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(74) Agent: **PROZOROVSKIY, Alexander Yurjevich**; 1
Basmanny Lane, 12/1, a/ya 265, Moscow, 107078 (RU).

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(71) Applicant (for all designated States except US): **"ARTER TECHNOLOGY LIMITED"**; P.O. Box 149, Mannor Place, St. Peter Port, Guernsey, GY1 4EW (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **LOMOVSKY, Oleg Ivanovich** [RU/RU]; Akademika Koptuga avenue, 5, Suite 33, Novosibirsk, 630090 (RU). **KOROLEV, Kirill Georgievich** [RU/RU]; Komsomolskaya street, 40, Suite 31, Berdsk, Novosibirskaya region, 633190 (RU). **POLITOV, Anatoly Aleksandrovich** [RU/RU]; Lesosechnaya street, 5-147, Novosibirsk, 630060 (RU). **BERSHAK, Olga Viktorovna** [RU/RU]; Uchenykh street, 8, suite 106, Novosibirsk, 630090 (RU). **LOMOVSKAYA, Tatyana Fedorovna** [RU/RU]; Akademika Koptuga avenue, 5, Suite 33, Novosibirsk, 630090 (RU).

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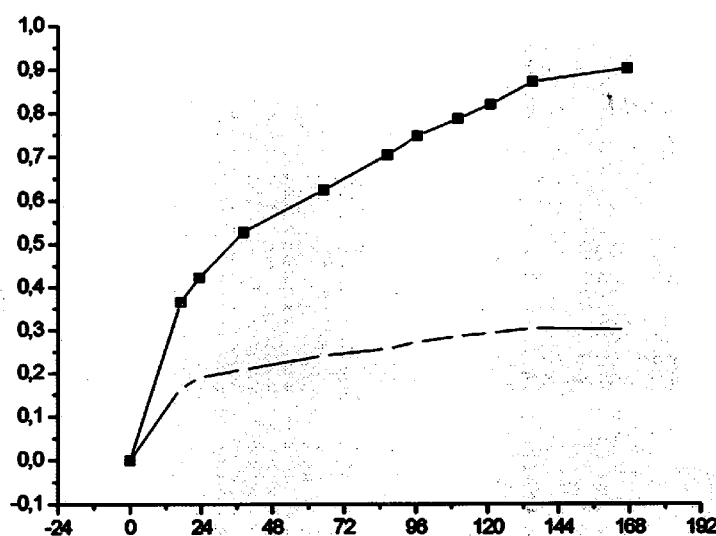


Fig.4

(57) Abstract: The method is intended to produce bioethanol from lignocellulose materials the plant origin; it is based on the mechanochemical (mechanoenzymatic) treatment of a solid mixture of the lignocellulose substrate and cellosolytic enzymatic complexes, fermentation of the resulting carbohydrates by ethanologenic microorganisms. The mechanoenzymatic treatment is mechanical treatment of the solid mixture of the lignocellulose substrate and complex enzymes that ensures a high reactive ability of the enzymatic hydrolysis that enables to preserve the activity of the enzymes and equally to save their consumption.

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Method of producing bioethanol from lignocellulose

Field of the Invention

5 The present invention relates to ethanol from lignocellulose materials. Production of ethanol from cellulose enjoys immense popularity due to a large available quantity of cellulose-containing waste because it is inadvisable to incinerate or bury it, besides ethanol-based fuel is environment friendly. The process of production of carbohydrates from cellulose materials is employed already to output bioethanol by sugar fermentation. The majority of prototypes of this process were tried during WW2 in Germany, Japan, and Russia after fuel prices leapt. Initially these processes were linked to acid hydrolysis, but their technology and equipment design were rather intricate they were vulnerable to slightest variations of parameters, such as temperature, pressure and acid concentration. Comprehensively these early processes and some contemporary methods are discussed in "Production of Sugars from Wood
10 Using High-pressure Hydrogen Chloride", *Biotechnology and Bioengineering*, 1983, vol. XXV, pp. 2757-2773.

Oil reserves were intensively developed during WW2. After the war until the 70s of the 20th century, studies of conversion of lignocellulose into ethanol were sluggish. After the oil crisis in 1973, efforts resumed to develop processes of converting wood and agricultural
20 waste into ethanol as an alternative energy source. These studies enabled to use ethanol as gasoline additive that increases the fuel octane number and reduces exhaust toxicity. The economic effect was less dependence, the USA in particular, on imported oil production. Recently these processes are becoming more and more challenging for conversion of renewable lignocellulose materials into other products, like ethanol. At present new method of hydrolysis of the biomass are attractive as a source of the alternative liquid fuel and to ease dependence on unreliable imports of the crude.

1. Lignocellulose stock.

Many types of biomass, such as wood, agricultural waste, grassy crops and solid rural waste are considered as a stock suitable to produce ethanol. These materials consist basically
30 of cellulose, hemicellulose, and lignin. The present invention relates to conversion of polysaccharides contained in the lignocellulose stock into ethanol. The present invention does not cover the well-known process of producing ethanol from the starch containing stock when the starch is converted into glucose by acid and/or fermentation hydrolysis and then fermented into ethanol.

35 This method permits to process many types of the lignocellulose stock into the liquid fuel. The main types of this stock are grain crops, quick-growing trees, agricultural waste, wooden waste, and cellulose fibers from solid rural waste and paper waste. It is preferable

that these vegetable materials be in the form of small particles, like sawdust, chips, or pulverized biomass.

The lignocellulose stock suitable for this method to produce bioethanol includes, without limitations, the following types: agricultural plants, corn stocks, corn ears, wheat, oat
5 straw, rice straw, sugar cane stocks (bogassa), flax straw (boon), soya bean stems, groundnut stems, pea stems, sugar beat stems, sorghum stems, tobacco stems, maize, barley straw, buckwheat straw, cassava stems, potato stems, bean stems, cotton and its stems, inedible parts of plants, grain shells (husk); wood of fir, pine, silver fir, cedar, larch, oak, ash, birch, aspen, poplar, beech, maple, nut-tree, cypress, elm, chestnut, alder, hickory, acacia, platan, pepperidge, butternut, apple-tree, pear-tree, plum-tree, cherry-tree, cornel, catalpa, box-tree, cam-
10 tree, red-wood, lanceolate oxandra, tall mora, primavera, rose tree, teak-wood, satinwood, mangrove-wood, orange-wood, lemon, logwood, scumpia, orange maclura, hedge wood cis-alpine, fragrant cisalpine, cam wood, sandal-wood, rubber-bearing wood, huta, mesquite, eucalyptus; shrubs, oleander, cypress, juniper, acanthus, lantana, bougainvillea, azalea, feijoa,
15 holly, hibiscus, stramonium, acutifolia, hydrangea, jasmine, rhododendron, common Palma Christi, myrtle, euonymus, aralias; algae, brown algae; herbs, creeping plants and flowers.

The waste of agricultural plants containing cellulose can be crushed into fine particles and used in the present invention. The commercial waste containing cellulose, such as paper, cotton fabric, timber can also be used in the present invention. Partially decomposed vegetable
20 materials, such as mowed grass, humus, peat, can be used in the present invention.

2. Biomass chemical composition

The biomass of vegetable materials consists of five major components: cellulose, hemicellulose, lignin, protein, and inorganic matter. The cellulose, hemicellulose, and lignin are
25 the most essential for ethanol production.

2.1. Cellulose

Cellulose is a linear polysaccharide consisting of elementary links of anhydro-D-glucose and represents a poly- β -1,4-D-glucopyranosyl-D-glucopyranose. The cellulose macromolecule can in addition to the anhydroglucose contain remnants of other monosacharrides (pentose and hexose) and uronic acids. The nature and the concentration of these remnants are determined by the conditions of biochemical synthesis. The degree of polymerization of the native cellulose can amount to over ten thousand monomeric units; the degree of polymerization of majority of grassy plants does not exceed one and a half thousand units.

Cellulose is the main component of the cellular walls of higher plants. It plays together
35 with the accompanying substances the role of the skeleton bearing the main mechanical loading.

Cellulose has a complex super molecular structure resulting from the ordering of its molecules. The smallest cellulose super molecular link is the primary fibril in which groups of arranged in parallel macromolecules are linked together by numerous hydrogen bonds. The cellulose macromolecules in the primary fibrils form highly ordered crystalline zones that alternate with inhomogeneous, less ordered amorphous zones. The crystalline zones in the primary fibrils stretch for 15 nm; their cross section is 3-7 nm.

The primary fibrils in the cellulose are linked together with hydrogen bonds into microfibrils that are the main links of the fibrous cellulose structure. According to the commonly accepted now Freigh-Wissling model a significant role in formation of the microfibrils is played by the occluded water and lignin and hemicellulose found in between the primary fibrils.

Such specific cellulose morphological structure makes its stable when exposed to significant mechanical loads. The cellulose is also quite stable to enzymes and microorganisms. The structural strength is because natural cellulose is a composite material with the crystalline matrix and amorphous fillers, hemicellulose and lignin acting as adhesives.

The intricacy of the process of conversion of the lignocellulose stock into the bioethanol is that it transforms stable cellulose into glucose. The latter is known to ferment easily by yeast into ethanol.

2.2. Hemicellulose

Hemicellulose are polysaccharides in the composition of the plant tissue cellular walls that together with the cellulose and lignin are branched polymers of different structures, the main monomeric units of the hemicellulose being galactose, glucose, mannose, xylose, arabinose, uronic acids.

Hemicellulose differs from cellulose by better solubility in alkaline solutions and the capability to be hydrolyzed quickly by the solutions of cellulosolytic enzymes and weak solutions of acids. The degree of polymerization of the hemicellulose is, as a rule, inferior to that of the cellulose. The monosaccharide units are usually combined by β -1,4-links, the latter having frequently lateral links of another type. The main component of the hemicellulose is xylose (50-70 % monomeric links); the main class of the hemicellulose is xylane.

2.3. Lignin

Lignin is an amorphous cross-linked phenol polymer that only vascular plants have and it account for up to 30 % of their mass. Microorganisms capable to produce ethanol do not digest lignin and so it is useless for ethanol production. Lignin remnant can be used as fuel for production facilities in order, for instance, steam and power generation. Lignin can be oxidized into a number of useful chemical substances, but so far there are no well-developed processes and they have not yet gained broad application.

The plant biomass consists of cellulose macrofibers coated with a hemicellulose layer. These layers are embedded into the lignin matrix. The diameter of the cellulose macrofibers is about 1-4 μm . Thus, the mechanical separation of cellulose from lignin can be achieved by crushing the material to particles 1-4 μm . Pulverization of the vegetable materials into a powder of the same size with the efficiency acceptable from the industrial viewpoint is linked with large difficulties.

2.4. Comparative reaction ability of polysaccharides in the lignocellulose stock to split hydrolytically producing simple carbohydrates

The amorphous cellulose and hemicellulose parts of lignocellulose materials are easily hydrolyzed yielding water-soluble carbohydrates in the process called saccharification leaving lignin and unhydrolyzed crystalline cellulose. The process of saccharification implies hydrolytic decomposition of the cellulose in the presence of a catalyst.

Prior Art

There are two principal catalysts for the saccharification process. The sulfuric acid is a common chemical catalyst. The residue of saccharification by sulfuric acid contains lignin and unhydrolyzed cellulose.

The common biochemical catalysts are cellulose enzymes that are obtained, as a rule, as a complex preparation by ultrafiltration of cultural fluids of definite microorganisms. The enzymes in the composition of these cellulolytic complexes have inherent specialization: some of them hydrolyze effectively internal glycoside links between monosaccharide units (endopolymerases, endoglucanases, endoenzymes); others split preferably the external glycoside links at the ends of the polysaccharide chain (exodepolymerase, exoglucanases, exoenzymes); still another glucosidases perform hydrolysis of glycoside links of di- and oligosaccharides.

3. Catalytic hydrolysis of polysaccharides in the lignocellulose stock

There are two main known processes of the catalytic hydrolysis of the polysaccharides in the lignocellulose stock to fermentable monosaccharides:

- Acid hydrolysis is attractive because it occurs quite quickly. However, it needs special acid resistant equipment; moreover, the acids during hydrolysis of carbohydrates and lignin produce by-products that are toxic for majority of the microorganisms generating ethanol. Therefore application of the acid hydrolysis to produce bioethanol demands special techniques of cleaning hydrolates leading to a considerably higher cost of the end product. Utilization of the acidic waste and regeneration of acids also complicate the process. There is a risk to personnel health and environment contamination;

• The enzyme hydrolysis of polysaccharides in the lignocellulose stock evolves with larger selectivity and larger yields characterize it. Until recently, the application of enzymes was limited by the duration of the hydrolysis processes and their costliness.

3.1. Acid hydrolysis

5 The oldest method of conversion of polysaccharides into monosacharrides was based on the acid hydrolysis (the review by Grethlein, Chemical Breakdown of Cellulose Materials, *J. Appl. Chem. Biotechnol.* 1978, no. 28, pp. 296-308). This process can include use of concentrated and diluted acids. The process using the concentrated acids presumes use of the 72 % sulfuric acid, 42 % hydrochloric acid at the room temperature to dissolve cellulose, then dilu-
10 tion to 1 % by acid and heating to 100-120 °C during 3 hours to hydrolyze cellulose oligomers into glucose. This method enables to achieve high glucose yield. Yet regeneration of acids, deployment of special materials in the equipment, the great amount of water used in the system, are serious shortcomings of this process. Similar problems can be confronted when using concentrated organic acids to convert cellulose into glucose.

15 The process with the diluted sulfuric acid 0.5-2 % at 180-240 °C lasts from several minutes to several hours (Brink, U.S. Patent Nos. 5,221,537 and 5,536,325). They describe a two-stage process of acidic hydrolysis of lignocellulose materials. The first stage is conducted under mild conditions and involves depolymerization of hemicellulose into xylose and other monosacharrides. The second stage is depolymerization of cellulose into glucose. A modest
20 consumption of acids obviates regeneration. The maximum glucose yield is 55 % of the cellulose concentration; the by-products of acid hydrolysis suppress the fermentation process inhibiting industrial use of diluted acids.

The state-of-the-art of acid processes is disclosed in the materials (FY 1997 Biochemical Conversion/Alcohol Fuels Program, *Annual Report*, page 85). This process employs concen-
25 trated sulfuric acid to convert corn straw into sugars. There is a diagram of separation of sugars that the concentrated sulfuric acid contains using a solvent with a high boiling point in order to dilute the sulfuric acid and a low boiling solvent to dilute the high boiling solvent. This method has losses of the solvent and the sulfuric acid neutralized by the lime.

Thus, the problem of sulfuric acid regeneration during acid hydrolysis remains unre-
30 solved; the cost of concentrated sulfuric acid effective regeneration is very high.

Another unresolved problem of the acid process is to obtain the lignin from the lignocel-
lulose stock free of sulfuric acid impurities. Only this lignin can serve as an environmentally friendly fuel and as a component in the formulas to feed animals.

3.2. Enzyme hydrolysis

35 Usually treatment with enzymes is performed during mixing of the substrate (the ligno-cellulose material) with water to obtain 5-12 % suspensions of the cellulose mass, afterwards

the enzymes are added. Hydrolysis is conducted during 24-150 hours at 37-50 °C, pH 4.5-5. Once the hydrolysis is over the soluble monosacharrides are in the liquid, unhydrolyzed portion of cellulose, lignin and other insoluble components of the substrate remain in the solid portion of glucose molasses. They are extracted by filtering the suspensions, the solid residue is washed through to increase the glucose yield. The glucose molasses are fermented into ethanol by yeast; ethanol is purified by distillation or other method. The ethanol fermentation and purification are a well-known process applied in alcohol production.

The availability of the substrate to enzymes is the main factor governing the effectiveness of cellulose enzymatic hydrolysis (Nazhad, M. M., L. P. Ramos, L., Pászner, and J. N. Sadler, Structural Constraints Affecting the Initial Enzymatic Hydrolysis of Recycled Paper, *Enz. Microb. Tech.*, 1995, no. 17, pp. 68-74.

The effectiveness of enzyme hydrolysis depends on the specific features and the mechanism of action of the enzymes. For instance, the cellulase *T. longibrachiatum* bonds strongly to the cellulose resulting in a reversible inactivation of the enzyme (Brooks, T. A., and Ingram, L. O., Conversion of Mixed Office Paper to Ethanol by Genetically Engineered *Klebsiella oxytoca* Strain P2, 1995, *Biotechnol. Prog.*, vol. 11, no. 6, pp. 619-625). The degree of bonding is governed by the stirring intensiveness (Kaya, F., J. A. Heitmann, Jr., and T. W. Joyce, Cellulase Binding to Cellulose Fibers in High Shear Fields, *J. Biotech.*, 1994, no. 36, pp. 1-10). The problem can be solved by applying the conditions ensuring intensive mass transfer. A very high rate of hydrolysis was achieved in the reactor with intensive mass transfer (Gusakov, A. V., Sinitsyn, A. P., Davydkin, I. Y., Davydkin, V. Y. and Protas, O. V., Enhancement of Enzymatic Cellulose Hydrolysis Using a Novel Type of Bioreactor with Intensive Stirring Induced by Electromagnetic Field, *Appl. Biochem. Biotechnol.*, 1996, no. 56, pp. 141-153).

4. Factors determining the effectiveness of using enzymes

Regretfully, so far the method of treatment of the cellulose containing stock with enzymes have failed to produce glucose and other fermentable sugars sufficiently cheaply that would make the process of ethanol production profitable. Even application of the most effective, so far known methods of pre-treatment, the degree of transformation does not exceed 77-84 % of soluble carbohydrates against the total concentration of polysaccharides in the lignocellulose stock (U.S. Patent No 5,196,069), meanwhile the amount of enzymes needed to convert the polysaccharides in the lignocellulose stock into fermentable carbohydrates is too large.

Several methods have been advised to save the enzyme.

When a lesser amount of cellulolytic enzymes is added, the quantity of glucose drops to the intolerable limit, treatment of the stock takes more time making the process unprofitable.

The method of saving the quantity of enzymes by combining hydrolysis with the process of fermentation is ineffective too. The process of combined saccharification and fermentation (CAF) yields no profit because the optimum 28-35 °C temperature to activate the yeast is much lower than the optimum 50-58 °C temperature of activation of the enzymes. The CAF at a moderate temperature 30-37 °C is ineffective and provokes development of vulgar microflora.

The urgency of development of a profitable process of ethanol production is the motive for numerous studies aiming at developing effective methods of pre-treatment. An effective pre-treatment method should combine the advantages of the known methods, including a high degree of cellulose processing, low yield of side-products and frugal consumption of cellulolytic enzymes.

The effect of the pre-treatment method is characterized by the degree of transformation of cellulose components into soluble sugars and the amount of the enzyme consumed to convert a definite amount of cellulose into glucose.

Pre-treatment in the present invention combines the known approaches to acceleration of enzymatic hydrolysis:

- splitting of the lignocellulose material into lignin, hemicellulose and cellulose as a result of destruction of the lignin membrane into cellulose fibers;
- dispersion of the treated material and significant expansion of the phase interface where the subsequent heterogeneous hydrolysis of cellulose takes place in the aqueous solutions;
- amorphization of the crystalline cellulose noticeably accelerating the preceding reaction of cellulose enzymatic hydrolysis ;
- implementation of the new approach, namely, direct introduction of enzymes into the lignocellulose substrate impossible using their aqueous solutions.

5. Pre-treatment stock prior to hydrolysis

The pre-treatment by all the methods employs steam energy, mechanical energy, and energy of radiation. One or several types of pre-treatment are used to increase the rate and degree of hydrolysis. The effect of pre-treatment is commonly explained by the fact that it increases the availability and the surface area of hydrolyzed polysaccharides, destroys the physical and molecular structure of the original material and splits up (reduces sharply the intermolecular interactions between the macrostructural components) lignocellulose materials into lignin, hemicellulose and cellulose components.

When additional chemical agents are used at the pre-treatment stage, they are to be eliminated in the end product.

The commonly acknowledged pre-treatment methods are exemplified in the review (Sinitsyn, A.P., Gusakov, A.V., and Chernoglazov, V.M., *Bioconversion of Lignocellulose Materials*, Moscow: *Publishing House of Moscow State University*, 1995, 220 pp., and the references in the list):

- 5 • dissolution with chemical agents, such as caustic alkalis, ammonia, chlorite, sulfur dioxide, amides, diluted and concentrated acids, and others commonly used to produce pulp and paper;
- steam treatment, steam explosive treatment (steam explosion, powerful steam extrusion, i.e. feeding steam under pressure into the stock and its destruction due to a sharp pressure drop when steam passes through the outlet hole);
- 10 • autohydrolysis in high-temperature steam (220-270 °C);
- mechanical treatment: crushing and grinding;
- microwave irradiation;
- ultrasound irradiation;
- 15 • electron bombardment;
- Gamma-irradiation.

5.1. Dissolution

Application of the chemical agents commonly implies heating of the lignocellulose stock in the presence of acids, alkalis, and solvents. Acids catalyze hydrolysis of polysaccharides into soluble carbohydrates, monosaccharides primarily. The hydroxides of alkaline metals serve to delignify the polysaccharides, then the polysaccharides undergo the acid hydrolysis into soluble carbohydrates, the sulfuric acid is used, as a rule. According to the improved method, biomass is first wetted by the solution of the alkaline metal hydroxide, and then it is stirred in order to distribute the catalyst over the substrate and destroy interactions between lignin and polysaccharides. The hydroxides of alkaline metals are introduced in the amount sufficient to initiate thermal reactions. The latter release carbon dioxide from the cellulose carbohydrates and modify the lignin.

The carbohydrates formed by the present method can serve to produce bioethanol as animal feed or in the synthesis, for instance, to synthesize high-molecular alcohols.

30 5.2. Steam explosive treatment

Steam treatment is one of the main methods of pre-treatment of the lignocellulose stock (U.S. Patent # 4,461,648). By this method, the biomass is charged into a vessel, the so-called steam gun. A solution of acids (up to 1 %) is added into the vessel with the biomass. Then the vessel is filled up rapidly with steam and kept under high pressure during the assigned time.

35 When the time expires, the pressure in the vessel is rapidly released, the treated biomass is thrown out, hence the method is called «steam explosion». The pre-treatment effect depends

on the time of exposure under treatment, temperature, the concentration of acids and particle sizes in the stock. The steam pressure ranges between 17 and 72 atm, the temperature between 208 and 285 °C.

Other researchers who tested different substrates and equipment later confirmed the optimum pre-treatment conditions disclosed in U.S. Patent # 4,461,648. For instance, U.S. Pat # 4,237,226 describes pre-treatment of oak, poplar wood, newspaper and corn straw in an impact flow-through continuous type reactor resembling an extruder. Rotating screws force the suspended stock through a small hole and the stock structure is destroyed mechanically at the outlet.

Modern publications study the pre-treatment mechanisms that improve the enzyme hydrolysis of the lignocellulose substrate. U.S. Patent # 5,628,830 describes pre-treatment of the lignocellulose material by steam explosion in order to destroy the hemicellulose followed by the cellulose hydrolysis. Knappert et al. in "A Partial Acid Hydrolysis of Cellulosic Materials as a Pretreatment for Enzymatic Hydrolysis, Biotechnology and Bioengineering", 1980, no. 23, pp. 1449-1463, report that the reactivity of enzymatic processes after pre-treatment is explained by formation of micropores when hemicellulose is removed and the crystallinity of the substrate is modified, and also by reduction of the degree of polymerization of cellulose molecules.

5.3. Mechanical grinding and amorphization

Mechanical treatment usually implies application of impact, shear, pressure, grinding, mixing, compression/expansion or other types of mechanical effects.

Expansion of the substrate surface area was considered as the effect of pre-treatment. Grethlein and Converse (Common Aspects of Acid Prehydrolysis and Steam Explosion for Preheating Wood, *Bioresource Technology*, 1991, vol. 36, no. 2, pp. 77-82) improved this explanation by showing that that surface area is essential that is available to cellulolytic enzymes having the size about 50 Å. The specific surface measured by sorption of small molecules, like those that nitrogen has, does not correlate with the rate of the substrate enzymatic hydrolysis. The method of determination of the surface from adsorption of gases considers also small pores too that are inaccessible to enzymes and are not involved in the enzymatic hydrolysis.

U.S. Patent # 5,366,558 describes an improved method of producing glucose when the stock is subjected to mild hydrolysis during which the hemicellulose splits without any substantial cellulose hydrolysis. The solid residue containing cellulose and lignin is subjected to fine grinding, for instance, by the method disclosed in U.S. Patent # 4,706,903. The ground substrate is subjected to acid hydrolysis under tougher conditions until the glucose solution is obtained.

According to U.S. Patent # 5,268,830, the fine ground solid residue resulting from the biomass after hydrolysis of the lignocellulose stock, as disclosed in U.S. Patent # 5,366,558, is subjected to enzymatic hydrolysis by cellulolytic enzymes producing an aqueous glucose solution that is later fermented into ethanol. Hydrolysis of polysaccharides into monosaccharides and their fermentation into ethanol can be conducted simultaneously in the presence of cellulolytic enzymes and special microorganisms, yeast or bacterial fermenting monosaccharides into ethanol.

Thus, the ethanol yield can be increased compared with the method by which the residue after removal of easily hydrolyzed polysaccharides (the hemicellulose) serves as the substrate without any further fine grinding.

The methods with steam explosion have limitations at the first stage by the size of particles. Too small particles resist this effect; hence, the optimum size of particles is 200 μm .

5.4. Microwave irradiation

Azuma J. et al. (Journal of Fermentation Technology, 1984, vol. 62, no. 4, pp. 377-384, and U.S. Patent No 5,196,069) proposed a method of microwave pre-treatment for enzymatic hydrolysis of polysaccharides in the lignocellulose stock. The enzymatic hydrolysis rate of polysaccharides accelerates in case of microwave pre-treatment of the stock at 160 °C; the maximum effect is reached at 223-228 °C. Such treatment enables to obtain 77-84 % of the reducing carbohydrates from the total concentration of polysaccharides in the lignocellulose stock.

5.5. Ultrasound irradiation

U.S. Patent # 6,333,181 considers the improved method of enzymatic hydrolysis of polysaccharides from the lignocellulose stock. The method is based on the ultrasound treatment of the lignocellulose stock in the presence of water and enzymes ensuring further hydrolysis of polysaccharides. The duration and conditions of the ultrasound treatment are selected such as to prevent heating of the mixture to the temperature at which a considerable portion of enzymes denaturizes. It is taken into account that ultrasound treatment leads to a considerable destruction of the cellulose crystalline structure. This method saves consumption of enzymes two-three times versus the common methods.

5.6. Electron bombardment, gamma-irradiation

Gamma-irradiation in high doses (150-200 Mrad) increases the reactivity of cellulose 2-4 times. About 20 % of the cellulose forms a mixture of soluble isomeric sugars that do not ferment and reduce the ethanol yield (Sinitsyn, A.P., Gusakov, A.V., and Chernoglazov, V.M., Bioconversion of Lignocellulose Materials, Moscow: Publishing House of Moscow State University, 1995, 220 pp.).

Electron bombardment was also proposed for pre-treatment of the lignocellulose stock (Petersen et al., The Engineering Society for Advancing Mobility Land Sea and Space (SAE International) technical paper 901282, Jul. 9 – 12, 1990). Apparently, due to the extremely expensive equipment and treatment with gamma rays and electrons, these approaches are applicable solely under specific conditions, for instance, in the outer space.

Numerous studies of methods of pre-treatment of the lignocellulose stock have provided the idea about the mechanisms on which subsequent acceleration of the enzymatic hydrolysis and laid grounds for optimization of the processes of biological conversion of polysaccharides into ethanol. However, there are still no profitable, environmentally friendly, and industrially applicable methods combining pre-treatment of the lignocellulose stock, enzyme hydrolysis of polysaccharides and fermentation of carbohydrates into ethanol.

6. Combined saccharification and fermentation (CAF, SSF)

Blotkamp, P. J. et al. (American Institute of Chemical Engineering (AIChE) Symposium 1981, Series # 181, vol. 74) described the process of combined saccharification of cellulose and fermentation of sugars into ethanol (CAF) using the enzymes of the fungi *Trichoderma reesea* and the yeast *Saccharomyces cerev.* The rate of hydrolysis of the cellulose stock increases in comparison to the process of consecutive stages of saccharification and fermentation due to reduction of the rivaling inhibition of enzymes by glucose and other soluble carbohydrates.

7. Fermentation

Any suitable method is applicable to fermentation of carbohydrates to produce ethanol according to the present method. Any yeast capable to induce conversion of carbohydrates into ethanol can be added to the aqueous solution of carbohydrates obtained under the present method. The mixture is fermented until the carbohydrates are fully consumed. Ethanol is purified by distillation.

Another method can be used, like microbic conversion, or combined saccharification and fermentation. There are several types of yeast used to produce ethanol on industrial scale, like Montrachet, Pasteur Chalmmpagne, Cote des Blancs, Pasteur Red, Lalvin K1-V-1116 and Lalvin 71 B-1122.

8. Mechanical activation and mechanochemical treatment

Unfortunately, so far there is no general theory that would well describe all mechanochemical reactions. Beyer, M.K., Clausen-Schaumann, H. in Mechanochemistry: the mechanical activation of covalent bond, *Chem. Rev.*, 2005, vol.105, no. 8, pp. 2921 – 2948, treat only individual aspects and possible phenomena:

- formation of active surface radicals,

- the role of interphase processes (Butyagin, P.Yu. The Role of Interphases in Low Temperature Reactions of Mechanochemical Synthesis, *Russian Colloidal Journal*, 1997, vol.59, no. 4, pp. 460-467),

- 5 - hydrothermal chemical processes in mechanical activation of heterogeneous systems containing water (the conditions of autoclaving resulting in particles from the constrained impact in the contact and these conditions are characterized by high temperatures and pressures) (Boldyrev, V.V., Hydrothermal reactions under mechanochemical action, *Powder Technol.*, 2002, vol. 122, pp. 247-254).

From the technological viewpoint, the mechanical activation is rated an effective method
10 of modification of physicochemical properties of solid phases. The mechanical activation implies enhance of the reactivity due to stable changes in the structure of a substance under the effect of mechanical loading. The mechanical and activated solid differs by the fact that its deformation process and physicochemical consequences of deformation are divided by the time insufficient for the relaxation processes to complete (Avakumov, E.G., Mechanical
15 Methods of Activation of Chemical Processes, Novosibirsk: *Nauka*, Siberian Branch, 1986, 303 pp.).

The mechanical strain applied to the solid can relax through several ways. The mechanical energy is expended primarily for formation of new surface and defects in the crystalline structure. These processes increase the free energy in the solid resulting in its enhanced reactivity. The latter circumstance has general nature. So, the mechanical and activated solid
20 phases are characterized by higher dissolution rates and easier react chemically with gases and liquids compared with the non-activated phases.

The main physicochemical result of mechanical activation of solids is their intensified reactivity and the following results are promising for practical considerations:

- 25
- expansion of the surface and related stronger dimensional effects;
 - disordering of the crystalline structure and amorphization ;
 - evolution of heterogeneous systems with a developed interface between the phases where physicochemical characteristics of the substance change sizably (the free energy, the crystalline structure, etc.).

30 The term *mechanical activation* implies activation of the subsequent physicochemical processes involving the products of mechanical activation, for instance, the solid phase synthesis of materials by baking, the processes of extraction, dissolution, chemical interaction with liquid or gaseous media.

A more general term of *mechanochemical treatment* is applied to heterogeneous systems
35 that have a complex phase composition and consist of numerous components. The mechanochemical treatment, like mechanical activation, induces stable changes in the system's phys-

icochemical properties. Stronger reactivity affects, as a rule, most of the phases and components of the system. During the mechanochemical treatment, the chemical reactions can evolve resulting from stronger mobility of the components and their larger free energy under effect on mechanical loading (Boldyrev, V.V., Mechanochemistry and Mechanical Activation, *Materials Sci. Forum*, 1996, vol.225-227, pp. 511-520).

The mechanoenzymatic treatment is the mechanochemical treatment in which enzymes participate. This type of effect is applicable to plant stock, natural polymers and organic materials. The mechanoenzymatic treatment is conducted in order to increase the substrate reactivity; in number of cases, chemical reactions can evolve catalyzed by enzymes directly at the time of treatment.

The reactivity of solid phases is restricted by low mobility of the components making up these phases. Under the effect of intensive mechanical loading the components mix up, arrange directly close one to another so that the paths of diffusion are reduced sharply. The mechanical treatment of the mixture of solid phases intensifies the mobility of the components in the time of treatment and increases mobility due to disordering of the crystalline structure of solid phases. The solid components can accumulate defects and amorphize resulting in stronger reactivity of both the components and the system in general.

Mechanical activation and the subsequent chemical involving the liquid phase, for instance, hydrolysis, extraction, chemical interaction between solid components due to full or partial dissolution in the liquid phase, can be combined. It is shown that during the mechanical treatment of the solid – liquid system various chemical transformations are initiated and accelerated. Evolution of these chemical reactions are favored by the phenomena typical for activation of mixtures of solid components (expansion of the interface between phases, accumulation of defects, amorphization, rise of free energy). High temperature and pressure appearing during mechanical treatment can also generate unusual conditions for chemical reactions. Intensive mechanical effect on heterogeneous systems containing a liquid leads in a number of cases to appearance of hydrothermal conditions and evolution of cavitation processes over local spots exposed to the effect.

The mechanical energy from the viewpoint of economics is an «expensive» type of energy. It should be consumed effectively. In some cases the mechanochemical treatment of the solid mixture can be suspended at an early stage of transformation of agents and full chemical transformation is achieved with other, energy-saving processes involving, as a rule, liquid phases.

In case of this approach, the mechanochemical treatment is achieved:

- by introducing defects into the crystalline structure of the agents,

- by reducing the degree of crystallinity and amorphization of the agents ,
- by producing mechanocomposites. The mechanocomposites are products of the mechanochemical treatment of solid heterogeneous mixtures and they represent a system, having the physicochemical properties significantly different from the original mixture and they are determined by substantial changes in the morphology of the components, the developed interface phases with pronounced interphase surface interaction. The interphase material possesses the physicochemical characteristics that are different for any of the original components or individual phases.

So far, numerous processes have been described employing mechanochemical treatment, for instance:

- Preparation of the mineral stock in order to increase yield in the processes of recovery of the useful component a (Tkacova, K., *Mechanical Activation of Minerals*, Amsterdam: Elsevier, 1989, 156 pp.).
- Intensification of hydrometallurgical processes (Balaz, P., *Mechanicka Aktivacia v Procesoch Extrakcijn Metalurgie*, Bratislava (Slovakia): Veda, 1997, 223 pp.).
- Chemical coal processing (Khrenkova, T.M., *Mechanochemical Activation of Coals*, Moscow: Nedra, 176 pp.).

The progress of development of mechanochemistry is attributed to the catalysis of organic reactions (Molchanov, V.V., Buyanov, R.A., *Mechanochemistry of Catalysts*, *Russian Chemical Reviews*, 2000, vol.69, no. 5, pp. 476-493) in pharmacology (Boldyrev, V. V., *Mechanochemical Modification and Synthesis of Drugs*, *J. Materials Science*, 2004, no. 39, pp. 5117-5120) and solution of environmental problems (Lomovsky, O.I., Boldyrev, V.V., *Mechanochemistry for Solving Environmental Problems*, Novosibirsk (Russia): GPNTB SO RAN, 2006, 221 pp.).

The effectiveness of mechanochemical reactions depends both on the chemical and mechanical properties of the agents. Mechanochemical processes in which soft substances and materials participate consume energy modestly (Avvakumov, E., Senna, M., and Kosova, E., *Soft Mechanochemical Synthesis: a Basis for New Chemical Technologies*, Boston: Kluwer Academic Publishers, 2001, 200 pp.). Organic substances are usually much softer than the inorganic. The mechanochemical reactions evolving in the organic systems yield 1000 times more energy than in the inorganic systems. It is shown that some organic reactions are more effective in the solid phase than in the liquid phase (Tanaka, K., Toda, F., *Solvent-Free Organic Synthesis*, *Chem. Rev.*, 2000, vol.100, no. 3, pp. 1025-1074). Thus, the mechanochemical processes evolving with participation of organic substances promise more in the view of technological application.

One of the additional advantages of the mechanical treatment in biotechnologies is the possibility of simultaneous destruction of cellular membranes that consumes more thermal energy and agents in case of other versions of the technology.

The mechanochemical treatment of the systems containing enzymes requires considering the fact that their complex structural set-up governs the catalytic activity of the enzymes. Secondary and tertiary structures can change in the enzymes under the mechanical effect affecting their activity. For instance, the mechanochemical synthesis of the immobilized enzymatic catalyst turned out a failure (Trevan, M.D., *Immobilized Enzymes*, Chichester – New York: *John Wiley*, 1982, 213 pp.). The mechanical treatment of the substrate inactivated the enzyme irreversibly.

Summary of the invention

The conditions are proposed in the present invention under which the lignocellulose stock can undergo mechanoenzymatic treatment turning it into heterogeneous systems consisting of just solid phases and systems containing water. These conditions enable to achieve the technological effect that comprises:

- To prepare by mechanoenzymatic treatment the mechanocomposites based on the lignocellulose stock and cellosolytic enzymes while preserving the activity of the enzymes. Unlike the systems prepared by regular mixing, the fermentative hydrolysis of polysaccharides evolves very fast when these mechanocomposites are in contact with water.
- To achieve the optimum ranges of intensity and duration of the mechanochemical treatment in order to produce the mechanocomposites with high reactivity needed to accomplish effective hydrolysis of polysaccharides into simple carbohydrates and fermentation of the latter into bioethanol as the end product.
- To intensify the process of heterogeneous hydrolysis of polysaccharides from the lignocellulose stock by treating the pulp and the solution of enzymes in the devices of intensive stirring, cavitators and/or ultrasound devices.

The main criterion of effectiveness of the mechanoenzymatic treatment in the present process is enhancing of hydrolysis of polysaccharides from the lignocellulose stock as promotion of yield of water-soluble carbohydrates.

The technical task of the invention is to develop a method of production of bioethanol that would enable to introduce idle biorenewable sources of polysaccharides, predominantly the lignocellulose stock fit to be processed in other spheres of chemical and biochemical technologies;

- To develop an environment friendly method of conversion of polysaccharides from the lignocellulose stock into bioethanol, namely the method that obviates the use of strong inor-

ganic acids in the process of hydrolysis of polysaccharides, the procedures of purification of hydrolates to remove toxic side-products and does not contaminate the intermediate, end products, including waste, with sulfur compounds;

- To develop an effective method of conversion of polysaccharides from the lignocellulose stock into bioethanol, namely the method based on preliminary and/or intermediate mechanoenzymatic treatment of the stock under the conditions ensuring saving of the activity of the enzymes, noticeable enhancing of hydrolysis of polysaccharides and promotion of the yield of fermentable carbohydrates ;
- To optimize the conditions of mechanoenzymatic treatment to ensure effective use of the enzymes in the process of conversion of polysaccharides from the lignocellulose stock into bioethanol ;
- To create a new method of processing the cellulose-containing substrates into the product capable of treatment by fermentation with a simpler and effective method;
- To create a method of producing ethanol with a simple technology and with a relatively cheap equipment, namely, the method that can be applicable both in small- and large-scale production;
- To create a method of producing ethanol from the cellulose-containing waste materials, such as lignocellulose, namely the method that does not consume a large quantity of agents.

Thus, the present invention is based on application of preliminary and/or intermediate mechanoenzymatic treatment that enhances noticeably the hydrolysis of polysaccharides, promotes the yield of fermentable carbohydrates, and reduces material and energy cost of the process of production of bioethanol from the lignocellulose stock. The preliminary (intermediate) treatment implies that the mechanochemical effect of certain intensity and duration acts on the mixture of the lignocellulose stock and enzymes (the solution of the enzymes) in the mechanochemical reactor (the cavitation or ultrasound device).

The inventors have discovered that implementation of definite conditions, such as utilization of the soft lignocellulose materials as the raw stock, the optimum intensity and duration of the mechanical effect that ensure formation of mechanocomposites and preservation of the activity of the enzymes, is sufficient and necessary for effective conversion of the polysaccharides from the lignocellulose stock into bioethanol .

The discovered facts served to advance an improved method of conversion of polysaccharides from the lignocellulose stock into ethanol. This method comprises several stages:

- mechanoenzymatic treatment of the mixture of 90-98 % of the lignocellulose stock having the concentration of natural moisture 0.5-15 % of the stock mass, with 0.2-2.0 % of cellulosolytic enzyme preparation (containing the optimum ratio of endo-1,4- β -glucanase, exo-1,4- β -glucanase, exo-1,4- β -glycosidase and β -glycosidase), 0.0-8 % of inorganic salt,

0.0-1.0 of the surfactant, during 0.5-10 min in the ball mill with the acceleration 60-400 m/s² or in the rotor mill with the speed of rotors 10-120 m/s or in the pneumatic vortex mill with the gas flow rate 10-120 m/s;

• mixing of the obtained mechanocomposite powder with water, hydrolyzing of a part of the cellulose and hemicellulose in soluble carbohydrates to improve susceptibility during the next processes of saccharification and fermentation into ethanol ;

• enzyme hydrolysis of polysaccharides to achieve 90 % conversion of polysaccharides into soluble carbohydrates in the reactors of periodic action or by the substrate hydrolysate counterflow or in stages in two reactors of the periodic type or intermediate treatment of the hydrolysate - solid residue system with ultrasound ;

• the mechanoenzymatic treatment of the mixture of the above composition instead or in addition to the preceding stage is performed in the presence of water (the hydromodulus is over 3) in the mixers with intensive stirring or in cavitation devices;

• the preliminary enzyme hydrolysis is performed instead of the above stage to achieve the degree of conversion of polysaccharides 20-40 %, the hydrolysisate - solid residue system is treated in the cavitation devices in the presence of solid residue, ethanologenic microorganisms are introduced to perform the process of saccharification and combined fermentation (SSCF);

• intermittent introduction of enzymatic complexes into the process of enzymatic hydrolysis ;

- microbiological conversion of the carbohydrates the hydrolysates contain into ethanol;
- distillation of the ethanol from the wash.

The preliminary and/or intermediate mechanoenzymatic treatment increase the degree of conversion of the cellulose raw stock to 90%, saves considerably the consumption of the cellosolytic enzymes needed for hydrolysis of the polysaccharides in comparison with the known methods. Application of the claimed method makes production of ethanol from lignocellulose materials much cheaper.

The mechanochemical treatment during conversion of the polysaccharides from the lignocellulose raw stock into ethanol is a significant improvement of the known processes. An additional advantage of the method is that the unconverted residue of the lignocellulose stock contains no unhydrolyzed polysaccharides, etc., or traditional impurities, sulfur in the first place, that inhibit use of this residue for production process needs, for instance, for combustion in order to generate heat, steam and power.

Microorganisms that the biomass contains are usually used to obtain additional products, such as feed protein or feed additives with biologically active properties for agricultural animals.

This method can be optimized further by changing the types of treatment, the intensity, and duration of the effects.

Thus, the present invention embodies the method of producing ethanol from the lignocellulose raw stock, namely the method that is applicable in production of bioethanol from lignocellulose plant materials that comprises the mechanoenzymatic treatment of the material in the mechanochemical reactor in the presence of cellulosolytic enzymes with or without additional water that follows hydrolysis of the polysaccharides or combined performance of fermentation of resulting carbohydrates into ethanol with the help of suitable ethanologenic microorganisms.

The ethanologenic microorganisms can be special strains of bacteria or yeast, including recombinant strains that are capable to ferment the main monomeric units of the polysaccharides from the lignocellulose raw stock, namely xylose and glucose that hydrolysates contain. The preferable ethanol-producing microorganisms include *Saccharomyces*, *Zymomonas*, *Erwinia*, *Klebsiella*, *Xanthomonas*, *Escherichia*, etc.

Brief description of drawings

Fig. 1. Mechanochemical introduction of the enzyme into the lignocellulose stock mass – into the reaction zone (right), for comparison, left – addition of the substrate into the aqueous solution of the enzyme.

Fig. 2. The chromatographic splitting of carbohydrates. The hydrolate of the wheat straw.

Fig. 3. The chromatographic splitting of carbohydrates. The artificial mixture of carbohydrates as a reference.

Fig. 4. Dependence of the degree of transformation of microcrystalline cellulose into soluble sugars on the duration of enzymatic hydrolysis (the lower curve – without mechanoenzymatic treatment, the upper curve – with mechanoenzymatic treatment).

Fig. 5. Diagram of hydrolysis processes in the counterflow mode using several reactors.

Fig. 6. The data of the optical microscopy demonstrating the fracture of conglomerates of particles of the substrate (*a-c* – formation of conglomerates of particles in the process of preliminary hydrolysis, *d* – the result of treatment in the cavitation device).

Brief description of the invention

The basis of the present invention is mechanoenzymatic treatment; Fig. 1 illustrates its technological sense. The mechanical treatment of the solid mixture of the substrate and the enzyme does not affect the structure and the activity of the introduced enzyme; it permits to
5 distribute the enzyme molecules in the substrate volume. As regards the case of the traditional methods of addition of the enzyme aqueous solution into the substrate (shown in Fig. 1,2 for comparison, left), a major portion of the enzyme molecules appears outside the substrate and cannot be used effectively.

Terminology

10 The presented invention and its preferable embodiments are disclosed using definite terms; their definitions are given below.

The lignocellulose stock implies any raw stock that can be used in the processes of conversion of cellulose and attendant polysaccharides into ethanol. The raw stock contains at least 20-35 % cellulose; most of it is hydrolysable into glucose. The concentration of water in
15 the so-called "air-dry" stock dehydrated without vacuum at the temperature below 50-100 °C is 8-15 % of the stock mass. There are no special limits of concentrations of lignin, starch, protein, or inorganic compounds in the raw stock. For instance, the lignocellulose stock to produce ethanol can be wood, grass, straw, waste of agricultural crops.

Conversion into ethanol means conversion of at least 90 % of cellulose and hemicellulose
20 into glucose and other soluble carbohydrates intended for further fermentation into ethanol.

Hemicellulose contains different monosacharrides. Different publications from different
0sources indicate the composition of the hemicellulose obtained by different methods. Thus, according to the publications, the composition of the stock can be determined approximately. Practical implementation of this invention demands that each substrate should be analyzed
25 with the same methods.

Mechanoenzymatic treatment is the mechanochemical treatment of the lignocellulose stock in the presence of enzymes or a solution of the enzymes. This type of effect is applicable both to the lignocellulose raw stock and to individual polysaccharides. The mechanoenzymatic treatment is conducted in order to increase the reactivity of the substrate, namely, to
30 accelerate the hydrolysis and to hydrolyze more polysaccharides in the stock. Without mechanoenzymatic treatment consumption of the cellosolytic enzymes grows considerably when it is necessary to achieve 90 % conversion of polysaccharides by the reaction of enzymatic hydrolysis.

The mechanoenzymatic treatment of the mixture (90-98 % - the lignocellulose stock
35 with the concentration of natural moisture 0.5-15 % of the stock mass; 0.2-2.0 % - the cellosolytic enzymatic preparations containing the optimum ratio of endo-1,4-β-glucanase, exo-

1,4- β -glucanase, exo-1,4- β -glycosidase and β -glycosidase; 0.0-8 % - inorganic salt; 0.0-1.0 - surfactant) is performed during 0.5-10 min in the ball mill with the acceleration of balls 60-400 m/s² or in the rotary mill with the speed of rotors 10-120 m/s or in the pneumatic vortex mill with the rate of the gas flow 10-120 m/s.

5 The product of the mechanoenzymatic treatment of the solid mixture of the lignocellulose stock and enzymes is a mechanocomposite containing the polysaccharides more reactive in respect to the enzymatic hydrolysis. This process effect is an essential attribute of the present invention and it is achieved by the combination of the following factors:

- essential modification of the original morphology of the lignocellulose stock, lessening of intermolecular interactions between main macrostructural components of the stock: lignin, hemicellulose and cellulose, and, finally, destruction of the cellulose lignin membrane. This change in the morphology is observed when the particles of the product have the size comparable with the dimensions of relevant regions of macrostructural components in the original stock ;
- 15 • dispersion of the original stock and expansion of the reactive surface over which the fermentative hydrolysis of polysaccharides when the appearing mechanocomposites came into contact with aqueous media;
- amorphization of the crystalline cellulose leading to acceleration of its subsequent enzymatic hydrolysis ;
- 20 • direct introduction of enzymes into the lignocellulose substrate that is impossible using aqueous solutions of the enzyme.

The preliminary mechanoenzymatic treatment forms a qualitatively new product, or mechanocomposite, the polysaccharides in which are hydrolyzed at a faster rate than by the known methods; it is characterized by a high degree of transformation into monosacharrides and smaller consumption of the enzymes.

25 The pre-treatment of the lignocellulose stock proposed in the present invention is preferably a part of a more complex process of conversion of the lignocellulose stock into the ethanol. The general process comprises pre-treatment of the substrate, enzyme hydrolysis of polysaccharides into monosacharrides, fermentation of the latter into ethanol and ethanol purification.

30 Complex preparations of cellulolytic enzymes are preferable for mechanoenzymatic treatment of the lignocellulose stock and subsequent hydrolysis. According to the present invention a smaller portion of the cellulose is hydrolyzed during pre-treatment, a larger portion is hydrolyzed in the process of saccharification. The method of implementation of the fermentative hydrolysis is not limited by the invention, but the following conditions are prefer-

able. The hydrolysis of the product of the mechanoenzymatic treatment is conducted in the water suspension with the hydromodulus 5-10, pH 4.5-5 at a temperature 50 °C.

The mass-produced preparations of Iogen Corporation, Novo Nordisk, Genencor International, Primalco, Sibbiopharm (Russia) or other manufacturers are preferable as complex preparations containing cellulolytic enzymes. The compositions of enzymes separated by ultrafiltration from the cultural fluid of *Trichoderma viride (reesei)* and/or *Aspergillus awamori* and/or *Bacillus subtilis* can serve as cellulolytic preparations directly obtained during production of bioethanol

In case it is necessary, β -glycosidase can be added into the enzymatic complexes to ensure fuller conversion of cellobiose into glucose. The following mass-produced preparations of enzymes with the β -glycosidase activity were used: Novozym 188 produced by Novo Nordisk and/or Glucolux produced by Sibbiopharm.

The quantity of the enzymes in the hydrolytic process determines the time of hydrolysis, the yield of fermentable carbohydrates and their concentration. All these values influence the profitability of the processes and can vary in response to the technology. The usual dosage of the enzymes is 1-50 U/g of the substrate for 12-128 hours. The preferable dosage of the enzymes was 1 – 10 U/g of the cellulose. Examples 2 and 3 describe the cellulose hydrolysis in more detail.

It is preferable to conduct a combined process comprising the preliminary hydrolysis to the degree of conversion of polysaccharides 10-40 % followed by saccharification combined with microbiological fermentation (SSCF).

Fermentation of carbohydrates into ethanol and its purification are conducted with the well-known traditional methods. The invention is not limited to the methods used to perform these operations.

25

Preferred embodiment

Detailed Description of the invention

The invention is illustrated with detailed examples showing its preferable embodiments, but they do not limit the method that can be used to produce carbohydrates and ethanol. The preferable embodiment is to mix up the enzyme with the lignocellulose material followed by hydrolysis to produce fermentable sugars. The invention ensures effective enzyme hydrolysis of polysaccharides in the hemicellulose stock to produce fermentable carbohydrates and the water insoluble solid residue.

The invention relates to the method accelerating the fermentative hydrolysis of polysaccharides in the lignocellulose stock; it comprises the mechanoenzymatic treatment of the lignocellulose stock, mixing of the product with water under the conditions sufficient for the hydrolysis of polysaccharides. The aqueous suspension of the lignocellulose stock can be subjected to the mechanoenzymatic treatment too.

Usually the mechanoenzymatic treatment is performed with the help of the known equipment. The mechanochemical reactors applicable for the purposes of the invention should possess definite working parameter, such as the effect intensity and duration of the operation. The examples of the relevant mechanochemical reactors include:

- 5 • mechanochemical reactors, such as a planetary ball mill or a vibration ball mill in which the intensity of the mechanical effect is characterized by the acceleration of the balls. The optimum range of the acceleration of the balls is 60-400 m/s². To compare, the coefficient of the acceleration of the balls in the usual gravitation mill is about 10 m/s². the standard vibration mills of the series VCM and CEM produced by Novic, Russia, or Tribochem, Ger-
10 many, are applicable for these processes ;
 - the rotor mills in which crushing is performed by collision of the particles with vanes rotating with a speed 10-120 m/s. The disintegrators and standard rotary mills Titan, Saint-Petersburg, Russia, or Arter, Moscow, are applicable for these processes;
 - vortex or jet mills in which particles of the original material are accelerated by the flow
15 of air or gas up to 10-120 m/s. The material is crushed by collision of the particles with deflecting obstacles. The following mills can be used: Vortex Mills of Hydan Technologies, USA, or Jet Micronizers of Sturtevent, Inc., USA, vortex mills VIT, of VIT Ltd., Novosibirsk, Russia.

The mechanochemical treatment can be conducted within a broad range of intensities that
20 yield close results. The duration and intensity of the mechanochemical treatment can be selected such as to avoid conditions when a considerable quantity of enzymes is denaturized. Usually the mechanochemical treatment lasts 1-10 minutes.

Continuous and discrete modes of treatment

The continuous mode is characterized by the fact that the material can be delivered into
25 the working chamber of the activator during indefinite time (tens and hundreds of seconds). The mass of the treated material is determined by the speed of passage through the working chamber. The discrete mode is characterized by the fact that the material is charged into the working chambers in a quantity the working chamber can accommodate while the activator is off.

30 The solid mixture after the mechanoenzymatic treatment and after adding of water or the solution of enzymes can be further subjected to the enzymatic hydrolysis. The cellulases can be used unpurified or as suspensions produced by filtrating the cultural fluid of the relevant producers. The suitable sources of the cellulases comprise standard cellulase preparations like Spezyme™ CP, Cytolase™ M104, and Multifect™ CL (Genencor International), Glucolux
35 (Sibbiopharm, Russia).

The conditions of the enzymatic hydrolysis are usually selected taking into account the source of the cellulases, i.e. bacteria or fungi. For instance, the cellulases of the fungi are usually more effective at temperatures 30-48 °C and pH 4.0-6.0 within the action range 30-60 °C and pH 4.0-8.0.

- 5 The microorganisms capable to ferment sugars or oligosaccharides into ethanol comprise yeast and bacteria. The microorganisms are capable to secrete one or more that individually or together convert sugars into ethanol. For instance, the *Saccharomyces* (such as *S. cerevisiae*) are well known to be used in the processes of conversion of glucose into ethanol. Other similar microorganisms comprise the following types: Schizosaccharomyces (such as
10 *S. pombe*), *Zymomonas* (including *Z. mobilis*), *Pichia* (*P. stipitis*), *Candida* (*C. shehatae*) and *Pachysolen* (*P. tannophilus*). The genetically modified strains of *E. coli* can also serve to convert carbohydrates into ethanol.

- The preferable example of the microorganisms capable to produce ethanol comprises the microorganisms secreting alcohol dehydrogenase and decarboxylase pyruvate; for instance,
15 *Zymomonas mobilis* (see U.S. Patents Nos. 5,000,000; 5,028,539; 5,424,202; and 5,482,846)

- It is preferable to use recombinant genetically modified microorganisms in the processes fermentation capable to ferment into ethanol, pentoses, and hexoses that produce one main enzyme and an additional complex of enzymes. The examples of such microorganisms include those disclosed in U.S. Patents Nos. 5,000,000; 5,028,539; 5,424,202; 5,482,846;
20 5,514,583; and Ho et al., WO 95/13362. The microorganisms including *Klebsiella oxytoca* P2 and *Escherichia coli* KO11 are specifically preferable.

The conditions of conversion of sugars into ethanol are usual conditions disclosed in the quoted patents; mainly the temperature is 30-40 °C and pH 5.0-7.0.

- Nutritive substances and/or cofactors for microorganisms and/or enzymes are added to
25 optimize the conversion. It is also desirable to add digestible carbon, nitrogen, and sulfur to accelerate proliferation of the microorganisms. Numerous nutritive media for growth of microorganisms are known, in particular, Luria broth (LB) (Luria and Delbruck, 1943).

It is possible to optimize action of the enzymes or standard complexes of enzymes and save the cost of application of the enzymatic preparations.

- 30 Membrane filtration can be applied at any stages of the claimed process. The systems of membrane filters are selective to the molecular weight or size of molecules. The membrane filter is used at the stage of saccharification, at the stage of reversion of side products and at the stage of fermentation trapping enzymes, carbohydrates, salt, yeast and allowing to water and ethanol molecules to penetrate through the membrane. Application of the membrane fil-
35 tration enables to use side products, such as glycerol, lactic acid and others and to reduce the quantity of solid substances reaching the evaporator. The process enables to save the cost and increase profitability of ethanol production.

The waste heat boiler serves to evaporate the remaining liquid from the lignin, and then the organic substances are incinerated to generate heat and steam, the combustion products are reduced into the environmentally tolerable condition.

Materials and methods

5 The materials and methods considered below were used in the experiments disclosed in the Examples.

The following mass produced preparations of cellulases were used: Spezyme™ CP (Genencor) or Cellolux (Sibbiopharm, Russia), a mixture of cellulase enzymes. Novozyme 188 – beta-glucosidase from *Aspergillus niger* (Novo-Nordisk) or Glucolux (Sibbiopharm,
10 Russia) were used at the stage saccharification.

The original stock analysis

The wheat straw, surface portion of corn without ears and microcrystalline cellulose were used as the raw stock. All vegetable raw stock was harvested in the Novosibirsk region, Russia. The microcrystalline cellulose complied with TU 6-09-10-1818-87, had the index of
15 crystallinity equal to 86 % (Segal, L., Tripp, V.U., Determination of Cellulose Crystallinity. In: Cellulose and its Derivatives. Ed. by N. Bicles, L. Seagull, Moscow: 1974, vol. 1, pp. 214-235.).

The concentrations of moisture and volatile components, lipids, water soluble substances, water soluble carbohydrates, easily hydrolysable and hardly hydrolysable polysaccharides, lignin and ash were determined in the original stock .
20

The stock was roughly crushed in the disintegrator to the size of particles 500 µm. Then the crushed stock was kept at a temperature 15-25 °C in sealed packs. The humidity was checked by drying to a constant weight at the temperature 100 °C. The moisture content in the specimens was 5-10 %. The ash content was determined by the residue after the specimens
25 were baked in porcelain crucibles at the temperature 560 °C during 3-4 hours.

The lipids were separated by exhaustive extraction of the dry stock with hexane in the Sockslet apparatus. The solvent was removed from the extract in a rotary evaporator with the vacuum in the water-jet pump at the temperature 50 °C. The extracted substance and the stock extracted with hexane were dehydrated in the vacuum dessicator to the constant weight.

30 The water-soluble substances were determined by triple aqueous extraction of the crushed, degreased, and dehydrated stock. The extraction was conducted in the ultrasound bath at the room temperature and the hydromodulus equal to 20, during 20 minutes. The solid residue was rinsed, filtered through a fine-pore glass filter, the aqueous extracts and rinsed water were combined; water was eliminated in the rotary evaporator in the vacuum in the
35 water-jet pump at the temperature 50 °C. The obtained residue was dehydrated in the vacuum

dessicator to the constant weight. The solid residue of the plant stock was also dehydrated in the vacuum dessicator and served to determine further the easily hydrolysable polysaccharides. Free disaccharides, hexoses, pentoses, and oligosaccharides were determined in the water-soluble substance. The disaccharides, hexoses, and pentoses were determined with the method of HPLC, as described below. The concentration of oligosaccharides was determined from the difference between the carbohydrates in hydrolysates and the sum of free di- and monosaccharides. The method of acid hydrolysis is described below in the section relating to determination of easily hydrolysable polysaccharides.

Easily hydrolysable polysaccharides were determined by the soft acid hydrolysis of the stock after the water-soluble substances. 50 ml of the 5 % solution of the sulfuric acid were added to a stock portion (2.0 gram) and heated without air during 3 hours at the temperature 95 °C, then the hydrolysate was decanted, a fresh portion of the sulfuric acid (30 ml) was added to the solid residue. The primary hydrolysate and the solid residue with the fresh acid portion were heated without air for 3 hours more. The solid residue was separated through a glass filter, washed with the acid solution; the acidic hydrolysates and rinsing water were combined, diluted with water up to 200.0 ml in the measuring flask. A part of the obtained solution was neutralized with barium carbonate in the ultrasound bath to shorten the time of neutralization and to prevent sorption of the carbohydrates by the solid residue.

After the neutralization reaction was over, the suspension was centrifuged. The obtained transparent solutions were diluted 10-20 times and analyzed with the HPLC method to check the concentration of disaccharides, hexoses, and pentoses. For this purpose 100 µl of the ethanol solution of the ethyl ether of the para-aminobenzoic (20 mg/ml) acid, 100 µl of the ethanol solution of sodium cyanoboron hydride (1 M) and 40 µl of the glacial acetic acid were added into 30 µl of the test solution. The obtained mixture was kept in airtight vessels for 6 hours at the temperature 50 °C. The reduced Schiff bases formed by reaction (1) were separated by HPLC the method with detection in the UV-band. The analysis was performed in the isocratic mode (25 % methanol in the aqueous 0.001 M solution of the chloral acid containing 2 % lithium perchlorate with the analytic chromatographer Milichrom A-02 equipped with a microcolumn with the inverted phase (ProntoSil C-18. 5 µm, 2×70 mm) and a spectrophotometric detector. The instrument was calibrated with the solution containing known quantity of carbohydrates (lactose, cellobiose, glucose, mannose, and xylose).

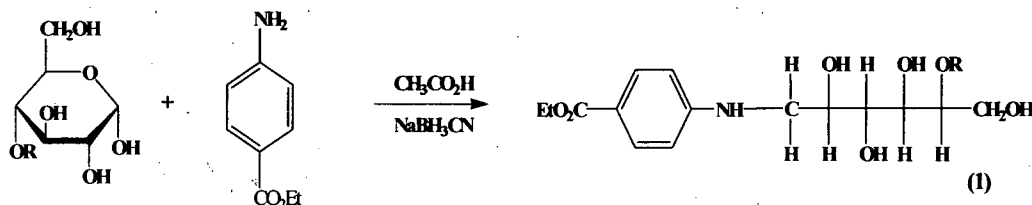


Fig. 2.3 exemplifies the calibration chromatogram and chromatogram recorded with the wheat straw hydrolysate.

The lignin concentration was assessed by treating the specimens with a 2 % hydrogen peroxide solution at pH = 11. After removal of the water soluble substances and easily hydrolysable polysaccharides the solid residues of the plant stock were poured with the alkyl hydrogen peroxide solution (the hydromodulus is 30) and kept during 1 hour at the temperature 80 °C while stirring 600 l/min. The vegetable stock residue was separated in the glass filter, rinsed with the alkaline hydrogen peroxide solution, then with a weak acetic acid solution in the water diluted to the neutral pH. The obtained residue was dried in a vacuum dessicator to the constant weight. The mass losses were assessed from the lignin concentration.

The residue obtained after degreasing, elimination of the water-soluble substances and easily hydrolysable polysaccharides, delignification, was thoroughly dried and weighed. The IR-spectroscopy, RFA and elementary residue analysis showed the cellulose with the crystallinity index 70 %. The ash content in the cellulose specimens was 1-2 %.

Table 1 shows the results of analysis of the plant stock.

Table 1. Stock group composition.

Component	Wheat straw, %	Corn straw, %
Water and volatiles	5,0	3,5
General ash content	4,9	5,9
lipids	2,5	2,0
Water-soluble substances	7,4	36,4
Water-soluble carbohydrates, converted into glucose	0,5	19,9
Including pentoses/hexoses	2,7/1	0,1/1
Easily hydrolysable polysaccharides	20,8	14,0
Including pentoses/hexoses	5,5/1	7,5/1
Cellulose	43,5	24,8
Lignin	19	12
Total concentration of carbohydrates converted into glucose	75	66

Determination of the enzymatic complex activity.

Determination of the activity of the enzymatic complex Cellolux (Sibbiopharm Co., Berdsk, the Novosibirsk Region, Russia) is described as an example.

- 5 The activity was determined by hydrolysis of the filtering paper Whatman # 1 with a partially modified the method (Ghose, T.K., Measurement of Cellulase Activity, *Pure Appl. Chem.*, 1987, vol. 59, pp. 257-268). The hydrolysis was the following: the hydromodulus was 30, temperature 50 °C, 0.05 M acetate buffer, pH = 4.5. The shredded filtering paper was placed into a plastic reactor (5.0 ml), the acetate buffer (2.0 ml) and thermostatted at 50 °C periodically until a
- 10 suspension. The solution of the enzymatic complex in the 0.05 M acetate buffer with the pH = 4.5 (0.5 ml) pre-heated to 50 °C was added to the obtained suspension. The activity of several specimens was measured with solutions of the complex with different concentrations within the range 0.6-5 mg/ml. The substrate was hydrolyzed during 60 minutes lightly shaking the reactors meanwhile, and then the reactors were heated in the water bath to 70 °C to inactivate the enzymatic
- 15 complex. The obtained hydrolysates were centrifuged, the solid residue in the hydrolysate was discarded, the concentration of carbohydrates (converted into glucose) with the phenol-sulfur oxide method (Ghose, T.K., Measurement of Cellulase Activity, *Pure Appl. Chem.*, 1987, vol. 59, pp. 257-268). The unit of activity was assumed equal to the hydrolysis of the quantity of soluble sugars equivalent to 1 mg glucose per hour.
- 20 The activity of the enzymatic complex of different batches was 2,000 units per gram of the complex on the average.

The stability of the enzymes

The enzymatic preparations were diluted with the 50 mM citrate buffer to the concentrations equivalent to those used in the processes of separation of sugars from paper 250 FPU Spezyme™, CP/L and 50 unit/l Novozyme 188. The solutions contained 0.5 g/l thymol, 40 mg/l chloramphenicol to prevent proliferation of bacteria. The enzymatic mixture was stirred during 15 minutes with the speed 120 r.p.m. until full distribution of the enzyme. Stirring continued for during 48 hours. Samples were taken every 0, 12, 24, 36, 48 h. The enzymatic activity was determined with the above described the method.

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The effect on the structure

Changes in the structure of the paper cellulose matrix were investigated by electron microscopy (the Hitachi S4000 microscope) and with the RFA. The samples were treating 2.5 l of the mixture containing 50 g/l of the MWOP paper in the 50 mM citrate buffer, pH 5.2 and 35 °C; mechanical crushing lasted 2 minutes with acceleration of the balls 20 m/s². Other samples were

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treated with cellulases for 4 hours. Control samples were left untreated. All the samples were dried and sputtered with gold before study under the electron microscope.

The RFA was performed with a diffractometer DRON-5 (Russia) in the CuK α -alpha emission. The crystallinity index was determined from the formula

$$IR = (I_{002} - I_a / I_{002}) 100\%,$$

where I_{002} – intensity of the diffraction reflex 002 of the cellulose, I_a – intensity of dissipation at $2\theta \sim 19^\circ$.

Mechanical treatment

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Mechanical treatment under controllable conditions was performed using laboratory mills with adjustable intensity and time: AGO - 2 (Novic, Russia) and Pulverizette -5 (Fritsch, Germany).

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Example 1. Acceleration of microcrystalline cellulose hydrolysis.

The enzyme hydrolysis of microcrystalline cellulose samples was conducted and the initial hydrolysis rate was measured as a function of cellulose pre-treatment conditions.

The cellulose sample was placed into the 0.1 M acetate buffer pH = 4.5 (the hydromodulus was 10) containing 0.1-0.2 % formaldehyde as a preservative. The obtained mixture was hydrolyzed while stirring in a magnetic mixer in a glass reactor at a temperature $51 \pm 1^\circ\text{C}$. The hydrolysis lasted for 8 hours. Then the reactors were rapidly heated to 70°C in order to inactivate the enzymatic complex.

Hydrolysates were centrifuged, diluted 10-20 times with water, and analyzed with the HPLC the method described above. The cellulose conversion was calculated by the concentration of soluble mono- and disaccharides with the formula:

$C = 100\% \times (m(\text{Di})/1.056 + m(\text{Mono})/1.11) / m_0(S)$, where $m(\text{Di})$ – the mass of the resulting disaccharides, $m(\text{Mono})$ – the mass of the resulting monosacharrides, 1.11 and 1.056 – the coefficients taking into account water participation in the cellulose hydrolysis.

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The original microcrystalline cellulose and the cellulose after treatment with the enzyme in AGO - 2 under different conditions served as the substrate. Table 2 shows the results. The rate of hydrolysis of the original MCC was assumed one.

Table 2. The rate of microcrystalline cellulose hydrolysis as a function of treatment conditions.

№	Treatment conditions	Seagull crystallinity index	Average size of particles, μm	Hydrolysis rate
1	Original microcrystalline cellulose	86 %	60	1
2	Microcrystalline cellulose treated in AGO-2 for 2 minutes	60 %	50-55	1,2
3	Cellulose and enzyme mixture (40 units per gram), activation in AGO-2 for 2 minutes	49 %	40	1,5
4	Mixture of cellulose (98 %), enzyme (40 units per gram) and calcium chloride (2 %), activation in AGO-2 for 2 minutes	25 %	25	2,1

According to the obtained results, the mechanical treatment of the cellulose jointly with the enzyme accelerates substantially the hydrolysis rate. The effect is achieved by producing the mechanocomposite consisting of amorphized cellulose particles with the enzyme distributed over its surface and in its body. When this mechanocomposite meets water, the enzyme turns out introduced directly in the zone in which it should function rather than being distributed in the entire solution volume. This condition accelerates the reaction rate.

The hydrolysis rate is accelerated additionally by increasing the share of the amorphous cellulose in the substrate. Water is known to stimulate cellulose recrystallization processes that evolve both during preservation of the mechanically activated cellulose and in the course of mechanical activation. In order to increase the amorphization effectiveness the mechanical treatment is conducted in the presence of dry inorganic salts capable to absorb water and produces complexes with carbohydrates, thus inhibiting the process of re-crystallization. Dry carbonate or calcium chlo-

ride served as these agents. The latter is because it is practically harmless for the pH in subsequent hydrolysis.

Extra advantages of application of the inorganic salts at the stage of mechanical treatment are that these materials are free of abrasive properties promoting the effectiveness of grinding of the organic material.

Example 2. Enzyme hydrolysis of microcrystalline cellulose after mechanochemical treatment

10 grams of the microcrystalline cellulose and 200 mg of calcium chloride were mixed with the enzymatic complex Cellolux, the enzyme consumption was 20 mg (40 units) per gram of the substrate. The obtained mixture was subjected to mechanical treatment in a planetary activator of the type AGO-2 (Novits, Novosibirsk, Russian) for 2 minutes.

The treated sample (4 grams) was placed into the 0.1 M acetate buffer pH = 4.5 (40 ml) containing 0.05-0.1 % polyethylenol ($M = 10^5$) and 0.1-0.2 % formaldehyde as a preservative. The obtained mixture was hydrolyzed while stirring in a magnetic mixer in a glass reactor at a temperature 51 ± 1 °C. The hydrolysis lasted for 6 days. After the first, second and third days of hydrolysis, fresh enzyme doses were added into the reaction mixture in the amounts 30, 20 and 10 unit per gram of the substrate, respectively.

During the hydrolysis, samples were taken from the reactor. Hydrolysates were centrifuged, diluted 10-20 times with water, and analyzed with the HPLC the method described above. The cellulose conversion was calculated by the concentration of soluble mono- and disaccharides with the formula:

$$C = 100\% \times (m(\text{Di})/1.056 + m(\text{Mono})/1.11) / m_0(\text{S}),$$
 where $m(\text{Di})$ – the mass of the resulting disaccharides, $m(\text{Mono})$ – the mass of the resulting monosaccharides, 1.11 and 1.056 – the coefficients taking into account water participation in the cellulose hydrolysis.

Fig. 3 shows the results of the enzymatic hydrolysis of the microcrystalline cellulose after mechanochemical treatment. According to the obtained data, the conversion is 87 percent. The results of the comparative experiments indicate that the effect is achieved due to the combination of the intensive mechanical treatment and subsequent separate addition of the enzymatic complex.

It is shown above that the mechanical treatment in the presence of calcium salts enables to increase the concentration of the amorphous phase in the substrate resulting in a faster reaction at the initial hydrolysis stages. Alongside with it a layer of strongly adsorbed endoglucanases appears on the surface of cellulose particles; they are known to possess low mobility. Diffusion is thus restricted during mass exchange between the

solvent and the substrate, specifically when the reaction products are eliminated from the reaction zone. These restrictions intensify by the appearance of low-molecular polysaccharides, their solutions being highly viscous.

Fig. 4 shows how the degree of transformation of microcrystalline cellulose into soluble sugars depends on the duration of enzymatic hydrolysis (the lower curve – without mechanoenzymatic treatment, the upper curve – with mechanoenzymatic treatment).

It is shown above that joint mechanical treatment of the enzymatic complex and the substrate results in a composite with the particles containing the enzyme. Due to this fact, all the enzymes, the cellulosolytic complex in the composition, including exogluconases too, concentrate in the reaction zone at the first hydrolysis stage. On the one part, they split up the oligosaccharides effectively and, on the other, reduce the irreversible adsorption of the endogluconases in the substrate (Sinitsyn, A.P., Gusakov, A.V., and Chernoglazov V.M., *Bioconversion of Lignocellulose Materials*, Moscow: Publishing House of Moscow State University, 1995, 220 pp.).

The stirring of the reactive mass drives the exogluconases into the solution so that their concentration under the surface drops. Addition doses of the enzyme are introduced into the reactor in order to compensate this lower concentration of exogluconases and the total reduction of the concentration of the enzymes due to their natural inactivation during the first three days.

Introduction of the high-molecular polyethylenol reduces positively the irreversible adsorption of endogluconases allowing the low-molecular polysaccharides concentrate near the surfaces of particles easing the diffusion constraints still more.

Example 3. Enzyme hydrolysis of the lignocellulose stock exposed to mechanoenzymatic treatment.

The dried and pre-crushed to particle size under 0.5 mm plant stock was mixed with calcium chloride (97:3 by mass); the cellulosolytic complex was added for 15 mg (30 units) per gram of the carbohydrates the stock contains. The obtained mixture was treated in the flow through mode in rotary mills at the speed of operating rotors 70 m/s; the mixture remained in contact with the rotors for 0.5 minute.

The hydrolysis was performed in a series of consecutive reactors. The hydrolysis scheme envisaged that the substrate remains in each reactor for 12 hours and then the substrate would be transferred for hydrolysis into the neighboring reactors in the flow through mode, as Fig. 5 shows it.

According to the presented scheme, the fresh substrate produced by mechanoenzymatic treatment of the lignocellulose stock comes into the first reactor. The substrate contacts for 12 hours the hydrolysate coming from reactor 2. This reactor receives the substrate that is free already of the amorphous cellulose and other polysaccharides easily hydrolysable by the enzyme. This substrate is subjected to treatment with the fresh solution of the enzymatic complex.

The lignocellulose stock is hydrolyzed in each reactor at a temperature $51 \pm 1^\circ\text{C}$ and the hydromodulus 7-10. The pH of the reaction mixture is maintained 4.5. The solution of the enzymatic complex delivered into reactor 10 contains 15 units of the complex per gram of carbohydrates. Reactors 3 and 6 receive fresh doses of the enzymatic complex in the amount 15 units per gram of carbohydrates. The solutions of the enzymes delivered into reactors 3, 6, and 10 were based on the cultural fluid of *Trichoderma viride (reesei)* as a cellulolytic complex producer.

The yield of monosacharrides under the conditions in Example 2 was determined from the obtained data conversion of polysaccharides amounting to 90 %.

Example 4. The effect of the surfactant additive during mechanoenzymatic treatment on the subsequent hydrolysis rate.

Wheat straw was subjected to three alternatives of the mechanoenzymatic treatment under the conditions of Example 3: without any surfactant, with the 1 % PEG additive ($M_r = 10^5$) and with the preparation Tween-20.

The plant stock was hydrolyzed in the reactors under periodic action during 8 hours while stirring in a magnetic mixer 600 1/min. The hydrolysis conditions were the same in all the alternatives: the temperature $51 \pm 1^\circ\text{C}$, the hydromodulus 10, the pH of the reaction mixture within the range 4.6 ± 0.1 , formaldehyde concentration 0.05-0.1 %.

After 8 hours of hydrolysis, samples were taken from the reactors and immediately analyzed by the HPLC the method. After water was removed from the soluble carbohydrates, the average hydrolysis rate was determined. Table 3 shows the results of the hydrolysis rate of the sample treated without any surfactant assumed one.

Table 3. Dependence of the relative hydrolysis rate on introduction of additives.

Alternative	Treatment with CaCl_2	Treatment with CaCl_2 and PEG	Treatment with CaCl_2 and TWEEN-20
Hydrolysis rate	1,0	1,36	1,32

Introduction of the above surfactants at the stage of mechanoenzymatic treatment accelerate hydrolysis noticeably. The most probable cause of this effect that lignin is blocked during the mechanoenzymatic treatment. The lignin is known to adsorb the enzymes of the cellulolytic complex irreversibly; meanwhile the surfactants introduced at the stage of mechanoenzymatic treatment reduce the effect off this unwanted process significantly.\

Example 5. The stepwise enzyme hydrolysis and intermediate ultrasound treatment

The mechanoenzymatic pre-treatment was conducted under the conditions of Example 3. The enzyme hydrolysis of polysaccharides was performed in steps.

The treated plant stock was subjected to pre-hydrolysis in the reactor during periodic action for 48 hours at the hydromodulus 7, $\text{pH} = 4.6 \pm 0.1$. To prevent irreversible inactivation of endoglucanases after 24 hours the cultural fluid (*Aspergillus awamori* and/or *Bacillus subtilis*) enriched with exoglucanases was added into the reactor. Addition was made from the calculation of 10 units per gram of the substrate.

After 48 hours, the suspension was exposed to ultrasound with the frequency 22 kHz for 5-15 minutes at a temperature $50-90^{\circ}\text{C}$, then the solid phase was separated from the hydrolysate. The hydrolysate was used in the fermentation process, the solid residue was subjected to another enzymatic hydrolysis for 48 hours at the hydromodulus 7, $\text{pH} = 4.6 \pm 0.1$, consumption of the cellulolytic complex if *Trichoderma viride* from the assessment of 30 unit per gram of polysaccharides.

According to the obtained data, the bi-step hydrolysis with intermediate ultrasound treatment yields 90-92 % conversion of the polysaccharides into water-soluble monosaccharides.

Example 6. Enzymee hydrolysis and combined fermentation of the lignocellulose stock after its mechanoenzymatic treatment.

Corn straw underwent mechanoenzymatic treatment under the conditions of Example 4. The required quantity of the preparation TWEEN-20 was introduced directly into the zone of contact between the crushing bodies and the vegetable stock. The preparation TWEEN-20 would increase the effectiveness of mechanoenzymatic treatment due to the adsorption of the surfactant on the lignin thus preventing partial inactivation of enzymes. The treated plant stock was subjected to pre-hydrolysis in the reactor during periodic action for 36 hours at the hydromodulus 7, $\text{pH} = 4.6 \pm 0.1$. To prevent reversible inactivation of endoglucanases after 24 hours the cultural fluid (*Aspergillus awamori* and/or

Bacillus subtilis) enriched with exogluconases was added into the reactor. Its introduction was made 10 units per gram of the substrate.

After the preliminary hydrolysis, the suspension of the plant stock and hydrolysate was pumped by the cavitators into the reactor for the following saccharification and combined fermentation (SSCF). Utilization of the cavitators as a pumping device altered the rheological characteristics of the pulp positively. This operation would reduce the viscosity of the solution 2-3 times. the optical microscopy in Fig. 6 shows the disintegration of the conglomerates of particles in the substrate, where: the sequence of structures *a-c* – formation of conglomerates of particles in the course of preliminary hydrolysis, the structure *d* – the result of treatment in the cavitations device.

Subsequent saccharification and combined fermentation are conducted at the temperature 37-38 °C in the presence of recombinant microorganisms *Zymomonas mobilis* capable to ferment glucose and xylose in the presence of the yeast *Saccharomyces cerevisiae*. In case of the yeast, xyloisomerase was introduced that would transform xylose into the yeast-fermentable ketopentose xylose.

The SSCF process is conducted for 7 days at the hydromodulus 8 (dilution by introduction of the components of the nutritive medium). In the process the enzymes are added separately calculated in units per gram of the substrate: 10 units of the cellulolytic complex *Trichoderma viride* (*reesei*) after two and four days, 5 units of the enzymatic complex *Aspergillus awamori* and/or *Bacillus subtilis* after three and six days.

The mechanoenzymatic treatment, preliminary enzymatic hydrolysis, and SSCF-process push to 90 % the conversion of polysaccharides from the stock into water-soluble carbohydrates.

The obtained hydrolysate contained 2.8-3.2 % ethanol that was separated by distillation. 1000 kg corn straw containing 66 % carbohydrates can yield 307 liters of ethanol; the wheat straw can yield 330 liters.

Thus, the invention provided a method of producing bioethanol enabling to utilize unused bio-renewable sources of polysaccharides, predominantly the lignocellulose inapplicable in other spheres of chemical and biochemical technologies.

Industrial Applications

The present invention is embodied with multipurpose equipment extensively employed by the industry.

Claims

1. The method of producing ethanol from the lignocellulose stock, comprising mechanochemical (mechanoenzymatic) treatment of a mixture of the lignocellulose stock and a cellosolytic enzyme preparation under the conditions ensuring formation of a mechanocomposite capable of hydrolysis when water or a solution of an enzyme and/or a mixture of enzymes are added that ensure partial hydrolysis of the lignocellulose stock and subsequent production of a solution sugars and then production of ethanol from the sugars using ethanologenic microorganisms.
2. The method according to Claim 1 characterized in that the enzymatic preparation used for drying in the form of a powder or a cultural fluid obtained by cultivating the microorganisms producing cellosolytic enzymes.
3. The method according to Claim 1 by which the mechanoenzymatic treatment is conducted in ball planetary or vibratory mills with acceleration of the treating balls from 60 to 400 m/s² during 0.5-2.0 minutes, or in rotary mills with the speed of the treating vanes 10-120 m/s during 0.2-2.0 minutes, or in pneumatic vortex mill with the gas flow speed 10-120 m/s during 0.2-2.0 minutes.
4. The method according to Claim 1 by which the product of the mechanoenzymatic treatment is subjected to counterflow enzymatic hydrolysis, with additional doses of the cellosolytic enzymatic complex being added at the intermediate stages.
5. The method according to Claim 1 by which the polysaccharides are prehydrolyzed to achieve the degree of conversion 10-40 %, the obtained suspension of the hydrolysate and the lignocellulose stock being subjected to treatment in cavitation devices and then undergoing the subsequent stage of saccharification and combined fermentation. In this case, the conversion of polysaccharides amounts to not less than 90 %.
6. The method according to Claim 1 by which the enzyme hydrolysis is conducted in steps in two stages and comprises preliminary hydrolysis, intermediate ultrasound treatment a suspension of the solid residue and hydrolysate, the repeated hydrolysis of the solid residue being conducted with a fresh solution of enzymes. In this case, the conversion of monosacharrides amounts to not less than 90 %.
7. The method according to Claims 4 and 5 by which a solution of the cultural fluid with a large concentration of exogluconases being added, in particular the cultural fluids of *Aspergillus awamori* and/or *Bacillus subtilis*, at intermediate stages rather than adding or in addition to the solution of the cultural fluid of *Trichoderma viride* (*reesei*)
8. The method according to Claim 1 by which a surfactant being introduced at the stage of mechanoenzymatic treatment.
9. The method according to Claim 1 by which ethanologenic bacteria or yeast serving as ethanologenic microorganisms.

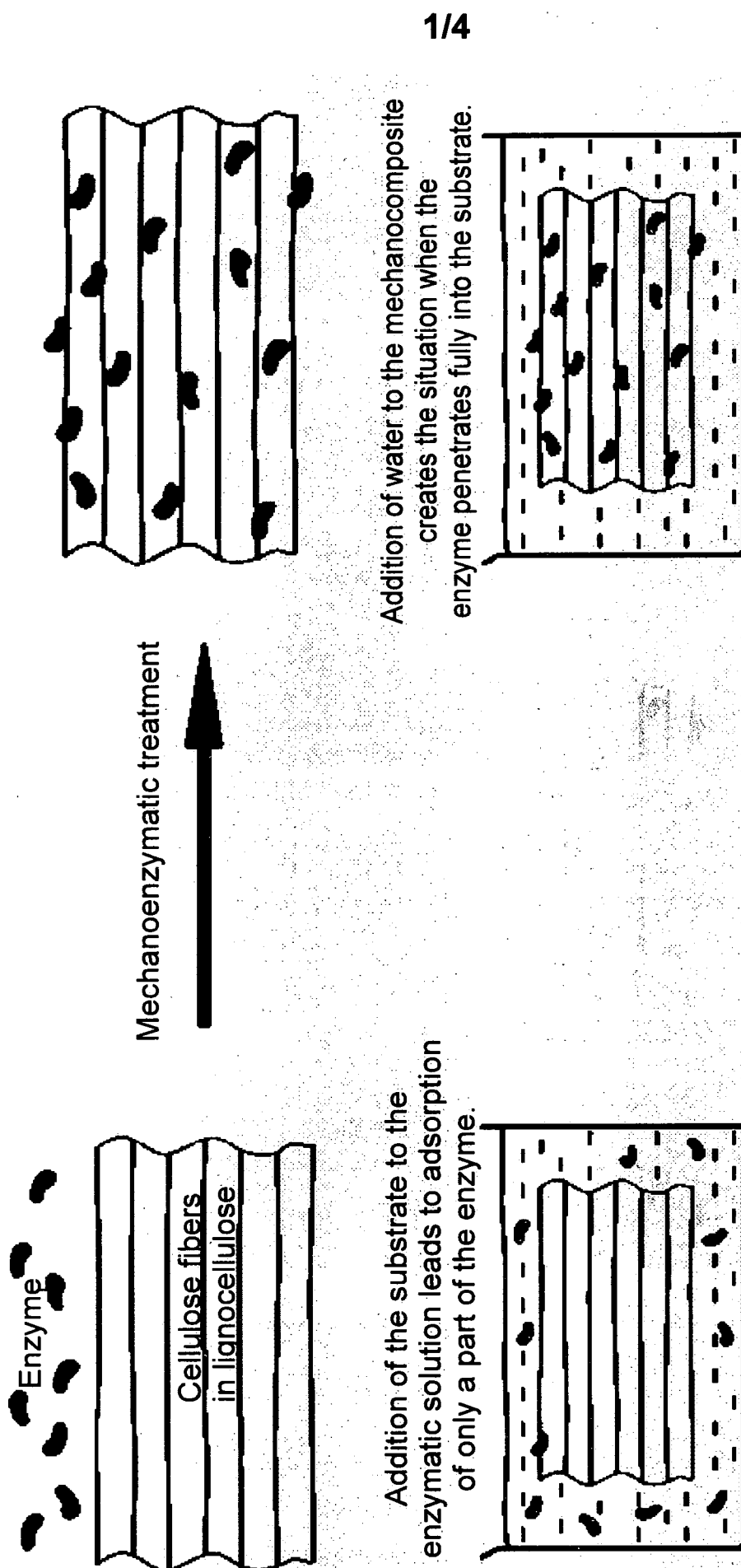


Fig.1

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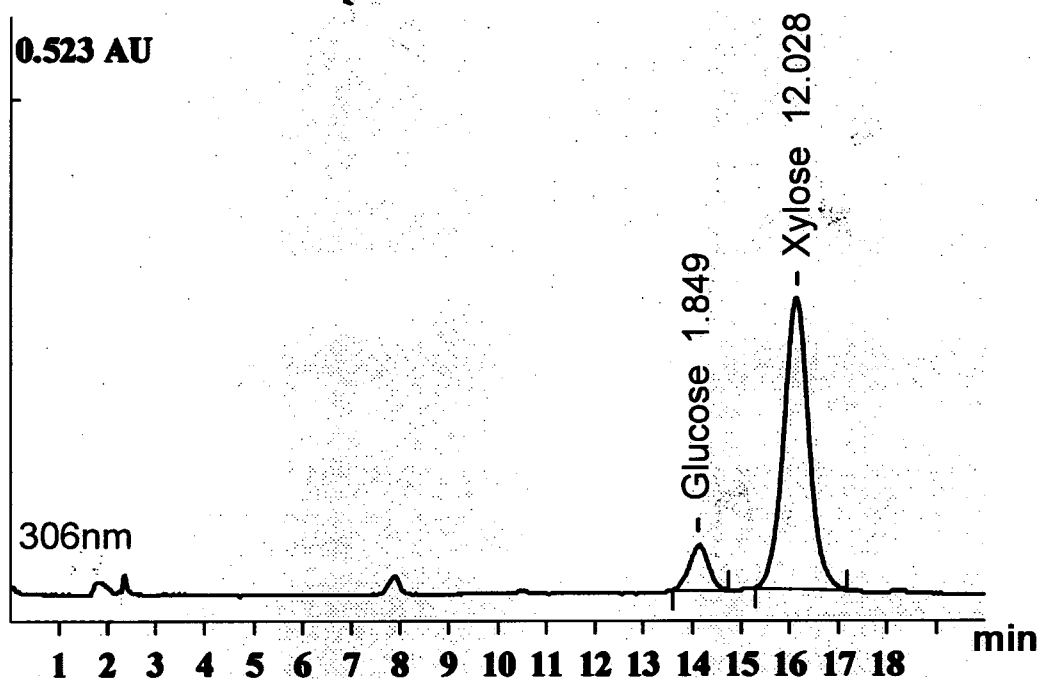


Fig.2

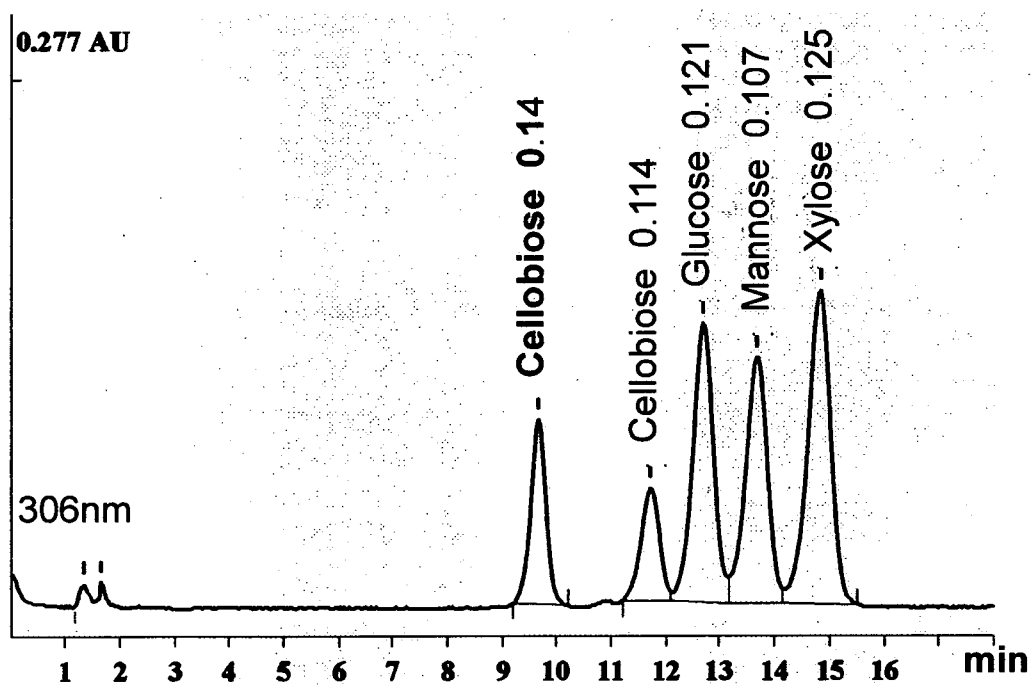


Fig.3

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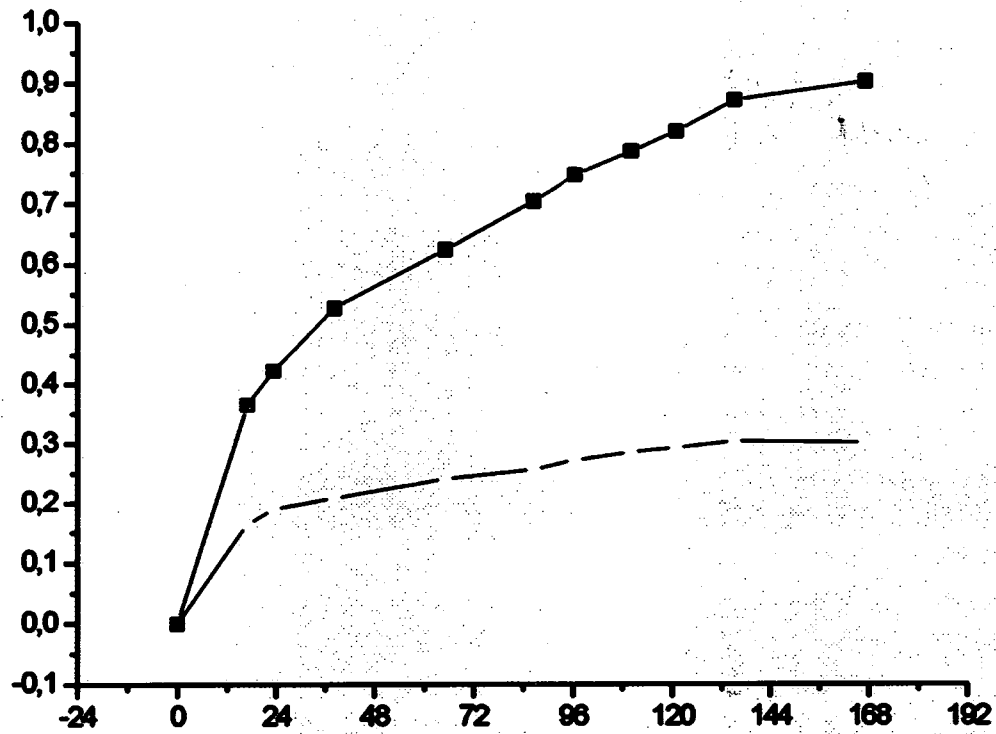


Fig.4

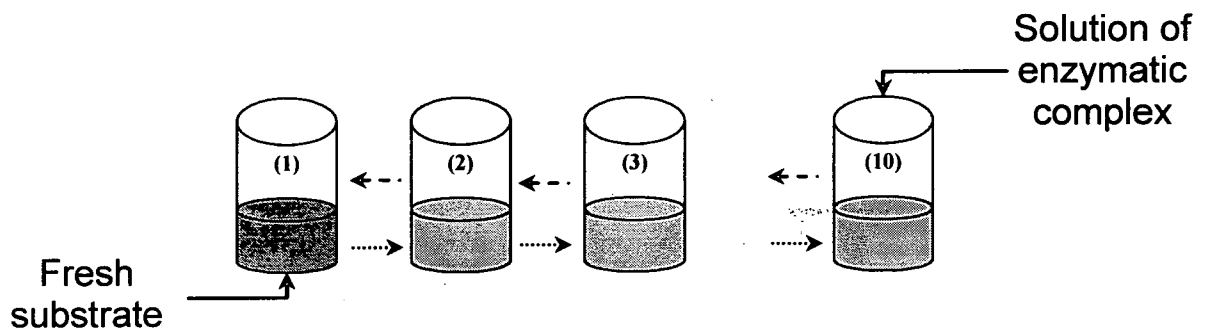


Fig.5

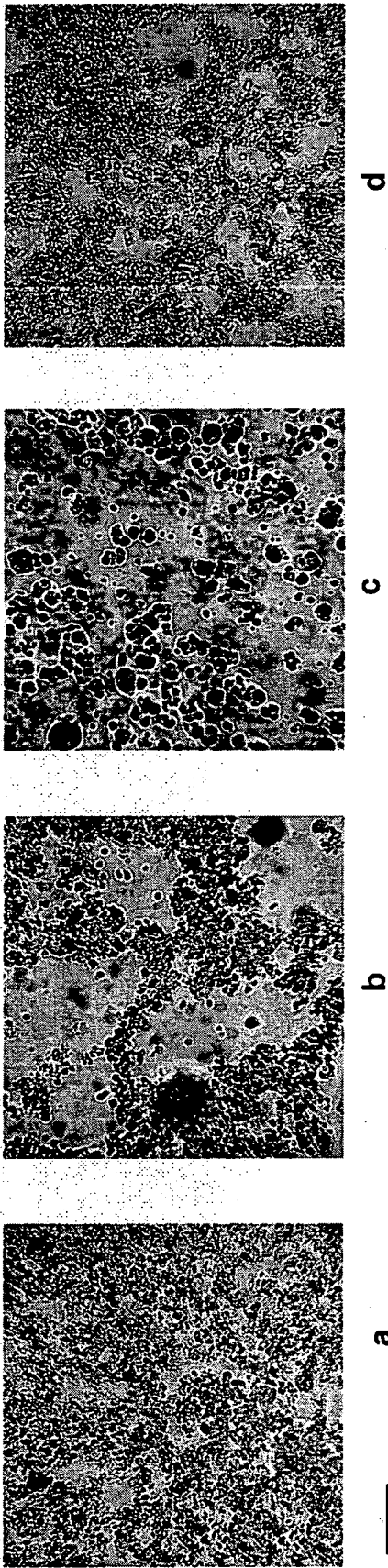


Fig.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/RU 2007/000365

A. CLASSIFICATION OF SUBJECT MATTER		<i>C07C 31/08 (2006.01)</i> <i>C12P 7/10 (2006.01)</i>
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) C07C 31/08, C12P 7/10, 7/08, 7/06		
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) RUPAT DB, PAJ, Esp@cenet, USPTO DB, DEPATIS, EAPATIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4321328 A (WILLIAM H. HOGE) 23.03.1982	1-9
A	US 2006/0177917 A1 (MICHEL WARZYWODA et al.) 10.08.2006	1-9
A	US 2006/0084156 A1 (ORESTE LANTERO et al.) 20.04.2006	1-9
A	RU 2162103 C1 (MOSKOVSKY GOSUDARSTVENNY UNIVERSITET PISCHEVYKH PROIZVODSTV) 20.01.2001	1-9
☐ 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 document 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 report 14 March 2008 (14.03.2008)		Date of mailing of the international search report 03 April 2008 (03.04.2008)
Name and mailing address of the ISA/RU FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. 243-3337		Authorized officer T. Krivoruchko Telephone No. (499) 240-2591