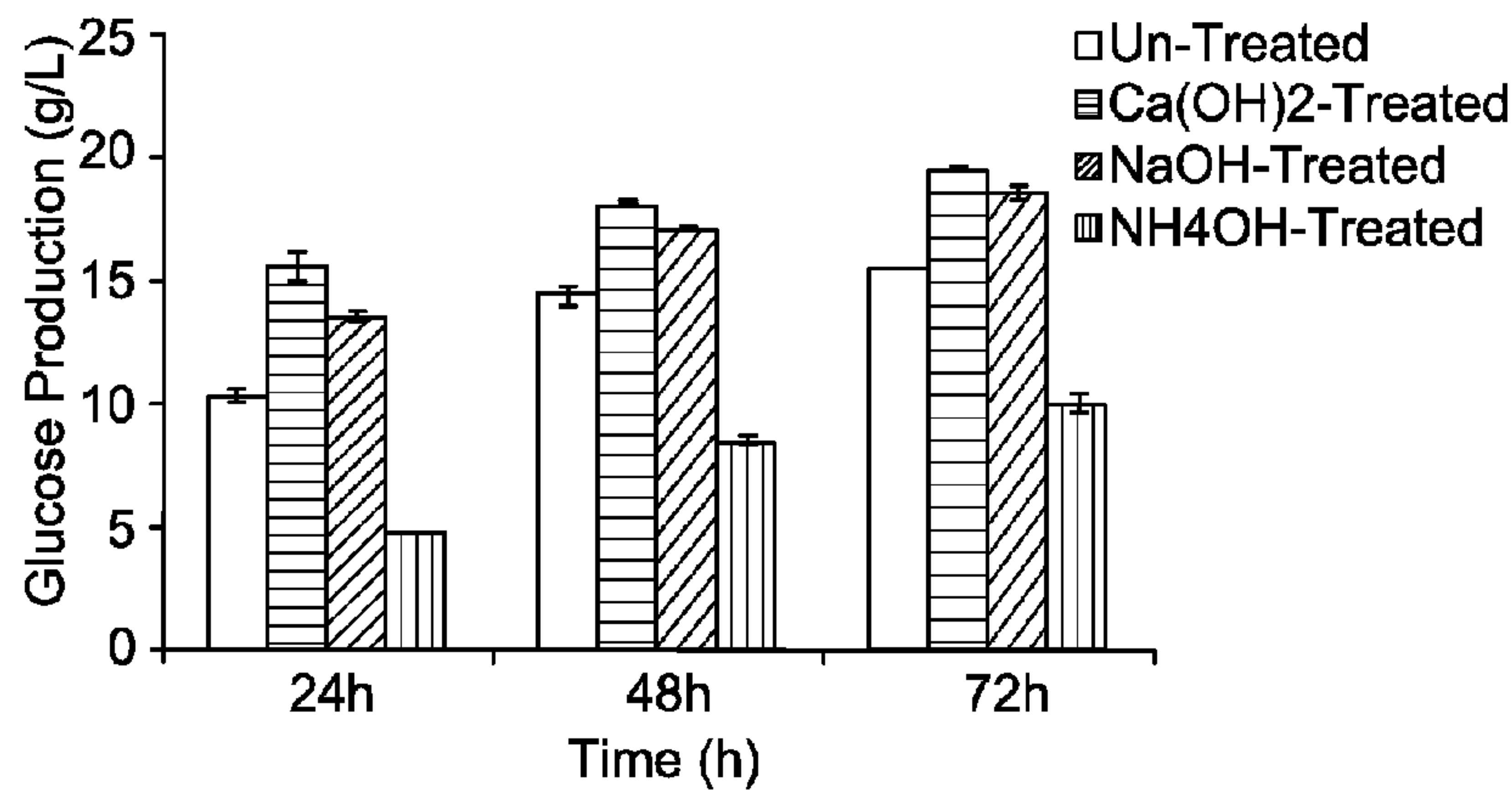




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(54) Title: METHODS FOR IMPROVEMENT OF ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC MATERIAL



(57) Abrégé/Abstract:

The present invention relates to a method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of: a) pretreating the lignocellulosic material to obtain a slurry having a pH of less than 6; b) adding NaOH, Ca(OH)₂ and/or CaO to the slurry to increase its pH to at least 8, said addition being carried out at a slurry temperature of at least 60 °C; c) reducing the pH of the slurry to below 7; and optionally cooling the slurry from step b) to a temperature below 60 °C; and d) adding hydrolytic enzymes to the slurry from c) and allowing the slurry to hydrolyze wherein no washing of the slurry is performed prior to step d)

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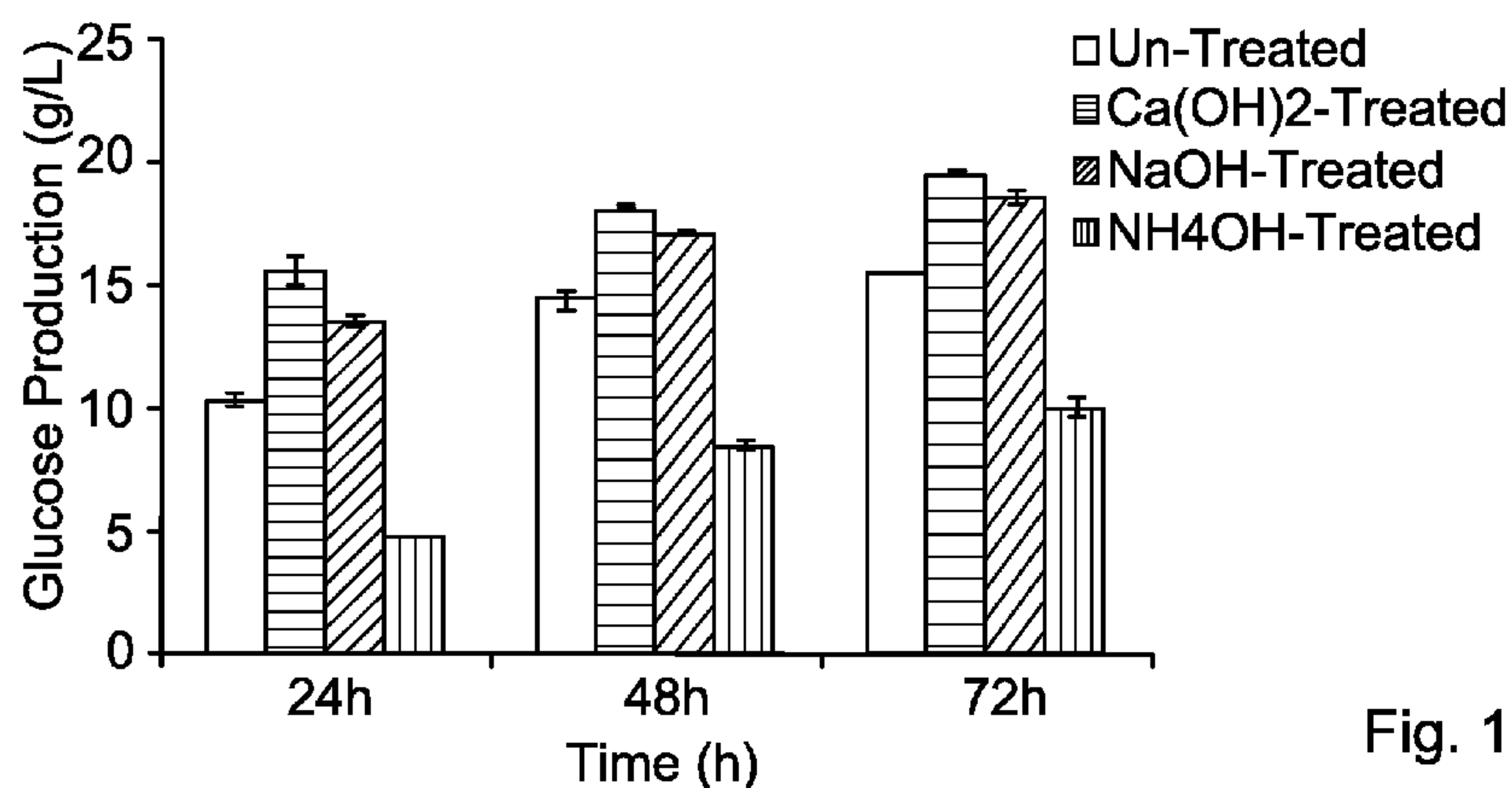


Fig. 1b

(57) Abstract: The present invention relates to a method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of: a) pretreating the lignocellulosic material to obtain a slurry having a pH of less than 6; b) adding NaOH, Ca(OH)₂ and/or CaO to the slurry to increase its pH to at least 8, said addition being carried out at a slurry temperature of at least 60 °C; c) reducing the pH of the slurry to below 7; and optionally cooling the slurry from step b) to a temperature below 60 °C; and d) adding hydrolytic enzymes to the slurry from c) and allowing the slurry to hydrolyze wherein no washing of the slurry is performed prior to step d)

METHODS FOR IMPROVEMENT OF ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC MATERIAL

Technical field

The present invention relates to improvements of the enzymatic hydrolysis in saccharification of a pretreated lignocellulosic material. The method provides sugars which are useful as substrates in the manufacture of various target compounds. The method is *inter alia* useful in the manufacture of a fermentation product, such as ethanol, from the lignocellulosic material.

Background

Biorefineries producing commodities from renewable resources offer an alternative to oil refineries based on dwindling supplies of petroleum and permit a move towards improved energy security. Lignocellulosic materials from forestry and agriculture are attractive as feedstocks, since they are abundant, relatively inexpensive, and are not used for food. Lignocellulose consists mainly of lignin and two classes of polysaccharides, cellulose and hemicellulose. The polysaccharides can be hydrolyzed to sugars and converted to various fermentation products, such as bioalcohols, in processes based on biocatalysts, such as the industrially important baker's yeast (*Saccharomyces cerevisiae*).

The hydrolysis of cellulose may be preceded by a pretreatment, in which the hemicellulose is degraded and the cellulose is made increasingly accessible to cellulolytic enzymes or acidic hydrolysis, see e.g. Alvira et al and Harmsen et al.

By using enzymatic hydrolysis, hydrolysis and fermentation can be performed simultaneously in a simultaneous saccharification and fermentation (SSF) process or in a consolidated bioprocess (CBP). Alternatively, separate hydrolysis and fermentation (SHF) can be used, a process configuration that may also include enzyme-based hydrolysis of the cellulose.

Summary

In order to obtain high yields of sugars from lignocellulosic substrates, the inventors consider dilute acid hydrolysis pretreatment and/or steam pretreatment with acid catalysts to be appropriate pretreatment methods. Furthermore, the inventors have realized that in industrial processes for converting lignocellulosic biomass to fermentation products, such as cellulosic ethanol, the whole slurry obtained after pretreatment will probably be used at a high solids concentration. However, the pre-treatment liquid is known to inhibit enzymatic hydrolysis. Previously, addition of surfactants has been considered for improving enzymatic saccharification of cellulosic substrates. Surfactants probably prevent unproductive binding of enzymes to complex lignocellulosic substrates, such as pretreated wood. The economical benefit of adding surfactants to reaction mixtures intended for production of yield-sensitive low value-added products such as liquid biofuels has, however, been questioned.

The addition of enzymes constitutes a considerable part of the total cost for the process of producing products from lignocellulosic material. The cost for enzymes is for instance regarded as one of the main obstacles for industrial implementation for conversion of lignocellulose to liquid biofuels. It would therefore be desirable to improve the efficiency of the enzymatic hydrolysis of lignocellulosic materials, e.g. to obtain more sugars from a certain enzyme dosage and time period, or to obtain the same amount of sugars from a lower enzyme dosage for the same time period. It is also desirable to achieve a certain amount of sugars with a certain enzyme dosage in a shorter time period, since this increases the production capacity and thereby allows for improved production and/or decreased costs of investment. Improving the efficiency of the enzymatic hydrolysis of cellulose may significantly contribute to commercialization of products based on lignocellulose-derived sugars.

It is an object of some aspects of the invention to improve the efficiency or production capacity of enzymatic hydrolysis of lignocellulosic materials. For this and other objects that will be evident to a person skilled in

the art from the following disclosure, the present invention provides according to a first aspect a method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of:

- 5
- a) pretreating the lignocellulosic material to obtain a slurry having a pH of less than 6,
 - b) adding NaOH, $\text{Ca}(\text{OH})_2$ and/or CaO to the slurry to increase its pH to at least 8, said addition being carried out at a slurry temperature of at least 60 °C;
 - c) reducing the pH of the slurry to below 7; and optionally cooling the
10 slurry from step b) to a temperature below 60 °C and
 - d) adding hydrolytic enzymes to the slurry from c) and allowing the slurry to hydrolyze.

The present invention further provides according to a second aspect a
15 method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of:

- 20
- a) pretreating the lignocellulosic material using SO_2 and/or sulfurous acid to obtain a slurry having a pH of less than 5;
 - b) adding $\text{Ca}(\text{OH})_2$ and/or CaO to the slurry to increase its pH to at least 8;
 - c) reducing the pH of the slurry from step b) to below 7; and
 - d) adding hydrolytic enzymes to the slurry from c) and allowing the slurry to hydrolyze.

25 According to a third aspect the present invention relates to a method of producing at least one target molecule comprising the steps of:

- 30
- a) pretreating a lignocellulosic material to obtain a slurry having a pH of less than 6;
 - b) adding Na(OH), $\text{Ca}(\text{OH})_2$ and/or CaO to the slurry to increase its pH to at least 8;
 - c) reducing the pH of the slurry to below 7;
 - d) adding hydrolytic enzymes to the slurry from c) and subjecting the slurry to enzymatic hydrolysis to obtain an at least partly hydrolyzed slurry.

e) utilizing the at least partly hydrolyzed slurry from step d) as a substrate in a fermentation process for production of at least one fermentation product; wherein the enzymatic hydrolysis in step d) and the fermentation process in step e) is performed separately in a separate hydrolysis and fermentation process.

In some embodiments of each of the above methods, no washing of the slurry is performed prior to the respective step d).

In a fourth aspect the present invention further provides a novel use of NaOH, Ca(OH)_2 or CaO for improving enzymatic hydrolysis of a lignocellulose-derived slurry derived from dilute acid pretreatment.

Brief description of the figures

15 Figure 1a:

The Y-axis shows the glucose production (g/l) during 72 h of enzymatic hydrolysis of alkali-treated and untreated spruce slurry. The treatment conditions were: NH_4OH , pH 9, 55 °C, 3 h; NaOH, pH 9, 80 °C, 3 h; Ca(OH)_2 , pH 11, 30 °C, 3 h. The error bars show the standard deviations of four measurements.

20

Figure 1b:

The Y-axis shows glucose production (g/l) during 72 h of enzymatic hydrolysis of cellulose (Avicel) in a l kali -treated and untreated pretreatment liquid. The treatment conditions were: NH_4OH , pH 9, 55 °C, 3 h; NaOH, pH 9, 80 °C, 3 h; Ca(OH)_2 , pH 11, 30 °C, 3 h. Error bars show the standard deviations of four measurements.

25

Figure 1c:

The Y-axis shows glucose production (g/l) during 72 h of enzymatic hydrolysis of alkali-treated and untreated spruce slurry. The treatment conditions were: NH_4OH , pH 9, 80 °C, 3 h; NaOH, pH 9, 80 °C, 3 h; Ca(OH)_2 , pH 9, 80 °C, 3 h. Error bars show the standard deviations of four measurements.

30

Detailed description

The present invention is based on the finding that enzymatic hydrolysis of cellulosic substrates in the presence of the liquid fraction obtained after pretreatment of a lignocellulose material can be enhanced by certain alkaline treatments. This approach differs from using alkaline treatment to achieve improved fermentability of lignocellulose hydrolysates (described in for example Alriksson et al 2005 and Alriksson et al 2006) since the target in the latter case is to alleviate the effect of inhibitors on the fermenting microorganism and not, as disclosed herein, on improving the enzymatic hydrolysis. Therefore, according to a first aspect, the invention relates to a method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of:

- a) pretreating the lignocellulosic material to obtain a slurry having a pH of less than 6,
- b) adding Na(OH), Ca(OH)₂ and/or CaO to the slurry to increase its pH to at least 8, said addition being carried out at a slurry temperature of at least 60 °C;
- c) reducing the pH of the slurry to below 7; and optionally cooling the slurry from step b) to a temperature below 60 °C and
- d) adding hydrolytic enzymes to the slurry from step c) and allowing the slurry to hydrolyze.

In step a), the lignocellulosic material is subjected to a pretreatment, in which the hemicellulose is degraded and the cellulose is made increasingly accessible to cellulolytic enzymes. The pretreatment may involve one or several pretreatment methods known to the skilled person. As an example, the pretreatment may be performed at elevated temperature with acid, typically dilute mineral acid, such as sulfuric acid. The pre-treatment may involve impregnation, which refers to impregnation of the cellulosic material with an impregnation fluid, followed by heating. In the case of acid pretreatment, the impregnation fluid may be an acid solution, such as a

mineral acid solution. The impregnation may also be performed with a gas, such as a SO_2 gas or CO_2 gas, or with the combination of a gas with a liquid to obtain e.g. sulfurous acid or carbonic acid. The elevated temperature may be achieved by steaming, a process used to drive air out from the cellulosic biomass to facilitate hydrolysis of the cellulose. Steaming is a well-known method for pretreating e.g. lignocellulosic biomass. As another example, the pretreatment may involve steam explosion, a process that combines steam, hydrolysis and rapid pressure releases for rupturing cellulosic fibers. However, the method of enzymatic hydrolysis according to the invention is especially suitable on lignocellulosic material subjected to dilute acid pretreatment since the effects of the alkaline treatment is most pronounced in this case. Therefore in one preferred embodiment the pretreatment in step a) is dilute acid pretreatment. The pH of the slurry obtained in step a) will depend on the pretreatment. Anyway, it is less than 6 according to the first aspect. In one embodiment, the pH of the slurry obtained in step a) is less than 5, such as less than 4, such as less than 3, such as less than 2. If the pretreatment is dilute acid pretreatment the pH of the slurry obtained in step a) would typically be in the range of 1 to 3. Therefore, in one embodiment the pH of the slurry obtained in step a) is in the range of pH 1.0 to 3.0.

In step b) $\text{Na}(\text{OH})$, $\text{Ca}(\text{OH})_2$ and/or CaO is added to the slurry to increase its pH to at least 8. An increase of the pH to at least 8.0 is sufficient to improve the enzymatic hydrolysis. The hydrolysis may however be even further improved if $\text{Na}(\text{OH})$, $\text{Ca}(\text{OH})_2$ and/or CaO is added to the slurry such that the pH of the slurry is increased further, such as for example to pH 9.0. Therefore, in one embodiment the pH of the slurry in step b) is increased to at least 8.5 preferably to at least 9.0. In one embodiment the pH of the slurry in step b) is increased to at least 8.0 but not above pH 12.0.

The present inventors have further demonstrated that, by having a relatively high temperature of the slurry in step b) prior to addition of $\text{Na}(\text{OH})$, $\text{Ca}(\text{OH})_2$ and/or CaO , a smaller increase in pH is needed compared to the case when the slurry temperature is below 60 °C. Thereby less $\text{Na}(\text{OH})$, $\text{Ca}(\text{OH})_2$ and/or CaO can be used in step b) which makes the method cheaper. If the slurry temperature is further increased the amount of added

Na(OH), Ca(OH)_2 and/or CaO can be even further reduced without reducing the efficiency of the hydrolysis. Therefore, in a preferred embodiment the addition of Na(OH), Ca(OH)_2 and/or CaO in step b) is being carried out at a slurry temperature of at least 70 °C or more preferably at a temperature of at least 80 °C. It may be easier to control the alkali detoxification process if the temperature is below the boiling point of the slurry. Thus, in one embodiment the addition of Na(OH), Ca(OH)_2 and/or CaO in step b) is being carried out at a slurry temperature in the range of 60 to 100 °C, such as 70 to 100 °C, such as 80 to 100 °C or 70 to 90 °C.

In step c) the temperature and pH is adjusted so that the conditions are suitable for the hydrolytic enzymes. Therefore the temperature is reduced below 60 °C and the pH is reduced below 7.0. The optimum pH and temperature differs between different enzymes. However, for some hydrolytic enzymes the optimal temperature is in the range of 40-60 °C and the optimal pH in a range of 3.0 to 7.0. For example the optimal temperature of the commercially available hydrolytic enzyme Cellic CTec2 is 45 to 50 °C and the optimal pH is about 5.0 to 5.5. Therefore, in a preferred embodiment, step c) comprises cooling the slurry from step b) to a temperature between 40 and 60 °C such as between 45 to 55 °C., for example 45 to 50 °C. In another preferred embodiment step c) comprises reducing the pH of the slurry to a pH in a range of 3.0 to 7.0 such as 4 to 6.5, for example 5.0 to 5.5.

Step d) involves addition of hydrolytic enzymes to the slurry from step c) and allowing the slurry to hydrolyze.

The hydrolytic enzymes may include at least one saccharification enzyme, which refers to at least one enzyme that can convert or hydrolyze cellulosic biomass into fermentable saccharides, such as monosaccharides and/or disaccharides and/or oligosaccharides. Such saccharification enzymes may be glycosidases, which hydrolyze polysaccharides. Examples of glycosidases include cellulose-hydrolyzing glycosidases, such as cellulases, endoglucanases, exoglucanases, cellobiohydrolases and β -glucosidases, hemicellulose hydrolyzing glycosidases, such as xylanases, endoxylanases, exoxylanases, β -xylosidases, arabinoxylanases, mannanases, galactanases,

pectinases and glucuronases, and starch hydrolyzing glycosidases, such as amylases, α -amylases, β -amylases, glucoamylases, α -glucosidases and isoamylases, or any enzymes in the group of enzymes found in EC 3.2.1.x, such as EC 3.2.1.4, where EC is the Enzyme Commission number.

5 It is surprising that the enzymatic hydrolysis of cellulosic substrates in the presence of the liquid fraction obtained after pretreatment of a lignocellulose material can be enhanced by alkaline treatment. The underlying mechanism for this effect is not known at present. The inventors have also surprisingly discovered that only some bases seem to be suitable for the
10 alkaline treatment. For example the inventors have demonstrated that conditioning with Na(OH) and Ca(OH)₂ increased the saccharification yield with about 20% while conditioning with ammonium hydroxide did not result in any improvement of the saccharification yield.

As well known by the skilled person, CaO reacts with water to form
15 Ca(OH)₂. Ca(OH)₂ and CaO are particular suitable bases for the alkaline treatment since they are relatively cheap. One problem with using CaO or Ca(OH)₂ in the alkaline treatment is that the calcium ions derived from the calcium based base easily react with sulfate ions from the sulfuric acid from the pre-treatment liquid. This leads to formation of gypsum which could cause
20 deposits and clog the equipment. The present inventors have realized that, in contrast to sulfuric acid, SO₂ or sulfurous acid does not give rise to substantial levels of sulfate ions and thus pre-treatment with SO₂ or sulfurous acid followed by alkaline treatment with Ca(OH)₂ and/or CaO is particularly suitable according to the present invention.

25 Accordingly, a second aspect of the invention relates to a method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of:

- a) pretreating the lignocellulosic material using SO₂ and/or sulfurous acid to obtain a slurry having a pH of less than 5;
- b) adding Ca(OH)₂ and/or CaO to the slurry to increase its pH to at
30 least 8;
- c) reducing the pH of the slurry from step b) to below 7; and
- d) adding hydrolytic enzymes to the slurry from c) and allowing the slurry to hydrolyze.

The embodiments of the first aspect apply to the second aspect *mutatis mutandis*.

According to one embodiment the method is for production of at least one target molecule from the lignocellulosic material and further comprises a
5 step e) of utilizing the at least partly hydrolyzed slurry from step d) as a substrate for production of at least one target molecule. According to one embodiment the at least one target molecule is a fermentation product, and the step e) of utilizing the at least partly hydrolyzed slurry comprises subjecting the at least partly hydrolyzed slurry to fermentation.

10 In one embodiment, the fermentation is a simultaneous saccharification and fermentation (SSF) of a pretreated lignocellulosic material. A SSF process refers to a process in which enzymatic hydrolysis and fermentation is performed simultaneously in a fermentor. Thus, in a SSF process, fermentable saccharides are prepared directly in a fermentor by
15 enzymatic hydrolysis of the pretreated lignocellulosic material, and the resulting saccharides are converted into a fermentation product. Further, the fermentation may be a consolidated bioprocess (CBP), in which the biocatalyst that convert the monosaccharides also produces the enzymes that hydrolyze the pretreated lignocellulosic material. In another embodiment, the
20 hydrolysate that is subjected to fermentation is obtained from a separate, preceding step of enzymatic hydrolysis. Consequently, the enzymatic hydrolysis and the fermentation may be performed as two separate process steps (separate hydrolysis and fermentation, SHF). This may e.g. be advantageous if the fermentation reaction and the enzymatic reaction have
25 different optimal temperatures. As an example, the temperature during enzymatic hydrolysis may be kept higher than the temperature during fermentation, thus facilitating the use of thermophilic enzymes.

In one embodiment fermentation is performed by a fermenting organism and the fermenting organism can for example be bacteria and/or
30 yeast. In one embodiment the fermenting organism is yeasts from the genera *Saccharomyces*, *Pichia* or *Candida*. . In one embodiment the fermenting organism is bacteria from the genera *Zymomonas* or *Escherichia*. Preferably, the fermenting organism may be wild type, mutant or recombinant

Saccharomyces cerevisiae. Using *S. cerevisiae* for producing a fermentation product is advantageous since *S. cerevisiae* is well established with regard to industrial fermentation and provides for a high product yield. According to one embodiment the fermentation product is selected from alcohols, acids, alkanes, alkenes, aromatics, aldehydes, ketones, biopolymers, proteins, peptides, amino acids or vitamins. In one embodiment the fermentation product is selected from ethanol, butanol, acetic acid, butyric acid and succinic acid.

Besides of fermenting the sugars, the sugars can also be utilized as substrates in various other chemical and biochemical (e.g. enzymatic) methods for production of desired target compounds. By way of example, the sugars can be used in a thermochemical process to produce levulinic acid, which in turn is an intermediate in the synthesis of polymers, plastics and pharmaceuticals. It is also a precursor in the industrial production of other chemical commodities such as methyltetrahydrofuran, valerolactone, and ethyl levulinate. Therefore, according to one embodiment the at least one target molecule is levulinic acid.

As discussed above several different hydrolytic enzymes can be used in the methods according to the invention. According to one embodiment the addition of hydrolytic enzyme in step d) comprises the addition of at least one glycosidases such as a cellulose-hydrolyzing glycosidase, hemicellulose hydrolyzing glycosidase and/or a starch hydrolyzing glycosidase. In one embodiment at least one of the hydrolytic enzymes added in step d) is a cellulase, endoglucanase, exoglucanase, cellobiohydrolase, β -glucosidase, xylanase, endoxylanase, exoxylanase, β -xylosidase, arabinoxylanase, mannanase, galactanase, pectinase, glucuronase, amylase, α -amylase, β -amylase, glucoamylase, α -glucosidase, isoamylase, and/or any enzymes in the group of enzymes found in EC 3.2.1.x, such as EC 3.2.1.4, where EC is the Enzyme Commission number. In one embodiment at least one enzyme originates from a filamentous fungus, such as *Hypocrea jecorina*.

In one embodiment at least one endoglucanase, at least one exoglucanase and at least one β -glucosidase is added in step d). For

example a combination of the commercial enzyme preparations Celluclast 1.5 L and Novozyme 188 can be added in step d)."

The method according to the invention is directed to treatment of
5 lignocellulosic materials. The term lignocellulosic material includes material comprising cellulose, lignin and possibly hemicellulose. The lignocellulose material may for example be a forestry residue, such as wood (e.g. wood chips), sawmill or paper mill by products or an agricultural residue, e.g. corn stover/cobs, sugarcane bagass or wheat straw. Depending on the
10 geographical location, wood, corn stover/ cobs, wheat straw or sugarcane bagass may be available in large quantities, making them attractive as raw materials.

In one embodiment the dry matter content of the slurry in step a) is in the range of from 5 to 40 % (w/v), such as from 8 to 30 % (w/v), such as from
15 12 to 20 % (w/v).

In the prior art washing of the slurry with water, which may comprise a chemical, has been described. For example Pan et al. describes that the enzymatic hydrolysis of a pretreated softwood with high residual lignin content can be enhanced by mild alkali extraction. This strategy is different from the
20 concept of the present invention since the purpose is to remove lignin. Importantly this method described in Pan et al. is dependent of an extraction of the lignin and it is described that the pretreated material is washed extensively with water subsequent to the alkaline extraction. The present invention does not relate to decreasing the lignin content of a pretreated
25 lignocellulosic biomass. Rather, the present invention is based on the surprising discovery that alkaline treatment of the pretreated slurry has a detoxifying effect on the enzymatic hydrolysis. Therefore the present invention does need a washing or extraction stage to remove lignin. In fact, such a washing is undesired since it may remove water soluble fermentable
30 saccharides from the slurry and thus decrease the overall yield in the process. Also, such a washing may increase the fresh water consumption in the method. Thus, in one embodiment no washing of the slurry is performed prior to step d).

For the same reason as described above, in the method according to the present invention, no separation of pre-treatment liquid from the solids is necessary prior to the hydrolysis step. Thus in one embodiment at least 50 %, preferably at least 90 % or 95 % of the liquid in the pretreated slurry from step a) is present during the enzymatic hydrolysis step d). In one embodiment, 100 % of the liquid is present. The percentage of liquid may, depending on the circumstances, be calculated by weight or by volume.

Other documents in the prior art, such as WO2005/099854, discloses acidic pre-treatment of a lignocellulosic feedstock followed by an adjustment of the pH to 4.0-6.0. Since the pH is not raised to at least 8, the pH adjustment described in WO2005/099854 will not be sufficient to have a detoxifying effect on the enzymatic hydrolysis.

A third aspect of the invention relates to a method of producing at least one target molecule comprising the steps of:

- a) pretreating a lignocellulosic material to obtain a slurry having a pH of less than 6;
- b) adding Na(OH), Ca(OH)₂ and/or CaO to the slurry to increase its pH to at least 8;
- c) reducing the pH of the slurry to below 7;
- d) adding hydrolytic enzymes to the slurry from c) and subjecting the slurry to enzymatic hydrolysis to obtain an at least partly hydrolyzed slurry; and
- e) utilizing the at least partly hydrolyzed slurry from step d) as a substrate in a fermentation process for production of at least one fermentation product,

wherein the enzymatic hydrolysis in step d) and the fermentation process in step e) are performed separately in a separate hydrolysis and fermentation (SHF) process.

A fourth aspect of the present invention relates to use of NaOH, Ca(OH)₂ or CaO for improving the enzymatic hydrolysis of a lignocellulose-derived slurry derived from dilute acid pretreatment. In one embodiment the dilute acid pretreatment is a pretreatment using SO₂ and/or sulfurous acid.

The embodiments of the first and second aspects apply to the third and fourth aspects *mutatis mutandis*.

Examples

5 Pretreatment of unbarked wood chips of Norway spruce (*Picea abies*) was performed in continuous mode in a 30-litre reactor. Sulfur dioxide was used as catalyst (one kg of sulfur dioxide per 40 kg of wood chips). The pretreatment was carried out at 203°C for 5 min. After the pretreatment, the pH of the slurry was 2, and the dry matter content was 13.8% (w/w). The
10 pretreated material was stored at 4°C until further use.

 The pretreatment liquid, i.e. the liquid fraction of the pretreated spruce slurry, was obtained by filtration. The content of monosaccharides, aliphatic acids, and furan aldehydes in the pretreatment liquid were determined by using high-performance liquid chromatography (HPLC) (MoRe Research,
15 Örnsköldsvik, Sweden) and the concentrations were: 26.5 g/l mannose, 29.0 g/l glucose, 12.5 g/l xylose, 5.8 g/l galactose, 3.1 g/l arabinose, 5.8 g/l acetic acid, <0.1 g/l formic acid, 1.3 g/l levulinic acid, 2.3 g/l 5-hydroxymethylfurfural (HMF), and 1.8 g/l furfural.

 The total concentration of phenols in the pretreatment liquid was
20 estimated to 6.6 g/l by using a spectrophotometric method (Singleton et al. 1999) based on the Folin and Ciocalteu reagent (Sigma-Aldrich, St Louis, MO, USA). Vanillin was used as the standard. Before phenol determinations, the pH of the samples was adjusted to 5.2 using 37% HCl or a 5 M solution of NaOH.

25

Alkaline treatment

 Prior to alkaline treatment, portions (72.5 g) of the slurry of pretreated spruce [13.8% (w/w) dry weight] were diluted with deionized water (27.5 g) to achieve a dry-matter content of 10.0% (w/w). The pretreatment liquid was
30 treated with alkali without any cellulosic substrate being present. The spruce

slurry and the pretreatment liquid were treated with NH_4OH , NaOH , and $\text{Ca}(\text{OH})_2$ as summarized in Table 1.

In a first set of experiments the treatment conditions were pH 9 and 55°C for the NH_4OH treatment, pH 9 and 80°C for the NaOH treatment, and
5 pH 11 and 30°C for the $\text{Ca}(\text{OH})_2$ treatment (Table 1). The samples were kept under alkaline conditions for 3 h with magnetic stirring. After completion of the alkaline treatment, the pH of the samples was adjusted to 5.2 using HCl (37%). For comparison, deionized water was added to untreated slurry and pretreatment liquid samples so that the dilution was the same as for the alkali-
10 treated samples. The pH of the untreated slurry and the pretreatment liquid was adjusted to 5.2 using the 5 M NaOH solution. After dilutions with water, alkali treatments and pH adjustments, the content of slurry was 69% (w/w) and the dry-matter content was 9.5% (w/w), while the content of pretreatment liquid was 94% (w/w).

15 In a second set of experiments performed with the pretreated spruce slurry, all alkaline treatments were carried out at pH 9 and 80°C (Table 1). Dilutions and pH adjustments were performed as in the first set of experiments.

20 *Enzymatic hydrolysis*

Hydrolysis experiments with the pretreatment liquid were performed by adding a cellulosic substrate, Avicel PH 101 (a preparation of microcrystalline cellulose purchased from Sigma-Aldrich). Avicel was added to the pretreatment liquid after the pH had been adjusted to 5.2, as described
25 above.

Two commercial enzyme preparations were used in the hydrolysis experiments, the *Trichoderma reesei* (*Hypocrea jecorina*) cellulase preparation Celluclast 1.5 L and Novozyme 188. Novozyme 188 was added to assure that sufficient amounts of β -glucosidase (cellobiase) were present in
30 the reaction mixtures. The enzyme preparations were purchased from Sigma-Aldrich and the stated activities were: Celluclast 1.5 L, 700 endoglucanase units (EGU)/g; Novozyme 188, 250 cellobiase units (CBU)/g.

Enzymatic hydrolysis experiments were conducted in 100-ml E-flasks containing reaction mixtures with a total mass of 25 g. The reaction mixtures with slurry consisted of 24.5 g alkali-treated or untreated slurry, pH 5.2, and 0.5 g of an enzyme cocktail consisting of equal amounts (w/w) of Celluclast 5 1.5 L and Novozyme 188. The content of slurry in the final reaction mixtures was 68% (w/w), the dry matter content was 9.3% (w/w), and the content of enzyme cocktail was 2% (w/w). The reaction mixtures with pretreatment liquid consisted of 22 g alkali-treated or untreated pretreatment liquid, pH 5.2, 2.5 g of Avicel, and 0.5 g of the enzyme cocktail. The content of pretreatment liquid 10 in the final reaction mixtures was 83% (w/w) and the content of enzyme cocktail was 2% (w/w). Duplicate experiments were performed for each hydrolysis reaction.

The E-flasks, which were covered with parafilm and aluminum foil, were incubated for 72 h at 45°C in an orbital shaker (Ecotron incubator 15 shaker, Infors, Bottmingen, Switzerland) set at 170 rpm. During the hydrolysis, 100 µl samples were collected, typically after 0, 24, 48, and 72 h. In the beginning and at the end of the hydrolysis experiments (i.e at 0 and 72 h), 1 ml samples were also taken. The samples were chilled on ice, and were then centrifuged at 14,100 g for 5 min. The supernatants were collected and used 20 for sugar analysis.

Glucose analysis

The glucose concentration of the samples that were collected during the hydrolysis experiments was determined using a glucometer (Glucometer 25 Elite XL, Bayer AG, Leverkusen, Germany). Each sample was analyzed twice. Analyses of the sugar content of samples collected at 0 and 72 h were also performed using ion chromatography (IC) and high-performance liquid chromatography (HPLC) (MoRe Research). The glucometer values were corrected using data obtained by chromatographic determination of glucose.

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Results

The effects of alkaline treatment on the enzymatic hydrolysis of two cellulosic substrates, pretreated spruce wood and Avicel, were investigated in

experiments with three different types of alkali; calcium hydroxide, sodium hydroxide, and ammonium hydroxide. The experimental series with pretreated wood and Avicel differ in the sense that Avicel was never exposed to alkaline conditions, and any improvements observed with Avicel must therefore be attributed to effects of alkali on the pretreatment liquid rather than on the solid phase. The experiments with spruce wood slurry indicate the effect of alkaline treatment on the saccharification of a realistic lignocellulosic substrate, which besides cellulose also contains other components, notably lignin.

10 The results obtained from the first set of experiments with spruce wood slurry and alkaline treatments are shown in Fig. 1a. After 72 h, conditioning with calcium hydroxide and sodium hydroxide increased the saccharification yield with 17 and 20%, respectively. Conditioning with ammonium hydroxide did, however, not result in any improvement. When the same treatment conditions were used for pretreatment liquid that was subsequently mixed with Avicel (Fig. 1b), calcium hydroxide and sodium hydroxide again gave improved saccharification yield (26 and 20% improvement after 72 h for calcium hydroxide and sodium hydroxide, respectively).

20 In a second set of experiments with the spruce wood slurry, all alkaline treatments were carried out in the same way, pH 9 and 80°C, (Fig. 1c). After 72 h, calcium hydroxide and sodium hydroxide treatments resulted in improvements of the saccharification yield amounting to 17% and 25%, respectively. It has thus been shown that an increased temperature may compensate for a decreased pH. Accordingly, lower amounts of base may be added if the process is performed at a higher temperature. As the pretreatment is normally performed at an elevated temperature, such as about 200 °C, no extra heating step will normally be required for reaching the appropriate temperature in the industrial setting. The saccharification yield obtained with ammonium hydroxide treatment was comparable to that of the untreated sample (Fig. 1c). In summary, treatments with calcium hydroxide or sodium hydroxide resulted in improvements in the range 17-26%, while ammonium hydroxide treatment did not improve the situation. The ammonium

hydroxide treatment shown in Fig. 1c was more powerful than the treatments shown in Figs. 1 and 2 in the sense that the pH was the same while the temperature was higher (80°C rather than 55°C).

5 **Table 1.** Summary of alkaline treatments used in the experiments.

Sample No	Medium	Alkali	pH	Temperature	Time (h)
1	S ^a and P ^b	NH ₄ OH	9	55°C	3
2	S	NH ₄ OH	9	80°C	3
3	S and P	NaOH	9	80°C	3
4	S and P	Ca(OH) ₂	11	30°C	3
5	S	Ca(OH) ₂	9	80°C	3
6	S and P	Untreated	—	—	—

^aS, Spruce slurry; P^b, Pretreatment liquid

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CLAIMS

1. A method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of:

- 5 a) pretreating the lignocellulosic material to obtain a slurry having a pH of less than 6;
- b) adding NaOH, Ca(OH)₂ and/or CaO to the slurry to increase its pH to at least 8, said addition being carried out at a slurry temperature of at least 60 °C;
- 10 c) reducing the pH of the slurry to below 7 ; and
- d) adding hydrolytic enzymes to the slurry from step c) and allowing the slurry to hydrolyze,
- wherein no washing of the slurry is performed prior to step d).

- 15 2. The method of claim 1, wherein step c) additionally comprises cooling the slurry from step b) to a temperature below 60 °C.

3. A method of enzymatic hydrolysis of a lignocellulosic material, comprising the steps of:

- 20 a) pretreating the lignocellulosic material using SO₂ and/or sulfurous acid to obtain a slurry having a pH of less than 5;
- b) adding Ca(OH)₂ and/or CaO to the slurry to increase its pH to at least 8;
- c) reducing the pH of the slurry from step b) to below 7; and
- 25 d) adding hydrolytic enzymes to the slurry from c) and allowing the slurry to hydrolyze.
- wherein no washing of the slurry is performed prior to step d).

4. A method of producing at least one target molecule comprising the steps of:

- 30 a) pretreating a lignocellulosic material to obtain a slurry having a pH of less than 6;

- b) adding NaOH, $\text{Ca}(\text{OH})_2$ and/or CaO to the slurry to increase its pH to at least 8;
c) reducing the pH of the slurry to below 7;
d) adding hydrolytic enzymes to the slurry from c) and subjecting the
5 slurry to enzymatic hydrolysis to obtain an at least partly hydrolyzed slurry; and
e) utilizing the at least partly hydrolyzed slurry from step d) as a substrate in a fermentation process for production of at least one fermentation product,
10 wherein the enzymatic hydrolysis in step d) and the fermentation process in step e) are performed separately and wherein no washing of the slurry is performed prior to step d).

5. A method according to any one of claims 1 – 3 wherein said method is for
15 production of at least one target molecule from the lignocellulosic material and further comprising a step e) of utilizing the at least partly hydrolyzed slurry from step d) as a substrate for the production of at least one target molecule.

6. A method according to claim 5 wherein the at least one target molecule is a
20 fermentation product, and the step e) of utilizing the at least partly hydrolyzed slurry comprises subjecting the at least partly hydrolyzed slurry to fermentation.

7. A method according to claim 6 wherein the enzymatic hydrolysis in step d) and the fermentation in step e) are performed separately in a separate
25 hydrolysis and fermentation process.

8. A method according claim 6 wherein the enzymatic hydrolysis in step d) and the fermentation in step e) is performed simultaneously in a simultaneous saccharification and fermentation process
30

9. A method according to claim 4 or 6 – 8 wherein the fermentation is performed by a fermenting organism.

10. A method according to any one of claims 4 or 6 – 9 wherein the fermentation product is selected from alcohols, acids, alkanes, alkenes, aromatics, aldehydes, ketones, biopolymers, proteins, peptides, amino acids or vitamins.

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11. A method according to any one of claims 1 – 10 wherein the addition of hydrolytic enzymes in step d) comprises the addition of at least one glycosidase, such as a cellulose-hydrolyzing glycosidase, a hemicelluloses hydrolyzing glycosidase and/or a starch hydrolyzing glycosidase.

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12. A method according to any one of claims 1 – 11 wherein the lignocellulosic material is a wood material or an agricultural residue.

13. A method according to any one of claims 1 – 12 wherein the dry matter
15 content of the slurry in step a) is in the range of from 5 to 40 % (w/v), or from 8 to 30 % (w/v), or from 12 to 20 % (w/v).

14. A method according to any one of claims 1 – 13 wherein at least 90 % of
the liquid in the pretreated slurry from step a) is present during the enzymatic
20 hydrolysis step d).

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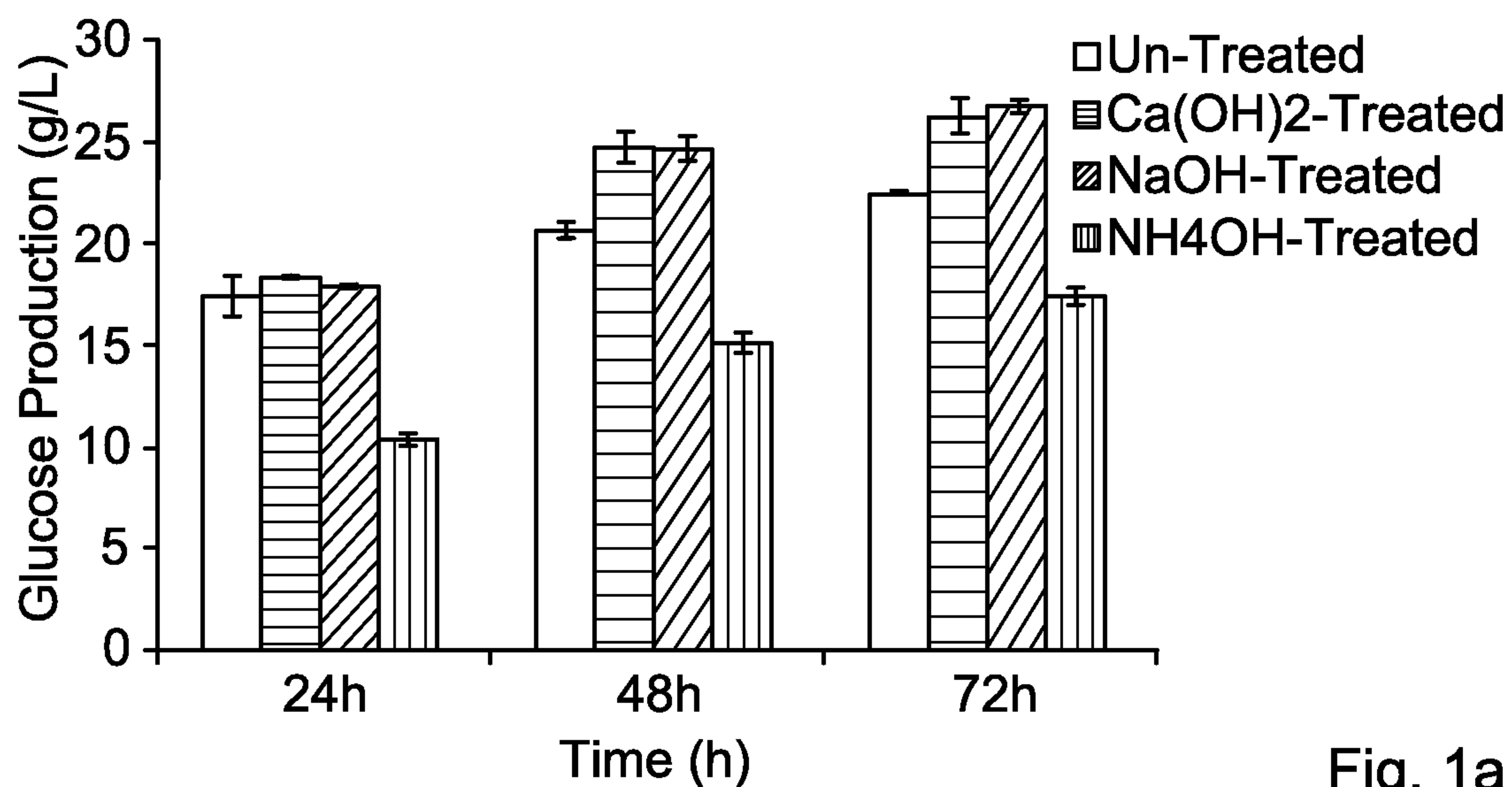


Fig. 1a

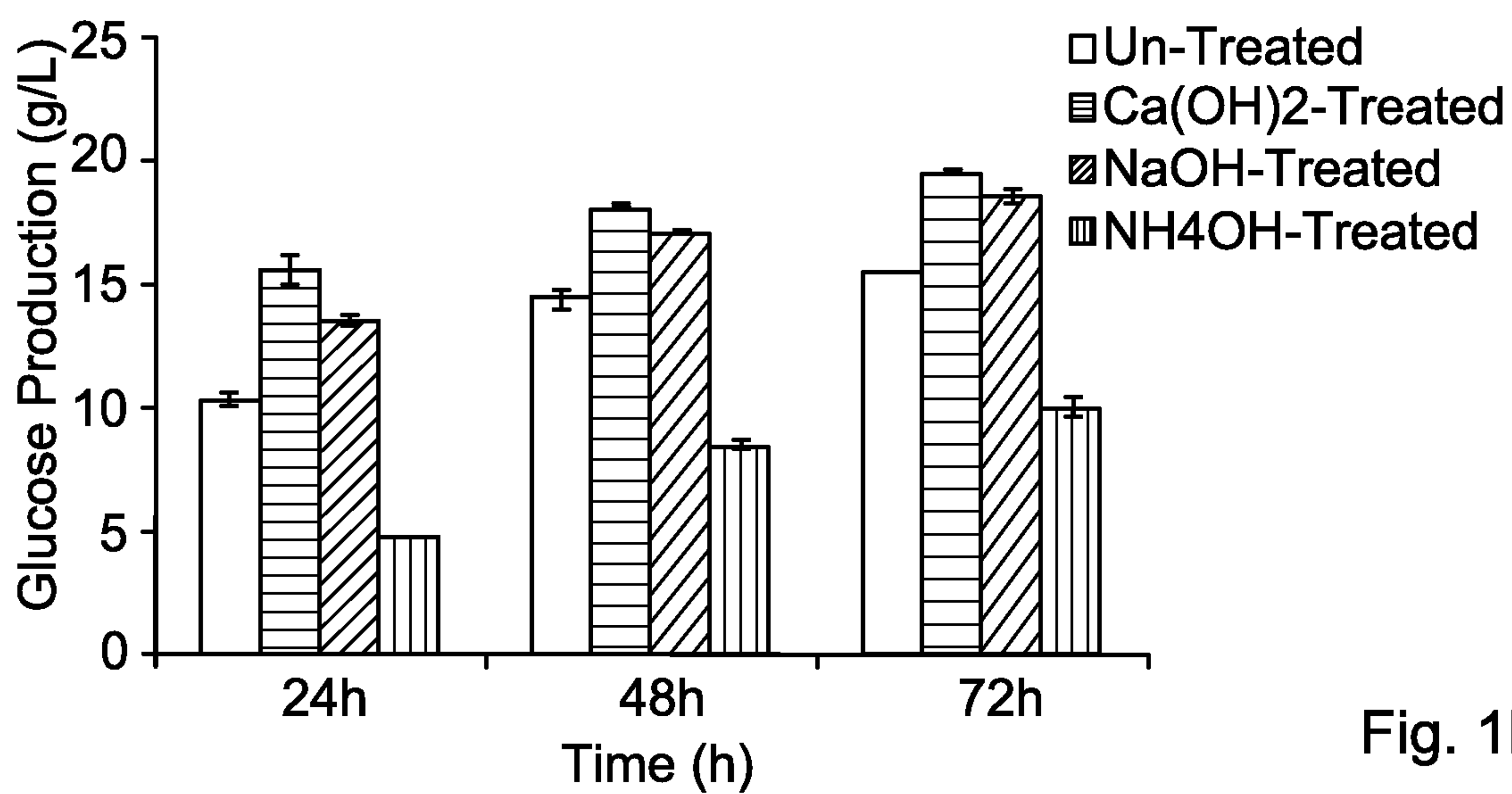


Fig. 1b

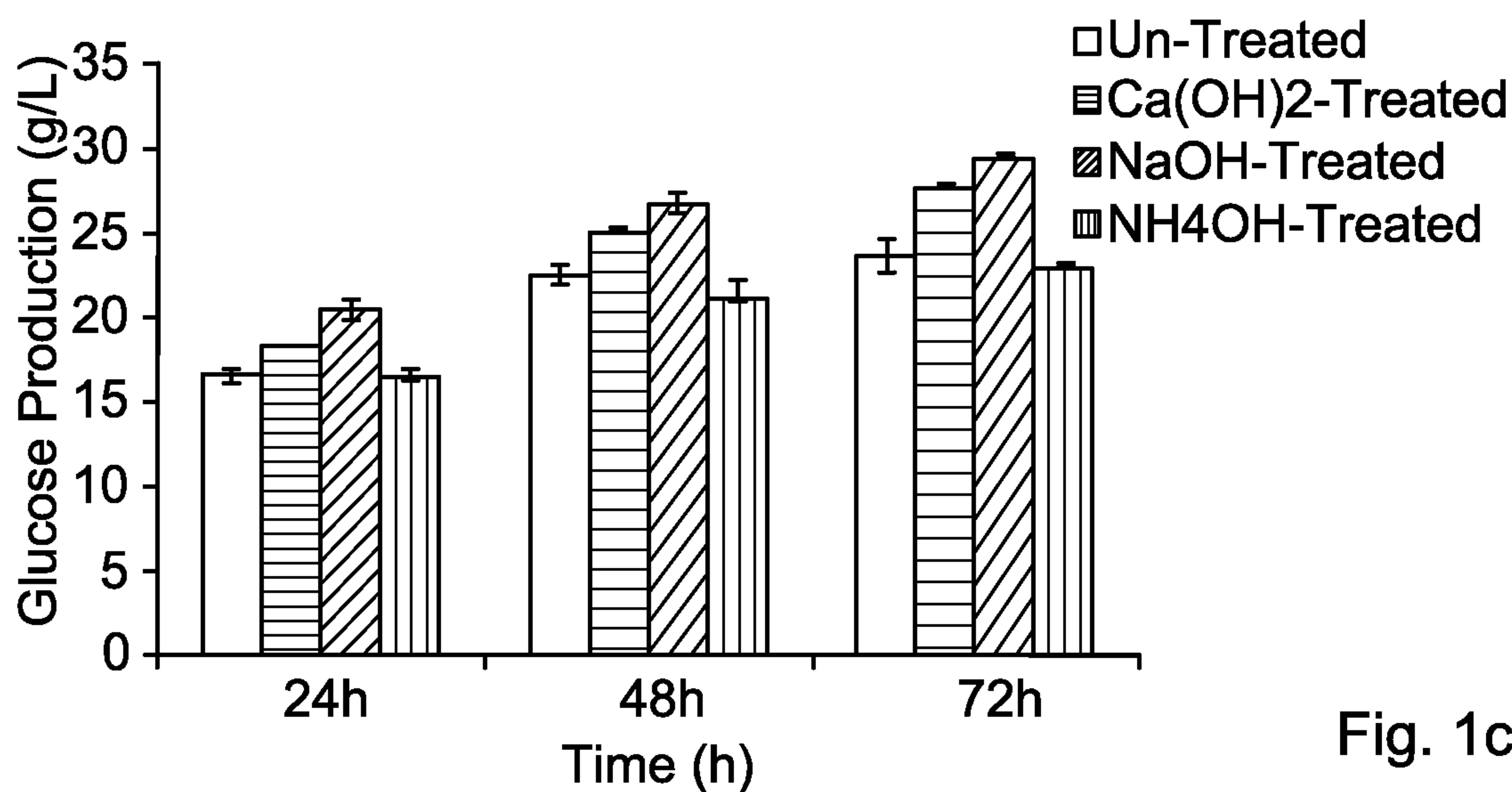


Fig. 1c

