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(54) **Title**: FERMENTATIVE PROCESS FOR LIGNOCELLULOSIC PLANT BIOMASS

(57) **Abstract**: The present invention refers to a fermentative process comprising a pre- treatment step and enzymatic hydrolysis of a lignocellulosic plant biomass, wherein this pre-treatment includes the use of steam, optionally in the presence of catalysts, an enzymatic pre-treatment and a fermented product from this process. Specifically, the plant biomass is cane bagasse and the product is ethanol.

Description

FERMENTATIVE PROCESS FOR LIGNOCELLULOSIC PLANT BIOMASS

Field of the Invention

The present invention refers to a fermentative process comprising pre-treatment steps of a lignocellulosic plant biomass, in which steam is used in the presence or absence of chemical and/or biochemical catalysts, an enzymatic hydrolysis of the aforesaid biomass and a fermentation of the hydrolyzed juice obtained by this enzymatic process. Specifically, the plant biomass is sugar cane bagasse and/or straw and the product is ethanol.

Background of the Invention

The possibility of producing ethanol from lignocellulosic materials has received a great deal of attention due to the high availability of these materials, especially in Brazil. There is also the fact that the method of obtaining ethanol is considered to be "green", when compared to the method employed in the production of fuels derived from petroleum, such as gasoline.

Cellulose is a linear polymer of D-glucose composed of β-1,4, glycosidic bonds with repeated units of cellobiose, forming a highly crystalline material that is insoluble in water. The degree of polymerization is located in the range of 7500-15000 molecules of glucose present in the cellulosic chain.

Cellulose is organized in fibers with a diameter of 2.0-4.0 nm. These fibers are associated with hydrogen bridges and van der Waals bonds, forming a rigid molecular structure (microfibrils), with diameters from 10 to 30 nm. The crystalline fraction is constituted from 50 to 90% of cellulose. In this fraction, the capillaries are small, which makes it exceedingly difficult for the penetration of the matrix by the enzymes (average size of 5 nm). Therefore, processes of enzymatic hydrolysis require a prior treatment (pre-treatment) of lignocellulosic biomass, seeking to "open" the cellulosic matrix to the action of the enzymes. The regions of low crystallinity (amorphous) existing in the microfibrils are

susceptible to enzymatic action, dispensing with the pre-treatment of the biomass.

Different from cellulose, hemicelluloses are heterogeneous polymers subdivided from different carbohydrates and linked by different chemical bonds. Several substitutes, e.g., acetyl groups and uronic acids are associated with the principal chain or with its respective subdivisions, in structures with a degree of polymerization varying from 20 to 300. Hemicelluloses do not present either the degree of crystallinity or the microfibril structure found in cellulose and they do not exercise effective influence over the structural properties of the plant tissue. In this manner, they present a greater susceptibility to acid and enzymatic hydrolysis, as well as higher solubility in aqueous-alkaline solutions. Generally, the term holocellulose is used to refer to the total saccharide fraction (cellulose and hemicelluloses) of the plant tissue free of extractives.

Hemicelluloses are associated with phenolic fraction (lignin) through covalent bonds and with cellulose via the hydrogen bond. Its composition varies according to the lignocellulosic material. Softwoods, such as *Pinus radiata*, contain a higher content of glucomannans, while hardwoods, such as birch, present a higher content of glucoxylans. The hemicelluloses of sugar cane bagasse are predominantly constituted of xylans, although they also present lower quantities of glucoxylans and arabinoxylans.

Together with cellulose, lignin is one of the most abundant organic polymers in the plant kingdom. Usually, lignin is seen as a "cement" or "incrusting substance" of the plant tissue, contributing significantly to the mechanical resistance of the tissue. On the other hand, while a relatively large quantity of microorganisms is capable of decomposing and converting cellulose and the hemicelluloses, only a significantly reduced number has the capability to decompose lignin effectively, which justifies the high resistance of the plants to deterioration.

Lignin is a complex tridimensional amorphous polymer, with a high molecular weight, generally associated with cellulose and hemicelluloses through ether and carbon-carbon bonds. Its chemical structure, in its natural

state, is highly aromatic, phenolic in character, and composed of units of phenyl-propane associated with the methoxyl and phenolic and aliphatic hydroxyl groups.

Due to the association of cellulose with the hemicelluloses and lignin, the access of several chemical agents (e.g. acids and alkalis) and biochemicals (e.g. enzymes and microorganisms) used in the production processes of ethanol from lignocellulosic biomasses by fermentation are significantly restricted.

Therefore, the need is evident to perform a prior treatment (pre-treatment) of the lignocellulosic biomass, seeking to remove non-cellulosic components, predominantly the hemicelluloses, in order to improve the accessibility of the enzymes to the cellulose.

The efficient conversion of the cellulose from the lignocellulosic material in fermentable sugars and subsequent fermentation of these in ethanol certainly represents an enormous challenge. Cellulose is the principal constituent of the lignocellulosic fibers and consists of a crystalline structure that is very resistant to breakage, as with hemicellulose, the second abundant component. The conversion of cellulose/hemicellulose to ethanol requires the separation of these components from the lignin and/or an increase in the access of the enzymes to these materials. In other words, it is necessary to provide a depolymerization of these components in free sugars for the subsequent fermentation of the sugars to ethanol.

Acid hydrolysis is a common method for the conversion of cellulose into glucose, which is a fermentable sugar. It generally involves the use of concentrated or diluted acids. This process produces moderate amounts of glucose, with problems of cost associated with the need to recuperate the acid and the use of special construction materials for the equipment and its corrosion.

The state of the art has already described several processes for the conversion of lignocellulosic raw material into glucose. The documents US 4,461,648 and US 7,198,925 describe methods of pre-treatment with a steam

explosion using diluted acid. In these documents, the biomass is positioned in the reactor ("steam gun") and acid is added to the biomass. Next, steam is rapidly injected into the tank which remains under high pressure for a determinate period of time. Finally, the tank is suddenly depressurized, making the biomass "explode". This biomass can then be submitted to the hydrolytic action of enzymes (particularly cellulases), which convert cellulose into glucose and other sugars.

The present invention differs fundamentally from the abovementioned documents because it includes the pre-treatment of the lignocellulosic biomass with steam until the final step of fermentation of the sugars available in the enzymatic hydrolysis step, particularly the glucose from the cellulose. Additionally, the present invention also embodies the use of a "pool" of enzymes (hemicellulases and cellulases) in the process of availability of the sugars, particularly glucose, in SHF (Separated Hydrolysis and Fermentation) and SSF (Simultaneous Saccharification and Fermentation) systems. The present invention specifically includes pre-treatments using non–catalytic and auto-catalyzed conditions, as well as catalytic systems using acids, metal salts (e.g. Lewis acids), alkalines (e.g. calcium salts) and neutral or weak acids (e.g. carbonates) in oxidative or non-oxidative systems. It also includes the enzymatic treatment of the residual hemicelluloses present in the pre-treated biomass by the physical-chemical processes of pre-treatment with steam. Such a sequence configures a pre-treatment in two steps, the first physical-chemical and the second enzymatic.

Document US 6,423,145 describes a production process of cellulose that comprises a step of hydrolysis of a lignocellulosic material in a steam explosion reactor (continuous or discontinuous) in the presence of a mixture of catalysts. The catalysts are diluted inorganic acids (e.g. H_2SO_4, SO_2, HCl and HNO_3) and metal salts of *acidic character*, selected from ferrous sulfate, ferric sulfate, ferric chloride, aluminum sulfate, aluminum chloride and magnesium sulfate.

In the present invention, the pre-treatment step employs not only inorganic acids and acid metal salts as chemical catalysts, but also organic

acids (e.g. acetic acid), carbonic acid (H_2CO_3), alkaline metal salts (e.g. sodium or calcium carbonate) and organic compounds (e.g. acetic acid). Furthermore, the present invention also employs hemicellulases as biochemical (enzymatic) catalysts. Another aspect that differentiates the present process from the abovementioned process is its global character. Accordingly, beyond the pre-treatment, it also embodies the steps of the enzymatic hydrolysis of the pre-treated biomass using cellulases and the fermentation of the sugars produced in the two prior steps, both in the SHF systems and the SSF systems.

Document US 7,189,306 describes a process to treat lignocellulosic material comprising steps of crushing, pre-treatment with steam in an alkaline ($pH \geq 8$) in two steps, in the presence or absence of an oxidizing agent (e.g. O2 and/or H_2O_2), followed by a fractionation step of the cellulose and subsequent fermentation of the carbohydrates by the SSCF (Separated Saccharification and Co-Fermentation) process.

The present invention differs from this document because the pre-treatment can be performed in acid (organic and inorganic), neutral and alkaline products, using metal catalysts (acid and alkaline salts) with the possibility of using an enzymatic step (hemicellulases) for the removal of the residual hemicelluloses. Furthermore, it includes steps of enzymatic hydrolysis of the cellulose and fermentation of the generated hydrolyzed juice, using yeast of the type *Saccharomices cerevisae*, for example, for the production of the cellulosic ethanol, in the SHF and SSF processes.

Document US 4,880,473 describes a process to produce fermentable sugars from cellulosic biomasses, comprising a step of pre-treatment (hydrolysis with diluted acid) followed by a separation of the solid phase that contains the cellulose, with subsequent steps of rapid pyrolisis of the pre-treated material and separation of the sugars present in the aqueous phase.

The present invention differs from the abovementioned document because it does not include any step relative to the pyrolisis process of the pre-treated biomass, among the other grounds already presented and discussed throughout the present text.

Therefore, based on what is available until now, it can be seen that the state of the art does not anticipate or suggest the embodiments of the present invention. Thus, there is a need of a process for the pre-treatment of lignocellulosic material for the production of sugars and also ethanol that provides a high yield.

Summary of the Invention

The object of the present invention is a fermentative process comprising the steps of:

a) Pre-treatment of the lignocellulosic plant biomass in one (steam explosion) or two (steam explosion followed by treatment with hemicellulases) steps;

b) Enzymatic hydrolysis of the pre-treated biomass using mixtures containing cellulases and/or β-glycosidases and/or hemicellulases

c) Fermentation of the carbohydrates produced during the step of the enzymatic hydrolysis of the pre-treated biomass.

Preferentially, the sugar cane bagasse that constitutes the process proposed in this invention is derived from a diffuser, when possible. However, the bagasse can also be derived from a crusher or a mixture from a diffuser and a crusher.

Optionally, chemical catalysts and oxidizing agents can be used in the pre-treatment of the biomass with steam.

Optionally, the pre-treatment process comprises a step of enzymatic hydrolysis of the hemicelluloses present in the pre-treated biomass with steam using a mixture of hemicellulases.

Optionally, the processes of the enzymatic hydrolysis of the hemicelluloses and of the cellulose occur simultaneously with the fermentation of the carbohydrates.

An additional object of the present invention is a fermented product obtained by the abovementioned fermentative process. Specifically, the fermented product is ethanol.

Brief Description of the Figures

Figure 1 displays a process for the production of ethanol from a lignocellulosic biomass, in which the pre-treatment can be performed by physical-chemical means and enzymatically (hemicellulases). The subsequent steps of the enzymatic hydrolysis of the cellulose (cellulases) and the fermentation (*Saccharomyces cerevisiae*) occur separately (SHF process).

Figure 2 displays a process for the production of ethanol, in which the pre-treatment of the lignocellulosic biomass can be performed in three steps: physical-chemical treatment, enzymatic hydrolysis (hemicellulases) for the removal of the residual hemicellulose and washing of the final pre-treated bagasse. Subsequently, the pre-treated bagasse is submitted to the simultaneous process of the enzymatic hydrolysis of the cellulose (cellulases) and the fermentation (*Saccharomyces cerevisiae*) - (SSF process).

Figure 3 displays a process for the production of ethanol from the lignocellulosic biomass, in which the pre-treatment step is performed in two steps: physical-chemical treatment and washing of the final pre-treated bagasse. Subsequently, the pre-treated bagasse is submitted to the simultaneous process of the enzymatic hydrolysis of the cellulose (cellulases) and of the residual hemicellulose (hemicellulases) and the fermentation (*Saccharomyces cerevisiae*) - (SSF process).

Figure 4 displays the chromatogram with respect to the liquid fraction after the pre-treatment of the biomass with steam. The presence of xylose is evidence of the partial removal of the hemicelluloses from the biomass after the process. Glucose is produced from the sucrose that is derived from the crushing of the cane and from the cellulose of the bagasse.

Figure 5 displays the chromatogram with respect to the compositional analysis of the pre-treated biomass (PTB) with steam. The presence of xylans (characterized in the form of xylose) in the biomass is evident, indicating the non-total removal of the hemicelluloses.

Figure 6 displays the chromatogram with respect to the SHF experiment. Enzymatic hydrolyzed product of the washed pre-treated biomass (PTB) using only cellulases. A lower production of glucose can be observed in relation to the condition presented in Figure 4, due to the recalcitrance exercised by the residual hemicelluloses present in the biomass.

Figure 7 displays the chromatogram with respect to the SHF experiment. Enzymatic hydrolyzed product of the washed pre-treated biomass (PTB) using a mixture of cellulases and hemicellulases. A higher production of glucose can be observed in relation to the condition presented in Figure 3, due to the action of the hemicellulases, which is evidence of the synergy between the different enzymes. The presence of sorbitol in the hydrolyzed enzymatic product characterizes a potential activation of the glucose in the presence of the hemicellulases, which remove the hemicelluloses from the biomass.

Figure 8 displays the chromatogram with respect to the fermented juice from the SSF experiments, using only hemicellulases (without cellulases) and washed pre-treated biomass (PTB). The significant production of xylose can be observed and the selective removal of the residual hemicelluloses present in the PTB (C_{xylose}: $C_{glucose}$ > 100). No production or consumption of glucose can be observed during the process, which is evidenced by the almost zero production of ethanol by the yeasts present in the reaction. The efficiency of the enzymatic pre-treatment (using hemicellulases) of the biomass is represented here, supplementing the pre-treatment process with steam, in the selective removal of the residual hemicelluloses, with minimal cellulosic loss.

Figure 9 displays the chromatogram with respect to the fermented juice from the SSF experiments, using only cellulases (without hemicellulases) and washed pre-treated biomass (PTB). A minimal concentration of glucose can be observed but with a high production and simultaneous consumption of the same, evidenced by the production of ethanol by the yeasts present in the reaction ($C_{ethanol}$>0.5 %m/m). The presence of xylose (C_{xylose} =1.20 g/kg) in the fermented liquid is evidence of the moderated xylanolitic activity of the cellulases, indicating the potential of the enzymatic pre-treatment of the

biomass with hemicellulases, supplementing the pre-treatment process with steam, in the selective removal of the residual hemicelluloses, with minimal cellulosic loss.

Figure 10 displays the chromatogram with respect to the fermented juice from the SSF experiments, of the PTB washed and treated with hemicellulases. A minimal concentration of glucose can be observed but with a high production and simultaneous consumption of the same, evidenced by the production of ethanol (0.5 %m/m) by the yeasts present in the reaction. The presence of xylose can be observed in the juice in a concentration of the order of 0.49 g/kg, but with a high production and consumption of the same. This is evidenced by the production of products distinct from ethanol, characterizing potential synergy from the hemicellulases and the yeasts. The production of ethanol is evidence that there is no inhibition to the activity of the yeasts due to the presence of the hemicellulases.

Figure 11 displays the chromatogram with respect to the fermented juice from the SSF experiments of the washed PTB, with cellulases complemented with a saccharidic solution (sugar cane juice). (A) Time 0 (zero), (B) Time 24 hours. The practically complete consumption of the glucose can be observed, evidencing that the addition of a solution rich in fermentable sugars (booster) does not inhibit the fermentative activity of the yeasts. From the values observed in Figure 10 for the concentrations of ethanol, there is evidence of a positive synergy between the hydrolyzed enzymatic product (produced during the SSF) and the solution rich in sucrose used as a booster. This evidence reinforces the use of the sugar cane molasses or juice as a booster in SSF processes.

Figure 12 displays the chromatogram with respect to the a: (A) SSF experiment using pre-treated bagasse with steam [CE_A = 0.531 %m/m] in the presence of cellulolytic enzymes. (B) Fermentation of the sugar cane juice [CE_B = 4.341 %m/m]. (C) SSF experiment using pre-treated bagasse with steam and complemented with a sugar cane juice (sucrose booster) in the presence of a mixture of cellulolytic products [CE_C = 4.885 %m/m].

C_{Ei} = concentration of ethanol from experiment i.

Detailed Description of the Invention

The examples described herein are intended only to illustrate the objects of the invention and should not be understood as to limit the scope of the present application.

Lignocellulosic Plant Biomass

The expression lignocellulosic plant biomass comprises any type of plant, namely: herbaceous biomass; cultivated plants such as C4 plants – belonging to the genera *Lolium*, *Spartina*, *Panicum*, *Miscanthus*, and combinations thereof; sugar cane bagasse (from crusher and/or diffuser, the bagasse from the diffuser being preferred); straws of cereals such as wheat, rice, rye, barley, oatmeal, corn and similar products (e.g. switchgrass); wood; trunks and stalks of the banana tree; cacti and combinations thereof. Furthermore, lignocellulosic materials can also comprise cardboard, sawdust, newspaper and agro-industrial residuals or similar products.

Vegetable biomasses from different origins can present specific differences although they may have a relatively similar global chemical composition. Some variations in the composition from different species and even from the same species are due to environmental and genetic variables, as well as the location of the tissue in different parts of the plant. Typically, approximately 35-50% is constituted of cellulose, 20-35% of hemicelluloses and approximately 20-30% of lignin. The remainder consists of smaller quantities of ashes, soluble phenolic compounds and fatty acids, as well as other constituents, denominated extractives. Cellulose and the hemicelluloses of the plant tissue are constituted of structural carbohydrates (e.g. glucans, xylans and mannans), generally denominated from the saccharidic fraction. Lignin is constituted in the phenolic fraction of the plant biomass.

The present invention comprises a fermentative process that has technical-operational advantages when compared to the processes disclosed in the state of the art and involves the following steps:

a) Pre-treatment of the lignocellulosic plant biomass with a steam explosion step; optionally followed by a second step of pre-treatment of the biomass using hemicellulases (enzymes);

b) Enzymatic hydrolysis of the pre-treated biomass using mixtures containing cellulases and/or β-glycosidases and/or hemicellulases;

c) Fermentation of the carbohydrates produced from the enzymatic hydrolysis of the pre-treated biomass.

Pre-treatment step

The removal of the hemicelluloses from the lignocellulosic biomasses such as the bagasse and the straw of the cane and the corn allow the increased accessibility of the cellulose to the chemical agents (e.g. acids or alkalis) or biochemicals (e.g. enzymes) that convert it into fermentable sugars, particularly glucose. The combination of the chemical processes (e.g. treatment with steam) and biochemical processes (e.g. hydrolysis with hemicellulases) allows the fragmentation and subsequent removal of the hemicelluloses present in the biomass with high selectivity, embodying a particularly efficient alternative in terms of pre-treatment. In the present invention, the pre-treatment is conceived as a group of operational steps that *follow* the preparation of the biomass and the feeding of the reactor and *precede* the enzymatic hydrolysis of the cellulose.

The pre-treatment of the lignocellulosic biomass with saturated steam or water under pressure at different levels of temperature and processing time promotes the partial removal of the hemicelluloses, increasing the accessibility of the cellulosic matrix to the cellulolytic enzymes (cellulases). The steam used can be generated in its own chamber or can be introduced into the chamber. The pre-treatment processes can be performed by the configuration of a steam explosion - STEX or a wet explosion - WEX, in which a rapid decompression occurs in the discharge of the material. A cooking configuration can also be used, in which a rapid decompression is not used after the process time. Generally, the steam explosion processes tend to produce a larger

fragmentation of the hemicelluloses, making the cellulose more accessible to the chemical and enzymatic agents.

In principle, more severe process conditions produce a larger extraction of hemicelluloses. However, these conditions tend to result in high losses of cellulose due to their fragmentation. Furthermore, degradation can occur in the carbohydrates made available in the process in compounds such as furfural and hydroxymethylfurfural (HMF).

The use of catalysts and adjuvants such as oxidants (e.g. O_2 and H_2O_2) in acid, alkaline or neutral systems allows the removal of large quantities of hemicelluloses under less severe process conditions. Furthermore, there is evidence of a high selectivity in terms of extraction predominantly of hemicelluloses, preserving the cellulosic content of the pre-treated biomass. Depending on the availability of water and the operational conditions, the deacetylation of the hemicelluloses can be intensified in order to produce acetic acid, which acts, in this context, as an hemicelluloses "autochthonous catalyst", characterizing an autocatalytic process.

The pre-treatment presented in the present invention can comprise of one or more catalysts, including, although not limited to, inorganic acids such as H_2SO_4, HCl, HNO_3, H_3PO_4 or combinations of these, organic acids such as acetic, formic and carbonic acid (H_2CO_3), oxides (SO_2, MnO_2, CO_2), sulfates ($FeSO_4$, $Al_2(SO_4)_3$), carbonates ($FeCO_3$, $CaCO_3$, Na_2CO_3) and metal chlorates ($FeCl_3$, $ZnCl_2$, $MnCl_2$, $CaCl_2$, $AlCl_3$).

Compounds that are generated during the pre-treatment can also act as catalysts and are denominated auto-catalysts. Examples of these compounds include organic acids, such as acetic acid and acids arising from the degradation of carbohydrates, as well as phenolic species derived from lignin. The catalysts can be present in a concentration that varies from 0.10% to 8% in relation to the dry mass of the biomass, but should preferentially be situated in the range from 0.25% to 6%.

Removal of the Residual Hemicelluloses

Despite the benefits associated with the use of catalysts in the pre-treatment step, the presence of residual hemicelluloses is frequently evidenced in the pre-treated biomass. In this context, the selective removal of these hemicelluloses under moderate process conditions results in pre-treated biomasses with a greater accessibility to cellulose, allowing the loading of the hydrolysis reactor with greater amounts of this component.

A selective enzymatic treatment of the pre-treated biomass using a physical-chemical process (e.g. STEX, WEX) under moderate conditions of temperature allows the production of a material with a high cellulosic content as well as the greater accessibility of cellulose to the enzymes, for example, mixtures of cellulases and β-glycosidases. The present invention presents the complementary enzymatic pre-treatment of the lignocellulosic biomass (pre-treated by physical-chemical processes) based on the use of hemicellulases (enzymes that fragment the hemicelluloses) under temperature conditions varying from 35°C to 60°C, preferentially from 45°C to 55°C, with reaction times varying from 6 hours to 48 hours, preferentially from 12 hours and 24 hours. These enzymes can be employed in SHF and SSF systems as a pre-treatment agent of residual hemicelluloses. The hemicellulases are in the proportions varying from 5.5% to 30%, preferentially from 12% to 22%, in relation to the quantity of the pre-treated biomass (dry base).

The data presented in Table 1 allows the observation, as previously commented, of the beneficial effects that the presence of the auxiliary enzymes such as β-glycosidases and, above all, hemicellulases bring to the hydrolytic system under examination. The auxiliary hemicellulase present in the reaction acts in removing the remaining hemicelluloses from the steam explosion process, conferring, therefore, greater accessibility of the cellulase to the cellulose. With this, larger conversions of cellulose into glucose are observed and, as a consequence, higher concentrations of glucose can be detected in the final hydrolyzed product that is destined for fermentation. Therefore, it is evident that the enzymatic treatment of the bagasse exploded by steam with auxiliary enzymes is, in fact, efficient.

TABLE 1 – Enzymatic hydrolysis of the pre-treated biomass. Concentration of glucose in the liquid fraction (hydrolyzed product).

Pre-Treatment Condition	Enzyme	Concentration of Glucose (g/L)
15 kgf/cm² 7 min	Cellulase	7.77
	Cellulase + β-Glycosidase	11.63
	Cellulase + β-Glycosidase + Hemicellulase	20.39
13 kgf/cm² 10 min	Cellulase	9.17
	Cellulase + β-Glycosidase	14.43
	Cellulase + β-Glycosidase + Hemicellulase	21.62
12 kgf/cm² 10 min	Cellulase	9.35
	Cellulase + β-Glycosidase	14.08
	Cellulase + β-Glycosidase + Hemicellulase	19.34

Base: Sugar cane bagasse pre-treated with steam.

Fermentative Process

The fermentation step can be performed after the enzymatic hydrolysis, by a process known as SHF (Separated Hydrolysis and Fermentation), or concomitantly with the hydrolysis, in a process known as SSF (Simultaneous Saccharification and Fermentation). Depending on the concentration of the sugars produced in the enzymatic hydrolysis, a concentrated saccharidic solution can be optionally added to the reaction, varying from 80 g/L to 820 g/L, preferentially from 120 g/L to 200 g/L (e.g. cane molasses or juice).

The present invention also allows the possibility of simultaneously performing the enzymatic pre-treatment of the hemicelluloses, the enzymatic hydrolysis of the cellulose and the fermentation, characterizing a Consolidated BioProcess (CBP) using the pre-treated biomass with steam or other agents as a base.

Figures 1, 2 and 3 display flowcharts concerning the different configurations contemplated in the present invention.

Figure 1 displays a typical SHF process, consisting of three distinct steps (pre-treatment, enzymatic hydrolysis and fermentation) performed separately. The cellulosic biomass is submitted to a physical-chemical pre-treatment (e.g. STEX, WEX, etc.), in the presence or absence of catalysts, oxidants and other inputs. The catalysts are present in a concentration that varies from 0.10% to 8% in relation to the dry mass of the biomass, preferentially from 0.25% to 6%. The charge of the solids varies from 5% to 60%, preferentially from 15% to 50%, in relation to the total mass.

The biomass remains in the reactor during a reaction time from 30 seconds to 60 minutes, preferentially from 5 minutes to 15 minutes, at a process temperature that can vary from 110°C to 240°C, preferentially from 180°C to 210°C. When the reaction time elapses, the reactor is preferentially unloaded by sudden decompression and optionally without sudden decompression.

Depending on the amount of the remaining or residual hemicelluloses, the pre-treated biomass can be submitted to a complementary pre-treatment using specific enzymes (hemicellulases). The hemicellulases are in proportions varying from 5.5% to 30%, preferentially from 12% to 22%, in relation to the mass of the pre-treated biomass (dry base). The biomass remains in the reactor for a reaction time from 6 hours to 48 hours, preferentially from 12 hours to 24 hours, at a process temperature varying from 35°C to 60°C, preferentially from 45°C to 55°C. The amount of solids varies from 2% to 20%, preferentially from 7.5% to 15%, in relation to the total mass. After the pre-treatment, the biomass can optionally be submitted to washing with water or acidic or alkaline solutions at an ambient temperature.

The pre-treated biomass is next submitted to an enzymatic hydrolysis, where it is sent to a reactor, which is preferentially discontinuous and optionally continuous, together with specific enzymes (cellulases and β-glycosidases). The cellulases are in proportions that vary from 5.5% to 30%, preferentially from 12% to 22%, in relation to the mass of the pre-treated biomass (dry base). The β-glycosidases are in proportions varying from 2.5% to 15%, preferentially from

5.5% to 7.5%, in relation to the mass of the pre-treated biomass (dry base). The charge of the solids varies from 2% to 20%, preferentially from 7.5% to 15%, in relation to the total mass.

The biomass remains in the reactor for a reaction time that varies from 12 hours to 72 hours, preferentially from 24 hours to 48 hours, at a process temperature varying from 35ºC to 60ºC, preferentially from 45ºC to 55ºC. When the reaction time elapses, the reactor is unloaded and the material is submitted to a separation process, preferentially filtration and optionally centrifugation or ultra-filtration, producing a liquid fraction (hydrolyzed enzymatic product) and a solid fraction (lignocellulosic residue or cellulignin).

The hydrolyzed enzymatic product is then submitted to an ethanolic fermentation preferentially using yeasts of the type *Saccharomices cerevisae* and optionally using bacteria of the type *Zymomonas mobilis*. The hydrolyzed enzymatic product is preferentially mixed with cane molasses or juice and optionally fermented individually, without any type of mixture with molasses or juice.

In accordance with that presented in Figure 1, the steps of enzymatic hydrolysis and fermentation occur separately.

Figure 2 presents a typical SSF process, in which the steps of enzymatic hydrolysis and fermentation are performed simultaneously. The cellulosic biomass is submitted to a physical-chemical pre-treatment (e.g. STEX, WEX, etc.), in the presence or absence of catalysts, oxidants and other inputs. When the pre-treatment includes catalysts, these are present in concentrations that vary from 0.10% to 8% in relation to the dry mass of the biomass, preferentially from 0.25% to 6%. The amount of the solids varies from 5% to 60%, preferentially from 15% to 50%, in relation to the total mass.

The biomass remains in the reactor during a reaction time from 30 seconds to 60 minutes, preferentially from 5 minutes to 15 minutes, at a process temperature varying from 110ºC to 240ºC, preferentially from 180ºC to 210ºC. When the reaction time elapses, the reactor is preferentially unloaded by sudden decompression and optionally without sudden decompression.

Depending on the remaining or residual amount of hemicelluloses, the pre-treated biomass can be submitted to a complementary pre-treatment using specific enzymes (hemicellulases). The hemicellulases are in proportions varying from 5.5% to 30%, preferentially from 12% to 22%, in relation to the mass of the pre-treated biomass (dry base). The biomass remains in the reactor for a reaction time varying from 6 hours to 48 hours, preferentially from 12 hours to 24 hours, at a process temperature varying from 35°C to 60°C, preferentially from 45°C to 55°C. The amount of the solids varies from 2% to 20%, preferentially from 7.5% to 15%, in relation to the total mass. After the pre-treatment, the biomass can optionally be submitted to washing with water or acidic or alkaline solutions at an ambient temperature.

The pre-treated biomass is submitted to an enzymatic hydrolysis, where it is sent to a fermentation reactor, which is preferentially discontinuous and optionally continuous, together with specific enzymes (cellulases and β-glycosidases). The cellulases are in proportions varying from 5.5% to 30%, preferentially from 12% to 22%, in relation to the mass of the pre-treated biomass (dry base). The β-glycosidases are in proportions varying from 2.5% to 15%, preferentially from 5.5% to 7.5%, in relation to the mass of the pre-treated biomass (dry base). The charge of the solids varies from 2% to 20%, preferentially from 7.5% to 15%, in relation to the total mass. When the enzymatic hydrolysis process produces sugars, preferentially glucose, these are submitted to an ethanolic fermentation using, preferentially, yeasts of the type *Saccharomices cerevisae* and, optionally, using bacteria of the type *Zymomonas mobilis*. Preferentially, a concentrated saccharidic solution ("booster") is added, preferentially cane molasses and, optionally, cane juice, to the fermentation at the start or during the enzymatic hydrolysis process although it can operate without the addition of the saccharidic solution. The saccharidic solution ("booster") presents a concentration of sugars varying from 80 g/L to 820 g/L, preferentially from 120 g/L to 200 g/L.

The biomass remains in the reactor during a reaction time varying from 12 hours to 72 hours, preferentially from 24 hours to 48 hours, at a process

temperature varying from 30°C to 50°C, preferentially from 37°C to 40°C, with the pH of the reaction varying from 4.2 to 5.5, preferentially from 4.8 to 5.0.

When the reaction time elapses, the reactor is unloaded and the material is submitted to a separation process, preferentially filtration and optionally centrifugation, producing a liquid fraction (must) and a solid fraction (lignocellulosic residue or cellulignin).

In accordance with that demonstrated in Figure 2, the steps of enzymatic hydrolysis and fermentation occur simultaneously.

Figure 3 presents an advanced SSF process, in which the enzymatic pre-treatment steps with hemicellulases, enzymatic hydrolysis with cellulases and fermentation are performed simultaneously. The cellulosic biomass is submitted to a physical-chemical pre-treatment (e.g. STEX, WEX, etc.), in the presence or absence of catalysts, oxidants and other inputs. The catalysts are present in a concentration that varies from 0.10% to 8% in relation to the dry mass of the biomass, preferentially from 0.25% to 6%. The amount of the solids varies from 5% to 60%, preferentially from 15% to 50%, in relation to the total mass.

The biomass remains in the reactor during a reaction time from 30 seconds to 60 minutes, preferentially from 5 minutes to 15 minutes, at a process temperature that varying from 110°C to 240°C, preferentially from 180°C to 210°C. When the reaction time elapses, the reactor is preferentially unloaded by sudden decompression and optionally without sudden decompression.

The pre-treated biomass is submitted to an additional pre-treatment using specific enzymes (hemicellulases). The pre-treated biomass by physical-chemical processes is sent to a fermentation reactor, which is preferentially discontinuous and optionally continuous, together with specific enzymes (hemicellulases). The hemicellulases are in proportions varying from 5.5% to 30%, preferentially from 12% to 22%, in relation to the mass of the pre-treated biomass (dry base). The biomass remains in the reactor for a reaction time varying from 6 hours to 48 hours, preferentially from 12 hours to 24 hours, at a process temperature varying from 35°C to 60°C, preferentially from 45°C to

55°C. The amount of the solids varies from 2% to 20%, preferentially from 7.5% to 15%, in relation to the total mass.

Concomitantly to the process of pre-treatment with hemicellulases or occurring from 1 hour to 6 hours, preferentially 4 hours, from the start of this step, the fermentation step begins with the enzymatic hydrolysis of the cellulose and the fermentation, characterizing an SSF process The cellulases are in proportions varying from 5.5% to 30%, preferentially from 12% to 22%, in relation to the mass of the pre-treated biomass (dry base). The β-glycosidases are in proportions varying from 2.5% to 15%, preferentially from 5.5% to 7.5%, in relation to the mass of the pre-treated biomass (dry base). The amount of the solids varies from 2% to 20%, preferentially from 7.5% to 15%, in relation to the total mass. When the enzymatic hydrolysis process produces sugars, preferentially glucose, these are submitted to an ethanolic fermentation using, preferentially, yeasts of the type *Saccharomices cerevisae* and, optionally, using bacteria of the type *Zymomonas mobilis*. Preferentially, a concentrated saccharidic solution ("booster") is added, preferentially cane molasses and, optionally, cane juice, to the fermentation at the start or during the enzymatic hydrolysis process although it can operate without the addition of the saccharidic solution. The saccharidic solution ("booster") presents a concentration of sugars varying from 80 g/L to 820 g/L, preferentially from 120 g/L to 200 g/L.

The biomass remains in the reactor during a reaction time varying from 12 hours to 72 hours, preferentially from 24 hours to 48 hours, at a process temperature varying from 30°C to 50°C, preferentially from 37°C to 40°C, with the pH of the reaction varying from 4.2 to 5.5, preferentially from 4.8 to 5.0.

When the reaction time elapses, the reactor is unloaded and the material is submitted to a separation process, preferentially filtration and optionally centrifugation, producing a liquid fraction (must) and a solid fraction (lignocellulosic residue or cellulignin).

In accordance with that demonstrated in Figure 3, the steps of pre-treatment with hemicellulases, enzymatic hydrolysis and fermentation occur simultaneously.

Claims

FERMENTATIVE PROCESS COMPRISING A PRE-TREATMENT STEP, ENZYMATIC HYDROLYSIS AND FERMENTED PRODUCT USING LIGNOCELLULOSIC PLANT BIOMASS

1. Fermentative process comprising a pre-treatment step that comprises the following steps:

a) Physical-chemical pre-treatment (steam explosion) of the plant biomass comprising, optionally, an additional pre-treatment step of the biomass with hemicellulases;

b) Enzymatic hydrolysis of the pre-treated biomass using cellulases or the mixture cellulases + β-glycosidases; and

c) Fermentation of the carbohydrates produced from the enzymatic hydrolysis of the pre-treated biomass.

2. Fermentative process, in accordance with claim 1, characterized by the plant biomass is chosen from the group that comprises herbaceous biomass, cultivated plants, cardboard, sawdust, newspaper and mixtures thereof.

3. Fermentative process, in accordance with claim 2, characterized by the cultivated plants are chosen from the group comprising:

i) plants belonging to the genera *Lolium*, *Spartina*, *Panicum*, *Miscanthus* and combinations of these;

ii) sugar cane bagasse;

iii) straws of cereals chosen from the group that comprises wheat, rice, rye, cane, barley, oatmeal, corn, switchgrass, wood, trunks and/or stalks of the banana tree, cacti and combinations thereof;

iv) combinations of i), ii) and/or iii).

4. Fermentative process, in accordance with claim 3, characterized by the cane bagasse being derived from a crusher and/or diffuser.

5. Fermentative process, in accordance with claim 1, characterized by the fact that the biomass plant comprises:

i) from 35-50% of cellulose;

ii) from 20-35% of hemicelluloses; and

iii) from 20-30% of lignin.

6. Fermentative process, in accordance with claim 1, characterized by the steam is saturated or super-heated, being generated in its own chamber and/or introduced into it.

7. Fermentative process, in accordance with claim 1, characterized by comprising catalysts selected from the group that comprises H_2SO_4, HCl, HNO_3, H_3PO_4, acetic, formic and carbonic acid (H_2CO_3), SO_2, MnO_2, CO_2, $FeSO_4$, $Al_2(SO_4)_3$, $FeCO_3$, $CaCO_3$, Na_2CO_3, $FeCl_3$, $ZnCl_2$, $MnCl_2$, $CaCl_2$, $AlCl_3$ and mixture thereof.

8. Fermentative process, in accordance with claim 7, characterized by the catalysts being generated during the pre-treatment step.

9. Fermentative process, in accordance with claim 8, characterized by the catalyst is acetic acid.

10. Fermentative process, in accordance with claim 7, characterized by the catalyst is present in a concentration ranging from 0.10% to 8% in relation to the dry mass of the biomass.

11. Fermentative process, in accordance with claim 10, characterized by the catalyst is present in a concentration that ranges from 0.25% to 6% in relation to the dry mass of the biomass.

12. Fermentative process, in accordance with claim 1, characterized by the temperature of the process of the steam explosion is within the range from 110°C to 240°C.

13. Fermentative process, in accordance with claim 12, characterized by the temperature of the process of the steam explosion is within the range from 180°C to 210°C.

14. Fermentative process, in accordance with claim 1, characterized by the reaction time of the steam explosion step is from 30 seconds to 60 minutes.

15. Fermentative process, in accordance with claim 14, characterized by the reaction time of the steam explosion step is from 5 minutes to 15 minutes.

16. Fermentative process, in accordance with claim 1, characterized by the hemicellulases are present in a concentration that varies from 5.5% to 30% in relation to the dry mass of the pre-treated biomass.

17. Fermentative process, in accordance with claim 16, characterized by the hemicellulases are present in a concentration that varies from 12% to 22% in relation to the dry mass of the pre-treated biomass.

18. Fermentative process, in accordance with claim 1, characterized by the hemicellulases are used at temperatures included in the range from 35°C to 60°C.

19. Fermentative process, in accordance with claim 18, characterized by the hemicellulases are used at temperatures included in the range from 40°C to 50°C.

20. Fermentative process, in accordance with claim 1, characterized by the reaction time with the hemicellulases ranges from 6 hours to 48 hours.

21. Fermentative process, in accordance with claim 20, characterized by the reaction time with the hemicellulases ranges from 12 hours to 24 hours.

22. Fermentative process, in accordance with claim 1, characterized by the pre-treatment step comprising from 5% to 60% p/p of total solid amount.

23. Fermentative process, in accordance with claim 22, characterized by the total solid amount is within the range from 3% to 25% p/p.

24. Fermentative process, in accordance with claim 1, characterized by comprising a step of washing with water or acid or alkaline solutions at room temperature after step a) and before step b).

25. Fermentative process, in accordance with claim 1, characterized by the cellulases are in proportions varying from 5.5% to 30% in relation to the mass of the pre-treated biomass (dry base).

26. Fermentative process, in accordance with claim 25, characterized by the cellulases are in proportions varying from 12% to 22% in relation to the mass of the pre-treated biomass (dry base).

27. Fermentative process, in accordance with claim 1, characterized by the β-glycosidases are in proportions varying from 2.5% to 15% in relation to the mass of the pre-treated biomass (dry base).

28. Fermentative process, in accordance with claim 27, characterized by the β-glycosidases are in proportions varying from 5.5% to 7,5% in relation to the mass of the pre-treated biomass (dry base).

29. Fermentative process, in accordance with claim 1, characterized by the fact that the hydrolysis step comprises from 2% to 20% p/p of total solid amount.

30. Fermentative process, in accordance with claim 29, characterized by the total solid amount is within the range from 2.5% to 25% p/p.

31. Fermentative process, in accordance with claim 1, characterized by the fermentation is performed with yeasts and/or bacteria.

32. Fermentative process, in accordance with claim 31, characterized by the yeast is *Saccharomices cerevisae*.

33. Fermentative process, in accordance with claim 31, characterized by the bacteria is *Zymomonas mobilis*.

34. Fermentative process, in accordance with claim 1, characterized by comprising the addition of a concentrated saccharidic solution with a concentration from 80 g/L to 820 g/L.

35. Fermentative process, in accordance with claim 34, characterized by the concentrated saccharidic solution is cane molasses and/or juice.

FIGURE 1

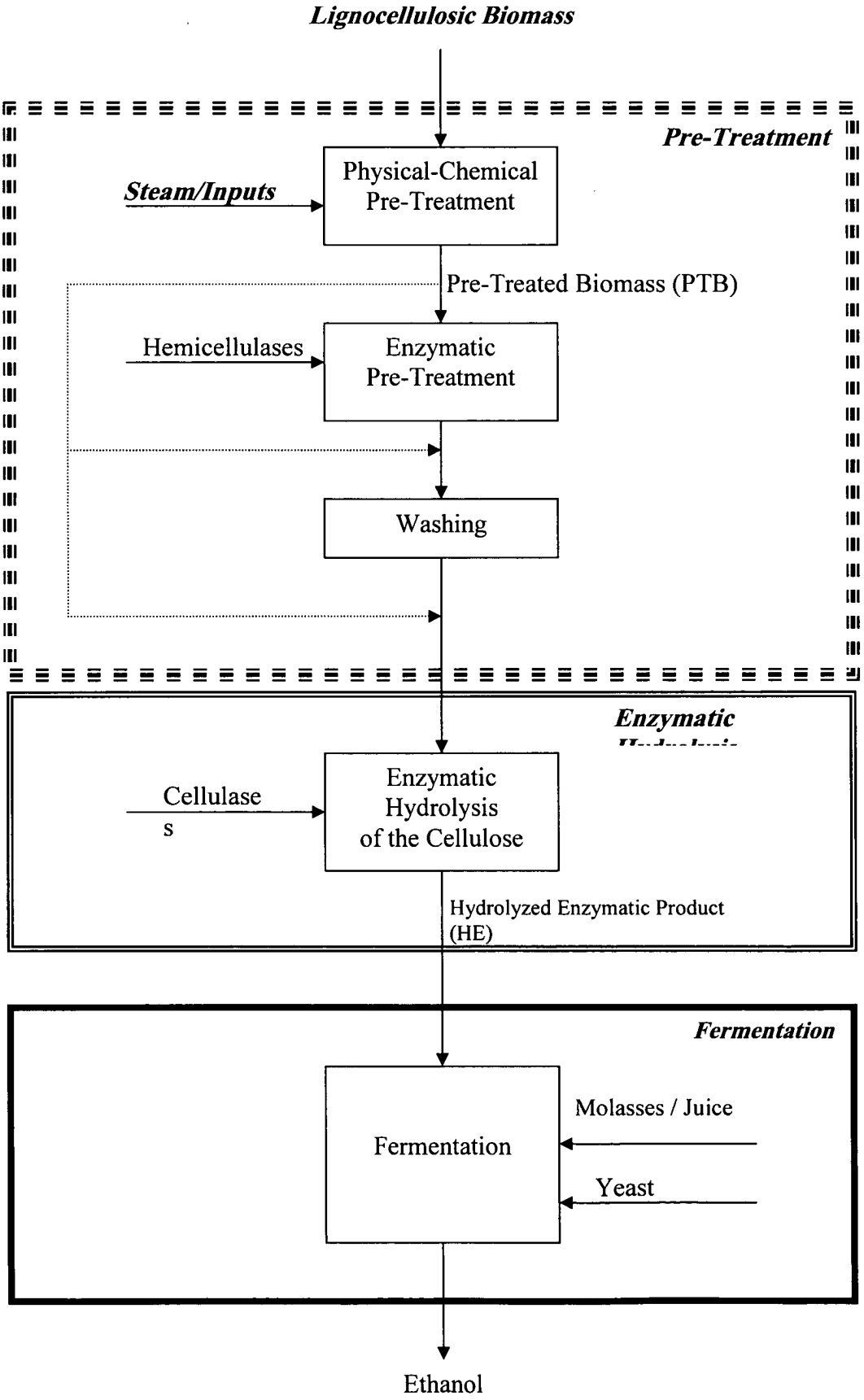

Lignocellulosic Biomass
Pre-Treatment
Steam/Inputs
Physical-Chemical Pre-Treatment
Pre-Treated Biomass (PTB)
Hemicellulases
Enzymatic Pre-Treatment
Washing
Enzymatic
Cellulase s
Enzymatic Hydrolysis of the Cellulose
Hydrolyzed Enzymatic Product (HE)
Fermentation
Molasses / Juice
Fermentation
Yeast
Ethanol

FIGURE 2

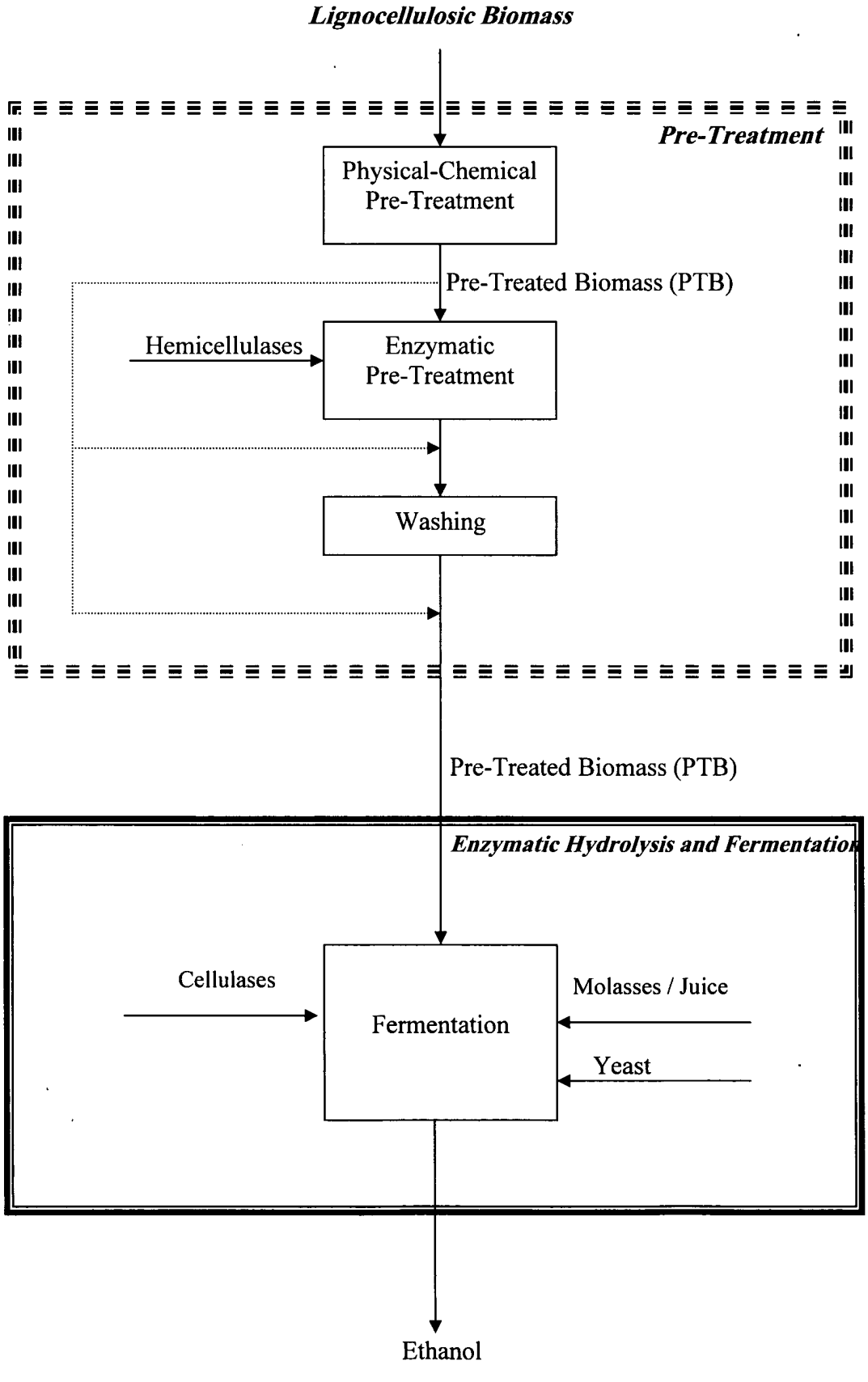

Lignocellulosic Biomass
Pre-Treatment
Physical-Chemical Pre-Treatment
Pre-Treated Biomass (PTB)
Hemicellulases
Enzymatic Pre-Treatment
Washing
Pre-Treated Biomass (PTB)
Enzymatic Hydrolysis and Fermentation
Cellulases
Fermentation
Molasses / Juice
Yeast
Ethanol

FIGURE 3

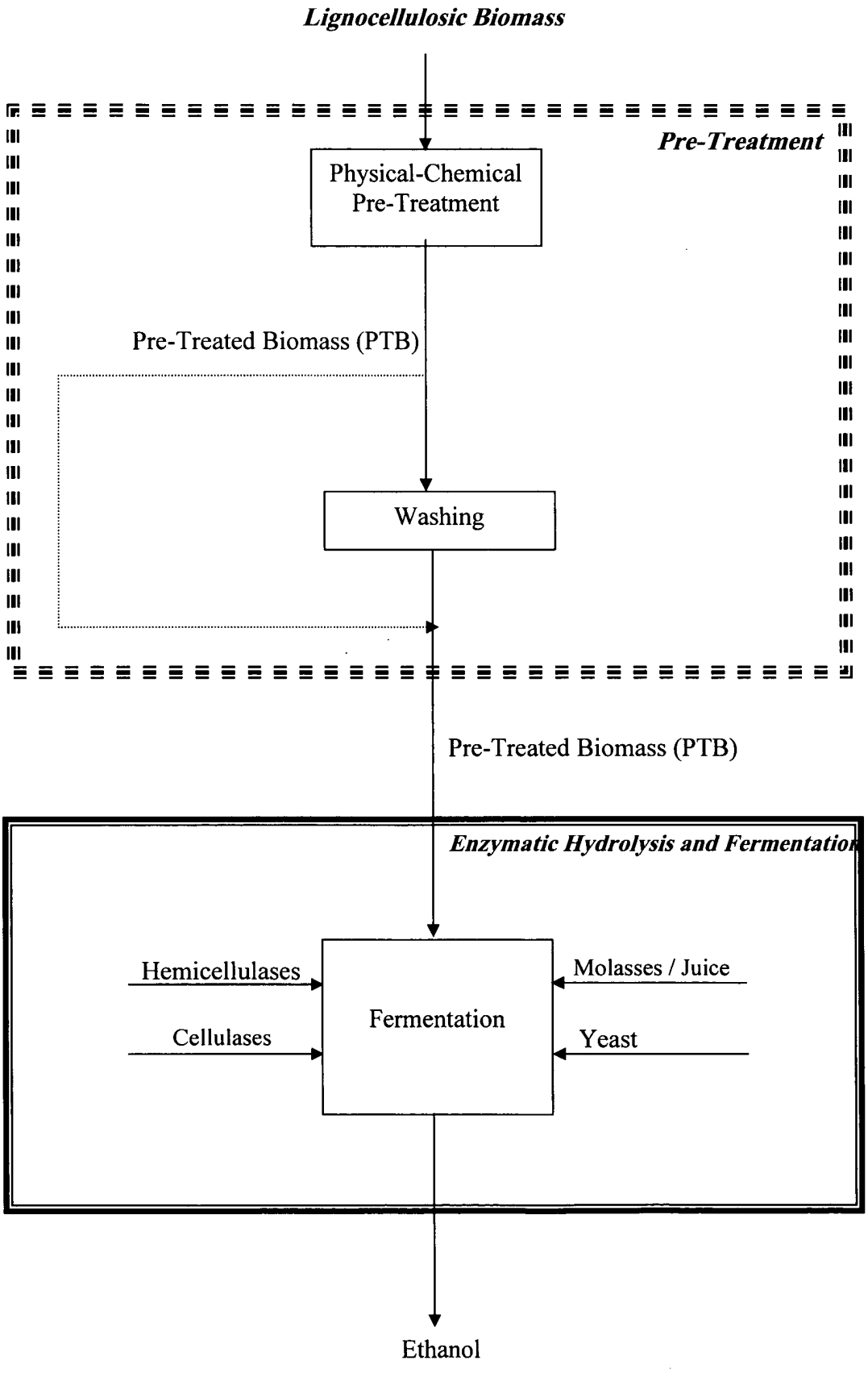
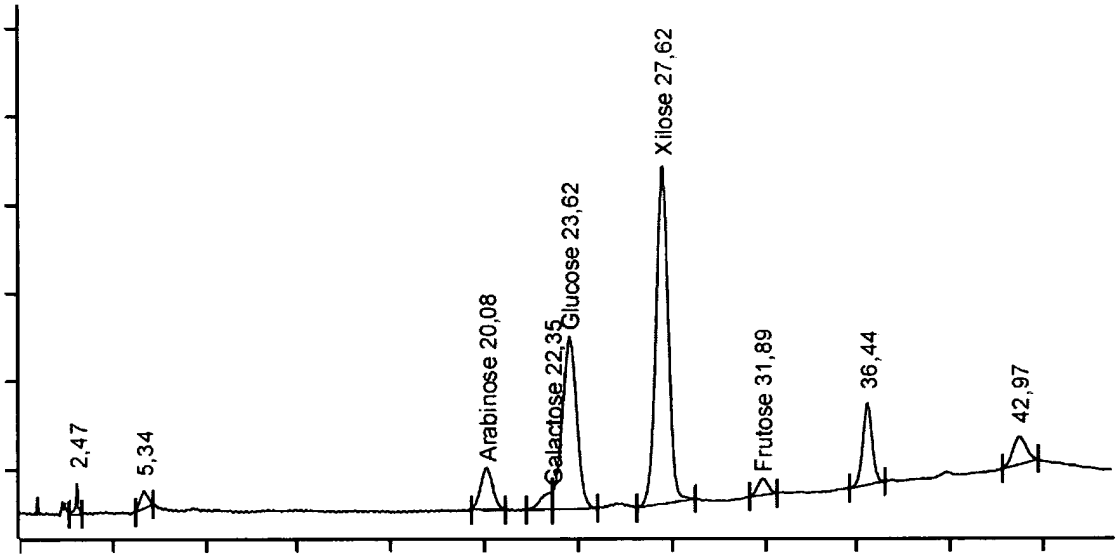
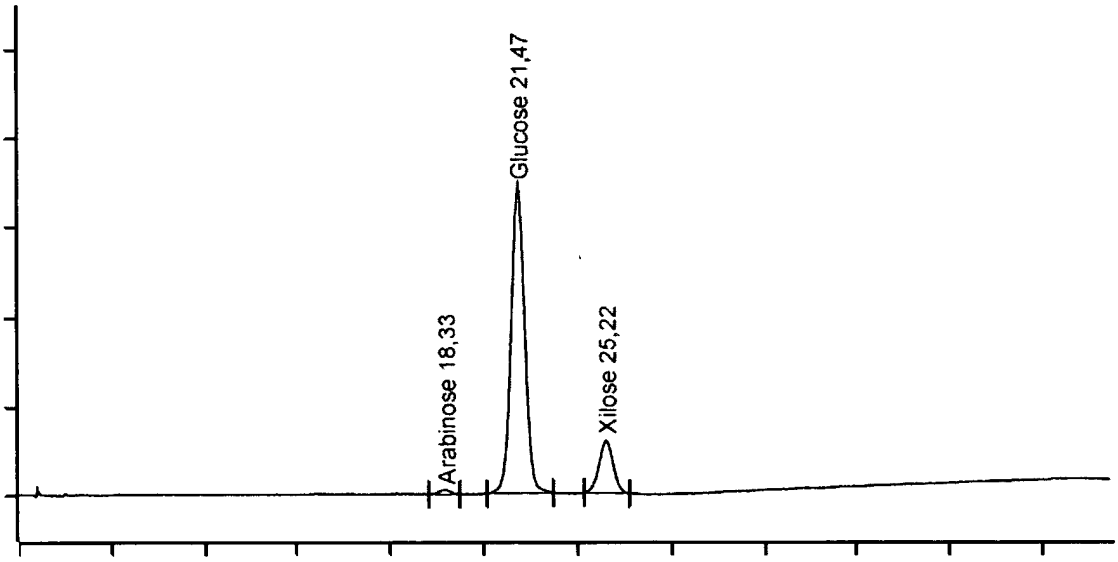

Lignocellulosic Biomass
Pre-Treatment
Physical-Chemical Pre-Treatment
Pre-Treated Biomass (PTB)
Washing
Pre-Treated Biomass (PTB)
Enzymatic Hydrolysis and Fermentation
Hemicellulases
Cellulases
Fermentation
Molasses / Juice
Yeast
Ethanol

FIGURE 4



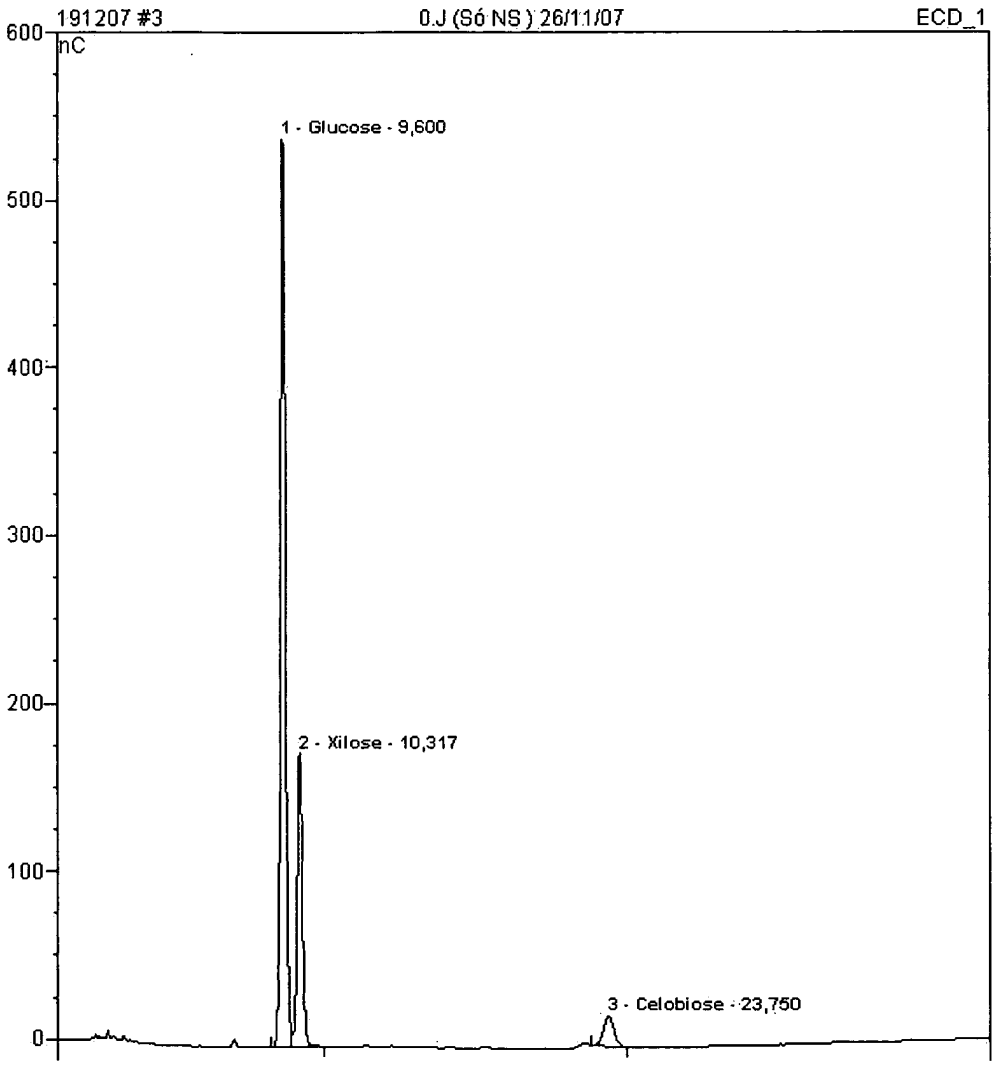
Key:
Xilose = Xylose
Frutose = Fructose

FIGURE 5



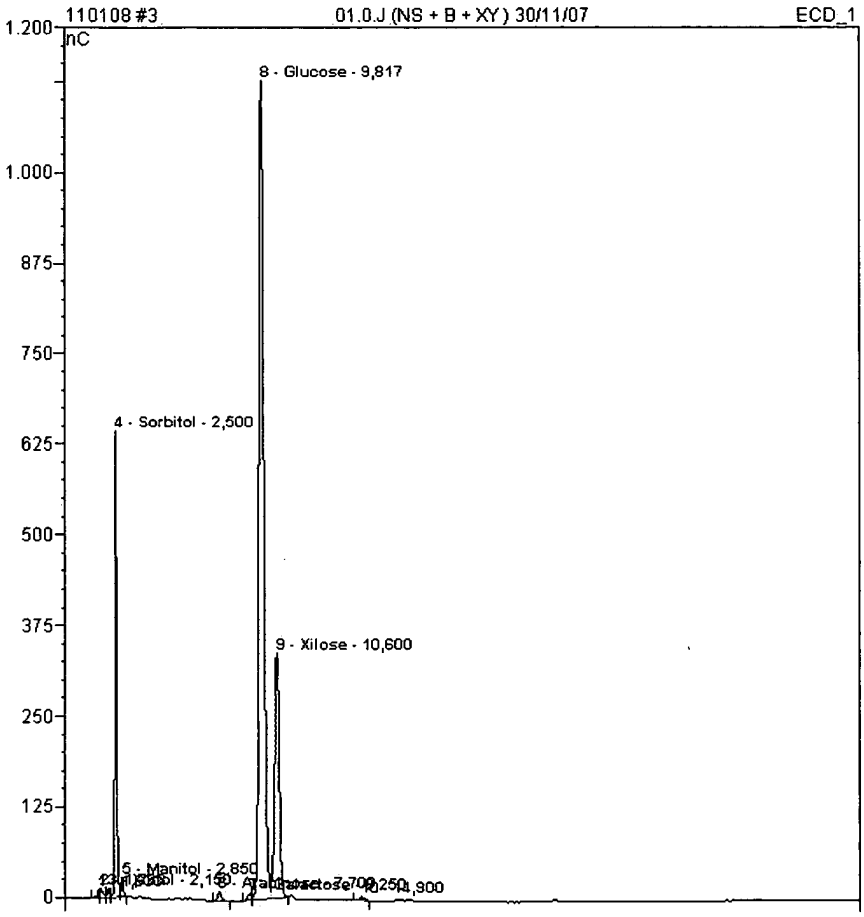
Key:
Xilose = Xylose

FIGURE 6



Key:
Xilose = Xylose
Celobiose = Cellobiose

FIGURE 7



Key:
Xilose = Xylose

FIGURE 8

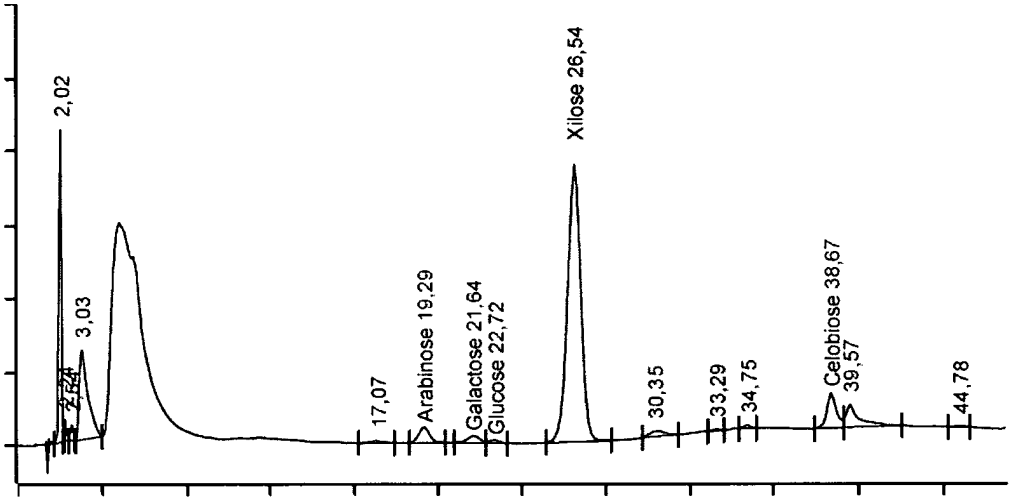
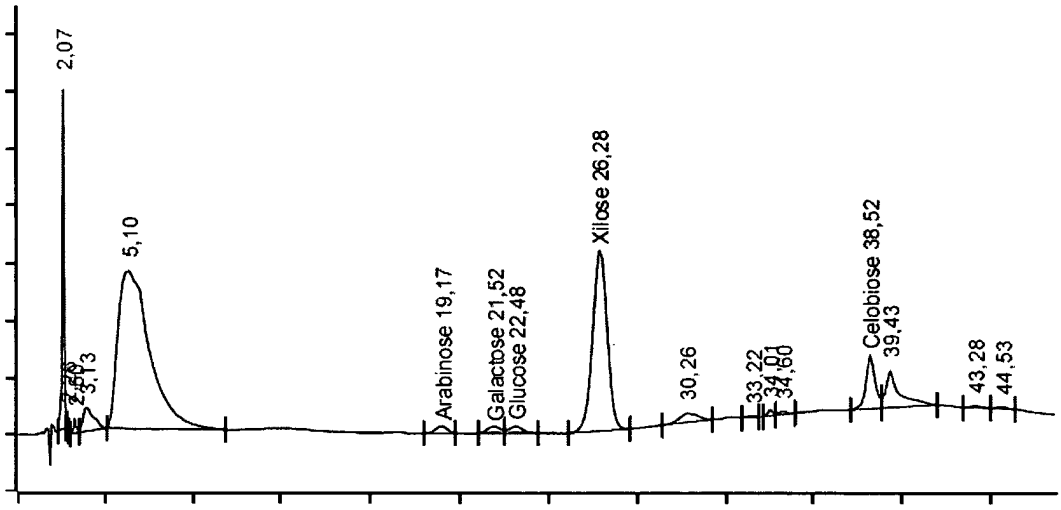


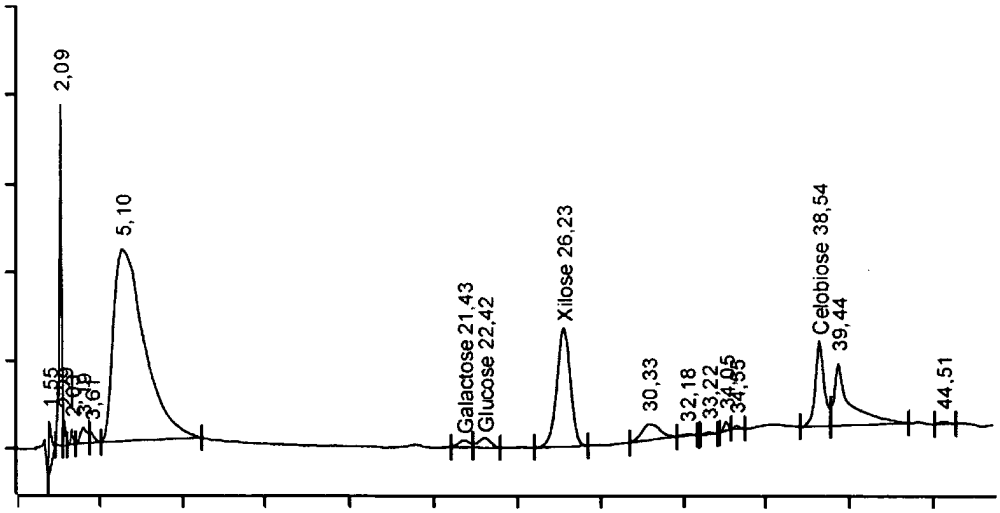
Figure 8
Xilose = Xylose
Celobiose = Cellobiose

FIGURE 9



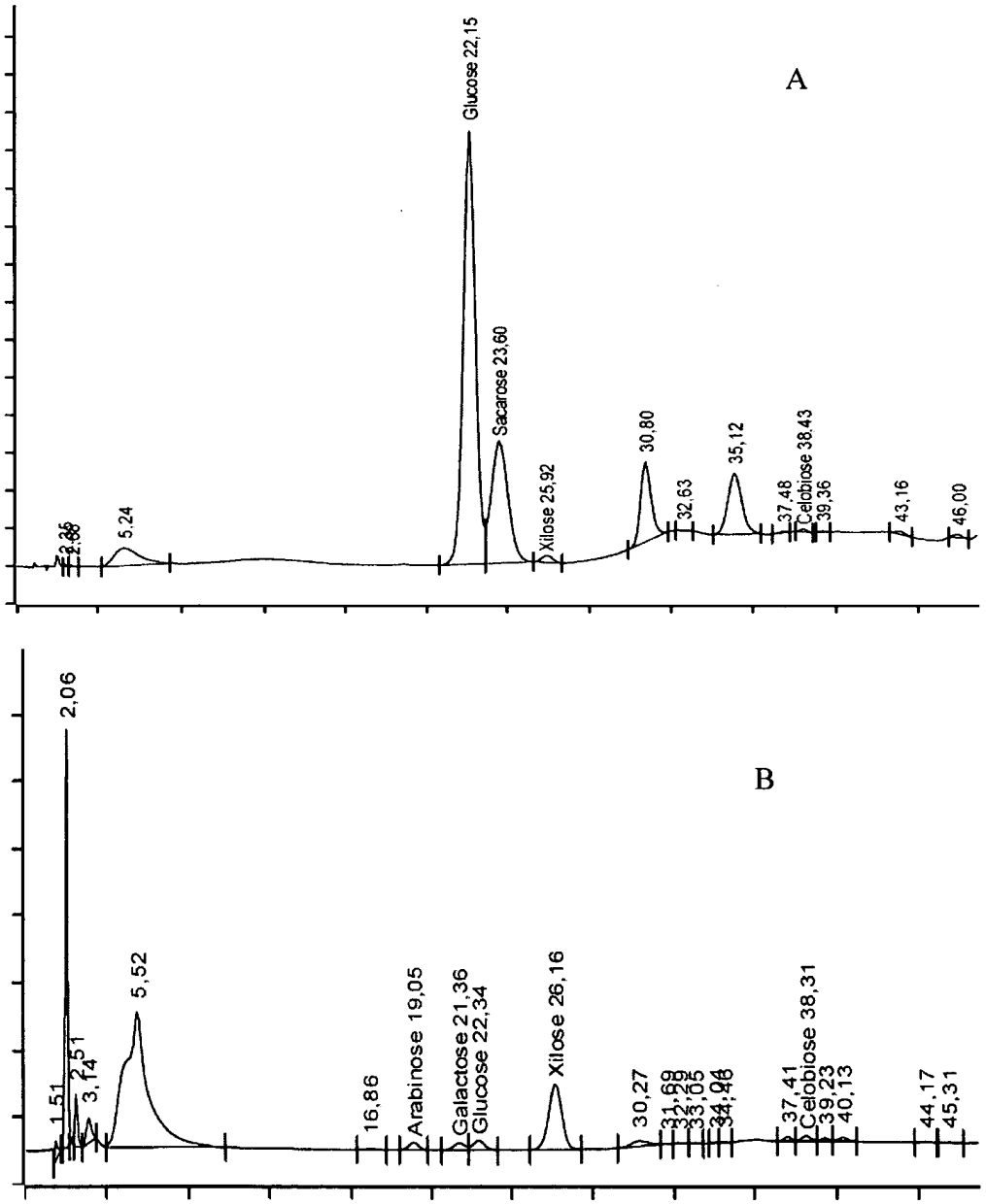
Key:
Xilose = Xylose
Celobiose = Cellobiose

FIGURE 10



Key:
Xilose = Xylose
Celobiose = Cellobiose

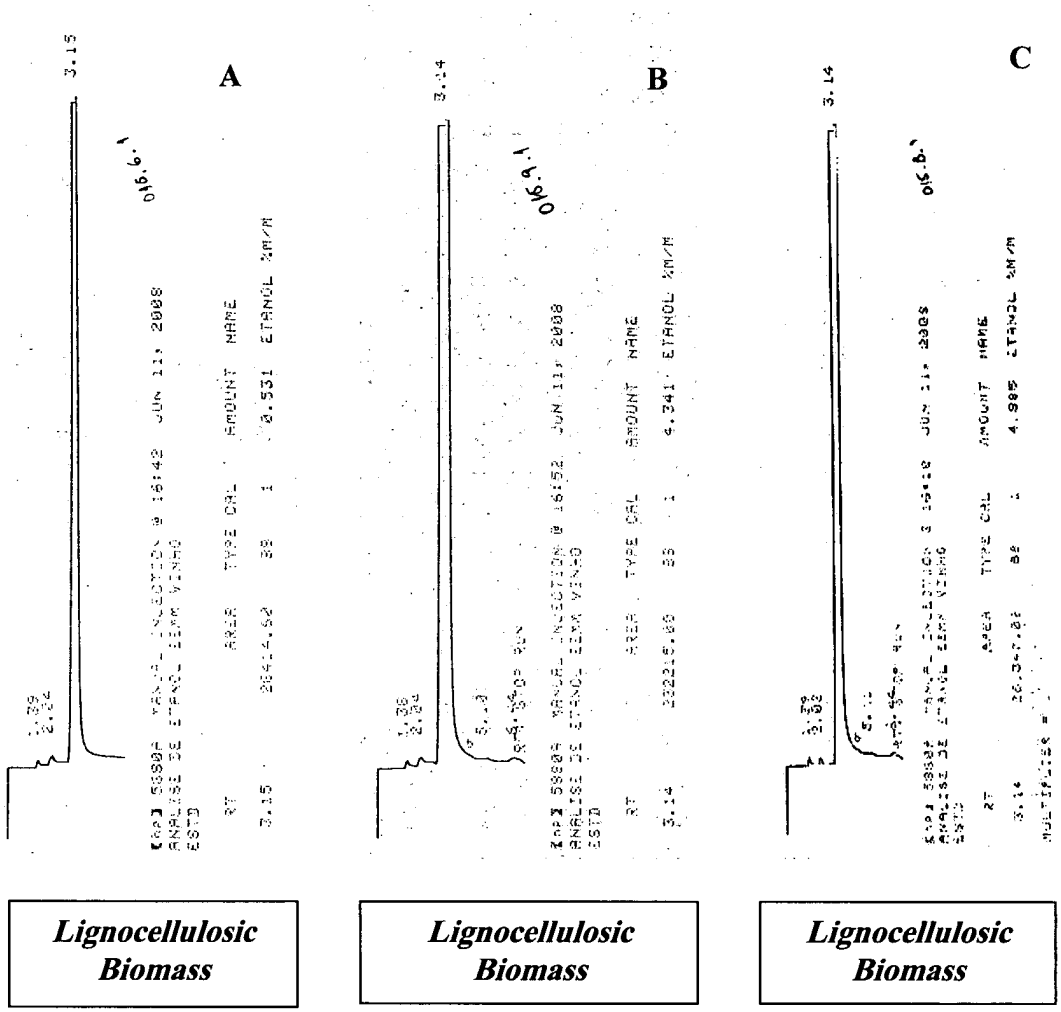
FIGURE 11



Key:
A
Sacarose = Sucrose
Xilose = Xylose
Celobiose = Cellobiose

B
Xilose = Xylose
Celobiose = Cellobiose

FIGURE 12


A
B
C
Lignocellulosic Biomass
Lignocellulosic Biomass
Lignocellulosic Biomass

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 2009/000177

A. CLASSIFICATION OF SUBJECT MATTER
IPC[8]: ***C12P 7/10*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC[8]: C12P

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)
WPI, Fulltext

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/136843 A2 (NORTH CAROLINA STATE UNIVERSITY) 29 November 2007 (29.11.2007) *page 4, lines 15-20; paragraph [016]; page 6, lines 5-15; paragraphs [022][023][043]; page 10, lines 18-23; page 15, lines 22-28; paragraph [072]; claims 1, 16, 22, 23, 28, 29, 33, 34* --	1-35
X	US 2008/0044877 A1 (PENTTILA et al.) 21 February 2008 (21.02.2008) *paragraphs [0006][0008][0009][0043][0045][0048]-[0052][0056]; clains 1, 2, 7, 8* ----	1-35

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 September 2009 (03.09.2009)	28 September 2009 (28.09.2009)
Name and mailing address of the ISA/ AT **Austrian Patent Office** Dresdner Straße 87, A-1200 Vienna Facsimile No. +43 / 1 / 534 24 / 535	Authorized officer MOSSER R. Telephone No. +43 / 1 / 534 24 / 437

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/BR 2009/000177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO A 2007136843		US A1 2008006536	2008-01-10
		WO A2 2007136843	2007-11-29
US A 2008044877		DK T3 1751296T	2009-08-24
		AT T 430204T	2009-05-15
		US A1 2008044877	2008-02-21
		JP T 2008501330T	2008-01-24
		EP A1 1751296	2007-02-14
		CA A1 2567824	2005-12-15