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(54) Title: IMPROVED METHODS OF TREATING A BIOMASS FOR ENZYMATIC HYDROLYSIS

(57) Abstract: The present invention is a process for treating a feedstock comprising holocellulose. The process comprises mixing the feedstock with a solution comprising cellulose binding domains to form a mixture. The mixture is then subjected to conditions sufficient to reduce the crystallinity of holocellulose. Subsequent enzymatic hydrolysis may show an improved rate and/or fermentable sugar yield as compared to processes which do not employ the process.



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IMPROVED METHODS OF TREATING A BIOMASS FOR ENZYMATIC HYDROLYSIS

Cross-Reference to Related Applications

[0001] For purposes of United States patent practice, the contents of U.S. Provisional Application No. 61/266,618 filed December 4, 2009 is herein incorporated by reference in its entirety to the extent that it is not inconsistent.

Field of the Invention

[0002] The present invention pertains to improved methods for treating a feedstock comprising holocellulose using, for example, a solution comprising cellulose binding domains prior to enzymatic hydrolysis.

Background and Summary of the Invention

[0003] The enzymatic hydrolysis of cellulose to glucose has gained increased interest over the last ten years, and growing demand for economically sustainable biofuels points to an urgent need for reducing costs in their production. Cellulose, a polysaccharide made by many plants, is one of the most abundant organic compounds on Earth and therefore represents a potential goldmine for the biofuel industry. However, current enzymatic degradation of cellulose faces major issues that prevent its wide utilization to produce, for example, economically competitive biofuel. Such issues may include, for example, low reaction and/or conversion rates.

[0004] Advantageously, the inventors have discovered a process and composition which accomplishes the aforementioned goals and more. In general terms the instant invention pertains in one embodiment to a process for treating a feedstock comprising holocellulose. The process comprises mixing the feedstock with a solution comprising cellulose binding domains to form a mixture. The mixture is then subjected to conditions sufficient to reduce the crystallinity of holocellulose. Subsequent enzymatic hydrolysis may show an improved rate and/or fermentable sugar yield as compared to processes which do not employ the CBD treatment process.

Brief Description of the Figures

[0005] Fig. 1 illustrates an SDS-PAGE gel of purified cel7A from *Trichoderma reesei* and cel7A catalytic domain after papain cleavage.

[0006] Fig. 2 illustrates the enzymatic hydrolysis of Avicel after CBD treatment.

[0007] Fig. 3 illustrates the effect of various CBD incubation times.

[0008] Fig. 4 shows the effect of buffer incubation on hydrolysis rate compared to CBD incubation (13 h).

[0009] Fig. 5 shows the effect of buffer incubation time on hydrolysis rates.

[0010] Fig. 6 shows the results of a CBD concentration optimization experiment.

[0011] Fig. 7 shows hydrolysis for various CBD treatment times.

[0012] Fig. 8 shows the effect of various concentrations of CBD.

[0013] Fig. 9 shows the effect of sequential addition and CBD removal.

[0014] Fig. 10 shows the effect of a 45°C treatment.

[0015] Fig. 11 shows the effect of a 50°C treatment.

[0016] Fig. 12 shows the amount of reducing ends produced from switchgrass using cellulases and β -glucosidase after treatment with CBD, treatment with buffer, no treatment with simultaneous addition of CBD, and no treatment (dry sample).

[0017] Fig. 13 shows the SDS-PAGE before and after papain cleavage of cellulases.

[0018] Fig. 14 shows the XRD spectra for Avicel samples treated with CBD_{Cel7A} (cellulose-binding domain from purified Cel7A), CD_{Cel7A} (catalytic domain) or buffer.

[0019] Fig. 15a shows the hydrolysis profile of Avicel after pretreatment with mixture of CBDs for 15 h at 42°C.

[0020] Fig. 15b shows the hydrolysis profile of fibrous cellulose (FC) after pretreatment with mixture of CBDs for 15 h at 42°C.

[0021] Fig. 16 shows the hydrolysis profile of Avicel after pretreatment with CBD_{Cel7A} for 15 h at 42°C.

[0022] Fig. 17 shows effect of various CBD concentrations in the pretreatment step (15 h at 42°C) on hydrolysis rate.

[0023] Fig. 18 shows the effect of stir bar during pretreatment of cellulose with CBDs on hydrolysis rates.

[0024] Fig. 19 shows amino acid sequences of various CBDs that may be useful in the present inventions.

Detailed Description of the Invention

[0025] In general terms the instant invention pertains in one embodiment to a process for treating a feedstock comprising holocellulose. The process comprises mixing the feedstock with a solution comprising cellulose binding domains to form a mixture. The mixture is then subjected to conditions sufficient to reduce the crystallinity of holocellulose. Subsequent enzymatic hydrolysis may show an improved rate and/or fermentable sugar yield as compared to processes which do not employ the CBD treatment process prior to a subsequent hydrolysis. Advantageously, the fermentable sugar in the hydrolyzed composition and/or the hydrolysis

rate may be at least about 10%, or at least about 15%, or at least about 20%, more than a comparable process which does not employ cellulose binding domains. Following hydrolysis to produce one or more fermentable sugars such as glucose, processes such as fermentation may be employed to produce, for example, bioethanol.

Feedstock Comprising Holocellulose

[0026] The nature of the feedstock employed in the processes and compositions herein is not particularly critical so long as the feedstock comprises holocellulose. As used herein, "holocellulose" means at least a portion of the water-insoluble carbohydrate portion of a biomass, i.e., the portion of the biomass that is not lignin, extractives, or ash, but rather, includes substances such as polysaccharides. The precise composition of holocellulose may vary depending upon the specific feedstock employed. However, holocellulose useful herein typically comprises various amounts of celluloses such as alpha-cellulose and hemicellulose and may contain various pentosan or hexosan polymers. Thus, virtually any lignocellulosic biomass that comprises cellulose may be employed as the feedstock in the processes and compositions of the instant invention. The invention may be advantageously employed on compositions comprising cellulose in which the crystallinity of the cellulose is in need of reduction.

[0027] In one embodiment a particularly preferable feedstock is a plant biomass. Biomass comes in many different types, which may be grouped into a few main categories: wood or forestry residues, including sawmill and paper mill discards, municipal paper waste, algae, agricultural residues, including corn stover (stalks and straw), and sugarcane bagasse, and dedicated energy crops, which are mostly composed of fast growing tall, woody grasses such as, for example, switchgrass. Any of the aforementioned may find use in the instant invention. A particularly preferable biomass comprises one with high cellulose content.

[0028] Depending upon the nature of the feedstock it may be desirable to reduce at least a portion of it in size in order to expose additional surface area for treatment. Such reduction may be done in any convenient manner such as by grinding, cutting, chopping, etc. The desired size of the feedstock varies depending upon the type of ingredients and other specifics of the instant inventions. Typically, smaller size feedstocks may react quicker but cost more to produce. Generally, it is advantageous if the feedstock is reduced prior to CBD treatment and/or prior to hydrolysis.

[0029] Similarly, it is often advantageous, or may even be necessary, in some situations to first purify at least a portion of the feedstock. By purify is meant to partially clean in order to remove at least some contaminants that may negatively effect downstream processes. This purification may assist in reducing or eliminating any undesired reactions in the following

steps. The type of purification will depend upon the source of the feedstock, as well as, the amount and nature of the impurities and the following steps to which it will be subjected. Often, simple washing of the lignocellulosic feedstock is sufficient. Such purification, if done, may be accomplished prior to, in conjunction with, or subsequent to any size reduction. Additionally, if desired or advantageous, at least a portion or all of the feedstock that does not comprise holocellulose may be separated. However, this is unnecessary for many processes and conditions to which the composition will likely be subjected.

Cellulose Binding Domain (CBD)

[0030] The cellulose binding domains employed herein are typically derived from cellulases. Cellulases are glycosyl hydrolases of varying structure which catalyze the hydrolysis of 1,4- β -D-glycosidic bonds. Most cellulases comprise three domains: (1) a catalytic domain which primarily serves as the locus of hydrolysis, (2) a cellulose-binding domain that often assists in anchoring the whole enzyme onto the cellulose surface and/or in orienting the cellulose fiber towards the tunnel containing the active site, and (3) a glycosylated flexible linker connecting the two other domains and often providing sufficient spatial separation between them. The linker may also assist in allowing processive motion and/or assisting in energy storage.

[0031] As used herein the term “cellulose binding domain” or “CBD” is a portion of cellulase that includes a substantial portion of the aforementioned (2) but lacks a substantial portion, the majority, or all of (1). Such cellulose binding domains in some embodiments include all or at least a portion of the linker (3) while in other embodiments the linker (3) is modified or absent from the cellulose binding domain employed. Thus, useful CBD for use herein may often be obtained from cellulase by cleaving CBD and, if desired, linker from the cellulase in a convenient method such as, for example, proteolytic cleavage. Depending upon the cleaving method, the CBD may then be recovered in a convenient manner such as, for example, filtration. In some embodiments, the ratio of cellulose binding domains to catalytic domain is greater than 1:1, or greater than 2:1, or greater than 3:1, or greater than 5:1, or greater than 10:1.

[0032] The nature and type of cellulose binding domain employed is not particularly critical so long as they function in some manner to reduce the crystallinity of cellulose and/or facilitate any further processing such as hydrolysis. Suitable CBSs may be derived from a suitable enzyme produced by a species like, for example, a *Trichoderma* species such as *T. reesei*, *T. viride*, as well as, for example, *Phanerochaete chrysosporium*, *Humicola insolens*, *Coprinopsis cinerea*, *Acremonium cellulolyticus*, *Hypocrea jecorina*, *Penicillium occitanis*, *Irpex lacteus*, *Penicillium echinulatum*, *Lentinula edodes*, *Penicillium decumbens*.

[0033] In one embodiment the cellulose binding domains are derived from cellobiohydrolase I (cel7A) such as produced by, for example, a *Trichoderma* species like *Trichoderma reesei*. In another embodiment the cellulose binding domains are derived from cellobiohydrolase II (cel6A). In other embodiment the cellulose binding domains are derived, for example, from an enzyme in Table 5 below. In another embodiment the cellulose binding domains employed may be a mixture of two or more of the aforementioned. The CBD

[0034] Cellulose-binding domains (CBDs) of cellulases from *Trichoderma reesei* (belonging to family I CBD) form a wedge-like fold, where a flat face provides key (aromatic) residues strongly interacting with crystalline cellulose. Such methods as those described in the below examples may be employed. In addition, with the benefit of the instant disclosure, one of skill may employ routine testing of other substances to identify useful materials. Alternatively or additionally, protein engineering may be conducted on CBD. Mutations of certain residues on CBD (such as those involved in binding or interacting with cellulose and/or any that may effect a faster hydrolysis rate) may then be exploited to develop CBD tailored to, for example, specific feedstocks and/or process conditions.

Mixing the feedstock with a solution comprising cellulose binding domains

[0035] The solution comprising cellulose binding domains may be made and mixed with the feedstock in any convenient manner. The concentration of the solution comprising cellulose binding domains varies depending upon the particular feedstock, other ingredients of the solution such as buffer, and the conditions to be employed. Generally, it should have a concentration at least sufficient to reduce the crystallinity of the cellulose at the treatment conditions but not so high that it interferes with any subsequent desired processing steps such as hydrolysis. Generally, a concentration of at least about 5, or at least about 10, or at least about 15 up to at most about 100, or at most about 75, or at most about 55, or at most about 45, or at most about 35 $\mu\text{g/ml}$ may be effective. A suitable buffer may also be employed in the solution if desired. It may be advantageous to employ a buffer which acts synergistically with the CBD to even further facilitate reducing the crystallinity of the cellulose. Suitable buffers often include salts or esters of lower alkyl acids such as acetic acid and typically have a pH of from about 4.5 to about 5.5. In one embodiment, the solution comprises a sodium acetate buffer at a concentration of from about 25 to about 75 mM.

CBD Treatment Conditions

[0036] Once a mixture comprising one or more feedstocks with a solution comprising cellulose binding domains is made, then the mixture is subjected to conditions sufficient to reduce the crystallinity of holocellulose and/or facilitate a subsequent hydrolysis. These conditions may

vary widely depending upon the nature and type of feedstock and the type and amount of ingredients in the solution comprising cellulose binding domains.

[0037] Typically, the mixture is subjected to an increased temperature for a time sufficient to reduce the crystallinity of holocellulose. Such temperatures vary but may be at least about 30°C, or at least about 35°C up to at most about 60°C, or at most about 50°C, or at most about 45°C. Generally, the higher the temperature employed, the shorter the time that the mixture must be heated and vice versa. At the previously described temperatures, suitable times to which to subject the mixture to increased temperature at least about 8 hours, or at least about 10 hours, or at least about 12 hours, or at least about 14 hours, up to at most about 48 hours, or at most about 24 hours, or at most about 16 hours. Using the present disclosure, one of skill in the art may use routine experimentation to determine proper times and temperatures for a particular feedstock and composition.

[0038] Without wishing to be limited to any particular theory, it is believed that the cellulose binding domains and similar proteins reduces the degree of crystallinity, and therefore renders the cellulose less recalcitrant to enzymatic attack. The aforementioned processes of the instant invention may be used alone or in conjunction with other techniques to decrease the crystallinity to a desired level. Such techniques may be used before, during, or after the processes of the instant invention and include, for example, (thermo)chemical and/or mechanical techniques (e.g. treatment with phosphoric acid, ionic liquids or organic solvents, AFEX, and ball mill grinding). In some instances purification may be beneficial before the hydrolysis step (e.g. with ionic liquids). In some instances it may be advantageous to subject the mixture to sufficient stirring such that substantially all solids are suspended. For example, in some instances the mixture is subjected to stirring, preferably vigorous, in a vial with a stir bar. In some embodiments the stir bar has a diameter of at least about 45%, preferably at least about 50%, preferably at least about 55%, of the inner vial diameter.

[0039] In another embodiment, the instant invention pertains to the use of a solution or composition comprising cellulose binding domains in a pre-treatment step in, for example, a cellulose hydrolysis process. The solution or composition comprising cellulose binding domains is as described above and below. Similarly, the pre-treatment step is as described, i.e., first mixing a holocellulosic feedstock with the solution or composition comprising cellulose binding domains to form a mixture and then subjecting the mixture to conditions sufficient to reduce the crystallinity of holocellulose. The specific ingredients, conditions, etc. may be as described elsewhere herein.

EXAMPLES 1-8

General Procedure for CBD treatment of Avicel

[0040] Cellobiohydrolase I (cel7A) is a component of a cellulase mixture which is an exoglucanase which attacks cellulose chains from the reducing-end side. The following inventive examples employ the cellulose-binding domains (CBD) from this enzyme, and more specifically cel7A from *Trichoderma reesei*, a commonly used organism in the production of, for example, bioethanol. The effect of CBD on cellulose was investigated, i.e. the consequence of cellulose incubation, i.e., treatment, in a solution comprising CBD on the degree of crystallinity, and subsequently on the enzymatic hydrolysis rate of the reaction performed with the CBD-treated cellulose.

[0041] Cel7A is constituted of three domains: a cellulose-binding domain (CBD), a catalytic domain (CD) and a highly glycosylated linker that joins the CBD to the CD. The examples involve:

- (1) producing CBD from cel7A, *via* proteolytic cleavage of that fragment (plus linker) from the CD using papain (a cysteine protease);
- (2) recovering CBD *via* filtration through a 30 kDa membrane (CBD + linker ~ 61 residues; theoretical size ~ 6 kDa; estimated size from experiments ~ 14 kDa due to *O*-glycosylation of the linker; estimated size of CD ~ 50 kDa due to *N*-glycosylation);
- (3) incubating, i.e., treating, cellulose (Avicel) with a solution comprising CBD; and
- (4) adding cellulase cocktail (from *Trichoderma reesei*) and monitoring hydrolysis rate (*via* glucose content).

General Procedure for CBD production

[0042] Following the procedure on CBD cleavage from the core protein as used in, for example, P. Tomme *et al.*, *Eur. J. Biochem.*, **1988**, 170, 575-581, papain was employed to perform the proteolytic digestion under the following conditions:

1. Activation of papain in 50 mM NH₄OAc buffer pH 6.0 at 30 °C for 30 min;
2. Addition of papain to cel7A solution (in 50 mM NaOAc buffer, pH 5.0) at 30 °C, ratio papain/cel7A 1:5 (w/w, v/v);
3. Stirring for 2 h at 30 °C; and
4. Filtration of the reaction mixture through 30 kDa membrane (4000 rpm for 15 min) to collect the cellulose binding domain (attached to the linker) in the filtrate.

[0043] The cleavage reaction was found to be complete after 2 h, and no remaining cel7A (~ 64 kDa) could be detected by SDS-PAGE as shown in Figure 1. The reaction led to the formation of a single strong band, with a molecular weight of ~ 50 kDa. The papain successfully cleaved off CBD + linker from the core protein (CD); however, this small protein fragment (~ 14 kDa) was not detected by SDS-PAGE (the sequence shows very few residues that can react with commonly used Coomassie dye-staining). However, CBD was detected *via*

micro BCA protein assay (bicinchoninic acid) in the filtrate (< 30 kDa) (Table 1, 100% recovery).

[0044]

Table 1. Cel7A fragments recovery after papain cleavage

Protein fragments	Total mass (mg)
Cel7A (+ papain) before reaction	4.6 mg
CD (+ papain) concentrate after filtration through membrane > 30 kDa	3.8 mg
CBD filtrate after filtration through membrane < 30 kDa	0.6 mg
Expected CBD (~ 10-14 kDa from 64 kDa)	0.6-0.9 mg

Example 1A – Avicel treatment with a solution comprising cellulose binding domain (CBD) and measurement of crystallinity via X-ray diffraction

[0045] While not wishing to be bound to any particular theory it is believed that the activity of CBD relates to non-hydrolytic disruption of cellulose and a weakening and splitting of the hydrogen-bonding network in cellulose. The following investigation determined whether that activity was strong enough to reduce cellulose crystallinity.

[0046] Avicel was treated with CBD solution (obtained as described above), and the degree of crystallinity of the recovered cellulose was subsequently determined *via* X-ray diffraction using our recently developed analytical method described in, for example, Bansal *et al.*, Multivariate statistical analysis of X-ray data from cellulose: A new method to determine degree of crystallinity and predict hydrolysis rates. *Bioresource Technol.*, 101, 4461-4471.

[0047] The cellulose treatment step was performed as follows: Avicel (20 mg/ml) in CBD solution (50 mM NaOAc buffer, pH 5) was stirred at 37 °C for 2 days. The mixture was centrifuged at 4000 rpm for 15 min and recovered cellulose was freeze-dried. The powder was analyzed by the X-ray diffraction method referred to above.

[0048] Avicel is a microcrystalline cellulose with an average degree of crystallinity of about 60%. Upon treatment with the CBD solution, a significant decrease in Avicel crystallinity was observed as shown in Table 2 below. The highest decrease of 13% was obtained with sample 4 which corresponded to 4.7 ml of filtrate obtained from the cleavage of 3.3 mg cel7A in 7 ml buffer.

[0049] Table 2. Degree of crystallinity (CrI) of Avicel after incubation (2 days) with cellulose-binding domain (CBD) or catalytic domain (CD)

CrI (%) [*]	CBD	CD	No treatment prior to hydrolysis
1	57.2	59.6	
2	57.4	60.3	
3	56	61	60%
4	52	62	

* samples 1-4 refer to different experiments. 1 and 2 were run at low CBD concentrations, 3-4 at higher CBD concentration

Example 1B – Enzymatic hydrolysis of CBD treated samples of Example 1A

[0050] The cellulose samples (3 and 4, Table 2) that presented a significant lower crystallinity value compared to untreated Avicel were submitted to enzymatic hydrolysis using the cellulase cocktail from *Trichoderma reesei* (supplemented with β -glucosidase to prevent product inhibition). A significant increase in rate was observed for the samples treated with CBD solution prior to hydrolysis, with an overall rate 2-fold higher than that of untreated Avicel over the course of the reaction as shown in Figure 2. Thus, the decrease in crystallinity observed after treating Avicel with CBD solution correlated well with a decreased recalcitrance to enzymatic attack, and therefore a faster conversion to glucose.

General Procedure to Measure Effect of Various CBD Treatment Conditions

[0051] The following shows the effect of various CBD treatment conditions on hydrolysis rates. As crystallinity was shown to decrease, no additional crystallinity measurement was conducted and therefore cellulases were directly added to the reaction mixture after CBD treatment time was over, i.e. the samples were not freeze-dried before investigation of hydrolysis rates, nor was the buffer exchanged or the solution diluted. The sequential CBD treatment and hydrolysis process was performed as followed:

1. Incubating Avicel with CBD solution over various time lengths at 37 °C;
2. Changing temperature to 50 °C;
3. Adding cellulase cocktails and β -glucosidase and stirring at 50 °C; and
4. Withdrawing reaction mixture aliquots for glucose quantification at various time intervals.

Example 2 – Effect of incubation time, CBD concentration, and buffer on hydrolysis rate

[0052] To find more optimum CBD treatment times that would lead to a higher increase in hydrolysis rate, the 48 h incubation time that led to the 13% decrease in crystallinity (see above) was varied from 8 h to 24 h and the enzymatic hydrolysis rates were examined. One single CBD solution was used throughout these experiments, so that they were performed at constant CBD concentration ($\sim 42 \mu\text{g/ml}$). Positive effects of CBD were obtained between 8 h and 13 h of incubation at 37°C . Beyond 13 h, no further increase in rates was observed as shown in Figure 3. Depending on the incubation time, a maximum of 20-25 percentage points difference was observed in glucose content (corresponding to 50-60% increase in rate between the 8h and $\geq 13\text{h}$ CBD treatment times).

[0053] However, incubation in buffer was found to also have a positive effect on hydrolysis rate. Despite lower conversion compared to incubation with CBD solution, conversion on sample incubated in buffer for 13 h was higher than after 1 h incubation in buffer as shown in Figure 4. When conversion on CBD treated sample was up to two-fold higher than that on buffer-treated sample for 1 h (after 1 h hydrolysis), the difference was less significant between the CBD treated sample and the buffer treated sample for 13 h (1.3-1.4 fold higher).

[0054] The effect of buffer on hydrolysis rate was shown to be time-dependent, however, no major difference was observed between 13 h and 48 h of incubation in buffer, although an incubation for 24 h led to the highest rates as shown in Figure 5. Interestingly, water incubation (18 h) showed a positive effect on rate (higher conversion than with 1 h incubation in buffer), however, less pronounced than with similar incubation time in buffer. A pH shift is suspected, as water had a pH of 5.5, 0.5 units higher than the pH of the buffer (50 mM NaOAc pH 5).

[0055] Crystallinity measurements were then conducted on cellulose samples preincubated in buffer only and water only, respectively. No significant change from the untreated Avicel was observed after 24 h incubation at 37°C (apart from a slight increase in crystallinity up to 63%, which should not relate to an increase in rate in many cases).

[0056] Next, the CBD concentration was varied, starting from a reference volume (V_r added to the reaction mixture, corresponding to a final CBD concentration of $24 \mu\text{g/ml}$). Higher concentrations (up to $4 V_r$) and lower concentrations (down to $0.14 V_r$) were examined. Overall, the effect of CBD was less pronounced than in the previous examples as shown in Figure 6.

[0057] The highest rates were obtained with $0.28 V_r$, whereas both lower and higher concentrations ($0.14 V_r$ and $4 V_r$ resp.) led to lower conversion. At higher CBD conversion, where cellulases added to perform hydrolysis have less binding spots available on cellulose surface due to the presence of multiply-bound CBDs, competitive adsorption between CBD

and cellulases occurs. No major difference was found between 0.5 and 2 Vr. The lowest CBD concentration (0.14 vr) performed similarly to water, and the highest CBD concentration (4 Vr) performed similarly to the buffer.

CBD production for the CBD of Examples 3 – 8

[0058] The CBD employed in Examples 3-6 was made as follows: Cel7A was obtained from a mixture of cellulases from *Trichoderma reesei* and purified by means of anion exchange column chromatography using a HiPrep Q FF column. The purified protein was cleaved by proteolysis using papain and the cellulose binding domain (attached to the linker) was separated from the catalytic domain by centrifugation through a 30 kDa membrane and collected in the filtrate at a concentration of 125-175 µg/ml.

Example 3 – Effect of CBD Treatment Time

[0059] Avicel (20 mg/ml) was incubated in buffer (NaOAc 50 mM pH 5.0) together with CBD (24 µg/ml) at 37 °C and under gentle stirring in a 24 ml glass vial. The effect of treatment was investigated over various time periods (8 h, 13 h, 24 h and 48 h).

[0060] Hydrolysis of cellulose was performed on the CBD treated Avicel (20 mg/ml) by addition of cellulases (24 µl/ml, 3.4 mg/ml total protein, 3.8 FPU/ml) and β-glucosidase (15 U/ml) to the mixture and the reaction was carried out at 50 °C. Glucose production was monitored by the DNS assay at various intervals (1 h, 2.5 h and 4 h). Controls were run on dry Avicel (no CBD treatment) and on samples incubated in buffer only (for 1 h, 13 h and 48 h).

[0061] The incubation in buffer was found to be beneficial to the hydrolysis, as a higher glucose concentration was obtained already after 1 h incubation compared to hydrolysis on dry cellulose. The effects were more pronounced after a 13 h incubation, and no further increase was obtained up to 48 h. Incubation of Avicel in CBD gave a surprising and unexpected increase compared to the buffer alone (20-35%). The CBD treatment reached a maximum effect after 13 h incubation. The results are shown in Figure 7.

Example 4 – Effect of CBD concentration

[0062] Avicel (20 mg/ml) was incubated in buffer (NaOAc 50 mM pH 5.0) together with CBD at various concentrations (3.4, 6.9, 12, 24, 48 and 96 µg/ml) at 37 °C and under gentle stirring for 18 h in a 24 ml glass vial. Hydrolysis of cellulose was performed on CBD treated Avicel (20 mg/ml) by addition of cellulases (24 µl/ml, 3.4 mg/ml total protein, 3.8 FPU/ml) and β-glucosidase (15 U/ml) to the mixture and the reaction was carried out at 50 °C. Glucose production was monitored by the DNS assay at various intervals (1 h, 2.5 h and 4 h). The effect of CBD incubation on cellulose hydrolysis was found to be better at 7 µg/ml, up to 48 µg/ml, whereas higher concentrations gave a lower increase in glucose production, especially

at 96 µg/ml where the treatment was less efficient than in buffer only. The results are shown in Figure 8.

Example 5 – Effect of sequential addition

[0063] Avicel (30 mg/ml) was incubated in buffer (NaOAc 50 mM pH 5.0) at 37 °C and under gentle stirring for 18 h in a 24 ml glass vial, followed by the addition of a CBD solution in same buffer (48 µg/ml), corresponding to a final concentration of Avicel of 20mg/ml. The mixture was stirred for another 12 h at 37 °C.

[0064] Hydrolysis of cellulose was performed on CBD treated Avicel (20 mg/ml) by addition of cellulases (24 µl/ml, 3.4 mg/ml total protein, 3.8 FPU/ml) and β-glucosidase (15 U/ml) to the mixture and the reaction was carried out at 50 °C. Glucose production was monitored by the DNS assay at various intervals (1 h and 2.5 h).

[0065] The sequential addition of CBD after first incubating in buffer only led to higher conversion than simultaneous incubation in buffer and addition of CBD. This effect was more pronounced at higher degrees of conversion (about 35% higher glucose production compared to incubation in buffer only after 2.5 h hydrolysis). The results are shown in combination with the results of Example 6 at Figure 9.

Example 6 – Effect of CBD removal

[0066] Avicel (20 mg/ml) was incubated in buffer (NaOAc 50 mM pH 5.0) together with CBD at various concentrations (6.9 and 48 µg/ml) at 37 °C and under gentle stirring for 18 h in a 24 ml glass vial. The CBD adsorbed onto cellulose was removed by heating up the cellulose (recovered by centrifugation) in 1.1% SDS solution, followed by multiple washings with EtOH (75%) and water.

[0067] Hydrolysis of cellulose was performed on CBD treated and washed Avicel by addition of cellulases (24 µl/ml, 3.4 mg/ml total protein, 3.8 FPU/ml) and β-glucosidase (15 U/ml) to a solution of cellulose (20 mg/ml) in buffer (NaOAc 50 mM pH 5.0) and the reaction was carried out at 50 °C. Glucose production was monitored by the DNS assay at various intervals (1 h and 2.5 h).

[0068] The CBD removal procedure had a detrimental effect on cellulose hydrolysis, as the amount of glucose produced was significantly lower than that obtained after treatment with CBD with no removal, and slightly lower than that obtained after incubation in buffer only. The results are shown in combination with the results of Example 5 at Figure 9.

Example 7 – Effect of Incubation temperature

[0069] Avicel (20 mg/ml) was incubated in buffer (NaOAc 50 mM pH 5.0) together with CBD at various concentrations (24 and 48 µg/ml) and temperatures (45 and 50 °C) and under gentle stirring for 24 h in a 24 ml glass vial.

[0070] Hydrolysis of cellulose was performed on CBD treated Avicel (20 mg/ml) by addition of cellulases (24 µl/ml, 3.4 mg/ml total protein, 3.8 FPU/ml) and β-glucosidase (15 U/ml) to the mixture and the reaction was carried out at 50 °C. Glucose production was monitored by the DNS assay at various intervals (1 h, 2.5 h and 4 h).

[0071] Treatment with CBD at 45 °C led to higher glucose production compared to 37 °C, whereas 50 °C showed lower glucose production compared to 45 °C. At 50 °C, the CBD concentration effect differed from the one at 37 °C: CBD incubation at 24 µg/ml was less efficient than at 48 µg/ml. In addition, CBD incubation at 48 µg/ml performed less good than at 45 °C. The results are shown in Figures 10 and 11.

Example 8 – Treatment of Switchgrass with CBD

[0072] The treatment based on cellulose-binding domain (CBD) from cellobiohydrolase I (Cel7A) from *Trichoderma reesei* was applied to lignocellulosic materials (switchgrass), followed by the addition of cellulases to the CBD treatment mixture to perform hydrolysis. This led to an increase in lignocellulose enzymatic hydrolysis compared to no treatment with CBD (“dry sample”) prior to hydrolysis, as measured as the amount of reducing ends produced over time.

[0073] Briefly, switchgrass (20 mg/ml) was incubated at 45 °C in sodium acetate buffer (pH 5.0, 50 mM) supplemented with CBD (24 µg/ml) and under gentle stirring in a 24 ml glass vial for 16 h. The hydrolysis was performed on CBD-treated switchgrass (20 mg/ml) by addition of cellulases (24 µl/ml, 3.4 mg/ml total protein, 3.8 FPU/ml) and β-glucosidase (15 U/ml) to the mixture and the reaction was carried out at 50 °C. The effect of CBD treatment was investigated by measuring the amount of reducing ends produced over time (as equivalents of glucose) using the DNS assay at various intervals (1 h, 2.5 h, 4 h and 6 h). Controls were run on dry switchgrass (no CBD treatment), dry switchgrass with simultaneous addition of CBD and cellulases and on samples incubated in buffer only (for 16 h).

[0074] The incubation in buffer was found to be slightly beneficial for hydrolysis, as a higher reducing end concentration was obtained after 16 h incubation compared to hydrolysis on dry switchgrass (maximum 25% increase after 1h conversion). The effects were much more pronounced when CBD was present in the incubation mixture (up to 65% increase in reducing end concentration after 2.5 h conversion compared to dry sample). After 16 h incubation of switchgrass with CBD, the reducing end concentration presented a substantial increase compared to the one obtained after incubation in buffer alone (40-45% after 2.5 h conversion). Simultaneous addition of CBD and cellulases did not yield any difference in conversion compared to the dry sample. The results are shown in Figure 12.

EXAMPLE 9 – CBD Treatment of Avicel and Fibrous Cellulose from Cotton Linters

[0075] Avicel PH-101, fibrous cellulose from cotton linters (Sigma C6288, medium), cellulases from *Trichoderma reesei* (159 FPU.mL⁻¹), and β -glucosidase (from almonds, 2.32 U.mg⁻¹) were obtained from Sigma (St Louis, MO, USA). The (micro) BCA protein assay kit and the Coomassie (Bradford) protein assay kit were obtained from Thermo Fischer Scientific (Rockford, IL, USA). Papain was obtained from Fluka as lyophilized powder from *Carica papaya* (9.84 U.mg⁻¹). Centrifugal devices with polyethersulfone membranes were from Pall Life Sciences (Ann Arbor, MI, USA). All experiments and assays were run at least in duplicate.

Cel7A purification

[0076] Cel7A was purified from *Trichoderma reesei* cellulase cocktail by means of anion-exchange chromatography as published in, for example, Hall *et al.*, 2010. Cellulose crystallinity - a key predictor of the enzymatic hydrolysis rate. *Febs Journal*, 277, 1571-1582. Purity was confirmed by SDS-PAGE. After purification, Cel7A buffer was exchanged to sodium acetate buffer (50 mM, pH 5) using a polyethersulfone membrane (molecular weight cut-off of 10 kDa) in a Macrosep device.

Cellulase proteolysis and CBD isolation

[0077] Cellulase cleavage using papain was adapted from procedures published at, for example, Lemos *et al.*, 2000. A simple method to separate cellulose-binding domains of fungal cellulases after digestion by a protease. *Biotechnology Letters*, 22, 703-707. Cellulase preparation (mixture from *Trichoderma reesei*) was diluted (5 X) in sodium acetate buffer (50 mM, pH 5) and concentrated in a Jumbosep centrifugal device (polyethersulfone membrane, molecular weight cut-off of 30 kDa) to remove smaller molecules. Papain was activated in ammonium acetate buffer (50 mM, pH 6) at 30°C and 170 rpm for 30 min and added to the cellulase solution (w/w 1:5, v/v 1:5). The digestion was performed at 30°C for 2 h (completion checked *via* SDS-PAGE). The mixture was then filtered through 30 kDa membrane and both filtrate and concentrate were analyzed for protein content using the micro BCA assay.

X-ray diffraction and crystallinity measurement

[0078] X-ray diffraction patterns of cellulose samples (buffer and CBD pretreated) obtained after freeze-drying (at -55°C and 0.03 mbar for 16 hours) were recorded with an X'Pert PRO X-ray diffractometer (PANalytical BV, Almelo, the Netherlands) at room temperature from 10 to 35°C, using Cu/K α_1 irradiation (1.54 Å) at 45 kV and 40 mA. The scan speed was 0.021425° .s⁻¹ with a step size of 0.0167°. The crystallinity index of cellulose was measured from X-ray diffraction data using an analytical method described at, for example, Bansal *et al.*, Multivariate statistical analysis of X-ray data from cellulose: A new method to determine degree of crystallinity and predict hydrolysis rates. *Bioresource Technol.*, 101, 4461-4471.

Cellulose pretreatment and hydrolysis

[0079] Cellulose-binding domains were added in various amounts (up to $12 \mu\text{g} \cdot \text{mg}^{-1}$) to cellulose (Avicel or fibrous cellulose, total 56 mg , $40 \text{ mg} \cdot \text{mL}^{-1}$) in sodium acetate buffer (50 mM , $\text{pH } 5$) in a glass vial (containing a stir bar). The mixture was incubated for 15 h at various temperatures in a shaker incubator at 170 rpm and was then diluted with buffer to lower cellulose concentration to $20 \text{ mg} \cdot \text{mL}^{-1}$ for subsequent hydrolysis. Hydrolysis was initiated after the pretreatment step by adding cellulases ($24 \text{ mL} \cdot \text{L}^{-1}$, $3.4 \text{ g} \cdot \text{L}^{-1}$ total protein) and β -glucosidase ($15 \text{ kU} \cdot \text{L}^{-1}$) to the reaction mixture, and was carried out at 50°C and 170 rpm . The course of the reaction was monitored by performing DNS assay on small aliquots taken after 30 min , 1 h , and 2.5 h as described in, for example, Hall et al., 2010. Cellulose crystallinity - a key predictor of the enzymatic hydrolysis rate. *Febs Journal*, 277, 1571-1582.

[0080] For the thermostability experiment, cellulases and catalytic domains were incubated at 50°C for 15 h and then added ($300 \mu\text{g} \cdot \text{mL}^{-1}$ and $125 \mu\text{g} \cdot \text{mL}^{-1}$, resp.) to a cellulose mixture (Avicel, $20 \text{ mg} \cdot \text{mL}^{-1}$ in sodium acetate buffer, 50 mM , $\text{pH } 5$) that had been incubated at 50°C for 1 h at 900 rpm . The hydrolysis was run at 50°C and 900 rpm and the conversion was monitored after 6 h and 22 h by performing DNS assay on the supernatant.

Enzyme adsorption experiments

[0081] Avicel samples ($20 \text{ mg} \cdot \text{mL}^{-1}$) in sodium acetate buffer (50 mM , $\text{pH } 5$) were incubated at 50°C for 1 h at 900 rpm , and then cooled down to 4°C . Cellulases (which had been incubated in sodium acetate buffer at 50°C for 15 h or stored at 4°C since purchase) were added in various amounts and the mixture was further agitated for 30 min at 4°C . After centrifugation, the supernatant was collected and protein content analysis was performed using the BCA protein assay kit.

Results of Example 9

Cellulase proteolysis and CBD isolation

[0082] Both cellulase mixture (endoglucanases and exoglucanases) and purified cellobiohydrolase Cel7A from *Trichoderma reesei* were used in the proteolysis experiment using papain as cleaving agent. Cleavage was complete in both cases (as observed by the disappearance of full-length enzymes) and isolation of CBD through filtration yielded a mixture of CBDs or pure $\text{CBD}_{\text{Cel7A}}$, and corresponding catalytic domains (CDs) from the retentate could be observed by SDS-PAGE as shown in Figure 13.

Cellulose incubation with CBDs and crystallinity

[0083] In Example 9, vigorous stirring was provided using a shaker incubator and the addition of a stir bar to the system. The high crystalline content of Avicel (CrI 60%) and fibrous cellulose (CrI 72%) allows for monitoring of changes occurring during pretreatment. After

pretreatment of cellulose with CBDs in buffer at 42°C for 15 h under vigorous agitation, the samples were collected and their crystallinity content was assessed by X-ray diffraction (XRD), while the supernatant was analyzed for the presence of soluble reducing ends. No reducing ends were released from the buffer control sample. While the amount of soluble reducing ends obtained with CBDs was very low ($< 120 \mu\text{g.mL}^{-1}$, starting from cellulose concentration at 20 mg.mL^{-1}), it was consistent and above buffer background. Family I CBDs most likely do not have any hydrolytic activity; rather, small soluble oligomers were released from the cellulose surface upon binding/processing of CBDs. No disrupting activity could be observed e.g., no agglomerate formation nor faster precipitation without CBDs). After incubation in buffer only (control), the crystallinity of both types of cellulose (Avicel and fibrous) was shown to have slightly increased as shown in Table 3 below.

[0084]

Table 3. Effect of 15 h incubation with buffer and cellulose-binding domains on cellulose crystallinity (CrI) as determined *via* X-Ray diffraction.

Cellulose substrate	Avicel	Fibrous cellulose
CrI on dry sample (untreated)	60%	72%
CrI after incubation in buffer ^a	65%	74%
CrI after pretreatment with CBDs	58%	68%
CrI after pretreatment with CBD _{Cel7A} ^b	53-56%	n.d.
CrI after incubation with CD _{Cel7A} ^b	64%	n.d.

^a Sodium acetate (pH 5.0, 50 mM). ^b Longer incubation times ($> 48 \text{ h}$). n.d. Not determined

[0085] While not wishing to be bound to any particular theory, the crystallinity increase could be due to a phenomenon referred to as “recrystallization” described in, for example, Ouajai, S., Shanks, R.A., 2006. Solvent and enzyme induced recrystallization of mechanically degraded hemp cellulose. *Cellulose*, 13, 31-44. The increase (from 60 % up to 65-66% on average for Avicel, and 72% up to 74% for fibrous cellulose) is not likely attributable to the removal of non-crystalline material from cellulose, as no soluble reducing ends were released in the buffer control sample. Also, the freeze-drying does not have effect on cellulose crystallinity so that only extended incubation in buffer resulted in such a dramatic increase in crystallinity index (additionally, both buffer and CBD pretreated samples were freeze-dried before XRD analysis). The use of CBDs (up to $10 \mu\text{g.mg}^{-1}$ at 40 mg.mL^{-1} cellulose in a typical

pretreatment step) resulted in a different trend, as it not only prevented the substrate from recrystallizing, but also reduced the initial crystallinity of cellulose. Avicel crystallinity reached 58% after incubation with a mixture of CBDs, while fibrous cellulose crystallinity reached 68%, a significant drop from the crystallinity indices obtained after incubation in buffer only. When cellulose was incubated with pure CBDs from cellobiohydrolase I Cel7A (CBD_{Cel7A}) over extensive periods of incubation (> 48 h), an even higher reduction in crystallinity was obtained with Avicel (down to 53%, as shown in Figure 14).

[0086] Though the difference between the spectra appears limited to one portion of the XRD patterns, the analytical method described above for interpretation of XRD data of cellulose allowed accurate determination of the crystallinity index. The drop in intensity observed at a Bragg angle of 22.5° ((200) plane) accounts for the overall reduction in crystallinity. Thus, CBDs from *Trichoderma reesei* (belonging to family I CBD, along with CBDs of cellulases from *Phanerochaete chrysosporium* or *Penicillium janthinellum*), though not catalytically active with cellulose, had a strong effect on the cellulose structure, as overall crystallinity was significantly reduced. Interestingly, the crystal plane (200) seems to be the only one affected by these changes as illustrated in Figure 14. Overall, CBDs are responsible for splitting of intermolecular hydrogen bonds to such an extent that the cellulose crystallinity is noticeably reduced. While not wishing to be bound to any particular theory this most likely proceeds *via* a dynamic process (processive motion on cellulose surface required), which is still poorly understood.

Hydrolysis of CBD pretreated cellulose

[0087] Cellulose incubated with CBDs for 15 h was hydrolyzed by adding cellulases (supplemented with β -glucosidase) to the pretreatment mixture (one-pot process). The pretreatment of Avicel at 40 mg.mL⁻¹ affected the conversion as shown in Figure 15a, as the amount of sugar released from CBD pretreated samples (reduced crystallinity of 58%, Table 3) was 25% higher than that released from buffer pretreated samples (increased crystallinity of 65%, Table 3) after 30 min hydrolysis time (7.9 mg.mL⁻¹ vs. 6.3 mg.mL⁻¹ glucose resp.). Such an increase could potentially lead to a reduction in cost of the enzymatic process which is one of the major cost contributing factors in biofuel production from biomass.

[0088] Interestingly, no significant difference in rate compared to incubation in buffer only could be monitored at 20 mg.mL⁻¹ Avicel concentration when using the mixture of CBDs, even when the CBD concentration was increased to 12 μ g.mg⁻¹ (Fig. 15a). Similar behavior was observed with fibrous cellulose: the incubation at 20 mg.mL⁻¹ FC concentration did not give a significant change in conversion compared to the incubation in buffer only (Fig. 15b), whereas 23% increase was obtained after incubation with CBDs at 40 mg.mL⁻¹ FC compared to buffer

incubation (relating to 68% vs. 74% crystallinity resp., Table 3). The much slower rate obtained in the conversion of FC compared to Avicel can be explained in part by higher initial crystallinity (72% vs. 60% for untreated samples; however, surface area and particle size may also contribute).

[0089] It was observed that pretreatment with CBDs for 8 h instead of > 15 h did not lead to significant change in the amount of glucose released during the hydrolysis step compared to the buffer incubated sample. Incubation with CBDs opens up new possibilities in cellulose pretreatment and avoids the use of harsh conditions (such as high temperature or acid concentration) or isolation of cellulose from the pretreatment mixture before hydrolysis with cellulases.

[0090] The exact amino acid sequence of family I CBDs seems less important for rate improvement: pretreatment with homogeneous CBD_{Cel7A} yielded results comparable to those with a mixture of CBDs: increased rates i) after pretreatment at 42°C (and also 37°C) with CBD_{Cel7A} compared to buffer only, ii) after 30 min hydrolysis (7.5 mg.mL⁻¹ vs. 6 mg.mL⁻¹): 25% increase in glucose concentration, and iii) under similar conditions (34 mg.mL⁻¹ Avicel concentration compared to 40 mg.mL⁻¹ with the mixture). However, pretreatment conducted at lower cellulose concentration (20 mg.mL⁻¹) also yielded faster conversion, as there was only a minor drop in rate in the case of the sample pretreated at 20 mg.mL⁻¹ (compared to 40 mg.mL⁻¹ as shown in Fig. 16).

[0091] While not wishing to be bound to any particular theory desorption of CBDs upon dilution performed prior to the hydrolysis might be responsible for the higher rates after pretreatment with a CBD mixture at 40 mg.mL⁻¹ (compared to no improvement at 20 mg.mL⁻¹). Such desorption would liberate binding spots for cellulases on the cellulose surface and thus increase overall hydrolysis rate. Though incubation in buffer led to an increase rather than decrease in overall cellulose crystallinity (Table 3), it provided hydration of the sample and a nearly two-fold increase in hydrolysis rate was observed compared to employing dry cellulose without pretreatment. Thus, the increase in rate obtained after pretreatment with CBDs may be a combination of hydration effect from the buffer and reduction in crystallinity from CBDs.

[0092] Increased CBD concentration during pretreatment led to enhanced production of glucose in the hydrolysis step as shown in Figure 17. The present loading of 9 µg.mg⁻¹ is not saturating the surface since 150 µg.mg⁻¹ cellulase at a total loading of 1230 µg.mg⁻¹ bind to the cellulose surface as shown by Hall, *et al*, 2010. Cellulose crystallinity - a key predictor of the enzymatic hydrolysis rate. *Febs Journal*, 277, 1571-1582. And there was no saturation of Avicel employing up to 470 µg.mg⁻¹ CBD_{Cel7A} (corresponding to 49 µg.mg⁻¹ bound CBD) as

shown by Stahlberg, *et al.*, 1991. A New Model for Enzymatic-Hydrolysis of Cellulose Based on the 2-Domain Structure of Cellobiohydrolase-I. *Bio-Technology*, 9, 286-290.

[0093] In the absence of stirring during pretreatment (but when shaking was still provided), overall reaction rates were 40% lower and incubation with CBDs surprisingly did not lead to an increase in glucose concentration as shown in Figure 18. The use of stir bars 72% as large as the inner diameter of the vials resulted in up to 1.5 fold higher conversion levels in buffer, a positive effect of CBD incubation, but reduced reproducibility (most likely due to inhomogeneous stirring owing to geometric constraints in the reaction vial). Henceforth, stir bars of 55 % of inner vial diameter were used. Likely, the lack of continual convection in the absence of stirring prevents lateral displacement of CBD molecules and thus limits their effectiveness, given the absence of catalytic events as a potential energy source for CBD movement along the cellulose surface.

[0094] Surprisingly and unexpectedly, preincubation of cellulose with CBDs lowered its index of crystallinity and resulted in higher glucose concentration in the subsequent hydrolysis reaction. Also, it was observed that simultaneous addition of CBDs to non-pretreated cellulose samples did not show any improvement in conversion levels. Thus, the use of CBDs in a pretreatment step rather than a one-step process combining addition of CBDs and full-length enzymes is surprisingly and unexpectedly effective. While not wishing to be bound to any particular theory,, it may be that simultaneous addition of CBDs to the hydrolysis mixture prevents reduction of crystallinity by CBDs due to competitive binding with cellulases and/or hydrolysis of cellulose by cellulases.

Thermostability of CBDs

[0095] Similarly to the conditions used in the enzymatic hydrolysis of pretreated cellulose, pretreatment with CBDs was also performed at 50°C. After 15 h pretreatment at 50°C and hydrolysis for 30 min, it was observed that conversion was only slightly lower than after pretreatment at 42°C indicating that CBDs can be considered relatively thermostable. This observation was confirmed with binding experiments, where full-length cellulases were shown to bind similarly to Avicel after 15 h of incubation at 50°C, compared to storage at 4°C as shown in Table 4 below.

[0096] **Table 4.** Effect of 15 h incubation at 50°C on catalytic activity of cellulases and catalytic domains (CDs) and binding of cellulases on Avicel (compared to standard storage at 4°C).

Incubation temperature	Activity (Glucose concentration in mg.mL ⁻¹)		Binding capacity
	Cellulase cocktail	CDs	(bound cellulases µg.mg ⁻¹)
4°C	After 6 h: 5.6	After 6 h: 1.2	37.3 (total added 90 µg.mg ⁻¹)
	After 22 h: 8.2	After 22 h: 2.2	38.4 (total added 150 µg.mg ⁻¹)
50°C	After 6 h: 3.2	After 6 h: 0.5	41.4 (total added 90 µg.mg ⁻¹)
	After 22 h: 3.6	After 22 h: 0.7	44.0 (total added 150 µg.mg ⁻¹)

[0097] The activity of cellulases, however, was affected by the same incubation period at 50°C (1.8 fold decrease after 6 h hydrolysis, up to 2.3 after 22 h). The catalytic activity of CDs was even more reduced after incubation at 50°C (2.4 and 3.1 fold decrease in glucose concentration after 6 h and 22 h resp.). It can thus be concluded that the observed thermolability of cellulases is mostly due to loss of catalytic activity rather than binding capacity, as pretreatment of cellulose with CBDs can be performed at 50°C.

[0098] CBD_{Cel7A} is a small peptide (37 residues) and made up of an irregular triple-stranded antiparallel β-sheet as reported in, for example, Kraulis, et al., 1989. Determination of the 3-Dimensional Solution Structure of the C-Terminal Domain of Cellobiohydrolase-I from *Trichoderma-Reesei* - a Study Using Nuclear Magnetic-Resonance and Hybrid Distance Geometry Dynamical Simulated Annealing. *Biochemistry*, 28, 7241-7257. While not wishing to be bound to any particular theory it may be that the stability at higher temperatures may be at least in part due to its compact structure and/or the two disulfide bridges.

Conclusions

[0099] Cellulose-binding domains had impacted cellulose structure upon incubation. The crystallinity of different types of cellulose (microcrystalline and fibrous) was reduced after pretreatment with CBDs, rendering the substrates less recalcitrant to enzymatic hydrolysis, and an increase up to about 25 % in glucose concentration during hydrolysis was obtained. These surprising and unexpected results show that cellulose binding domains can be used as a pretreatment method for cellulosic material. The inventive pretreatment employs biological molecules and relatively mild conditions instead of harsh chemicals and conditions. In addition, the inventive pretreatment may be used in a manner such that it does not require a change of solvent. Moreover, the binding capacity of CBDs is retained after extensive incubation at 50°C while there is significant loss of catalytic activity.

Figure Legends

- [0100]** Fig. 1 SDS-PAGE of purified cel7A from *Trichoderma reesei* and cel7A catalytic domain after papain cleavage. Lane 1: ladder, lane 2: purified cel7A, lane 3: catalytic domain (CD) in concentrate after filtration through 30kDa membrane (Coomassie staining).
- [0101]** Fig.2 Enzymatic hydrolysis of Avicel after CBD treatment (numbers refer to cellulose sample from Table 2). Reactions were run as follows: 20 mg/ml Avicel, 50 mM NaOAc pH 5, 50 °C (#3 and 4 were used as freeze-dried powder, generated for X-ray diffraction analysis). All samples were incubated 1 h at 50 °C before addition of the enzyme to the reaction mixture.
- [0102]** Fig. 3. CBD incubation time effects (after reported time period, enzymes were added to the mixture of CBD and Avicel and hydrolysis was monitored by measuring glucose content, expressed as conversion %).
- [0103]** Fig. 4. Effect of buffer incubation on hydrolysis rate compared to CBD incubation (13 h). Avicel was incubated in CBD solution or in buffer for 13 h (also 1 h in buffer). Cellulases were then added to the reaction mixture and glucose content was monitored for up to 2.5 h.
- [0104]** Fig. 5. Effect of buffer incubation time on hydrolysis rates. Avicel was incubated in buffer over various periods of time. Cellulases were then added to the reaction mixture and glucose content was monitored for up to 4 h.
- [0105]** Fig. 6. CBD concentration optimization experiment (V_r represents the starting concentration). Graph b consists of the same data as graph a but for the lines connecting the data points.
- [0106]** Fig. 7. The hydrolysis for various CBD treatment times.
- [0107]** Fig. 8. The effect of various concentrations of CDB.
- [0108]** Fig. 9. The effect of sequential addition and CBD removal.
- [0109]** Fig. 10. The effect of a 45°C treatment.
- [0110]** Fig. 11. The effect of a 50°C treatment.
- [0111]** Fig. 12. Production of reducing ends from switchgrass using cellulases and β -glucosidase after treatment with CBD, treatment with buffer, no treatment with simultaneous addition of CBD and no treatment (dry sample).
- [0112]** Fig. 13. SDS-PAGE before and after papain cleavage of cellulases. Lane 1: ladder, lane 2: cellulase cocktail from *Trichoderma reesei* after filtration through 30 kDa membrane, lane 3: catalytic domains (CDs) of cellulases, lane 4: purified Cel7A after anion-exchange chromatography, lane 5: CD of purified Cel7A, lane 6: ladder, lanes 7 and 8: higher

loading of cellulase cocktail from *Trichoderma reesei* after filtration through 30 kDa and corresponding CDs, lane 9: purified Cel7A (lower loading).

[0113] Fig. 14. XRD spectra for Avicel samples treated with CBD_{Cel7A} (cellulose-binding domain from purified Cel7A), CD_{Cel7A} (catalytic domain) or buffer. The spectral data have been normalized according to Bansal et al., 2010. Multivariate statistical analysis of X-ray data from cellulose: A new method to determine degree of crystallinity and predict hydrolysis rates. *Bioresource Technology*, 101, 4461-4471. 1 and 2 refer to duplicated experiments.

[0114] Fig. 15a. Hydrolysis profile of Avicel after pretreatment with mixture of CBDs for 15 h at 42°C (the hydrolysis was run at 50°C and 20 mg.mL⁻¹ cellulose. The cellulose concentration refers to the actual concentration during pretreatment).

[0115] Fig. 15b. Hydrolysis profile of fibrous cellulose (FC) after pretreatment with mixture of CBDs for 15 h at 42°C (the hydrolysis was run at 50°C and 20 mg.mL⁻¹ cellulose. The cellulose concentration refers to the actual concentration during pretreatment).

[0116] Fig. 16. Hydrolysis profile of Avicel after pretreatment with CBD_{Cel7A} for 15 h at 42°C (the hydrolysis was run at 50°C and 20 mg.mL⁻¹ cellulose. The cellulose concentration refers to the actual concentration during pretreatment).

[0117] Fig. 17. Effect of various CBD concentrations in the pretreatment step (15 h at 42°C) on hydrolysis rate (the hydrolysis was run at 50°C and 20 mg.mL⁻¹ cellulose. The cellulose concentration refers to the actual concentration during pretreatment).

[0118] Fig. 18. Effect of stir bar during pretreatment of cellulose with CBDs on hydrolysis rates. The pretreatment was run at 42°C for 15 h without stir bar (10 µg.mg⁻¹), or with large stir bars (7.5 µg.mg⁻¹). The hydrolysis was run at 50°C and 20 mg.mL⁻¹ cellulose concentration.

[0119] Fig. 19. Amino acid sequences of the CBD of major organisms. The sequences of Figure 19 were identified *via* a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi#>) (Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic Local Alignment Search Tool. *Journal of Molecular Biology* 1990, 215:403-410). The sequences were aligned using clustalW (Thompson JD, Higgins DG, Gibson TJ: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994, 22(22):4673-4680). The names of enzymes and species, of which the sequences in Fig. 19 are a part of (according to the number in the alignment xx| where xx denotes the number; e.g., the *Trichoderma reesei* Cel7A CBD sequence is designated 1|1/1-36 outlined in Fig. 19 is number 1 in Table 5 below).

[0120] Table 5

Number	Enzyme (species)
1	Cel7A [<i>Trichoderma reesei</i>]
2	cellobiohydrolase I [<i>Trichoderma viride</i>]
3	cellubiohydrolase I [<i>Trichoderma</i> sp. XST1]
4	bifunctional chitosanase-cellulase [<i>Trichoderma viride</i>]
5	Cip1 [<i>Hypocrea jecorina</i>]
6	cellobiohydrolase I [<i>Hypocrea virens</i>]
7	cellobiohydrolase [<i>Hypocrea lixii</i>]
8	cellobiohydrolase I [<i>Neolentinus lepideus</i>]
9	exocellobiohydrolase [<i>Phanerochaete chrysosporium</i>]
10	endo-1,4-xylanase D [<i>Penicillium funiculosum</i>]
11	endoglucanase [<i>Aspergillus fumigatus</i> Af293]
12	xylanase/cellobiohydrolase [<i>Penicillium funiculosum</i>]
13	cellobiohydrolase I [<i>Penicillium occitanis</i>]
14	glycosyl hydrolase family 45 protein [<i>Neosartorya fischeri</i> NRRL 181]
15	cellulose binding protein [<i>Phanerochaete chrysosporium</i>]
16	CBHI [<i>Volvariella volvacea</i>]
17	endo-1,4-beta-xylanase [<i>Aspergillus fumigatus</i> Af293]
18	cellulose binding iron reductase [<i>Coprinopsis cinerea</i> okayama7#130]
19	Acetyl xylan esterase [<i>Hypocrea jecorina</i>]
20	cellulase [<i>Irpex lacteus</i>]
21	endoglucanase IV [<i>Trichoderma viride</i>]
22	endoglucanase IV [<i>Trichoderma</i> sp. SSL]
23	endoglucanase I [<i>Penicillium echinulatum</i>]
24	endoglucanase II [<i>Trichoderma viride</i>]
25	endoglucanase III [<i>Trichoderma viride</i>]
26	endoglucanase III [<i>Hypocrea jecorina</i>]
27	endoglucanase II [<i>Hypocrea jecorina</i>]

Number	Enzyme (species)
28	glycoside hydrolase family 5 [Nectria haematococca]
29	glycosyl hydrolase family 61 [Glomerella graminicola M1.001]
30	cellulase [Irpep lacteus]
31	endo-1,4-beta-xylanase C precursor [Phanerochaete chrysosporium]
32	endoglucanase I [Penicillium oxalicum]
33	endoglucanase I [Penicillium decumbens]
34	cellulose binding protein [Talaromyces emersonii]
35	cellulose-binding beta-glucosidase [Phanerochaete chrysosporium]
36	Acetyl xylan esterase [Aspergillus fumigatus Af293]
37	glycosyl hydrolase family 62 protein [Coprinosia cinerea okayama7#130]
38	Family 61 endoglucanase [Phanerochaete chrysosporium]
39	Fungal cellulose binding domain protein [Aspergillus clavatus NRRL 1]
40	cellulase CEL7A [Lentinula edodes]
41	exoglucanase [Verticillium albo-atrum VaMs.102]
42	cellobiohydrolase II [Acremonium cellulolyticus Y-94]
43	glycosyl hydrolase family 7 protein [Neosartorya fischeri NRRL 181]
44	cellulase CEL6B [Lentinula edodes]

[0121] The claimed subject matter is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

[0122] All references cited herein are incorporated herein by reference in their entirety to the extent that they are not inconsistent and for all purposes to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

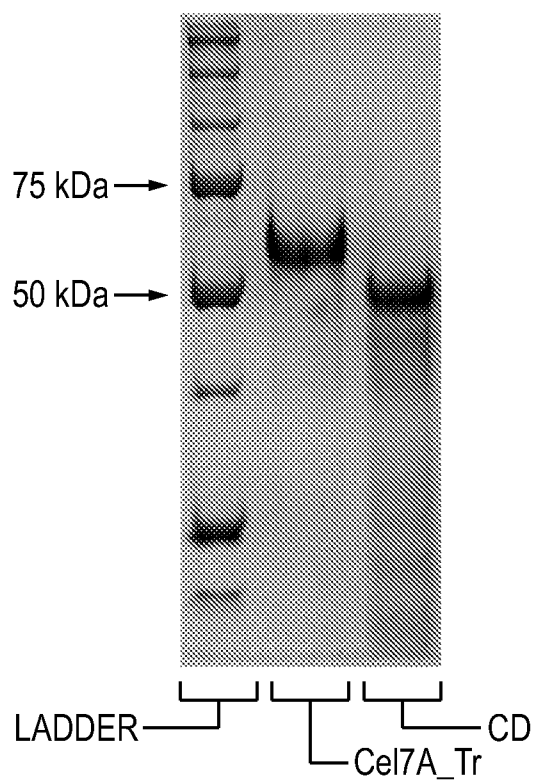
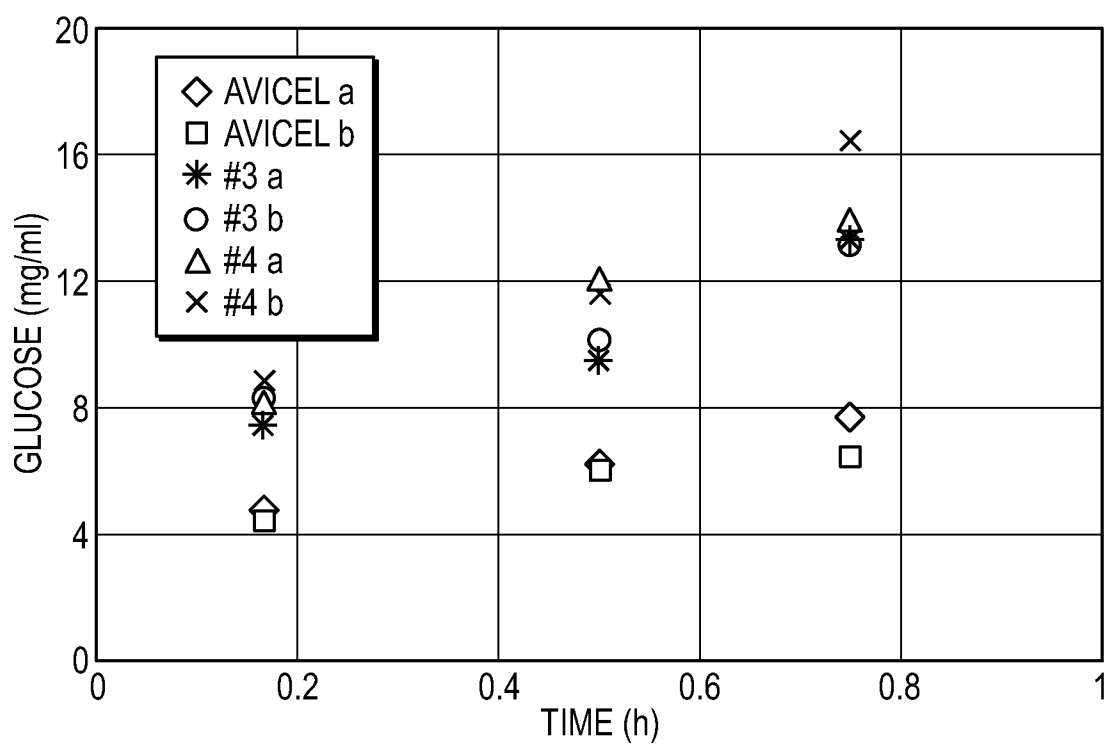
[0123] The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

What is claimed is:

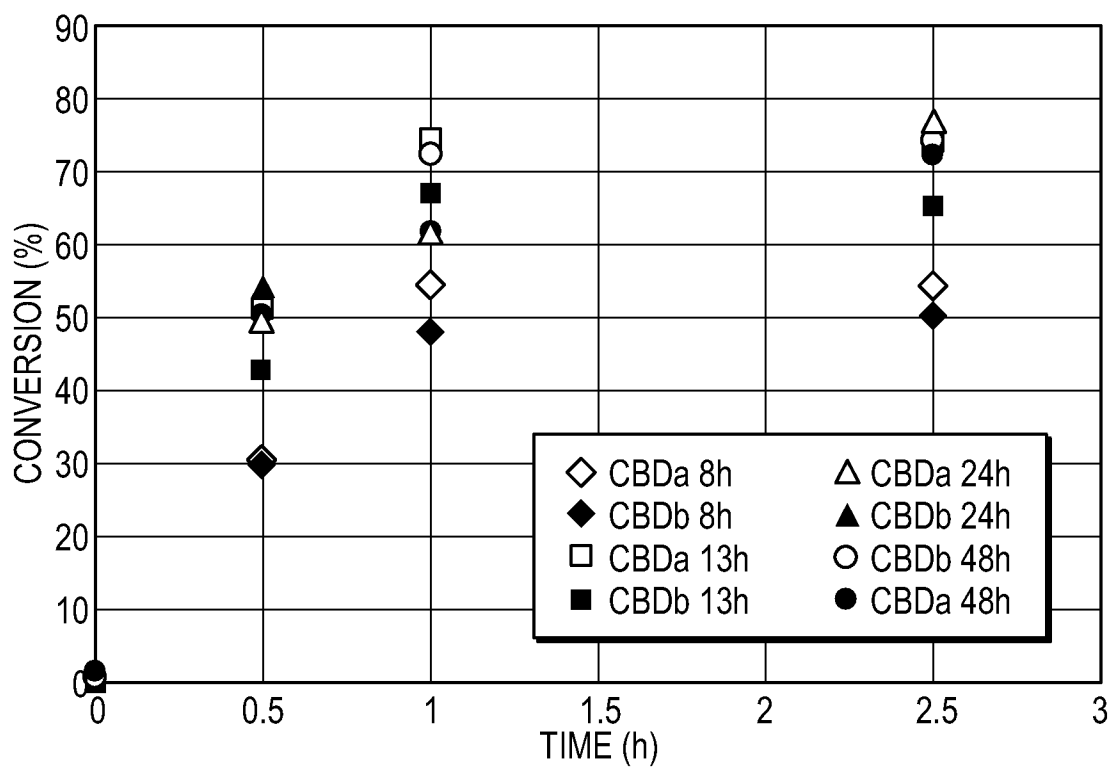
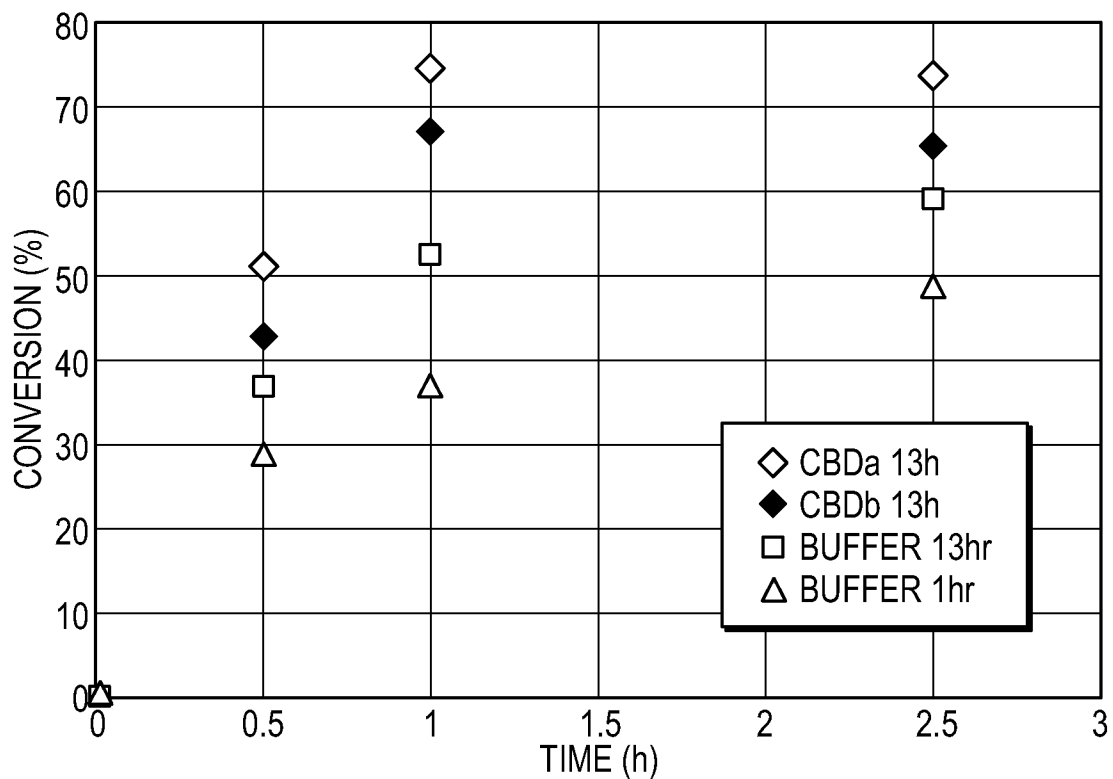
1. A process for treating a feedstock comprising holocellulose wherein the process comprises:
 - a) mixing the feedstock with a solution comprising cellulose binding domains to form a mixture; and
 - b) subjecting the mixture to conditions sufficient to reduce the crystallinity of holocellulose.
2. The process of claim 1 wherein the cellulose binding domains are derived from cellobiohydrolase I (cel7A) or cellobiohydrolase II (cel6A).
3. The process of claim 1 wherein the cellulose binding domains are derived from cellobiohydrolase I (cel7A) produced by *Trichoderma reesei*.
4. The process of any one of Claims 1-3 wherein the mixture is subjected to a temperature of greater than 30°C for a time sufficient to reduce the crystallinity of holocellulose.
5. The process of any one of Claims 1-3 wherein the mixture is subjected to increased temperature for at least 8 hours.
6. The process of any one of Claims 1-3 wherein the mixture is subjected to stirring such that substantially all solids are suspended.
7. The process of any one of Claims 1-3 wherein the solution comprising cellulose binding domains has a concentration of cellulose binding domains of from 5 to 55 µg/ml.
8. The process of any one of Claims 1-3 wherein the solution comprising cellulose binding domains further comprises a suitable buffer and wherein the solution has a pH of from 4.5 to 5.5.
9. The process of any one of Claims 1-3 which further comprises hydrolyzing the product of step 1b in Claim 1 to produce a composition comprising at least one fermentable sugar and wherein said at least one fermentable sugar in said composition is at least 10% more than a comparable process which does not employ cellulose binding domains.

10. The process of any one of Claims 1-3 wherein the ratio of cellulose binding domains to catalytic domain is greater than 1:1.
11. A composition suitable for reducing the crystallinity of cellulose comprising cellulose, a suitable concentration of a solution comprising cellulose binding domains, and a suitable buffer.
12. The composition of Claim 11 wherein the cellulose binding domains are derived from cellobiohydrolase I (cel7A) produced by *Trichoderma reesei*.
13. The composition of any one of Claims 11-12 wherein the solution comprising cellulose binding domains has a concentration of from 5 to 55 µg/ml wherein the buffer comprises a 25 to 75 mM sodium acetate buffer and wherein the solution has a pH of 5.
14. The composition of any one of Claims 11-12 wherein the solution comprising cellulose binding domains has a ratio of cellulose binding domains to catalytic domain which is greater than 1:1.
15. The composition of any one of Claims 11-12 wherein the solution comprising cellulose binding domains has a ratio of cellulose binding domains to catalytic domain which is greater than 5:1.

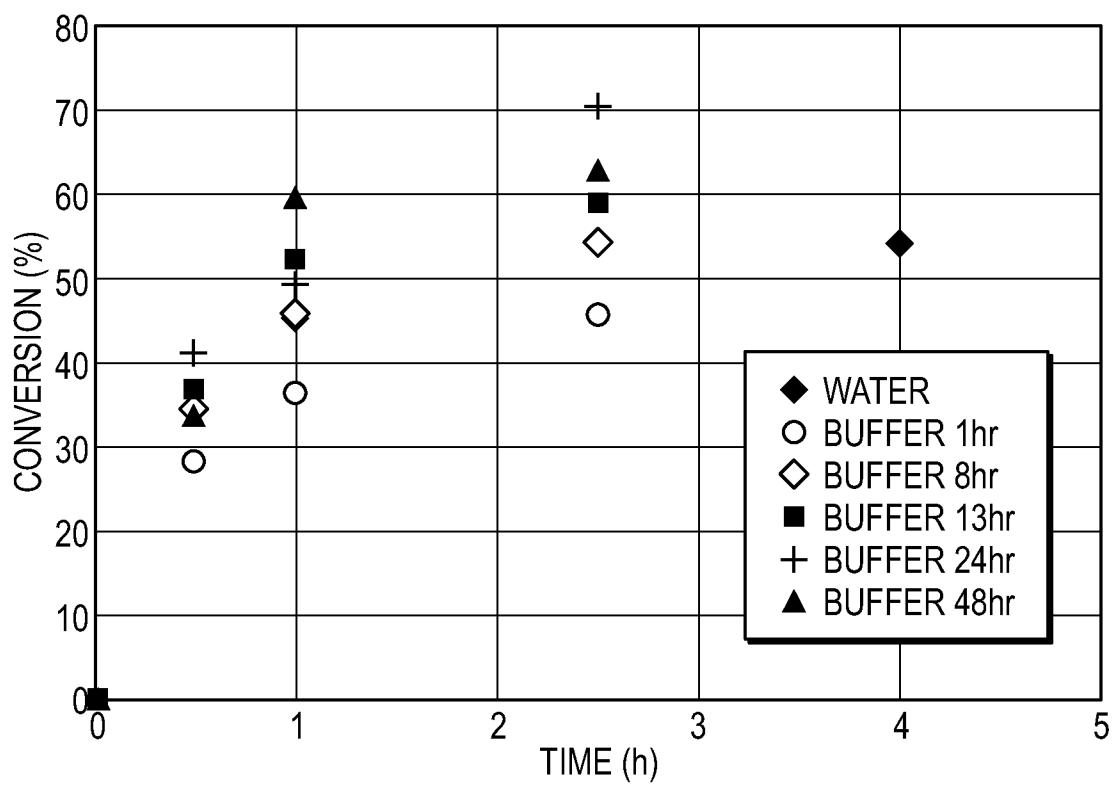
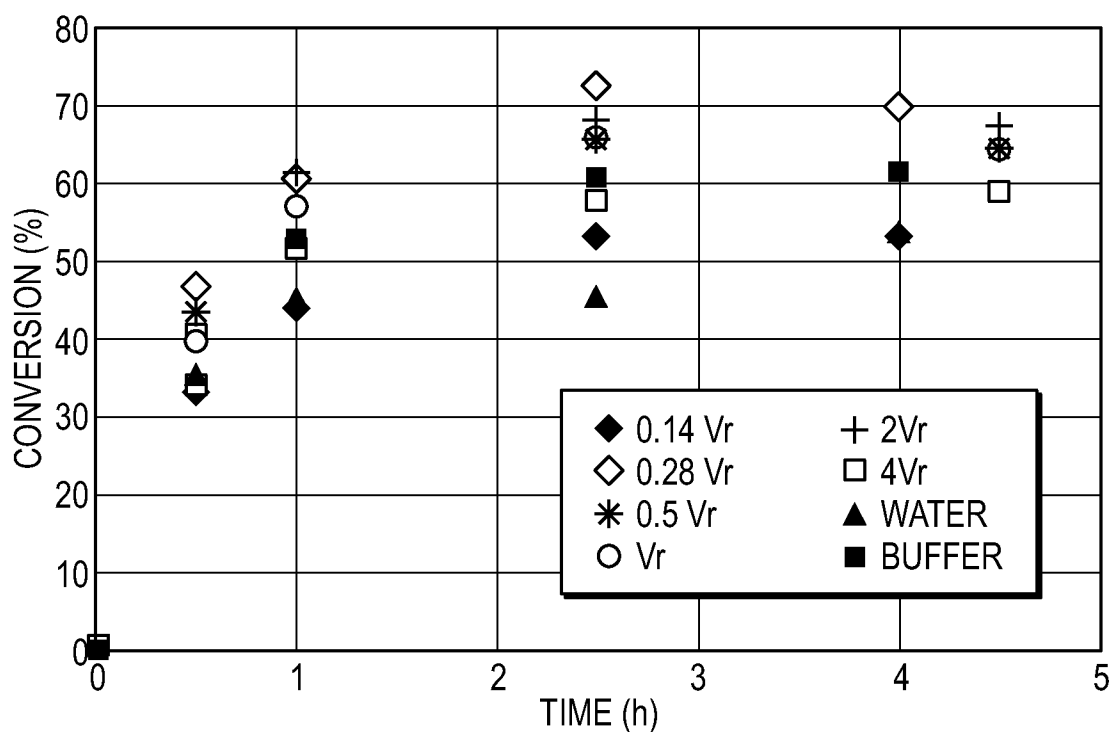
1/13

FIG. 1**FIG. 2**

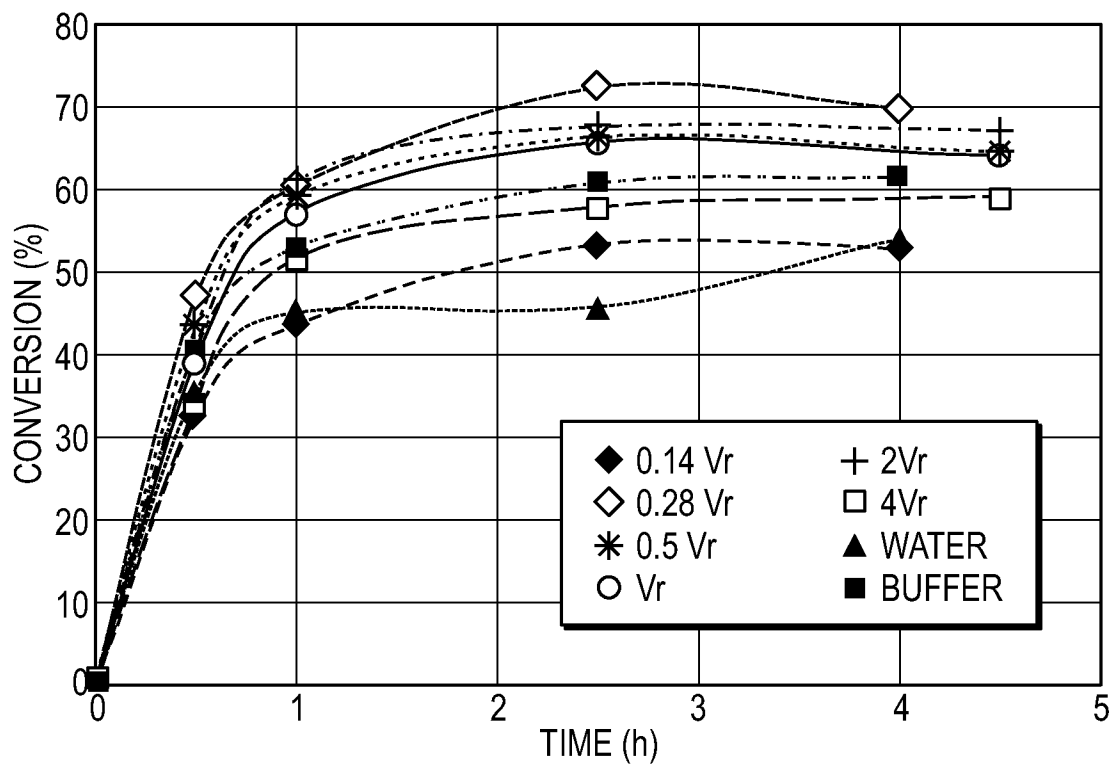
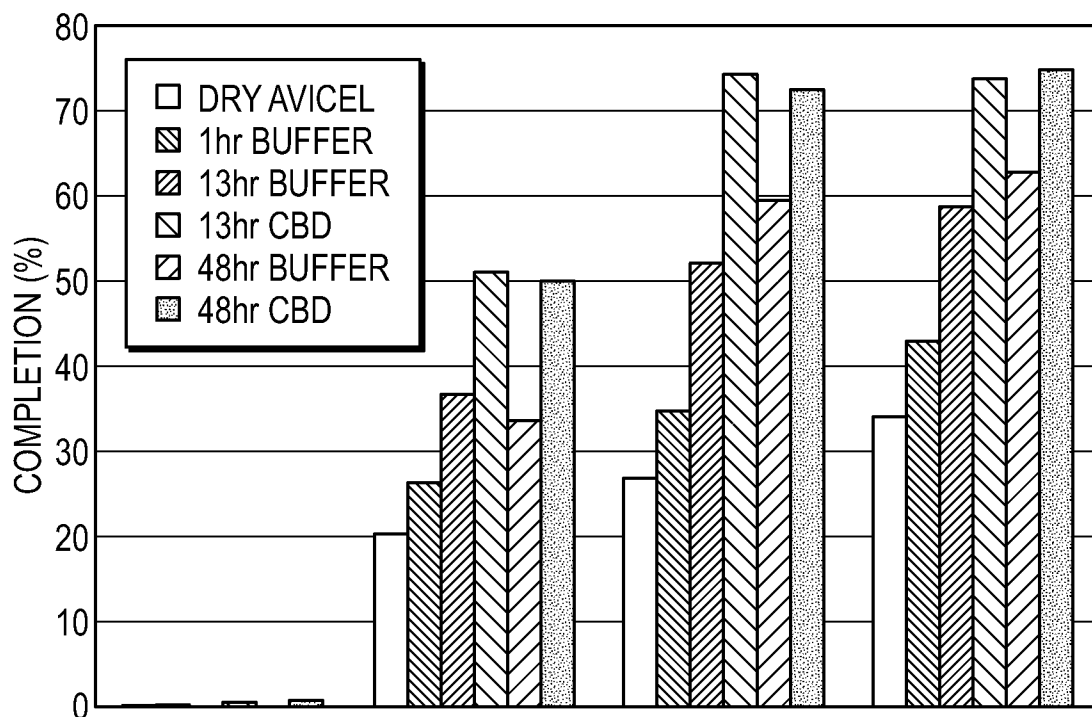
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FIG. 3**FIG. 4**

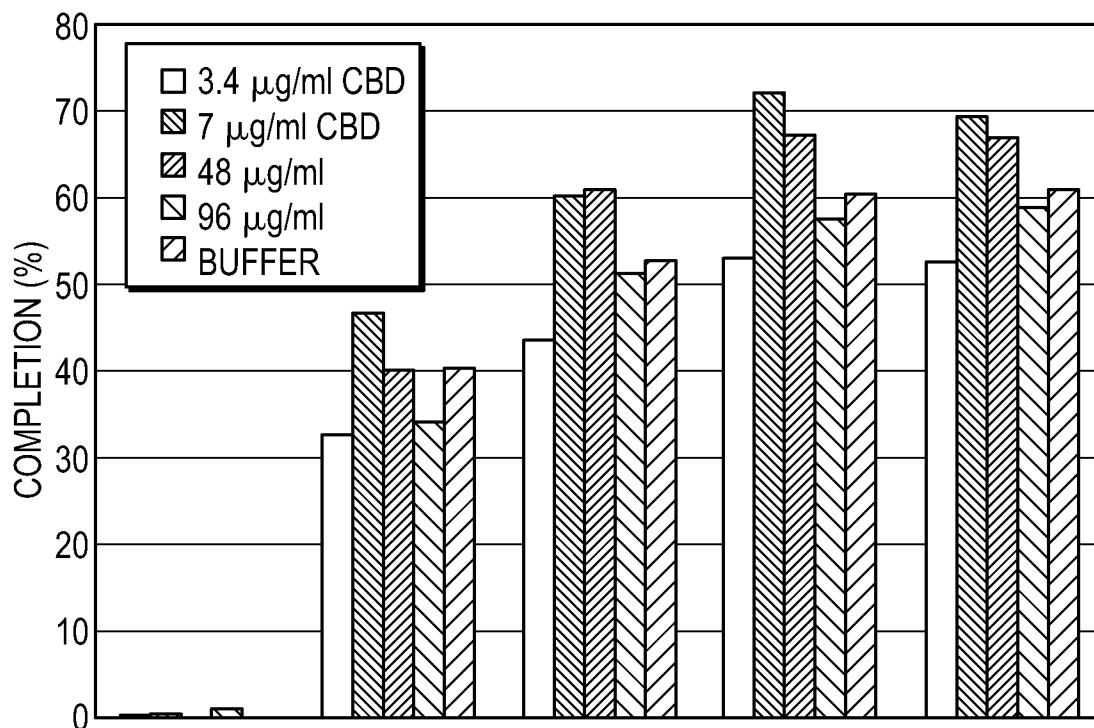
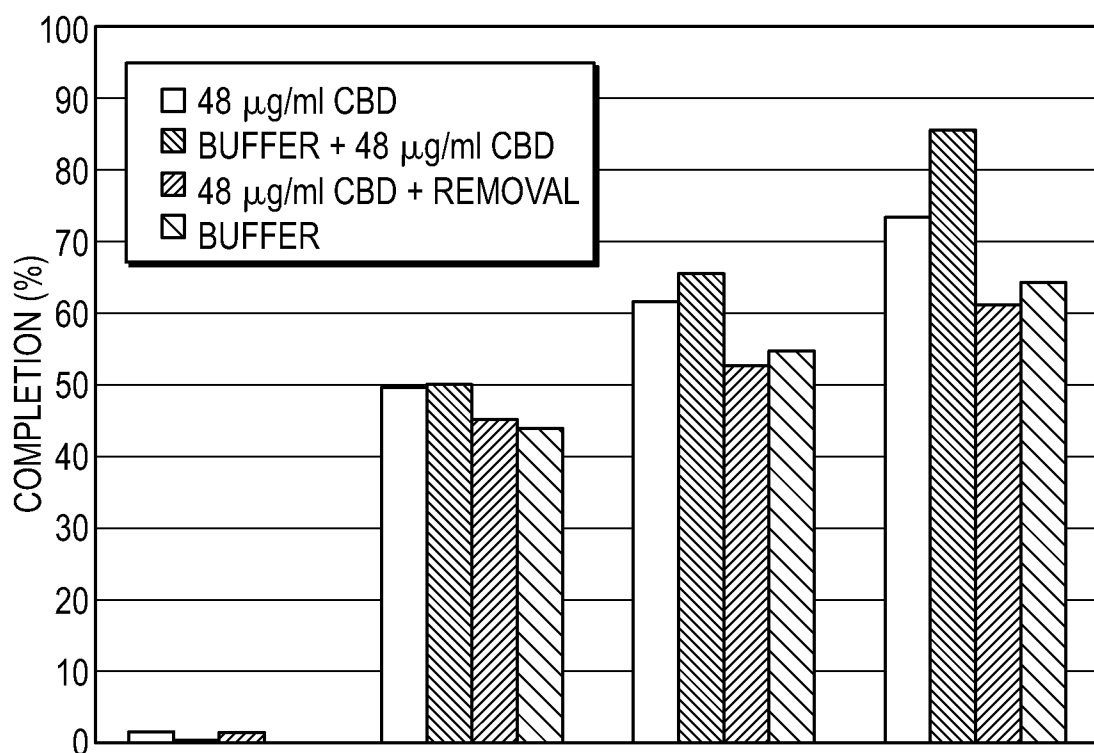
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FIG. 5**FIG. 6A**

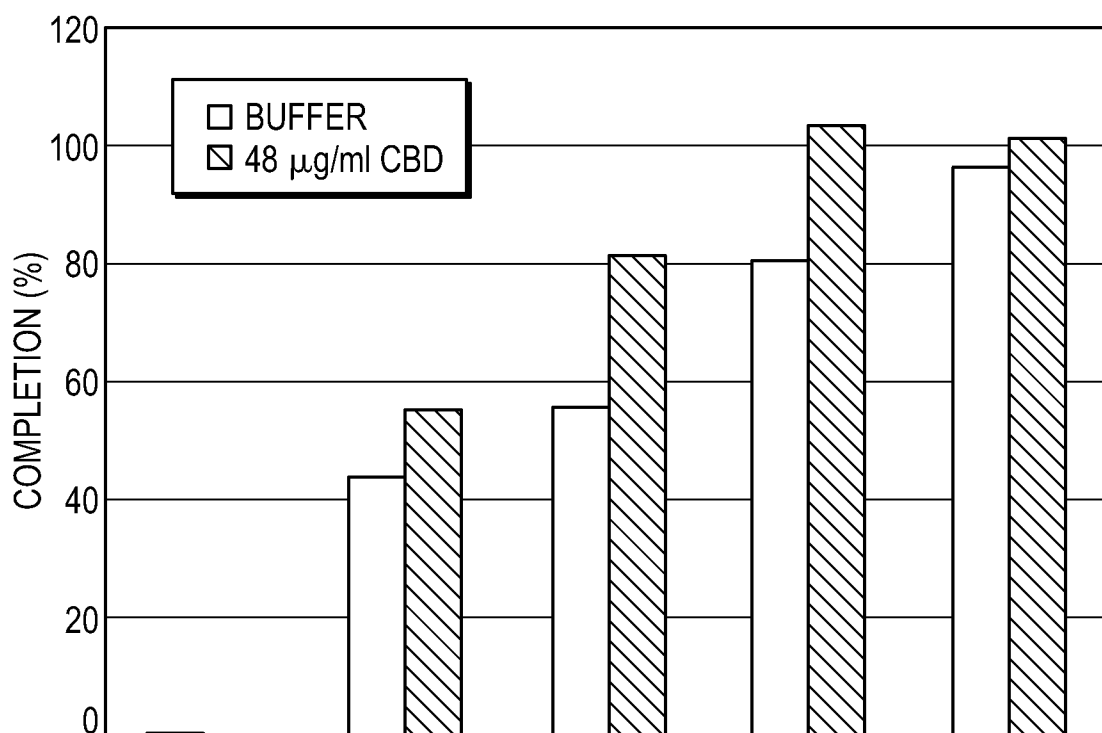
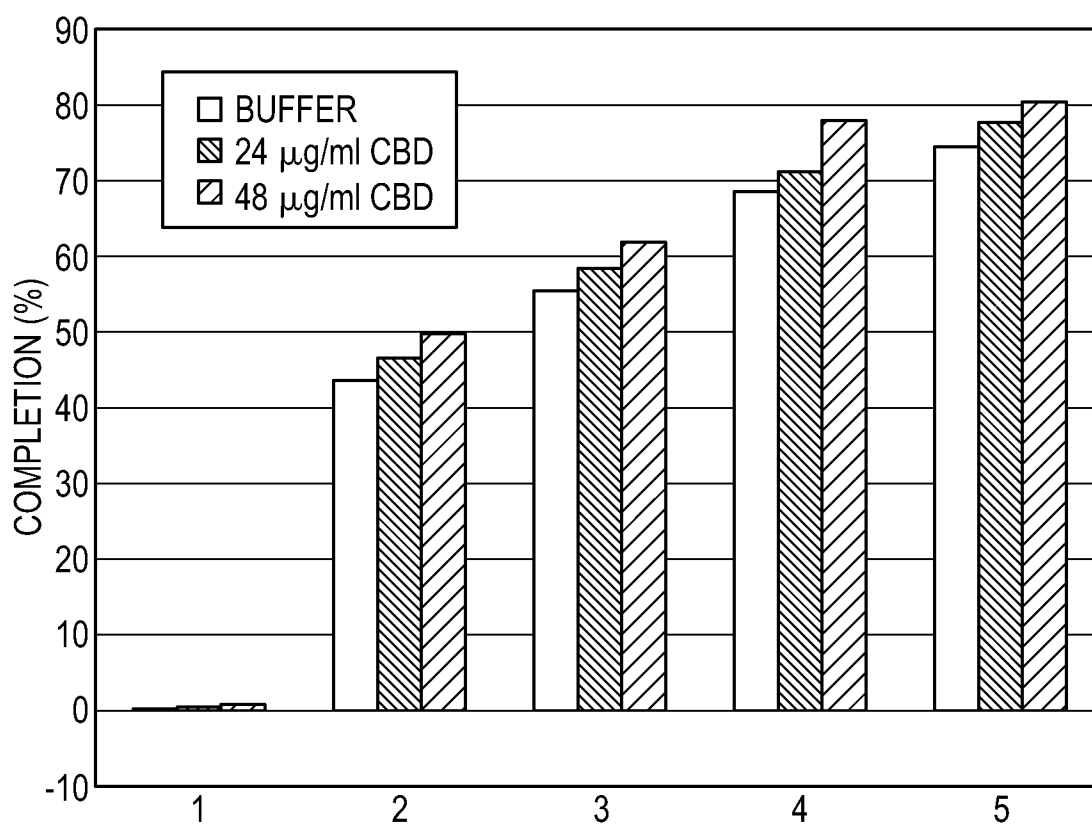
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FIG. 6B**FIG. 7**

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FIG. 8**FIG. 9**

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FIG. 10**FIG. 11**

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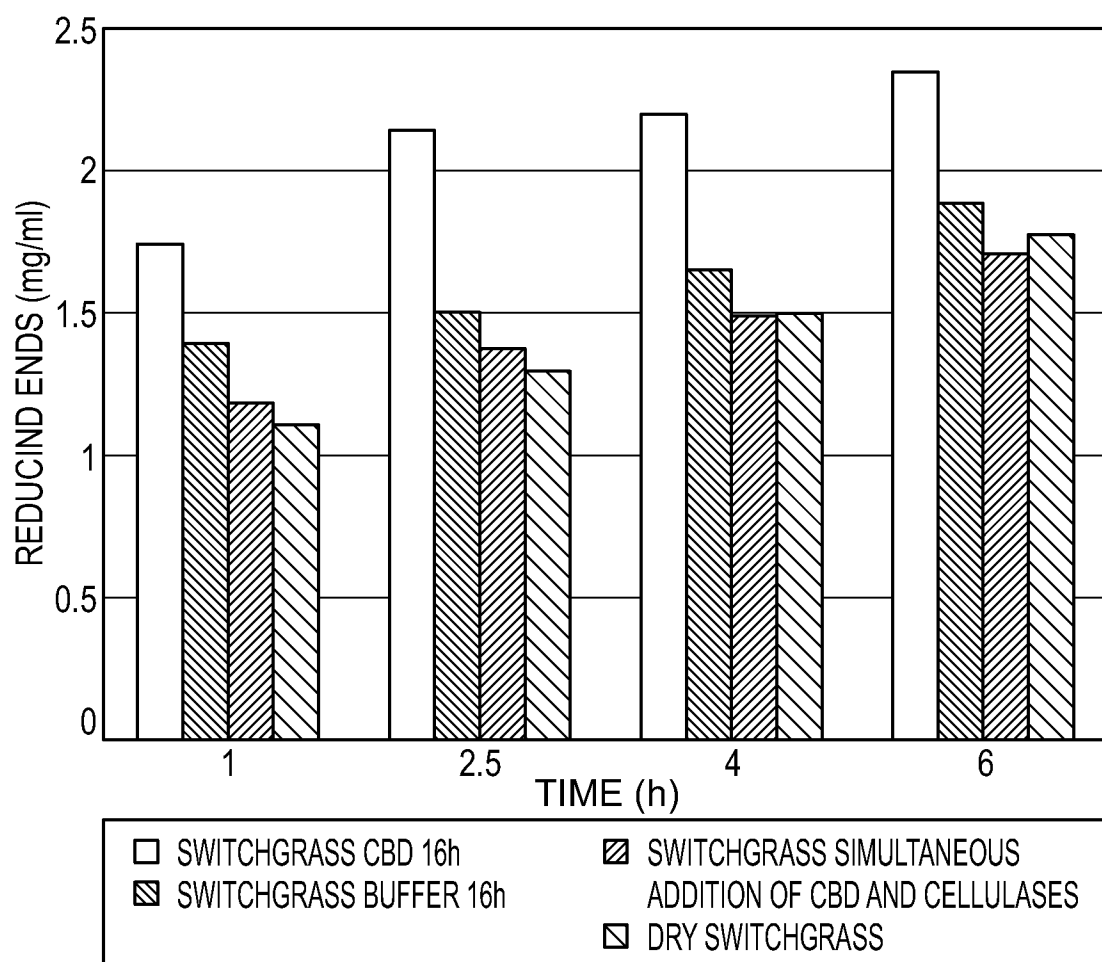
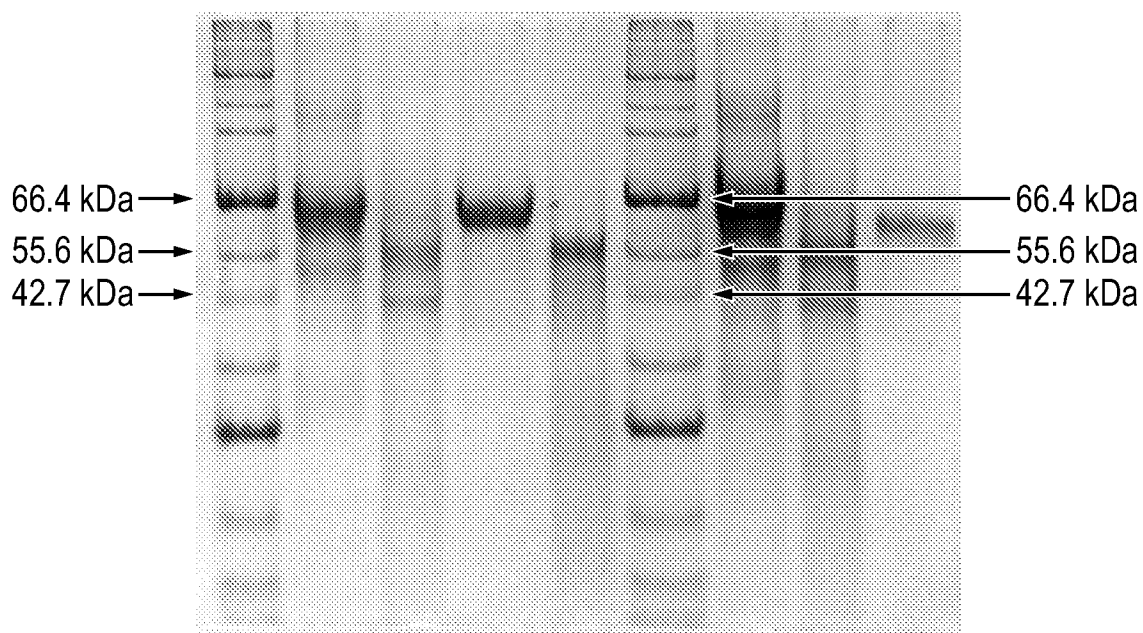
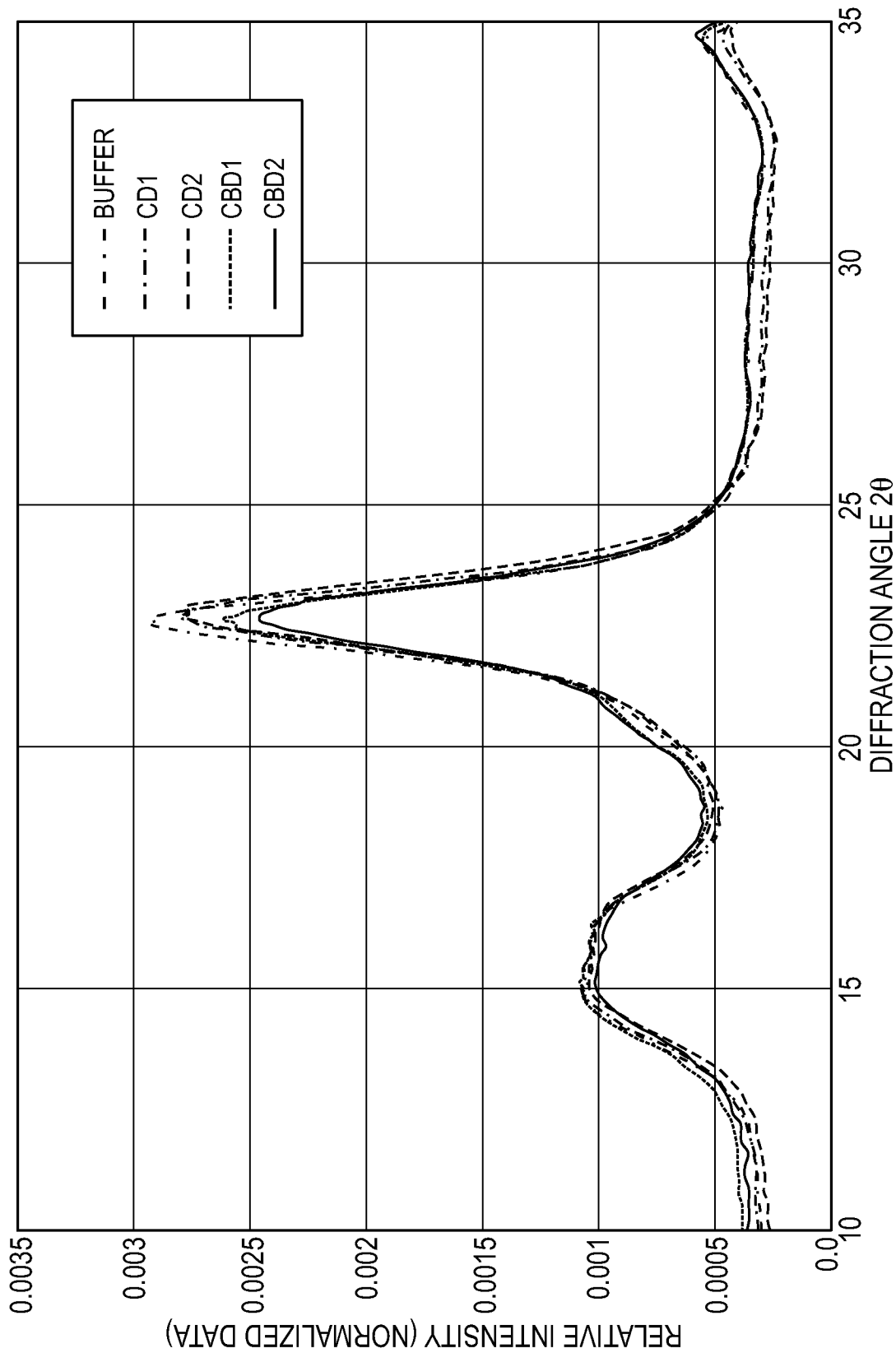
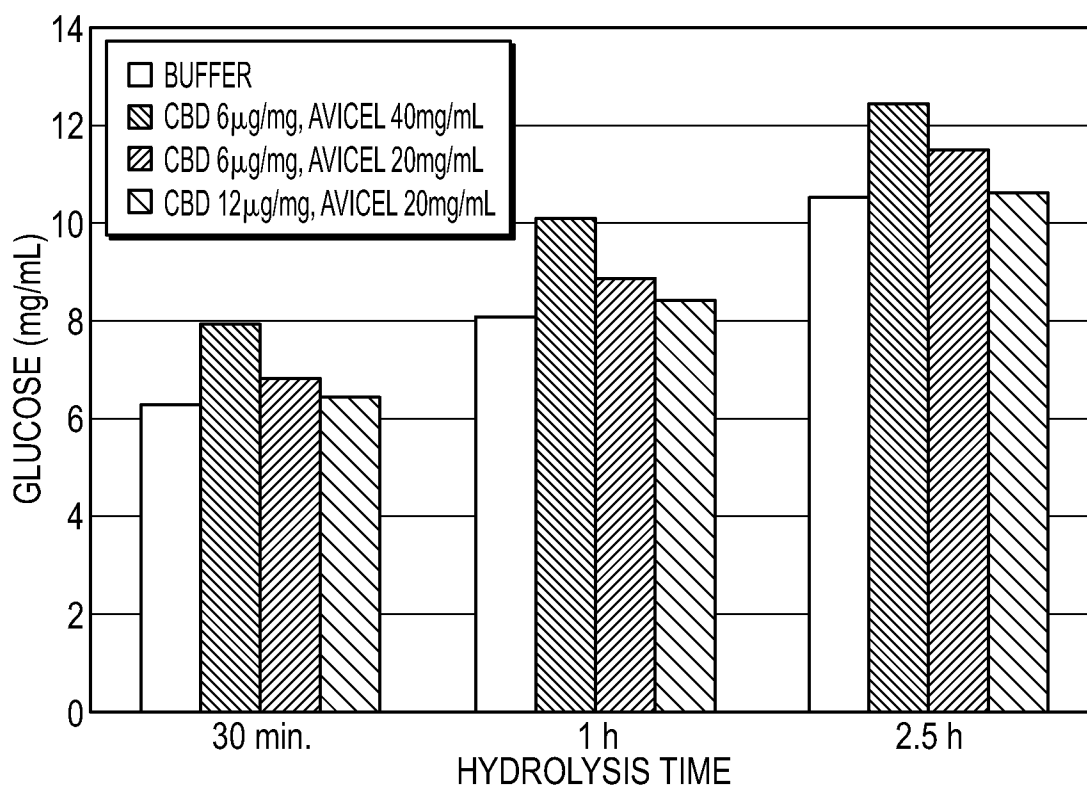
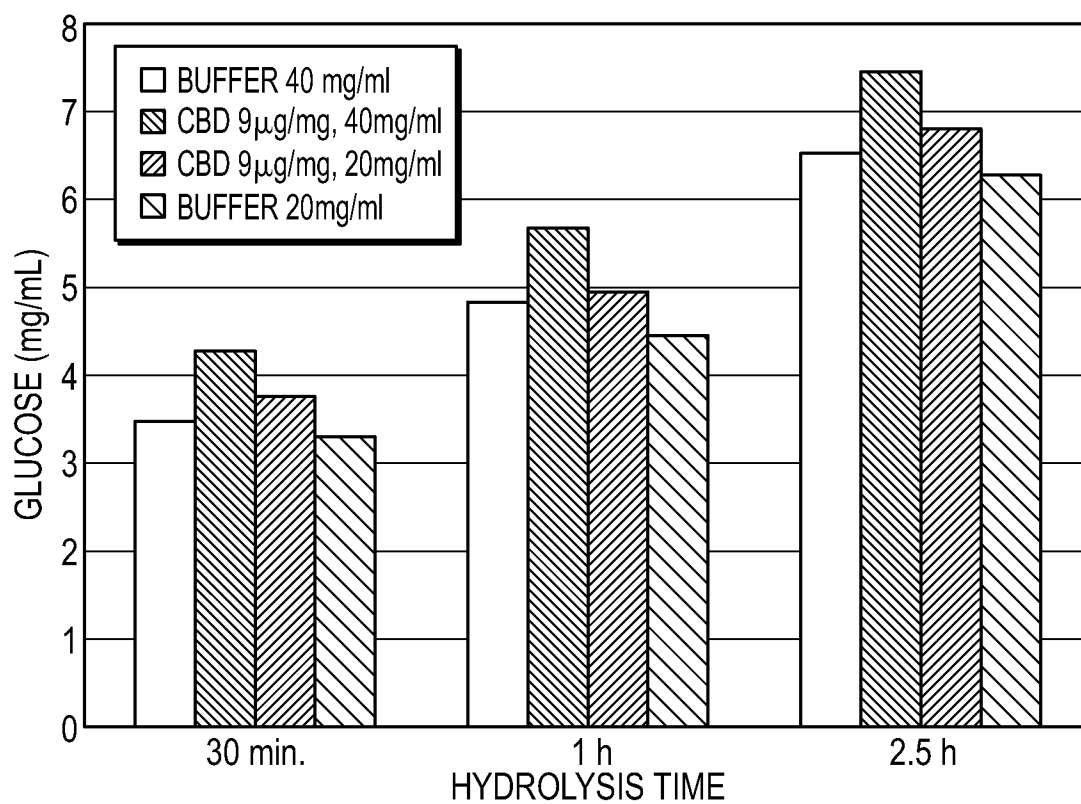
FIG. 12**FIG. 13**

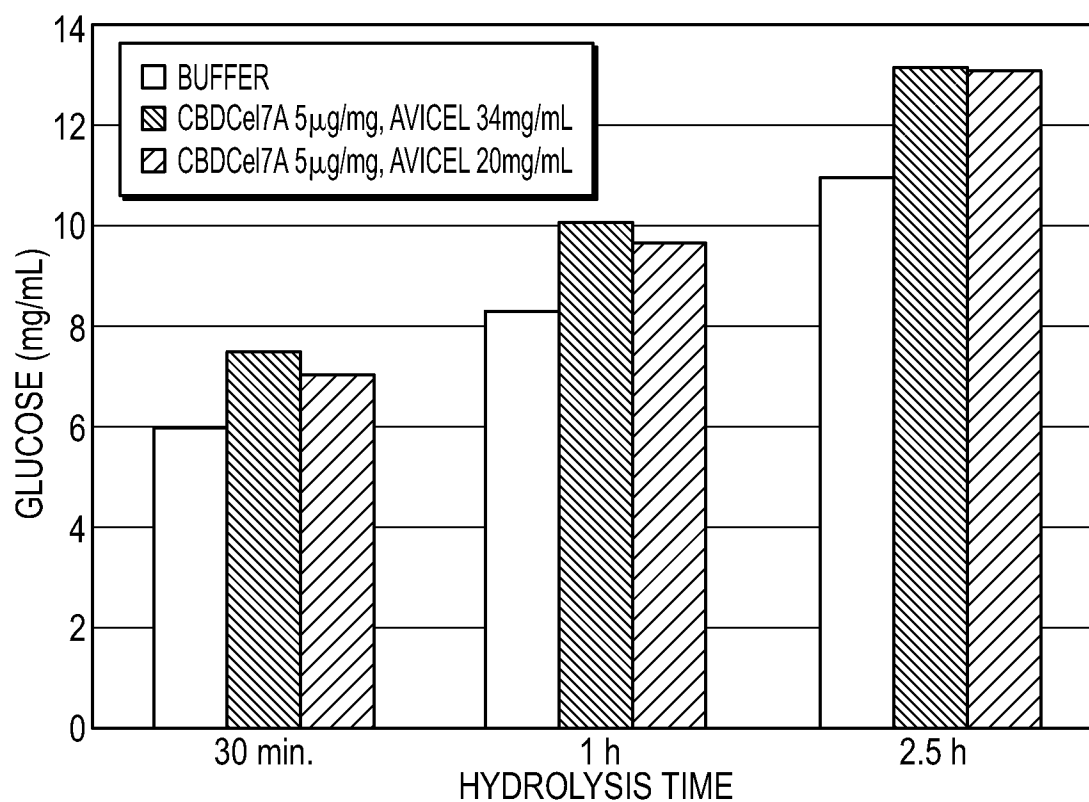
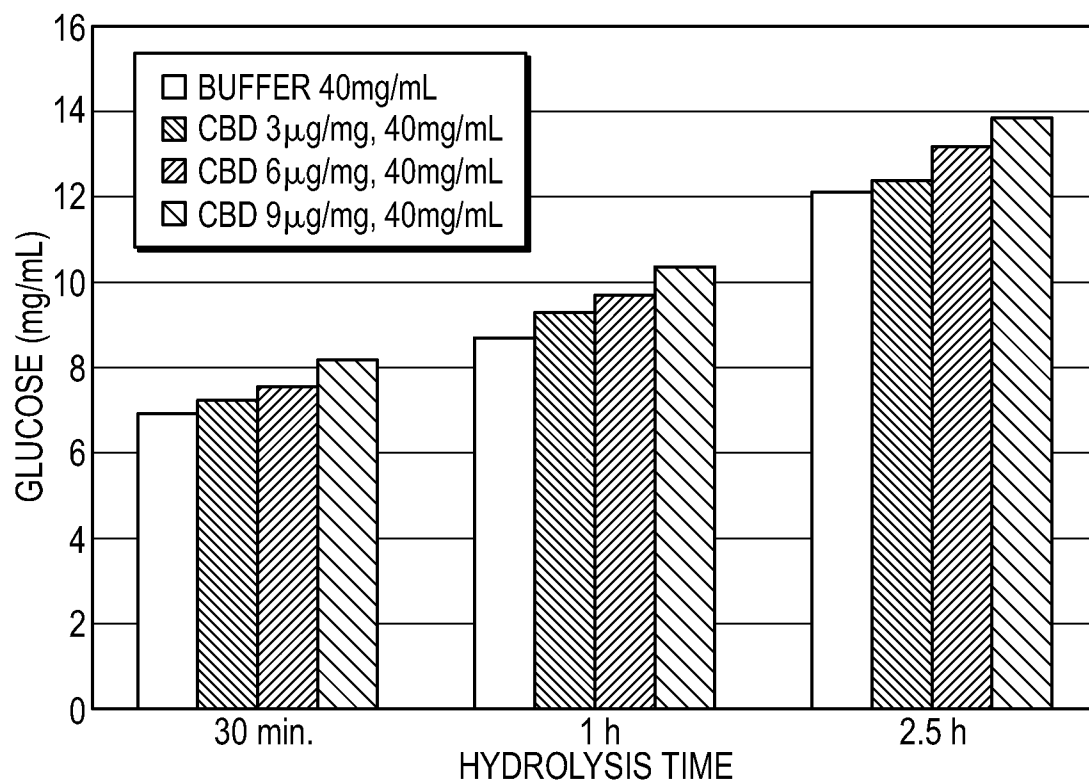
FIG. 14



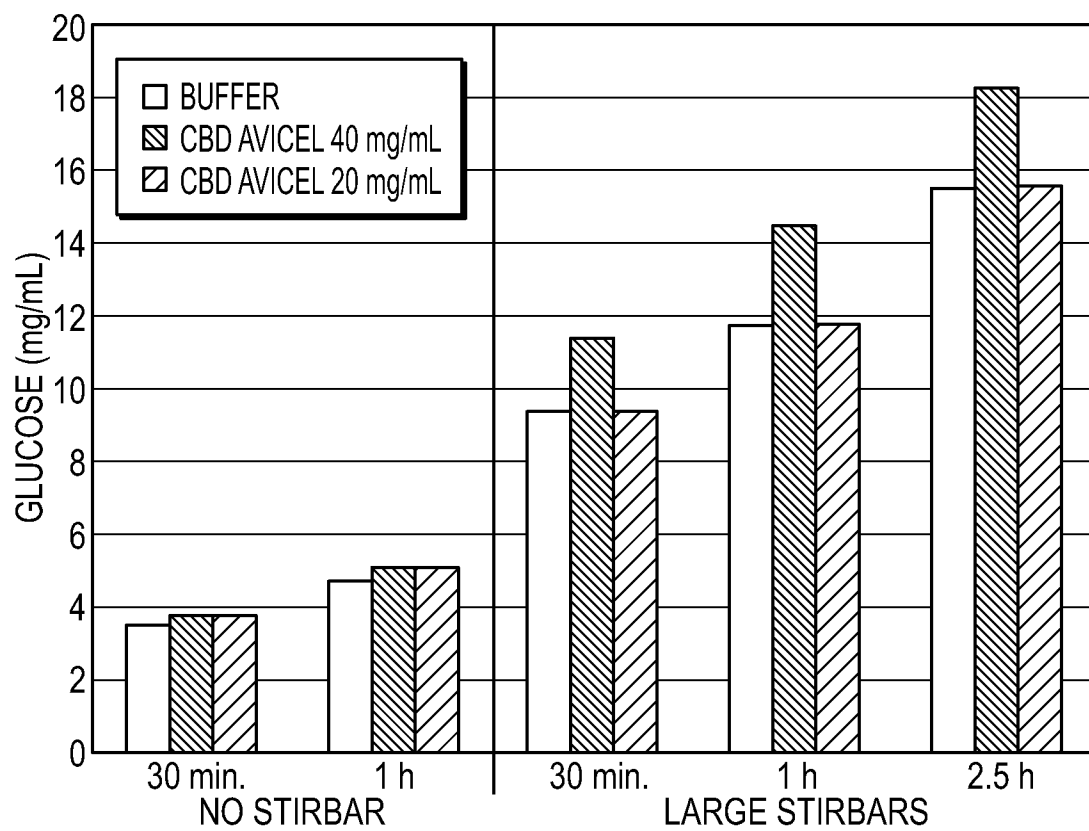
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FIG. 15A**FIG. 15B**

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FIG. 16**FIG. 17**

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FIG. 18

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FIG. 19

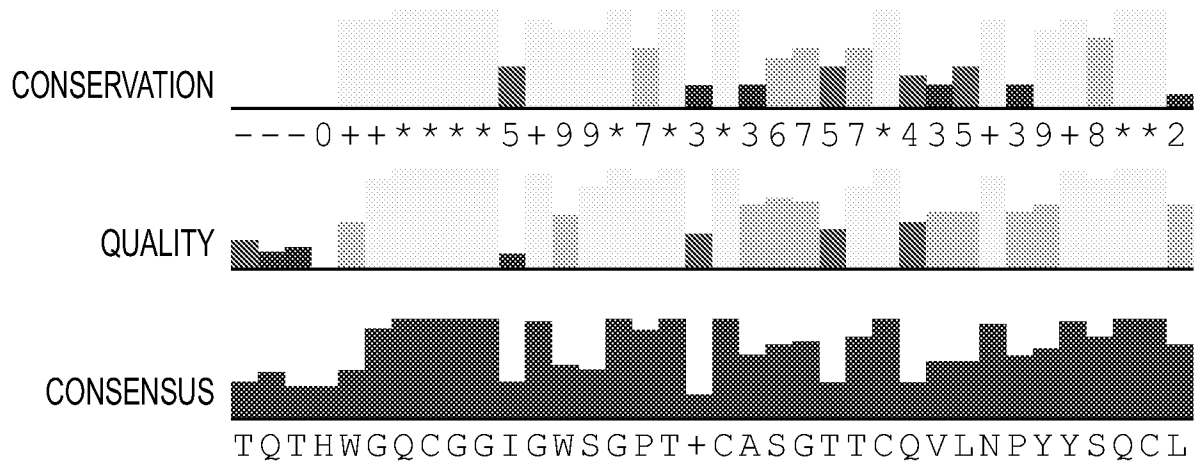
	10	20	30
7 7/1-36	TQTHYGQCGGTGWTGPTRC	CASGFTC	QVLNPFYYSQCL
11 11/1-36	TQTHYGQCGGQGWGTGPTAC	CASPYTC	QVLNPPWYYSQCL
29 29/1-36	TVPKYGQCGGQGWSGPTTC	CASGSTC	QALNDFYYSQCV
43 43/1-33	---HYGQCGGNWQGP	TTCCASPYTC	QKLNDFYYSQCL
19 19/1-36	TQTHWGQCGGQGWGTGPTQC	ESGTTCC	QVISQWYYSQCL
28 28/1-35	-QVKYGQCGGS	SGWTGPTLCESGSTC	QVQNQWYYSQCL
39 39/1-35	-QSLYGQCGGNW	SGPTECTAGACC	QVQNPWYYSQCL
41 41/1-34	--SQWGQCGGQGW	SGPTCCPSGTTCC	QLQNAWYYSQCL
1 1/1-36	TQSHYGQCGGI	GYSGPTVCASGTTCC	QVLNPYYYSQCL
2 2/1-36	TQSHYGQCGGI	GYSGPTVCASGTTCC	QVLNPYYYSQCL
5 5/1-33	---HYGQCGGI	GYSGPTVCASGTTCC	QVLNPYYYSQCL
3 3/1-36	TQTHYGQCGGI	GYSGPTVCASGTTCC	QVLNEYYSQCL
4 4/1-36	TQTHYGQCGGI	GYSGPTVCASGTTCC	QVLNEYYSQCL
6 6/1-36	TQTHYGQCGGI	GYSGPTQCVSGTTCC	QVLNPFYYSQCL
10 10/1-33	---HWGQCGGI	IGWSGPTICVSPYTC	QVLNPYYYSQCL
20 20/1-35	TAAQWAQCGGM	GFPTGPTVCASPFTC	CHVLNPYYYSQC-
30 30/1-35	TVAQWGQCGGT	GFTGPTVCASPFTC	CHVVNPYYYSQC-
15 15/1-31	----WGQCGGI	GYTGPTVCASGFTC	CHVLNPYYYSQC-
16 16/1-30	-----GQCGGI	GYTGPTTCCASPFTC	CHVLNPYYYSQC-
9 9/1-34	--SEWGQCGGI	IGWTGPTTCCVSGTTCC	TVLNPYYYSQCL
31 31/1-33	---EWGQCGGI	IGWTGPTTCCVAGTTCC	VESNPYYYSQCL
12 12/1-34	--AHWGQCGGQ	GWGTGPTTCCASGTTCC	TVVNPYYYSQCL
13 13/1-33	---HWGQCGGQ	GWGTGPTTCCVSGTTCC	TVVNPYYYSQCL
32 32/1-32	----WGQCGGQ	GYTGPTACVSGTTCC	KAQNPYYYSQCL
33 33/1-32	----WGQCGGQ	GYTGPTACVSGTTCC	KAQNPYYYSQCL
17 17/1-33	---KWGQCGGI	IGWTGPTTCCVSGTTCC	QKLNDWYYSQCL
38 38/1-34	--AKYGQCGGL	TYTGPTTCCVSGTTCC	TALNDYYSQCL
23 23/1-33	---HWAQCGGV	GYSGPTACASPYTC	KVQNDYYSQCL
37 37/1-36	TADHWAQCGGR	GFSGPTTCCASGAVC	TVVNEWYSQCI
34 34/1-32	----WGQCGGL	LGWTGPTVCASGFTC	TVINEYYSQCL
36 36/1-34	--EHWGQCGGN	GWGTGPTACASGYTC	TVINEWYSQCL
35 35/1-32	----YQQCGGI	IGWTGATTCCVSGATC	TVLNPYYYSQCL
44 44/1-32	----YGQCGGI	IGWSGATTCCVSGATC	TVVNAYYSQCL
14 14/1-36	TQTLYGQCGGS	SGWTGPTACASGATC	CVLNSYYSQCL
8 8/1-36	TQTKYGQCGGQ	GYSGPTVCASGSTC	QTSNPYYYSQCL
40 40/1-36	TQTKYGQCGGQ	GWGTGATVCASGSTC	TSSGPYYYSQCL
18 18/1-36	TQTIYGQCGGT	GYSGPTVCAGGSRCK	QVNPYHFSQCL
21 21/1-36	TQTLYGQCGGS	GYSGPTRCAPPATC	STLNPYYAQCCL
22 22/1-36	TQTLYGQCGGS	GYSGPTRCAPPATC	STLNPYYAQCCL
25 25/1-35	-QTVWGQCGGI	IGWSGPTNCAPGSAC	STLNPYYAQCCL

(Continued on next sheet)

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FIG. 19
(Continued)

26 26/1-35	-QTVWGQCGGIGWSGPTNCAPGSACSTLNPYYAQCI
27 27/1-35	-QTVWGQCGGIGWSGPTNCAPGSACSTLNPYYAQCI
24 24/1-35	-QTVWGQCGGIGWSGPTSCAPGSACSTLNPYYAQCI
42 42/1-35	-QSVWGQCGGQGWSGATSCAAGSTCSTLNPYYAQCI



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/58960

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - D21C 1/00 (201.01)

USPC - 435/277

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)

USPC: 435/277, 274, 267, 262, 72, 41

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)
 WEST-PGPUB, USPT, USOC, EPAB, JPAB; Google Scholar--cellulose binding domains, cellulose-binding domains, CBDs, cellulose-binding domains AND Trichoderma, pretreatment of biomass with cellulose-binding domains, treating biomass with CBD derived from trichoderma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2009/0042266 A1 (VEHMAANPERA et al) 12 February 2009 (12.02.2009) para[0061, [0062], [0092], [0186], [0194], [0237], [0241], [0245], and Table 30	1-15
A	US 2008/0299613 A1 (MERINO et al) 04 December 2008 (04.12.2008) para [0467]	10, 14, 15
A	LEVY et al. Cellulose-binding domains Biotechnological applications. Biotechnology Advances, 2002, Vol. 20, pages 191-213.	1-15
A	MEDVE et al Hydrolysis of Microcrystalline Cellulose by Cellobiohydrolase I and Engoglucanase II from Trishoderma reesei: Adsorption, Sugar Production Pattern, and Synergism of the Enzymes. Biotechnology and Bioengineering, 5 September 1998 (05.09.1998), Vol. 59, pages 621-634.	1-15
A	SZENGYEL et al. Cellulase Production of Trichoderma reesei Rut C 30 Using Steam-Pretreated Spruce. Applied Biochemistry and Biotechnology, 2000, Vol. 84-86, pages 679-691.	1-15
A	LEMOIS et al. The enhancement of the cellulolytic activity of cellobiohydrolase I and endoglucanase by the addition of cellulose binding domains derived from Trichoderma reesei. Enzyme and Microbial Technology, 2003, Vol. 32, pages 35-40.	1-15
A	SRISODSUK et al. Trichoderma reesei cellobiohydrolase I with an endoglucanase cellulose-binding domain: action on bacterial microcrystalline cellulose, 1997, Vol. 57, pages 49-57	1-15



Further documents are listed in the continuation of Box C.



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"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

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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

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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

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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

13 January 2011 (13.01.2011)

Date of mailing of the international search report

28 MAR 2011

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P.O. Box 1450, Alexandria, Virginia 22313-1450

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