolysate prewashing step. In other cases, a portion (0-100%) of the insoluble solids can be separated from the biomass to minimize the impact. In some embodiments, at least a portion of the compound(s) that are reacted in the biomass are capable of buffering the hydrolysate and/or neutralizing acid. By removing the buffering and/or neutralizing capabilities of such compounds, the amount of acid that is required for acid hydrolysis of the biomass may be reduced, and/or the amount of base that is required to neutralize and/or bring the pH of the hydrolysate to a suitable level for a downstream process such as fermentation may be reduced.

[58] Typically, the acid hydrolysate prewashing is performed under conditions (*e.g.*, temperature, pressure, pH, etc.) in which no or substantially no hydrolysis of the biomass occurs. For example, in some embodiments, the pH of the acid hydrolysate that is used to prewash biomass is about 1 to about 2. The process can cause an increase in pH to about 3 to about 4, depending on the washing conditions. No appreciable, *e.g.*, less than 10% of xylose yield, hydrolysis occurs within the washing step, owing to the lower reaction temperature, and relatively shorter contact time.

[59] In some embodiments, the acid hydrolysate that is used to prewash biomass is generated in a downstream process in which another portion of biomass is hydrolyzed by one or more acid. The biomass that is hydrolyzed to provide acid hydrolysate for prewashing fresh biomass is generated either from prewashed biomass, as described herein, or from unwashed biomass. The biomass that is hydrolyzed to provide acid hydrolysate for prewashing fresh biomass may be the same or different type of biomass as the biomass that is washed with the hydrolysate. In some embodiments, acid hydrolysate washed biomass is hydrolyzed with one

or more acid(s) to produce an acid hydrolysate, at least a portion of which is then used to wash fresh biomass. In some embodiments, acid hydrolysis of biomass, washing of fresh biomass with the acid hydrolysate, and recycling of acid hydrolysate for the washing process, are conducted in a continuous process. In other embodiments, batch processes are employed.

[60] Acid hydrolysate washed biomass (or unwashed biomass) may be contacted with fresh acid and treated under conditions sufficient to depolymerize at least one polymeric carbohydrate component (*e.g.*, hemicellulose, and optionally cellulose) from the biomass, thereby producing: (i) an acid hydrolysate that contains soluble sugar molecules; and (ii) residual solids. Typically, less acid is required for the depolymerization from acid hydrolysate washed biomass than from the same biomass that has not been prewashed, when both the washed and unwashed biomass are treated with acid under identical conditions. For example, in some embodiments, about 20% to about 50% less acid is required for depolymerization of at least one polymeric component (*e.g.*, hemicellulose, and optionally cellulose) from acid hydrolysate washed biomass. In some embodiments, less base is required to raise the pH of the acid hydrolysate to a level that is suitable for microbial fermentation (*e.g.*, about 4.5 to about 7) than a hydrolysate that is produced from the same biomass that has not been prewashed, when both the washed and unwashed biomass are treated with acid under identical conditions. For example, in some embodiments, about 20% to about 40% less base is required to adjust the pH of acid hydrolysate from acid hydrolysate washed biomass to a level that is suitable for microbial fermentation. In some embodiments of the methods disclosed herein, reduction in acid required for hydrolysis of biomass or base required for neutralization of hydrolysate may reduce total dissolved solids, facilitating downstream wastewater treatment.

[61] In some embodiments, at least a portion of the acid hydrolysate produced by acid hydrolysis of a portion of biomass is used to prewash fresh biomass. For example, in some embodiments, about 65% to about 85% of hydrolysate that is produced from acid hydrolysis of a portion of biomass is used to prewash fresh biomass (*e.g.*, another portion of biomass), although more or less acid hydrolysate may be used depending on various factors such as the amount of biomass hydrolyzed, the amount of biomass to be prewashed, the acid hydrolysis conditions used, or the volume of hydrolysate produced. Nonlimiting examples of acid hydrolysate production and biomass prewash processes are shown in Figure 2 for bagasse and Figure 3 for rice straw, and in Figures 4 and 6, which show acid hydrolysate production and biomass prewash processes exemplified in Examples 1 and 2, respectively.

[62] In some embodiments, the biomass to be prewashed is subjected to one or more upstream processes prior to washing with acid hydrolysate. For example, the biomass may be

deconstructed prior to prewashing with acid hydrolysate. In some embodiments, deconstruction may include mechanical disintegration in the presence of water and under pressure, thereby producing liquid and/or vapor and disintegrated biomass solid. In some embodiments, mechanical disintegration may be performed at a pressure and residence time sufficient to shear apart the biomass to render the carbohydrate polymers therein more accessible for acid-catalyzed depolymerization. In some embodiments, mechanical disintegration may include particle size reduction of the biomass.

[63] In some embodiments, one or more substance(s) may be removed from the biomass prior to washing with acid hydrolysate. For example, a high silica lignocellulosic biomass, such as, but not limited to, rice straw, wheat straw, rice husks, and/or corn stover, may be subjected to alkaline extraction of at least a portion of the silica prior to washing with acid hydrolysate. One nonlimiting example of a method for de-silicating feedstock, in which pre-pulping and low-consistency refining steps are used in conjunction with alkaline extraction, is described in U.S. Patent No. 7,364,640. In the methods described herein, residual alkali may be removed into the acid hydrolysate when the biomass is prewashed as described herein, for example, by reacting with acid to form a neutralization product of acid and residual alkali. An example of such a process is shown schematically in Figure 3.

[64] In embodiments of the methods described herein, biomass may be washed in a single stage with acid hydrolysate, or may be washed in multiple stages with acid hydrolysate and optionally one or more other liquid(s). In some embodiments, effective performance may be achieved in contactor systems in which the liquid (acid hydrolysate) and solids (biomass) are brought into intimate contact in a well-mixed environment and subsequently effectively separated into a liquid containing stream (with low suspended solids) and a relatively lower moisture solids fraction.

[65] In some embodiments of the methods disclosed herein, biomass is washed in a contactor/mixer system. In one embodiment, the contactor/mixer is a tornado pulper. In another embodiment, the contactor/mixer is a multi-stage mixer (*e.g.*, a mixer in which there is one unit operation with multiple parts), such as a multi-stage paddle mixer. In other embodiments, existing biomass and liquid contacting devices may be modified to facilitate the mixing and separation of the biomass and acid hydrolysate. One such embodiment includes the use of a sugarcane diffuser (*e.g.*, currently used for the extraction of sugarcane juice from sugarcane fiber) and associated roller mill equipment for the treatment of biomass (*e.g.*, bagasse) with acid hydrolysate. In another embodiment, a sugarcane roller mill (or series of roller mills) and associated equipment may be used. Additional non-

limiting examples include ribbon blending equipment, inclined screw mixers (*e.g.*, a screw inside a cylinder where the cylinder is angled (so excess liquid flows)), roller drums (*e.g.*, such as a sprayed bagasse washer (*e.g.*, a rotating drum, where biomass is transferred axially while the drum rotates with water being sprayed onto the biomass and excess water drains down and can be recirculated)), a sugar beet diffuser (*e.g.*, water is fed from top down and solids are conveyed 'upward'), or a conveyor belt with a sprayer. Other devices may be used, for example, any industrial device in which solids and liquid are contacted and then solids and liquids are separated. Batch systems, in which solids and liquid are added to a tank, and the liquid is drained may also be used (such as, for example, batch systems that have been used in the pulp and paper industry). For any of the contacting/mixing systems described herein, materials, configurations, and/or operating parameters may be altered and/or adapted for use in the methods described herein, and are not limited to materials, configurations, and/or operating conditions of previously known systems.

[66] In some embodiments, biomass may be washed with water and/or vinasse and/or process condensate (*e.g.*, from an evaporator), *e.g.*, in multiple unit operations in series, prior to washing with acid hydrolysate, optionally in a contactor/mixer system such as, for example, a tornado pulper or a multi-stage paddle mixer. In one embodiment, biomass is conveyed into a multi-stage contactor/mixer system such as a paddle washer, where it is contacted with water, then vinasse, and then acid hydrolysate. Different sequences and/or liquids may be used prior to the acid hydrolysate wash in other embodiments. The washed biomass is conveyed to a pretreatment reactor, where it is hydrolyzed with acid, and then at least a portion of the liquid acid hydrolysate is used to wash another portion of unwashed biomass.

Biomass feedstock

[67] A biomass feedstock is a substance that provides the base material from which sugar molecules are generated. Feedstock used in the methods described herein contains cellulose and hemicellulose. The material may contain cellulose and hemicellulose with or without lignin. In some embodiments, the feedstock is lignocellulosic biomass, which contains hemicellulose, cellulose, and lignin.

[68] Cellulose, which is a β -glucan built up of D-glucose units linked by $\beta(1,4)$ -glycosidic bonds, is the main structural component of plant cell walls and typically constitutes about 35-60% by weight (%w/w) of lignocellulosic materials.

[69] Hemicellulose refers to non-cellulosic polysaccharides associated with cellulose in plant tissues. Hemicellulose frequently constitutes about 20-35% w/w of lignocellulosic materials, and the majority of hemicelluloses consist of polymers based on pentose (five-carbon) sugar units, such as D-xylose and D-arabinose units, hexose (six-carbon) sugar units, such as D-glucose and D-mannose units, and uronic acids such as D-glucuronic acid.

[70] Lignin, which is a complex, cross-linked polymer based on variously substituted *p*-hydroxyphenylpropane units, typically constitutes about 10-30% w/w of lignocellulosic materials.

[71] In some embodiments, the biomass feedstock is bagasse (*e.g.*, sugarcane or sorghum bagasse), cane trash (*e.g.*, straw), rice straw, rice husks, empty fruit bunches, wheat straw, barley straw, corn stover, switchgrass, palm biomass (*e.g.*, chips, empty fruit bunches, fronds), Kenaf, energy cane, wood chips and/or pulp, municipal solid waste, rapeseed, mustard, canola straw, beet pulp, cassava pulp, or energy cane. In some embodiments, the feedstock contains grass, for example, sugar cane, miscanthus, and/or switchgrass, and/or straw, for example, wheat straw, barley straw, and/or rice straw.

[72] In some embodiments, an amount of feedstock that is used in a method disclosed herein is calculated as dry weight of biomass.

[73] In some embodiments, the feedstock is an agricultural residue. For example, bagasse may be used as the feedstock. Bagasse is the residual fiber generated as part of the sugar extraction process from sugarcane or sorghum, for example, in a sugar mill or biorefinery. Bagasse contains hemicellulose, cellulose, lignin, and some residual sugars. In some embodiments, bagasse may contain residual sucrose that was not removed during sugarcane processing. Residual sucrose may be extracted along with hemicellulose sugars in a method disclosed herein and during acid hydrolysis, the sucrose may be hydrolyzed to glucose and fructose, which will be included in the soluble sugar molecules in the hydrolysate, in addition to sugar molecules extracted from hemicellulose and cellulose carbohydrate polymers. In some embodiments of the methods disclosed herein, at least a portion of residual sucrose may be removed into the acid hydrolysate that is used for prewashing the biomass. In some embodiments of the methods disclosed herein, a portion of the residual sucrose is removed into the acid hydrolysate that is used for prewashing the biomass and a portion of the residual sucrose remains with the solid biomass material, and at least a portion of the sucrose that is retained with the solid biomass material is hydrolyzed to glucose and fructose in a downstream hydrolysis process.

[74] In some embodiments, a high silica lignocellulosic biomass is used as the feedstock. Nonlimiting examples of high silica biomass include rice straw, wheat straw, rice husks, sugar cane bagasse and straw, and corn stover. In some embodiments, silica is removed, for example, via alkaline extraction, prior to prewashing with acid hydrolysate as described herein.

[75] In some embodiments, the feedstock is a lignocellulosic material in the form of wood chips, sawdust, saw mill residue, or a combination thereof. In some embodiments, the lignocellulosic material is from a feedstock source that has been subjected to some form of disease in the growth and/or harvest production period. In one embodiment, the feedstock source is mountain pine beetle infested pine. In another embodiment, the feedstock source is sudden oak death syndrome infested oak, *e.g.*, coastal live oak, tanoak, etc. In another embodiment, the feedstock source is Dutch elm disease infested elm. In other embodiments, the feedstock source is lignocellulosic material that has been damaged by drought or fire.

[76] Lignocellulosic biomass may be derived from a fibrous biological material such as wood or fibrous plants. Examples of suitable types of wood include, but are not limited to, spruce, pine, hemlock, fir, birch, aspen, maple, poplar, alder, salix, cottonwood, rubber tree, marantii, eucalyptus, sugi, and acase. Examples of suitable fibrous plants include, but are not limited to, corn stover and fiber, flax, hemp, cannabis, sisal, hemp, bagasse, straw, cereal straws, reed, bamboo, mischantus, kenaf, canary reed, *Phalaris arundinacea*, and grasses.

[77] Other lignocellulosic materials may be used such as herbaceous material, agricultural crop or plant residue, forestry residue, municipal solid waste, pulp or paper mill residue, waste paper, recycling paper, or construction debris. Examples of suitable plant residues include, but are not limited to, stems, leaves, hulls, husks, cobs, branches, bagasse, cane trash, fronds, wood chips, wood pulp, wood pulp, and sawdust. Aquatic plants such as kelp, algae, lily, and hyacinth, which contain proportionately higher levels of hemicellulose, can also be used.

[78] Other lignocellulosic materials may be byproducts of other biomass industries such as soybean meal from soy oil extraction, rapeseed meal from rapeseed oil processing, empty fruit bunches from palm oil processing, or palm kernel meal from palm kernel oil extraction.

Acid hydrolysis of biomass

[79] Biomass feedstock is pretreated with an acid hydrolysis process, to generate acid hydrolysate that is used for prewashing additional biomass and/or to provide soluble sugar molecules for fermentation and bioproduct production by a microorganism.

[80] Acids that may be used for hydrolysis include, but are not limited to, nitric acid, formic acid, acetic acid, phosphoric acid, hydrochloric acid, sulfuric acid, sulfurous acid, or any combination thereof.

[81] Any acid concentration may be used that is suitable for depolymerization of sugar molecules from at least one polymeric component, and that will produce soluble sugar molecules that will support a microbial fermentation process. For example, an acid (*e.g.*, nitric acid) may be used for hydrolysis at a concentration of about 0.1% (w/w) to about 8.5% (w/w), for example, any of about 0.2% to about 1.5%, about 1.5% to about 3.0%, about 3.0% to about 4.5%, about 5.0% to about 6.5%, or about 6.5% to about 8.5%, or any of about 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0%, 7.5%, 8.0%, or 8.5%.

[82] In some embodiments of any of the above methods, hemicellulose and optionally some cellulose may be depolymerized from the biomass material, and the hydrolysate contains soluble sugar molecules from hemicellulose and optionally some sugar molecules from cellulose.

[83] Liquid hydrolysate that includes soluble sugar molecules (*e.g.*, from depolymerization of hemicellulose) may optionally be separated from residual solids prior to inclusion in a fermentation medium.

Further processing of solid residue from acid hydrolysis

[84] Residual solids remaining after acid hydrolysis of biomass as described herein may be subjected to one or more downstream processes. The residual solids may be further hydrolyzed to release additional soluble sugar molecules (*e.g.*, from depolymerization of cellulose) and/or may be used as a fuel source, *e.g.*, as fuel for a boiler and/or for electricity generation. For example, residual solids may be hydrolyzed in one or more process, including, but not limited to acid hydrolysis, enzymatic hydrolysis, or supercritical fluid hydrolysis. In embodiments in which residual solids are used as a fuel source, removal of ash by prewashing biomass material with acid hydrolysate (de-ashing) may improve the burning characteristics of the residual solids.

[85] In one embodiment, further hydrolysis of residual solids, *e.g.*, containing cellulose and lignin, may be conducted with one or more enzyme that is capable of depolymerizing cellulose, *e.g.*, hydrolysis of 1,4-beta-D-glycosidic linkages in cellulose. For example, enzymatic hydrolysis may be performed with one or more cellulase enzyme(s). Nonlimiting examples of cellulase enzymes include endocellulases, exocellulases, cellobiases, oxidative cellulases, and cellulose phosphorylases.

[86] In another embodiment, further hydrolysis of residual solids, *e.g.*, containing cellulose and lignin, may be conducted with acid, *e.g.*, under conditions suitable for depolymerization of cellulose. For example, hydrolysis is performed with an acid, *e.g.*, nitric acid, at a concentration of about 0.05% to about 0.1%, about 0.1% to about 0.5%, about 0.5% to about 1%, about 1% to about 4%, about 1.3% to about 3.5%, or about 1.3% (w/w of dry feedstock) at a temperature of about 190° to about 230°C, and at the saturation pressure for steam at the reactor temperature. In any of the biomass hydrolysis methods described herein, solids may optionally be separated from liquids to produce a hydrolysate and residual solids in a screw press, belt filter press, roller press, centrifuge, settling tank, vacuum filter, sieve screen, or rotary drum dryer.

[87] In some embodiments, the hydrolysate from the second hydrolysis (*e.g.*, hydrolysate containing depolymerized cellulose from residual solids) is combined with the hydrolysate from the first hydrolysis (*e.g.*, hydrolysate containing depolymerized hemicellulose from biomass) and the combined hydrolysates are included in a fermentation to produce one or more bioproduct(s) of interest in a bioproduct production facility as disclosed herein. In other embodiments, the hydrolysate from the second hydrolysis (*e.g.*, hydrolysate containing depolymerized cellulose from residual solids) and the hydrolysate from the first hydrolysis (*e.g.*, hydrolysate containing depolymerized hemicellulose from biomass) are separately fed to separate bioreactors for production of one or more bioproduct(s) of interest. The separate bioreactors may contain the same or different microorganisms and may produce the same or different bioproduct(s). In one embodiment, the first hydrolysate is fed to a bioreactor that contains a microorganism that is optimized for growth in the presence of this hydrolysate (*e.g.*, hydrolysate containing C5 and C6 sugar molecules), and the second hydrolysate is fed to a bioreactor than contains a microorganism that is optimized for growth in the presence of this second hydrolysate (*e.g.*, hydrolysate containing C6 sugar molecules).

[88] In some embodiments, the solid residue is prepared for use in other downstream processes, such as burning for fuel or papermaking. Solid residue that has been prepared from

de-ashed biomass, prepared as described herein by prewashing biomass with acid hydrolysate, and consequent removal of at least a portion of ash into the hydrolysate that is used for prewashing, may have advantageous characteristics as a fuel.

[89] In some embodiments, acid hydrolysate washed biomass from which ash has been removed (*e.g.*, biomass that has been washed with acid hydrolysate as described herein) is subjected to hydrolysis and the residual solids remaining after hydrolysis have higher heat value and/or less formation of ash in a combustor or boiler than the same biomass that has not been acid hydrolysate washed, when both the washed and unwashed biomass are hydrolyzed under identical conditions.

Processing of acid hydrolysate washed biomass and acid hydrolysate used to wash the biomass

[90] The acid hydrolysate washed biomass may be processed in one or more downstream operations. For example, acid hydrolysate washed biomass may be subjected to acid hydrolysis, auto hydrolysis (*e.g.*, hydrolysis by acetic acid released from biomass), enzymatic hydrolysis, or hydrolysis with a supercritical fluid. When subjected to acid hydrolysis, a stream of acid hydrolysate thus produced may be used to wash further biomass.

[91] After use for washing biomass, the acid hydrolysate that contains one or more non-carbohydrate compounds from the biomass, as described herein, may be used, for example, for microbial fermentation to produce one or more bioproduct(s) of interest, and/or may be reused to wash further unwashed biomass.

Conditioning of hydrolyzed biomass

[92] In some embodiments, hydrolysate is “conditioned” to remove inhibitors of microbial growth and/or bioproduct production and/or to adjust one or more parameters of the hydrolysate to render it more suitable for addition to a microbial growth medium, for example, adjustment of pH and/or temperature to a physiologically acceptable level for growth of a microorganism when added to microbial growth medium. In some embodiments, acid hydrolysate that has been used for prewashing biomass, as described herein, is conditioned after the prewashing and before inclusion in a microbial fermentation medium.

[93] In some embodiments, conditioning includes evaporation, steam stripping, charcoal adsorption, ion exchange resin treatment, electrodialysis, and/or reverse osmosis.

[94] In some embodiments of the methods disclosed herein, a biomass hydrolysate is rendered fermentable, *i.e.*, suitable for microbial fermentation, after raising the pH to a physiologically acceptable level for growth of a particular microbial culture, for example, from the pH of the hydrolysate after acid hydrolysis (*e.g.*, about pH 1.7) to about pH 6 to pH 7, or about pH 5 to about pH 7 (*e.g.*, about 6.7). In some embodiments, no further conditioning processes are required, other than the pH adjustment, for the hydrolysate to support microbial growth and/or bioproduct production (*i.e.*, treatment of the hydrolysate to remove microbial growth and/or fermentation inhibitors is not required). Although not wishing to be bound by theory, raising the pH may result in deprotonation of certain organic acid inhibitor compounds, rendering them less inhibitory.

[95] In some embodiments, conditioning processes are included for removal of inhibitors from the hydrolysate. Inhibitors of microbial growth and/or bioproduct production may include, but are not limited to, organic acids, furans, phenols, soluble lignocellulosic materials, extractives, and ketones. Inhibitors present in hydrolysates may include, but are not limited to, 5-hydroxy-methyl furfural (HMF), furfural, aliphatic acids, levulinic acid, acetic acid, formic acid, phenolic compounds, vanillin, dihydroconiferylalcohol, coniferyl aldehyde, vanillic acid, hydroquinone, catechol, acetoguaiacone, homovanillic acid, 4-hydroxy-benzoic acid, Hibbert's ketones, ammonium nitrate and/or other salts, *p*-coumaric acid, ferulic acid, vanillic acid, syringaldehyde, sinapyl alcohol, and glucuronic acid.

[96] In some embodiments, microbial growth and/or bioproduct titer, yield, and/or productivity, is increased when conditioned hydrolyzed feedstock is used, in comparison to identical hydrolyzed feedstock which has not been subjected to the conditioning process.

[97] In some embodiments, a microorganism that is tolerant to inhibitors in hydrolyzed feedstock is used, or the microorganism used for bioproduct production develops increased tolerance to inhibitors over time, *e.g.*, by repeated passaging, rendering the conditioning step unnecessary or uneconomical.

Microbial fermentation for production of bioproducts

[98] Methods are provided for producing one or more bioproduct(s) of interest in a microbial fermentation. The methods include culturing a microorganism that produces the bioproduct of interest in a medium that contains soluble sugar molecules produced from biomass (*e.g.*, in an acid and/or enzymatic hydrolysate of biomass) to support microbial growth for production of one or more bioproduct(s) of interest. In the methods herein, fermentable sugar molecules that are generated from biomass that has been prewashed with

acid hydrolysate, as described herein, are included in the fermentation medium for production of bioproduct(s).

[99] In some embodiments, the bioproduct is a biofuel, for example, butanol, acetone, and/or ethanol. In some embodiments, the bioproduct is solvent (*e.g.*, a polar protic or aprotic solvent), biomolecule, organic acid, alcohol, fatty acid, aldehyde, lipid, long chain organic molecule, vitamin, or sugar alcohol. In some embodiments, the bioproduct is a solvent or organic acid.

[100] The methods for bioproduct production herein include fermentation with a bioproduct-producing microorganism in a bioreactor in a growth medium that contains liquid sugar-containing extract from biomass, such as a hydrolysate or conditioned hydrolysate of biomass. In some embodiments, a liquid sugar containing extract such as cane juice and/or molasses is included in the growth medium.

[101] In some embodiments, the bioproduct production includes fermentation with a bioproduct-producing microorganism in an immobilized cell bioreactor (*i.e.*, a bioreactor containing cells that are immobilized on a support, *e.g.*, a solid support). In some embodiments, an immobilized cell bioreactor provides higher productivity due to the accumulation of increased productive cell mass within the bioreactor compared with a stirred tank (suspended cell) bioreactor. In some embodiments, the microbial cells form a biofilm on the support and/or between support particles in the growth medium.

[102] In other embodiments, for example but not limited to, embodiments in which a hydrolysate composition containing both liquid hydrolysate and solid residues is used, microorganisms may be grown in a non-immobilized system, such as an agitated fermentation reactor, *e.g.*, designed to provide adequate conditions for fermentation, including but not limited to mixing of components, gas removal, temperature control, and/or the ability to add and/or remove material from the reactor. Several fermentation operational moieties exist, including but not limited to batch, fed-batch, and continuous in single or multiple reactor configurations. Exemplar reactor types include but are not limited to agitated tanks, *e.g.*, where agitation is effected by a mechanical impeller, the addition and withdrawal of material, the addition of gas, and/or the recirculation of fermentation gas; corn and/or cane ethanol fermentation tanks; pharmaceutical fermentation vessels; vacuum fermentation systems; air-lift type reactors; fluidized bed reactors; anaerobic digestors; and activated sludge reactors. In some embodiments, an extractive fermentation process is used (*e.g.*, gas stripping, liquid extraction, vacuum fermentation, extraction by absorption and/or adsorption by a solid material such as a polymeric material).

[103] In some embodiments, the bioproduct production process herein includes continuous fermentation of a microorganism (continuous addition of conditioned hydrolyzed feedstock and withdrawal of product stream). Continuous fermentation minimizes the unproductive portions of the fermentation cycle, such as lag, growth, and turnaround time, thereby reducing capital cost, and reduces the number of inoculation events, thus minimizing operational costs and risk associated with human and process error.

[104] Fermentation may be aerobic or anaerobic, depending on the requirements of the bioproduct-producing microorganism.

[105] As known in the art, in addition to an appropriate carbon source, fermentation media must contain suitable nitrogen source(s), mineral salts, cofactors, buffers, and other components suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for the production of the desired bioproduct. In some embodiments, salts and/or vitamin B12 or precursors thereof are included in the fermentation media. In some cases, hydrolyzed biomass (*e.g.*, bagasse and/or cane straw) may contain some or all of the nutrients required for growth, minimizing or obviating the need for additional supplemental material.

[106] One or more microorganism that is capable of producing one or more bioproduct(s) of interest is used in the fermentation methods described herein. In embodiments in which two or more microorganisms are used, the microorganisms may be the same or different microbial species and/or different strains of the same species.

[107] In some embodiments, the microorganisms are bacteria or fungi. In some embodiments, the microorganisms are a single species. In some embodiments, the microorganisms are a mixed culture of strains from the same species. In some embodiments, the microorganisms are a mixed culture of different species. In some embodiments, the microorganisms are an environmental isolate or strain derived therefrom.

[108] The following examples are intended to illustrate, but not limit, the invention.

EXAMPLES

Example 1. Acid hydrolysate washing of sugar cane bagasse

Materials and methods

[109] Biomass: Sugarcane bagasse was reduced in size to less than one centimeter by knife milling.

[110] Neutralization (buffering) effect: A 100 g bagasse/deionized water mixture was prepared from 2.5 dry grams of bagasse. Three drops of 1N KCl was added to the bagasse/water mixture by small diameter pipette to increase the conductivity for pH measurement, and the pH of the bagasse/water mixture was measured with a portable pH meter. Two hundred micro liters of 2 wt% HNO₃ was added in increments to the bagasse/water mixture, magnetically stirred for 1 min, and then pH was measured and recorded at ambient temperature. The results are shown in Fig. 2.

[111] Acid hydrolysis (pretreatment) reactor: A 1-inch diameter x 8-inch length tubular stainless steel reactor with flange sealing on each end was used, heated in a sand bath.

[112] Acid hydrolysis and washing of bagasse with acid hydrolysate: This process is shown schematically in Fig. 4. Bagasse (about 10 g dry bagasse) and acid were well mixed in a beaker before loading into the reactor. The pH before pretreatment was 1.22. Bagasse was hydrolyzed with 0.028 g nitric acid/g unwashed dry solids at 20 wt% solids concentration at 140°C. After hydrolysis, the reactor was unloaded, and the pH of the slurry was recorded. The pH after hydrolysis was 1.52.

[113] Three hundred grams of water (water 2) was used to wash the slurry (slurry 1) in a Buchner funnel (three times, 100 g water each time). All liquid streams (liquid 1) were collected and the weight was recorded. A 2 mL sample of liquid 1 was taken for HPLC analysis. The volume loss caused by sampling was less than 1%. This completed Cycle #0.

[114] Cycle #1 started with washing (de-ashing) fresh bagasse with the hydrolysate (liquid 1) produced Cycle #0. Ten dry grams of bagasse were mixed with liquid 1 at room temperature for 15 minutes. Cheesecloth and manual pressing was used to separate liquids from solids. To remove soluble sugars and acid that were carried by the hydrolysate, 400 grams of water (water 3) was used to wash the de-ashed bagasse, producing liquid 2.

[115] The de-ashed bagasse was mixed with fresh acid solution (acid 2). The mixture was then loaded into a reactor and hydrolyzed with nitric acid as described above. After hydrolysis, the reactor was unloaded and the pH of slurry 2 was recorded. Three hundred grams of water (water 5) was used to wash slurry 2 (100 g of water three times). All liquid streams (liquid 4) were collected and the weight was recorded. A 2 mL liquid sample was taken for HPLC analysis. This completed Cycle #1.

[116] Cycles #2, 3, and 4 repeated the operations of Cycle #1.

Results

[117] Fig. 5 shows the concentration of free hydrogen ion in bagasse-acid mixtures as a function of acid loading. When unwashed bagasse (“Fresh bagasse 1” in Fig. 4) was used, approximately 5.5 mg of nitric acid was neutralized by the ash in one gram of bagasse (the offset in pH response in Fig. 5). When the hydrolysate de-ashed bagasse was used, adding nitric acid to the mixture led to an immediate and nearly linear increase of free hydrogen ion concentration. No offset in pH response was observed, indicating little neutralizing capacity.

[118] Table 1 summarizes the pH and xylose yield from the pretreatment of bagasse as shown in Fig. 1.

Table 1

	Cycle#	Acid loading (g/g dry bagasse)	pH before pretreatment	pH after pretreatment	Xylose yield (g /g-dry bagasse)
Test 1	0	2.8 %	1.21	1.55	0.170
	1	2.0 %	-	1.34	0.171
	2	2.0 %	-	1.34	0.175
	3	2.0 %	-	1.41	0.171
	4	2.0 %	-	1.43	0.170
Test 2	0	2.8 %	1.23	1.56	0.173
	1	1.7 %	-	1.50	0.176
	2	1.7 %	-	1.39	0.177
	3	1.7%	-	1.51	0.178
	4	1.7%	-	1.49	0.172
	Water washed	2.0 %	1.32	1.50	0.163
	Unwashed	2.0%	1.38	1.72	0.166

Note: Sugar was measured by HPLC according to NREL method NREL/TP-510-42623 (Determination of sugars, byproducts, and degradation products in liquid fraction process samples: Technical Report, Golden, CO: National Renewable Energy Laboratory; 2008) – using an Aminex HPX-87P column, BioRad, Hercules, CA)

[119] The xylose and monomeric sugar yields from baseline pretreatment (acid hydrolysis) of unwashed bagasse were 0.170 – 0.173 and 0.219 – 0.222 g/g dry bagasse, respectively (Cycle #0). The hydrolysate from the baseline pretreatment was used to wash the next batch of bagasse, followed by water washing to recover soluble sugars and acids in the

solids. The washed bagasse was then subjected to pretreatment at the same temperature and time but at a reduced acid loading (2% in Test 1 and 1.7% in Test 2). The cycle was repeated. In comparison with hydrolysate in the baseline pretreatment, the pH of hydrolysates from pretreatment of de-ashed bagasse was lower, even at a reduced fresh acid dose.

[120] To determine sugar yield with water washed bagasse (no de-ashing with hydrolysate), an experiment was performed with water washed bagasse at 2% acid loading. The results are shown in Table 1. The xylose yield decreased by about 6-7% in comparison to acid hydrolysate washed bagasse. As a control, unwashed bagasse was pretreated with the same acid loading (2%). The yield was almost the same as that from the water washed bagasse. This was consistent with the finding that water washed and unwashed bagasse had similar acid neutralizing capacities (4.9 and 5.5 mg nitric acid/g solids, respectively. See Fig. 5).

Example 2. Acid hydrolysate washing of rice straw

Materials and Methods

[121] The experimental process is shown schematically in Fig 6. Knife milled rice straw (<3mm; 85% dry matter content) and nitric acid solution (0.035 g nitric acid/g rice straw) were well mixed in a 1000 mL beaker. The mixture was loaded into four 1 inch diameter by 8 inch length stainless steel tubular reactors with flanges sealing at each end. Each reactor was loaded with 50 grams of rice straw/acid mixture at a solids concentration of 20% (g/g). A fluidized sand bath (SBL-2D, Techne, Princeton, NJ) was used as the heating source for the pretreatment reactors. The sand bath was preheated to 2°C higher than the desired pretreatment temperature. Immediately before pretreatment, the sand bath temperature controller was set to the desired pretreatment temperature. The reactors were then submerged in the sand bath. Due to the cold reactor bodies, the sand bath temperature dropped by about 5°C in 2 minutes, but stabilized to the set level in about another 3-5 minutes. It took approximately 5 minutes for the reactor centers to reach 5°C from the desired point. (The cold reactors were placed in the hot sand bath. The reactor outer wall was heated up first, and it took time for the heat to transfer from the reactor outer wall to the center of the biomass bed. When the center reached 5°C below the sand bath temperature, it was estimated that >90% of the biomass bed was heated up to within 2°C of the sand bath temperature (pretreatment temperature), when the pretreatment effectively started.) The

pretreatment temperature in this experiment was 145°C. After pretreatment, the reactors were taken out of the sand bath and quenched in cool water. The reactors were then unloaded and the unloaded materials from the four reactors were mixed together (slurry 0) for subsequent operations.

[122] 30 g of slurry 0 was saved for enzymatic hydrolysis (EH). The remaining slurry was pressed to collect hydrolysate (C5 liquid 0). The C5 liquid 0 was filtered through 0.7 µm filter paper to remove insoluble solids. This completed Cycle #0.

[123] Cycle #1 started with washing rice straw (Fresh rice straw 1) with acid hydrolysate from Cycle #0 (C5 liquid 0). The feedstock rice straw was mixed with C5 liquid 0 at a ratio of 3.59 g liquid/g dry rice straw at room temperature for 10 min. The acid hydrolysate washed rice straw was then washed with 50 mL deionized water/g dry rice straw to remove soluble sugars and acid that were carried by the hydrolysate.

[124] The acid hydrolysate washed rice straw 1 was mixed with fresh acid solution as well as some C5 liquid 0 at a ratio of 0.228 g C5 liquid /g dry rice straw. (It was estimated that 6.36% of the hydrolysate from the previous pretreatment was carried over to the next pretreatment due to incomplete washing of the soluble sugars from the solids, which is equivalent to 0.228g hydrolysate/g rice straw.) The mixture was loaded into two reactors for pretreatment using the same procedure as described above for Cycle #0. After pretreatment, the two reactors were unloaded and slurry 1 was collected. 30 g of slurry 1 was saved for enzymatic hydrolysis, and the remaining slurry 1 was pressed to collect acid hydrolysate (C5 liquid 1). The C5 liquid 1 was filtered through 0.7 µm filter paper to remove insoluble solids. This completed Cycle #1.

[125] The following Cycle #2 repeated the operation of Cycle #1, except only one reactor was used.

[126] Water washed rice straw: 100g rice straw was mixed with 2L deionized water at room temperature. Solid and liquid was separated by cheesecloth and hand pressing. This was repeated 3 times. Then the solid was pressed by hydraulic press, resulting in washed rice straw with dry matter of about 50%.

[127] Buffering capacity measurement: To measure the buffering capacity, 2.5 g (dry matter) of rice straw was mixed with 97.5 g deionized water to make 2.5% solids mixture. Three drops of 3M KI was added to the mixture to increase the conductivity for pH measurement. Dilute nitric acid solution (5 wt%) was added to about 100 mL of the mixture in increments of 200 µl or 500 µl. The mixture was mixed for 30 seconds and then

pH was measured and recorded at room temperature. Free hydrogen ion values were calculated from pH.

Results

[128] The following assumptions were used in this example. . Total solids concentration loaded to the pretreatment reactor is 20% (g/g). Forty percent of the loaded solids are solubilized in pretreatment. Solid-liquid separation (pressing) after pretreatment results in a cake containing 45% insoluble solids. With these assumptions, 3.59 g of acid hydrolysate is produced from each gram of rice straw and is used for washing the fresh rice straw feedstock in the next cycle. It was also assumed that 6.36% of the acid hydrolysate is carried over to the next pretreatment cycle due to incomplete washing of the soluble sugars from the solids, which is equivalent to 0.228 g hydrolysate/g rice straw.

[129] Fig. 7 shows the concentration of free hydrogen ion concentration in the liquid phase of rice straw slurry (2.5 wt.% solids) as a function of acid loading. The acid hydrolysate washed rice straw in this figure was feedstock that was mixed with acid hydrolysate (C5 liquid 0) and then thoroughly washed with deionized water, as described above. The intercepts of the dashed lines with the x-axis were read as buffering capacities, which are shown in Table 2.

Table 2

Rice straw	Buffer capacity (g nitric acid/g dry raw rice straw)
As-is	0.024
Water washed	0.018
Acid hydrolysate washed	0.010

[130] The pH of pretreated rice straw (hydrolysate/biomass mixtures) are shown in Table 3.

Table 3.

	Temperature (°C)	Acid input (g-acid/g-straw)	pH before pretreatment	pH after pretreatment
PT0	145	3.5%	1.44	1.87
PT1	145	2.0%	1.40	1.75
PT2	145	2.0%	1.38	1.74

C6: glucose; C5: xylose/mannose/galactose (XMG) + arabinose; Total: glucose+ XMG +arabinose

[131] The pH's of liquid acid hydrolysates and hydrolysate-rice straw mixtures are shown in Table 4. The pH of the C5 liquor increased significantly by reacting with biomass, suggesting that significant reduction in the amount of base required to raise the pH to a suitable level for downstream fermentation may be achieved.

Table 4

	C5 liquid 0	Mixture 1	C5 liquid 1	Mixture 2
pH	1.76	4.00	1.60	3.89

Example 3

[132] The effects of sugar that is washed from raw feedstock and carried over through pretreatment on sugar production and fermentation were investigated. The experimental process for this example is shown schematically in Fig. 8.

Preparation of deashed rice straw

[133] To prepare sufficient amount of deashed rice straw for the following experiment, knife milled and water washed rice straw (<3mm; 50% w/w dry matter content) was mixed (by hand) with nitric acid solution at pH of 1.7 followed by large amount of water washing. The mixing was done in a beaker at a ratio of 3.59 g acid solution/g dry rice straw at room temperature for 10 min. The nitric acid impregnated rice straw was then washed with 50 mL deionized water/g dry rice straw to remove residual acid. The resulting material is termed "deashed rice straw" in the following experiment.

Collection of soluble sugar solution

[134] Knife milled rice straw (<3mm; 85% w/w dry matter content) and deionized water were mixed at 10.5% total solids loading and incubated at 55° C, 155 rpm for 1 hour to extract soluble sugars. Liquid was then separated from residual solids through cheese cloth. Solids were further pressed to a dry matter content of approximately 50% w/w and more liquid was collected. All collected liquid was combined and filtered through 0.7 µm filter paper to remove insoluble solids. The resulting liquid is termed “soluble sugar solution” in following experiment.

Baseline pretreatment

[135] For “Cycle #0” as depicted schematically in Fig. 8, knife milled and water washed rice straw (<3mm; 50% w/w dry matter content) and nitric acid solution at predetermined amounts (See Table 5, control_water washed) were well mixed in a 1000 mL beaker. The mixture was loaded to four 1-inch diameter x 8-inch length stainless steel tubular reactors with flanges sealing at each end. Each reactor was loaded with 50 grams of mixture at a solids concentration of 20% (g/g-mixture). A fluidized sand bath (SBL-2D, Techne, Princeton, NJ) was used as the heating source for the pretreatment reactors. The temperature in the center of the reactor reached 5°C from the set level within 5 min. The pretreatment conditions in this experiment were 145° C, 35 min (not including the 5 min ramp up time).

[136] After pretreatment, the reactors were taken out of the sand bath and were quenched in room temperature water. The reactors were then unloaded and the unloaded materials from the four reactors were mixed together (“Slurry 0”) for the next operations.

[137] Thirty grams of Slurry 0 was saved for enzymatic hydrolysis. The remaining slurry was pressed to collect hydrolysate (“C5 Liquid 0”). The C5 Liquid 0 was filtered through 0.7 µm filter paper to remove insoluble solids.

[138] So far the first baseline pretreatment (control_water wash) was finished.

[139] The other baseline pretreatment (control_deashed) was carried out in the same way, but deashed rice straw was used instead of water washed rice straw, and acid loading was adjusted, targeting for a pH ~ 1.7 after pretreatment.

Dilute nitric acid pretreatment with carry-over sugars (sugars remaining with solids after acid hydrolysate and water washing).

[140] Three levels of carry-over sugar were tested: 10%, 15% and 20%, based on the following assumptions: (1) Total solids concentration (water washed or deashed solids not including the carry-over sugars) loaded to the pretreatment reactor is 20% (g/g). Forty percent of the loaded solids are solubilized in pretreatment. Solid-liquid separation (pressing) after pretreatment results in a cake containing 45% insoluble solids. With these assumptions, 3.59 g of hydrolysate is produced from each gram of rice straw. (2) Approximately 10-20% of the hydrolysate separated from the pretreated slurry (0.36 – 0.72 g hydrolysate/g deashed rice straw) is carried over to the next pretreatment cycle due to incomplete liquid-solid separation before pretreatment. The same percentage of soluble sugar from raw rice straw feedstock is also assumed to be carried over to the next pretreatment cycle.

[141] For 10% carry-over sugar, in Cycle #1, depicted schematically in Fig. 8, deashed rice straw was mixed with fresh acid solution as shown in Table 5, C5 Liquid 0 at ratio of 0.36g liquid/g deashed rice straw, and soluble sugar solution at a ratio of 0.9 g liquid/g deashed rice straw. Mixing was performed by hand in a beaker. The mixture was then loaded into a reactor for pretreatment using the same procedure as described above for Cycle #0. After pretreatment, the reactor was unloaded and “Slurry 1” was collected. Thirty grams of Slurry 1 was saved for enzymatic hydrolysis, and the remaining Slurry 1 was pressed to collect hydrolysate (“C5 Liquid 1”). The C5 Liquid 1 hydrolysate was filtered through 0.7 μ m filter paper to remove insoluble solids. This completed Cycle #1. Cycles #2 and #3 were performed in the same manner as Cycle #1, and as shown schematically in Fig. 8.

[142] For 15% carry-over sugar, the deashed rice straw was mixed with fresh acid solution as shown in Table 5, C5 Liquid 0 at a ratio of 0.54 g liquid/g deashed rice straw, and soluble sugar solution at a ratio of 1.35 g liquid/g deashed rice straw. Cycles #1, 2, and 3 were performed as described above for 10% carry-over sugar.

[143] For 20% carry-over sugar, the deashed rice straw was mixed with fresh acid solution as shown in Table 5, C5 Liquid 0 at a ratio of 0.72 g liquid/g deashed rice straw, and soluble sugar solution at a ratio of 1.8 g liquid/g deashed rice straw. Cycles #1, 2, and 3 were performed as described above for 10% carry-over sugar.

Table 5

% Carry-over sugar	Cycle	Acid loading (g/g washed biomass)	pH before pretreatment	pH after pretreatment	CSF
10%	1	2 %	1.47	1.67	1.20
	2		1.46	1.68	1.19
	3		1.48	1.68	1.19
15%	1	2.05%	1.46	1.68	1.19
	2		1.48	1.70	1.17
	3		1.48	1.69	1.18
20%	1	2.1%	1.45	1.69	1.18
	2		1.48	1.70	1.17
	3		1.50	1.70	1.17
Control _deashed	/	1.75%	1.51	1.73	1.14
Control _water washed	/	2.5%	1.46	1.63	1.23

Enzymatic hydrolysis

[144] Solids from all pretreatment cycles were subjected to enzymatic hydrolysis to compare net sugar production. The following conditions were applied in enzymatic hydrolysis: 12.5% w/w total solid loading, 30mg commercially available cellulytic enzyme/g total solid, operated at manufacturer recommended temperature, agitated for 48 hrs.

Fermentation

[145] A butanol-producing *Clostridium* strain was used in a fermentation test. 15mL seed media (60 g/l sugar from molasses, and other growth nutrients) was loaded into a 50 mL conical vial in an anaerobic chamber. The medium was de-oxygenated for at least 24hr.

[146] 2mL glycerol stock of the *Clostridium* strain was thawed in the anaerobic chamber. 0.25mL glycerol stock was inoculated into 20mL seed media. The solution was swirled gently and allowed to incubate in the anaerobic chamber overnight. When culture was at appropriate growth conditions, the seed culture was used for inoculation.

[147] Enzymatic hydrolysate, prepared as described above, was subjected to solid-liquid separation by centrifugation. Liquid was then filtered through 0.2 μ m filter in a laminar hood for sterilization. Hydrolysate growth media was prepared under laminar hood to contain 55 g/L total sugar, with 50 g/L from hydrolysate and 5 g/L from molasses, and other growth nutrients.

[148] 4mL hydrolysate growth media was pipetted into 15 mL conical vials and de-oxygenated in an anaerobic chamber for at least 24 hours. Duplicates were prepared.

[149] 0.44 mL seed media was added into 4 mL hydrolysate media and fermented for 48 hours anaerobically at the appropriate temperature conditions for this butanol producing strain. 1.5mL samples were taken from cultures and filtered through 0.2 μ m membrane. The liquid was collected for HPLC analysis. Results are shown in Fig. 9.

[150] Sugar recovery, enzymatic hydrolysis, and fermentation performance were not adversely affected by virtue of the biomass pretreatment process exemplified in this example.

[151] Although the foregoing invention has been described in some detail by way of illustration and examples for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced without departing from the spirit and scope of the invention. Therefore, the description should not be construed as limiting the scope of the invention.

[152] All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entireties for all purposes and to the same extent as if each individual publication, patent, or patent application were specifically and individually indicated to be so incorporated by reference.

CLAIMS

We claim:

1. A method for reacting and/or removing non-carbohydrate compounds from biomass, comprising:

(a) washing a second biomass with a first acid hydrolysate that is produced by acidic hydrolysis of a first biomass, thereby producing acid hydrolysate washed second biomass; and

(b) separating the acid hydrolysate washed second biomass from at least a portion of the first acid hydrolysate that was used for washing the second biomass.

2. A method according to claim 1, wherein at least a portion of at least one non-carbohydrate compound is removed from the second biomass, and wherein the first acid hydrolysate that is separated from acid hydrolysate washed second biomass in (b) comprises said at least one non-carbohydrate compound.

3. A method according to claim 1, further comprising:

(c) contacting the acid hydrolysate washed second biomass with acid and treating under conditions sufficient to depolymerize at least one polymeric carbohydrate component of the second biomass, thereby producing: (i) a second acid hydrolysate that comprises soluble sugar molecules; and (ii) residual solids,

wherein less acid is required for said depolymerization from acid hydrolysate washed second biomass than from biomass that has not been prewashed as in (a), when treated with acid under identical conditions.

4. A method according to claim 3, further comprising:

(d) separating the second acid hydrolysate produced in (c) from the residual solids produced in (c); and

(e) using at least a portion of the second acid hydrolysate separated in (d) to wash a third biomass.

5. A method according to claim 4, wherein acid hydrolysis of biomass, separation of acid hydrolysate from residual solids, and use of the acid hydrolysate for washing of

additional unhydrolyzed biomass are conducted in a continuous process.

6. A method according to claim 1, wherein less base is required to raise the pH of the first acid hydrolysate to a level that is suitable for enzymatic hydrolysis and/or microbial fermentation than a hydrolysate produced under identical conditions but that has not been used for prewashing biomass as in (a).

7. A method according to claim 3, wherein the acid in the first acid hydrolysate used for washing second biomass in (a) and the acid used for production of second acid hydrolysate in (c) comprises at least one acid selected from nitric acid, sulfuric acid, sulfurous acid, SO₂, hydrochloric acid, phosphoric acid, formic acid, and acetic acid.

8. A method according to claim 2, wherein said at least one non-carbohydrate compound that is removed from the second biomass comprises an inorganic salt, a mineral oxide, and/or an organic acid.

9. A method according to claim 2, wherein at least one non-carbohydrate compound that is removed from the second biomass is capable of buffering and/or neutralizing acid.

10. A method according to claim 3, wherein said second biomass comprises lignocellulosic biomass.

11. A method according to claim 10, wherein said at least one polymeric component that is depolymerized in (c) comprises hemicellulose.

12. A method according to claim 11, wherein said at least one polymeric component further comprises cellulose.

13. A method according to claim 10, wherein said second biomass comprises rice straw, rice husks, wheat straw, barley straw, corn stover, switchgrass, sugar cane bagasse, sugar cane trash, palm empty fruit bunches, and/or Kenaf.

14. A method according to claim 10, wherein said lignocellulosic biomass is a high silica lignocellulosic biomass.

15. A method according to claim 14, wherein said high silica lignocellulosic biomass comprises rice straw, wheat straw, rice husks, and/or corn stover.
16. A method according to claim 14, wherein at least a portion of the silica is removed by alkali extraction prior to step (a),
wherein acid in the first acid hydrolysate neutralizes at least a portion of residual alkali in the second biomass, and
wherein the first acid hydrolysate that is separated from acid hydrolysate washed second biomass in (b) comprises the neutralization product of acid and residual alkali.
17. A method according to claim 4, wherein said residual solids separated in (d) are subjected to a further hydrolysis process.
18. A method according to claim 17, wherein said further hydrolysis comprises acid hydrolysis, enzymatic hydrolysis, or hydrolysis with a supercritical fluid.
19. A method according to claim 18, wherein the residual solids are hydrolyzed with one or more enzyme(s) to produce additional soluble sugar molecules in an enzymatic hydrolysate.
20. A method according to claim 19, wherein said enzyme(s) comprise at least one cellulase.
21. A method according to claim 4, wherein said residual solids separated in (d) are subjected to one or more process steps selected from catalytic processing, preparation for other purposes such as paper production, or use as a fuel.
22. A method according to claim 3, wherein soluble sugar molecules in the first acid hydrolysate that is separated in (b) and/or the second acid hydrolysate that is produced in (c) are fermented by a microorganism to produce a bioproduct of interest.
23. A method according to claim 22, wherein the first acid hydrolysate that is separated in (b) and/or the second acid hydrolysate that is produced in (c) is conditioned to remove at

least a portion of at least one substance that inhibits microbial growth and/or bioproduct production, prior to fermentation.

24. A method according to claim 23, wherein conditioning comprises at least one process selected from evaporation, steam stripping, charcoal adsorption, electrodialysis, and reverse osmosis.

25. A method according to claim 22, wherein said bioproduct comprises a solvent.

26. A method according to claim 19, wherein soluble sugar molecules in the enzymatic hydrolysate are fermented by a microorganism to produce a bioproduct of interest.

27. A method according to claim 26, wherein said bioproduct comprises a solvent.

28. A method according to claim 1, wherein the second biomass comprises non-structural sugar molecules, and wherein at least a portion of the non-structural sugar molecules are washed into the first acid hydrolysate.

29. A method according to claim 28, wherein the first acid hydrolysate that is separated in (b) comprises about 50% to about 95% of the non-structural sugar molecules from the second biomass.

30. A method according to claim 3,
wherein the second biomass comprises non-structural sugar molecules,
wherein a portion of the non-structural sugar molecules are washed into the first acid hydrolysate,
wherein the acid hydrolysate washed second biomass in (c) comprises a portion of the non-structural sugar molecules,
and wherein the second acid hydrolysate comprises the non-structural sugar molecules from the second biomass.

FIGURE 1

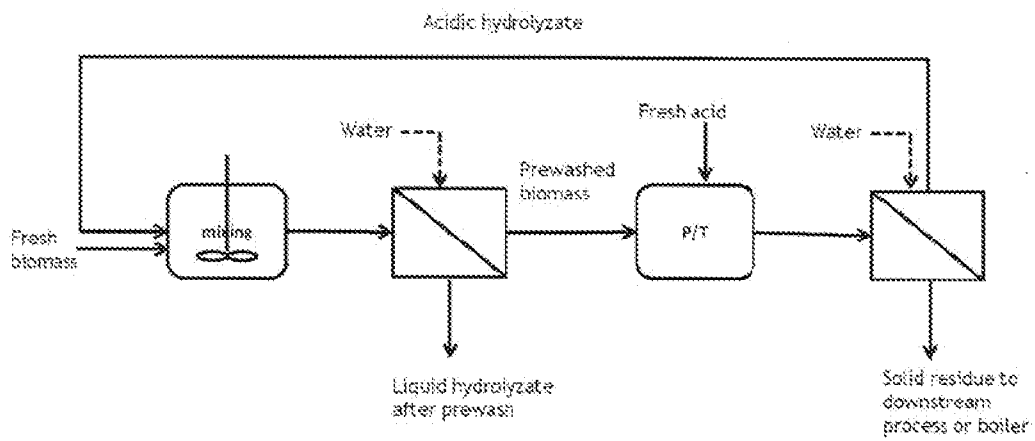


FIGURE 2

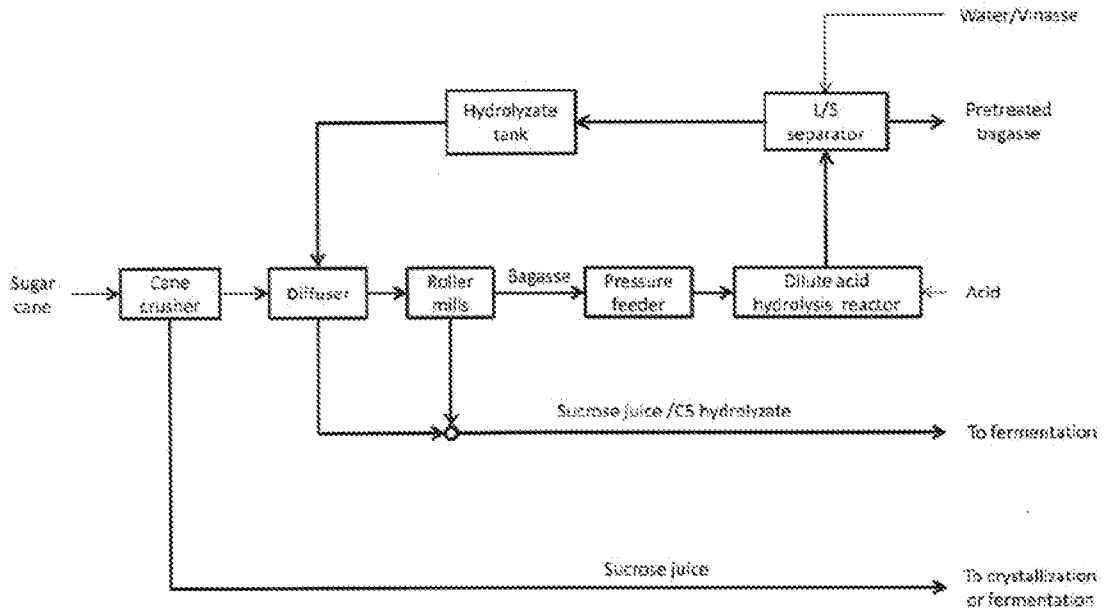


FIGURE 3

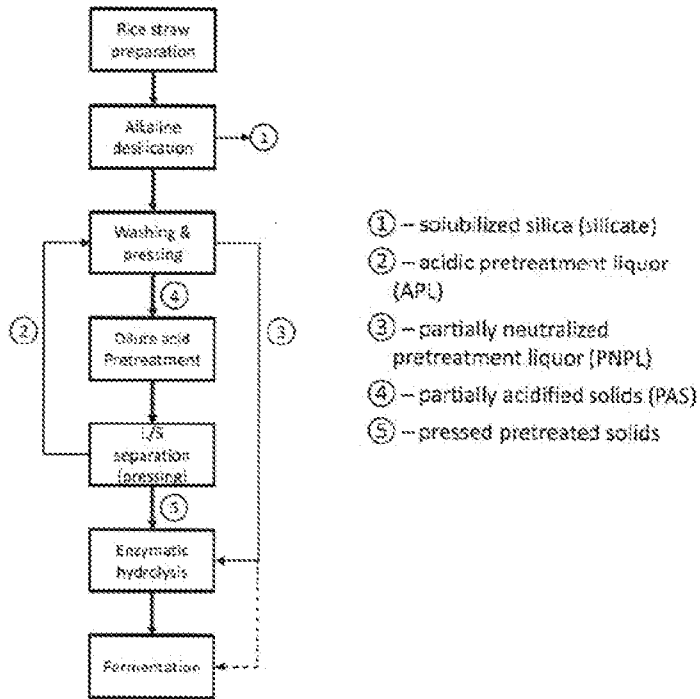


FIGURE 4

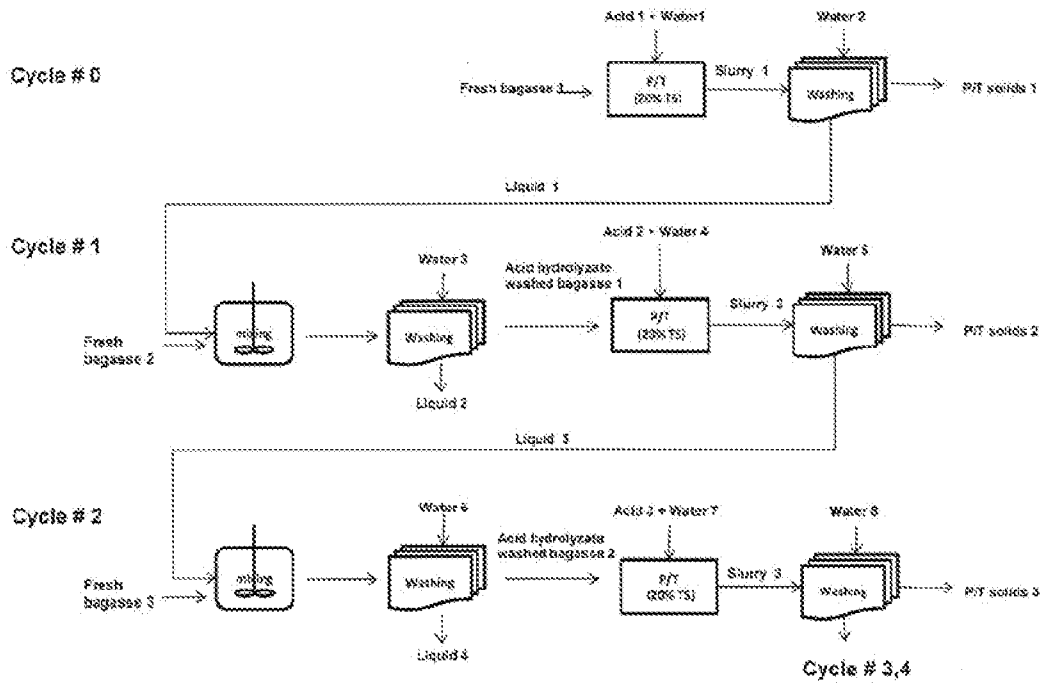


FIGURE 5

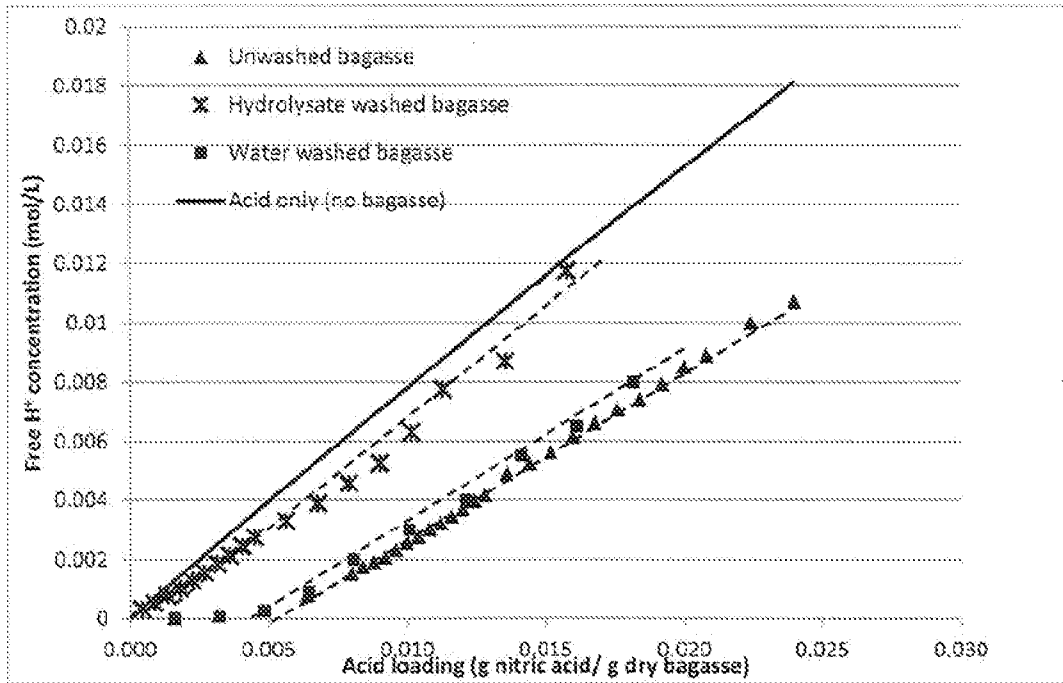


FIGURE 6

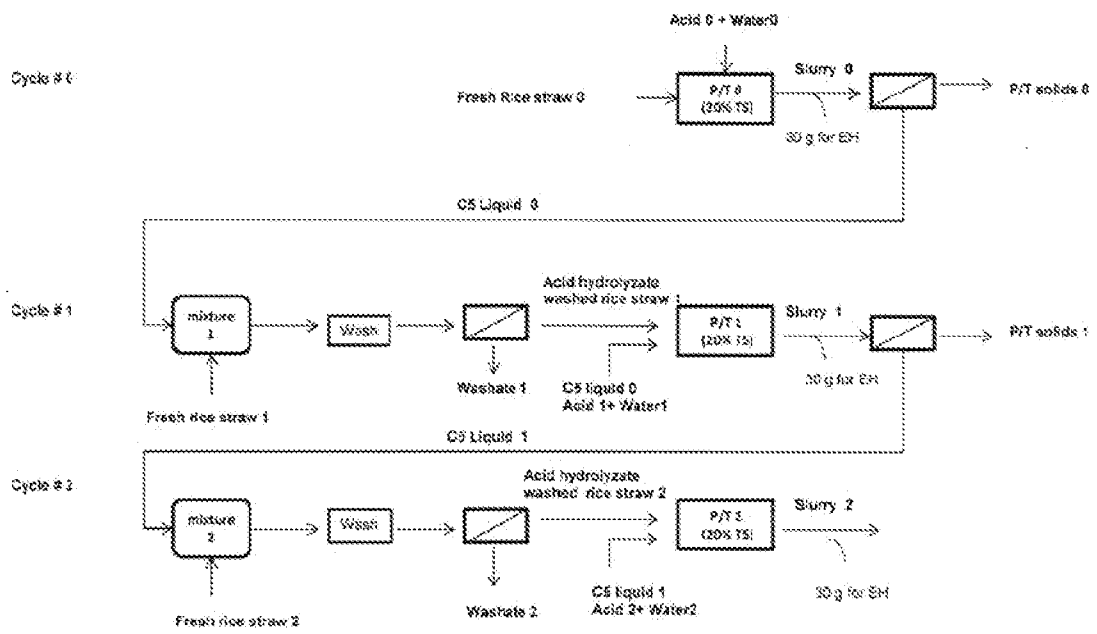


FIGURE 7

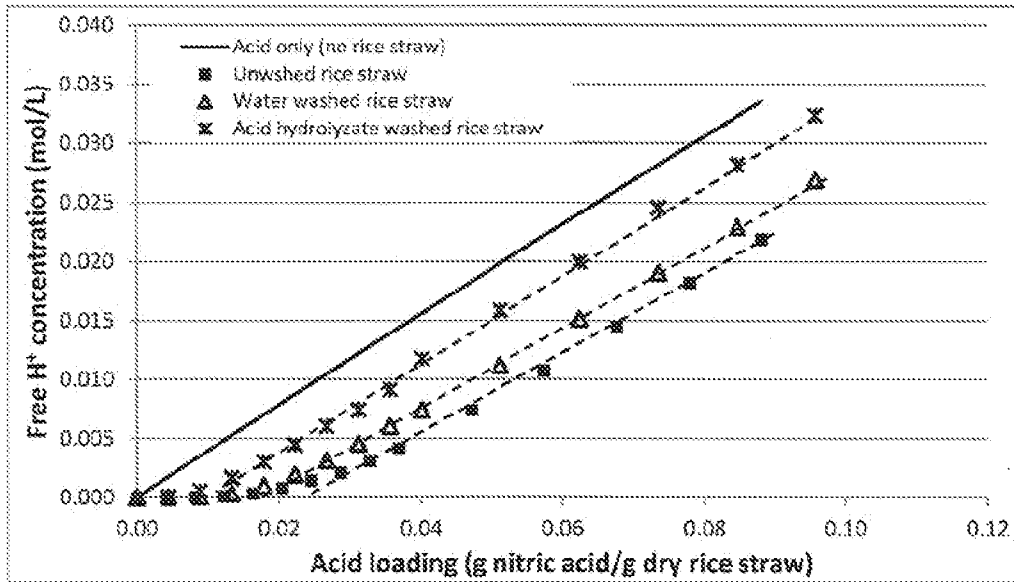


FIGURE 8

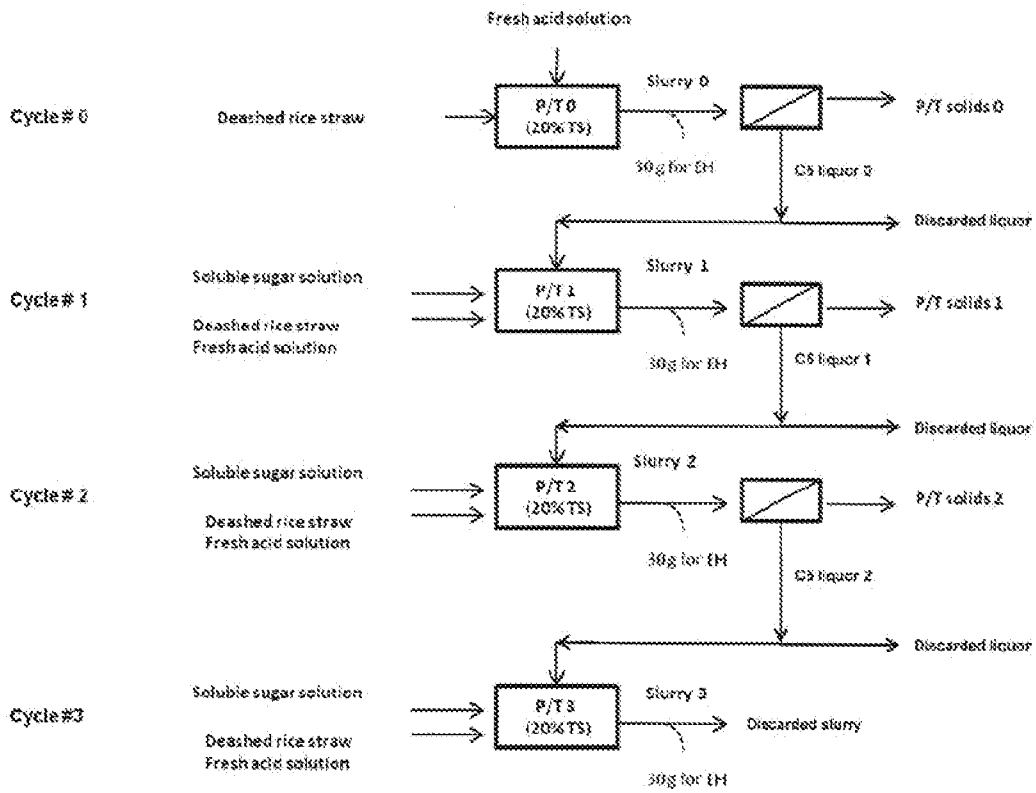
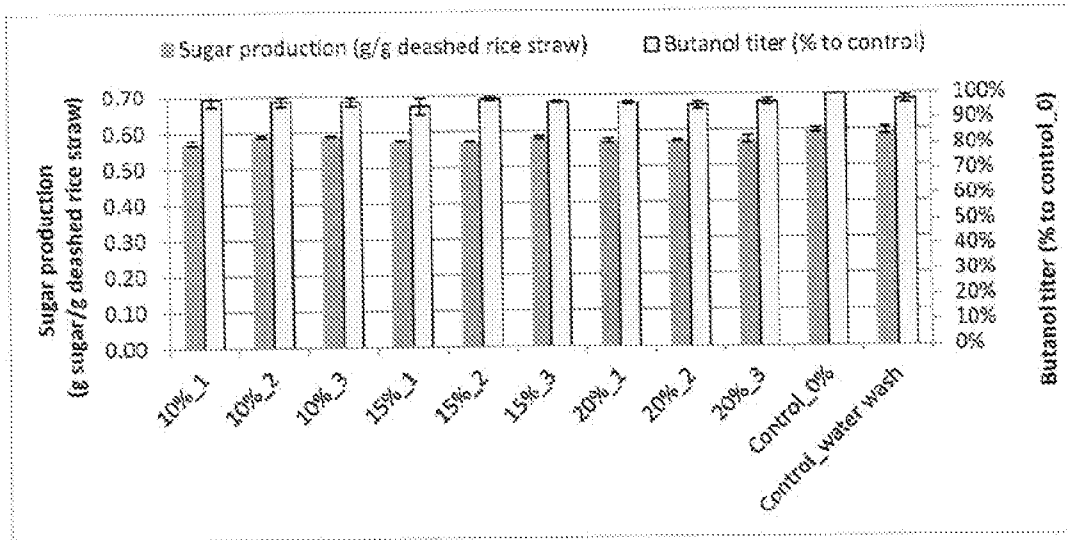


FIGURE 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/72278

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12P 7/06, 7/14, 19/00 (2015.01)

CPC - C12P 7/06, 7/14, 19/00

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)

IPC(8): C12P 7/06, 7/14, 19/00; D21C 1/04, 1/06 (2015.01)

CPC: C12P 7/06, 7/14, 19/00; D21C 1/04, 1/06; USPC: 162/14; 426/60; 435/99, 161, 162

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer; Google; Google Scholar; IP.com; PubMed; Cobalt Technologies, INC., Zhu, Walther, Gao, pre-wash, pre-treatment, pre-soak, remove, non-carbohydrate, biomass, wash, acid, hydrolysate, separate, depolymerize, residual solids, nitric acid, sulfuric acid, sulfurous acid, SO₂, hydrochloric acid, phosphoric acid, formic acid, acetic acid, hydrolysis, enzymatic hydrolysis, supercritical fluid, inorganic salt

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	WO 2012/162443 A2 (GEOSYNFUELS, LLC); November 29, 2012; abstract; paragraphs [0006]-[0007], [0024], [0026]-[0029], [0036], [0040], [0057], [0062]-[0065], [0067]; claim 1; figures 2-4	1-5, 7-14, 16-19, 21-30 --- 15, 20 --- 6
Y	GRAHAM, RL et al. Current and Potential U.S. Corn Stover Supplies. Agronomy Journal, 2007, Vol. 99, pp. 1-11 [online], [retrieved on 2015-03-03]. Retrieved from the Internet <URL: http://web.ornl.gov/info/ornlreview/v40_1_07/graham_2007_agronomy_journal.pdf >; page 1, column 2, paragraph 1	15
Y	GALAZKA, JM et al. Cellodextrin Transport in Yeast for Improved Biofuel Production. Science, October 1, 2010, Vol. 330, pp. 84-86 [online], [retrieved on 2015-03-03]. Retrieved from the Internet <URL: http://124.16.173.210/bitstream/0/125/2/Cellodextrin%20Transport%20in%20Yeast%20for%20Improved%20Biofuel%20Production.pdf >; page 84, column 1, paragraph 1; page 84, column 2, paragraph 1	20
A	US 7604967 B2 (YANG, B et al) October 20, 2009; claim 10	6
A	US 2014/0045226 A1 (DAKOTA STAR CAPITAL, LLC) February 13, 2014; figure 8	6

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

4 March 2015 (04.03.2015)

Date of mailing of the international search report

24 MAR 2015

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