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(54) Title: HOST CELL CAPABLE OF PRODUCING ENZYMES USEFUL FOR DEGRADATION OF LIGNOCELLULOSIC MATERIAL

(57) Abstract: The invention relates to a host cell comprising at least four different heterologous polynucleotides chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases, wherein the host cell is capable of producing the at least four different enzymes chosen from the group of cellulases, hemicellulases and pectinases, wherein the host cell is a filamentous fungus and is capable of secretion of the at least four different enzymes. This host cell can suitably be used for the production of an enzyme composition that can be used in a process for the saccharification of cellulosic material.



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**HOST CELL CAPABLE OF PRODUCING ENZYMES
USEFUL FOR DEGRADATION OF LIGNOCELLULOSIC MATERIAL**

Statement of Federally Sponsored Research and Development

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Field of the invention

The invention relates to a host cell capable of producing at least four different enzymes chosen from the group of cellulases, hemicellulases and pectinases, a process for the preparation of said host cell, an enzyme composition produced by said host cell, a process for the production of an enzyme composition using said host cell, a process for the saccharification of lignocellulosic material using the enzyme composition and a process for the preparation of a fermentation product using the enzyme composition.

Background of the invention.

Carbohydrates constitute the most abundant organic compounds on earth. However, much of this carbohydrate is sequestered in complex polymers including starch (the principle storage carbohydrate in seeds and grain), and a collection of carbohydrates and lignins known as lignocellulose. The main carbohydrate components of lignocellulose are cellulose, hemicellulose, and pectins. These complex polymers are often referred to collectively as lignocellulose.

Bioconversion of renewable lignocellulosic biomass to a fermentable sugar that is subsequently fermented to produce alcohol (e.g., ethanol) as an alternative to liquid fuels has attracted an intensive attention of researchers since 1970s, when the oil crisis broke out because of decreasing the output of petroleum by OPEC. Ethanol has been widely used as a 10% blend to gasoline in the USA or as a neat fuel for vehicles in Brazil in the last two decades. More recently, the use of E85, an 85% ethanol blend has been implemented especially for clean city applications. The

importance of fuel bioethanol will increase in parallel with increases in prices for oil and the gradual depletion of its sources. Additionally, fermentable sugars are being used to produce plastics, polymers and other biobased products and this industry is expected to grow substantially therefore increasing the demand for abundant low cost fermentable sugars which can be used as a feed stock in lieu of petroleum based feedstocks.

The sequestration of large amounts of carbohydrates in plant biomass provides a plentiful source of potential energy in the form of sugars, both five carbon and six carbon sugars that could be utilized for numerous industrial and agricultural processes. However, the enormous energy potential of these carbohydrates is currently under-utilized because the sugars are locked in complex polymers, and hence are not readily accessible for fermentation. Methods that generate sugars from plant biomass would provide plentiful, economically-competitive feedstocks for fermentation into chemicals, plastics, and fuels.

Regardless of the type of cellulosic feedstock, the cost and hydrolytic efficiency of enzymes are major factors that restrict the commercialization of the biomass bioconversion processes. The production costs of microbially produced enzymes are tightly connected with a productivity of the enzyme-producing strain.

With productivity of a strain is meant the amount of total protein or enzyme activity produced in the fermentation broth per time, for example kg protein/(m³ broth.year) or enzyme units /(m³ broth.year).

An enzyme unit is the ability of a defined amount of protein to convert a defined substrate under certain defined conditions.

Summary of the invention

The presence of a separate 'cellulase inducer' is usually needed to produce the enzymes for biomass conversion in a microorganism. A cellulase inducer is disadvantageous for the following reasons. First, the inducer, such as a plant material, may have a variable composition, which is disadvantageous for the controllability of the expression of the heterologous polynucleotides chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases. Secondly, energy is required to sterilise plant material before the inducer can be used for inducing the production of the at least four different enzymes chosen from the group of cellulases, hemicellulase and pectinases. Thirdly, plant material will heavily pollute fermentation equipment. Fourthly, the inducer may result in a higher viscosity of the medium wherein the host cell expresses

the at least four different polynucleotides chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases. Fifthly, the presence of a 'cellulase inducer', in particular when it has been pre-treated, may result in the production of inhibitors that may be detrimental to the host cell.

"Cellulase inducer" is herein defined as a compound that induces the production of cellulase in the host cell. Examples of cellulase inducers include pure cellulose, cellobiose, sophorose and gentiobiose or any lignocellulosic material.

It would therefore be highly desirable to provide a simple and economically attractive enzymatic process for the production of sugars from plant biomass.

This object has been achieved by the provision of a host cell comprising at least four different heterologous polynucleotides chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases, wherein the host cell is capable of producing the at least four different enzymes chosen from the group of cellulases, hemicellulases and pectinases, wherein the host cell is a filamentous fungus and is capable of secretion of the at least four different enzymes

Since all enzymes necessary for degradation of lignocellulosic material are produced in and secreted by one and the same host cell, a ready-to-use enzyme composition can be produced in only one fermentation. The enzyme composition produced by the host cell of the invention can suitably be used in a process for the saccharification of lignocellulosic material.

Furthermore, the host cell of the invention may not need a cellulase inducer to produce the at least four different enzymes chosen from the group of cellulases, hemicellulases and pectinases.

A possible advantage of using the host cell of the invention in a process for the saccharification of lignocellulosic material is that it is not necessary to blend/mix the enzymes needed for the degradation of the lignocellulosic material in the desired ratios. Furthermore, there is no need to store separate enzymes before using them in a process for the degradation of lignocellulosic material.

Furthermore, by selecting the host cell and the genetic modifications of the host cell according to the preferred embodiments of the invention, a high productivity of the enzymes can be achieved.

Furthermore, the fermentation broth (comprising the at least four different enzymes chosen from the group of cellulases, hemicellulases and pectinases)

may be used without any purification in a process for the degradation of lignocellulosic material. Therefore, the invention provides a very simple and cost-effective solution.

Brief description of the drawings

- Figure 1:** Map of pGBTOPEBA205 for expression of *T. emersonii* cellobiohydrolase I (CBH I) in *T. emersonii*. Depicted are EBA205 expressed from the glucoamylase promoter (PglaA). In addition, the glucoamylase flank (3'glaA) of the expression cassette is depicted. pGBTOPEBA205 is representative for pGBTOPEBA8, which comprises the DNA-sequence encoding the *T. emersonii* EG, and pGBTOPEBA4, which comprises DNA-sequence encoding the *T. emersonii* BG.
- Figure 2:** Map of pGBFINEBA176 for expression of *T. emersonii* cellobiohydrolase II (CBHII) in *T. emersonii*. pGBFINEBA176 is a pGBFIN11-based plasmid. Depicted is EBA176 expressed from the glucoamylase promoter (PglaA). In addition, the selectable marker (amdS), expressed from the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd) and the glucoamylase flanks (3'glaA and 3''glaA) of the expression cassette are depicted.
- Figure 3:** Detection of multiple recombinant *T. emersonii* cellulases in *A. niger*. (3A). SDS-PAGE detection of *T. emersonii* cellulases expressed in *A. niger*. *A.niger* was transformed with a mix of pGBTOPEBA4, pGBTOPEBA8, pGBFINEBA176, and pGBTOPEBA205. Transformants were grown in shake flasks containing maltose containing medium and proteins in supernatants harvested from 96 hours cultures were analysed by SDS-PAGE analysis. (3B). Graph showing WSU activity in transformants. Transformants were grown for 96 hours in maltose containing medium and WSU activity was determined in supernatants of the cultures.
- Figure 4.** Detection of multiple recombinant *T. emersonii* cellulases in *T. emersonii*. (4A). SDS-PAGE detection of *T. emersonii* cellulases expressed in *T. emersonii*. *T. emersonii* was transformed with a mix of pGBTOPEBA4, pGBTOPEBA8, pGBFINEBA176, and pGBTOPEBA205. Approximately 400 transformants were grown in

96-well plates and screened for expression of at least one cellulase by E-PAGE gel analysis. Interesting transformants were grown in shake flasks containing glucose-based medium and proteins in supernatants harvested from 72 hours cultures were TCA-precipitated and analysed by SDS-PAGE analysis. FBG142 is the empty strain. (4B). Graph showing WSU activity in transformants. Transformants were grown for 72 hours in glucose-based medium and WSU activity was determined in 16-times diluted supernatants of the cultures. FBG142 is the empty strain.

Brief description of the sequence listings

- SEQ ID NO: 1 sets out the amino acid sequence of a cellobiohydrolase I from *Talaromyces emersonii*
- SEQ ID NO: 2: sets out the polynucleotide from *Talaromyces emersonii* encoding the amino acid sequence of SEQ ID NO. 1.
- SEQ ID NO: 3 sets out the amino acid sequence of a β -glucanase CEA from *Talaromyces emersonii*.
- SEQ ID NO: 4 sets out the polynucleotide from *Talaromyces emersonii* encoding the amino acid sequence of SEQ ID NO. 3.
- SEQ ID NO: 5 sets out the amino acid sequence of a β -glucosidase from *Talaromyces emersonii*.
- SEQ ID NO: 6 sets out the polynucleotide from *Talaromyces emersonii* encoding the amino acid sequence of SEQ ID NO. 5.
- SEQ ID NO: 7 sets out the amino acid sequence of a cellobiohydrolase II from *Talaromyces emersonii*.
- SEQ ID NO: 8 sets out the polynucleotide from *Talaromyces emersonii* encoding the amino acid sequence of SEQ ID NO. 7.
- SEQ ID NO: 9 sets out the amino acid sequence of a Size 209 aa unknown protein from *T. emersonii*.
- SEQ ID NO: 10 sets out the coding sequence of an unknown protein from *T. emersonii* having amino acid sequence according to SEQ ID NO: 9.
- SEQ ID NO: 11 sets out the amino acid sequence of *T. emersonii* swollenin.
- SEQ ID NO: 12 sets out the coding sequence of *T. emersonii* swollenin.

SEQ ID NO: 13 sets out the aminoacid sequence of *T. emersonii* acetyl xylan esterase.

SEQ ID NO: 14 sets out the coding sequence of *T. emersonii* acetyl xylan esterase.

SEQ ID NO: 15 sets out the aminoacid sequence of *T. emersonii* xylanase.

SEQ ID NO: 16 sets out the coding sequence of *T. emersonii* xylanase.

As used herein the expression "heterologous polynucleotides" means polynucleotides that are not present in the untransformed host cell, whereas "homologous polynucleotides" means polynucleotides that are present in the untransformed host cell. Synthetic polynucleotides are herein considered heterologous polynucleotides, independent of their potential presence in the untransformed host cell.

As used herein "transformant" means a cell that has been the object of transformation. "Transformant", "host cell" and "recombinant cell" are herein used as synonyms.

"Transformation" herein means the genetic alteration of a cell by means of recombinant technology. It may result in the uptake, incorporation, and expression of genetic material (DNA, RNA or protein) or mutation or deletion of genetic material in the cell, through human intervention.

The host cell

The host cell according to the invention may be prepared from any cell. For specific uses of a compound produced in a host cell according to the invention, the selection of the cell to be transformed may be made according to such use. Where e.g. the compound produced in a host cell according to the invention is to be used in food applications, a host cell may be selected from a food-grade organism such as *Saccharomyces cerevisiae*. Specific uses include, but are not limited to, food, (animal) feed, pharmaceutical, agricultural such as crop-protection, and/or personal care applications.

According to one embodiment, the host cell according to the invention is a eukaryotic host cell. Preferably, the eukaryotic cell is a mammalian, insect, plant, fungal, or algal cell. Preferred mammalian cells include e.g. Chinese hamster ovary (CHO) cells, COS cells, 293 cells, PerC6TM cells, and hybridomas. Preferred insect cells include e.g. Sf9 and Sf21 cells and derivatives thereof. More preferably, the eukaryotic cell is a fungal cell, i.e. a yeast cell, such as a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* strain, more preferably from

Kluyveromyces lactis, *S. cerevisiae*, *Hansenula polymorpha*, *Yarrowia lipolytica* and *Pichia pastoris*, or a filamentous fungal cell. Most preferably, the eukaryotic cell is a filamentous fungal cell.

"Filamentous fungi" include all filamentous forms of the subdivision *Eumycota* and *Oomycota* (as defined by Hawksworth *et al.*, In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. Filamentous fungal strains include, but are not limited to, strains of *Acremonium*, *Agaricus*, *Aspergillus*, *Aureobasidium*, *Chrysosporium*, *Coprinus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Panerochaete*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, and *Trichoderma*.

Several strains of filamentous fungi are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL). Examples of such strains include *Aspergillus niger* CBS 513.88, *Aspergillus oryzae* ATCC 20423, IFO 4177, ATCC 1011, ATCC 9576, ATCC14488-14491, ATCC 11601, ATCC12892, *P. chrysogenum* CBS 455.95, *Penicillium citrinum* ATCC 38065, *Penicillium chrysogenum* P2, *Talaromyces emersonii* CBS 393.64, *Acremonium chrysogenum* ATCC 36225 or ATCC 48272, *Trichoderma reesei* ATCC 26921 or ATCC 56765 or ATCC 26921, *Aspergillus sojae* ATCC11906, *Chrysosporium lucknowense* ATCC44006 and derivatives thereof.

Preferred filamentous fungal cells belong to *Aspergillus*, *Chrysosporium*, *Penicillium*, *Talaromyces* or *Trichoderma* genus, and most preferably *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus sojae*, *Aspergillus fumigatus*, *Talaromyces emersonii*, *Aspergillus oryzae*, *Chrysosporium lucknowense*, *Trichoderma reesei* or *Penicillium chrysogenum*.

Most preferably, the cell to be transformed to form the host cell is an *Aspergillus*.

An advantage of expression of the enzymes (the at least four different cellulases, hemicellulases and/or pectinases) in *Aspergillus* may be a high enzyme yield on sugar.

When the cell to be transformed to form the host cell according to the invention is an *Aspergillus* strain, the *Aspergillus* strain is preferably CBS 513.88 or a derivative thereof, more preferably the host cell is *Aspergillus niger* CBS 513.88.

According to another embodiment, the cell to be transformed to form the host cell according to the invention is a prokaryotic cell. Preferably, the prokaryotic host cell is bacterial cell. The term "bacterial cell" includes both Gram-negative and Gram-positive microorganisms. Suitable bacteria may be selected from the group of *Escherichia*, *Anabaena*, *Caulobacter*, *Gluconobacter*, *Rhodobacter*, *Pseudomonas*, *Paracoccus*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Rhizobium* (*Sinorhizobium*), *Flavobacterium*, *Klebsiella*, *Enterobacter*, *Lactobacillus*, *Lactococcus*, *Methylobacterium*, *Staphylococcus* and *Streptomyces*. Preferably, the bacterial cell is selected from the group of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilis*, *B. megaterium*, *B. halodurans*, *B. pumilus*, *G. oxydans*, *Caulobacter crescentus* CB 15, *Methylobacterium extorquens*, *Rhodobacter sphaeroides*, *Pseudomonas zeaxanthinifaciens*, *Paracoccus denitrificans*, *E. coli*, *C. glutamicum*, *Staphylococcus carnosus*, *Streptomyces lividans*, *Sinorhizobium meliloti* and *Rhizobium radiobacter*.

The enzymes

In the invention, the at least four different enzymes are chosen from the group of cellulases, hemicellulases and pectinases, preferably from the group of cellulases and hemicellulases and more preferably from the group of cellulases.

Cellulases are enzymes that hydrolyze cellulose (β -1,4-glucan or β D-glucosidic linkages) resulting in the formation of glucose, cellobiose, cellooligosaccharides, and the like.

Cellulases have been traditionally divided into three major classes: endoglucanases (EC 3.2.1.4) ("EG"), exoglucanases or cellobiohydrolases (EC 3.2.1.91) ("CBH") and β -glucosidases ([β]-D-glucoside glucohydrolase; EC 3.2.1.21) ("BG"). See e.g. Knowles et al., TIBTECH 5, 255-261, 1987; Shulein, Methods Enzymol., 160, 25, pp. 234-243, 1988. Endoglucanases act mainly on the amorphous parts of the cellulose fibre, whereas cellobiohydrolases are also able to degrade crystalline cellulose (Nevalainen and Penttila, Mycota, 303-319, 1995). Thus, the presence of a cellobiohydrolase in a cellulase system is required for efficient solubilization of crystalline cellulose (Suurnakki, et al. Cellulose 7:189-209, 2000). β -glucosidase acts to liberate D-glucose units from cellobiose, cello-oligosaccharides, and other glucosides (Freer, J. Biol. Chem. vol. 268, no. 13, pp. 9337-9342, 1993).

Cellulases are known to be produced by a large number of bacteria, yeast and fungi. Certain fungi produce a complete cellulase system capable of degrading crystalline forms of cellulose, such that the cellulases are readily produced in large quantities via fermentation. Filamentous fungi play a special role since many yeast, such as *Saccharomyces cerevisiae*, lack the ability to hydrolyze cellulose. See, e.g., Aubert et al., 1988; Wood et al., *Methods in Enzymology*, vol. 160, no. 9, pp. 87-116, 1988, and Coughlan, et al., "Comparative Biochemistry of Fungal and Bacterial Cellulolytic Enzyme Systems" *Biochemistry and Genetics of Cellulose Degradation*, pp. 11-30 1988.

Fungal cellulase classifications of CBH, EG and BG can be further expanded to include multiple components within each classification. For example, multiple CBHs, EGs and BGs have been isolated from a variety of fungal sources including *Trichoderma reesei* which contains known polynucleotides coding for 2 CBHs, i.e., CBH I and CBH II, at least 8 EGs, i.e., EG I, EG II, EG III, EGIV, EGV, EGVII, EGVIII and EGVIII, and at least 5 BGs, i.e., BG1, BG2, BG3, BG4 and BG5.

Accordingly, an enzyme composition of the invention may comprise any cellulase, for example, a cellobiohydrolase, an endo- β -1,4-glucanase, a β -glucosidase or a β -(1,3)(1,4)-glucanase.

Herein, a cellobiohydrolase (EC 3.2.1.91) is any polypeptide which is capable of catalysing the hydrolysis of 1,4- β -D-glucosidic linkages in cellulose or cellotetraose, releasing cellobiose from the non-reducing ends of the chains. This enzyme may also be referred to as cellulase 1,4- β -cellobiosidase, 1,4- β -cellobiohydrolase, 1,4- β -D-glucan cellobiohydrolase, avicelase, exo-1,4- β -D-glucanase, exocellobiohydrolase or exoglucanase.

Herein, an endo- β -1,4-glucanase (EC 3.2.1.4) is any polypeptide which is capable of catalysing the endohydrolysis of 1,4- β -D-glucosidic linkages in cellulose, lichenin or cereal β -D-glucans. Such a polypeptide may also be capable of hydrolyzing 1,4-linkages in β -D-glucans also containing 1,3-linkages. This enzyme may also be referred to as cellulase, avicelase, β -1,4-endoglucan hydrolase, β -1,4-glucanase, carboxymethyl cellulase, celludextrinase, endo-1,4- β -D-glucanase, endo-1,4- β -D-glucanohydrolase, endo-1,4- β -glucanase or endoglucanase.

Herein a β -(1,3)(1,4)-glucanase (EC 3.2.1.73) is any polypeptide which is capable of catalyzing the hydrolysis of 1,4- β -D-glucosidic linkages in β -D-glucans containing 1,3- and 1,4-bonds. Such a polypeptide may act on lichenin and cereal β -D-glucans, but not on β -D-glucans containing only 1,3- or 1,4-bonds. This

enzyme may also be referred to as licheninase, 1,3-1,4- β -D-glucan 4-glucanohydrolase, β -glucanase, endo- β -1,3-1,4 glucanase, lichenase or mixed linkage β -glucanase. An alternative for this type of enzyme is EC 3.2.1.6, which is described as endo-1,3(4)- β -glucanase. This type of enzyme hydrolyses 1,3- or 1,4-linkages in β -D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at C-3. Alternative names include endo-1,3- β -glucanase, laminarinase, 1,3-(1,3;1,4)- β -D-glucan 3 (4) glucanohydrolase; substrates include laminarin, lichenin and cereal β -D-glucans.

Herein, a β -glucosidase (EC 3.2.1.21) is any polypeptide which is capable of catalysing the hydrolysis of terminal, non-reducing β -D-glucose residues with release of β -D-glucose. Such a polypeptide may have a wide specificity for β -D-glucosides and may also hydrolyze one or more of the following: a β -D-galactoside, an α -L-arabinoside, a β -D-xyloside or a β -D-fucoside. This enzyme may also be referred to as amygdalase, β -D-glucoside glucohydrolase, cellobiase or gentobiase.

The host cell may produce and secrete endoglucanase activity and/or cellobiohydrolase activity and/or β -glucosidase activity. The host cell may produce and secrete more than one enzyme activity in one or more of those classes. For example, the host cell may produce and secrete two endoglucanase activities, for example, endo-1,3(1,4)- β glucanase activity and endo- β -1,4-glucanase activity.

Herein, a hemicellulase is any polypeptide which is capable of degrading or modifying hemicellulose. That is to say, a hemicellulase may be capable of degrading or modifying one or more of xylan, araban, glucuronoxylan, arabinogalactan, arabinoxylan, glucomannan, galactomannan and xyloglucan. A polypeptide which is capable of degrading a hemicellulose is one which is capable of catalysing the process of breaking down the hemicellulose into smaller polysaccharides, either partially, for example into oligosaccharides, or completely into sugar monomers, for example hexose or pentose sugar monomers. A hemicellulase may give rise to a mixed population of oligosaccharides and sugar monomers when contacted with the hemicellulase. Such degradation will typically take place by way of a hydrolysis reaction.

A composition of the invention may comprise any hemicellulase, for example, an endoxylanase, a β -xylosidase, an α -L-arabinofuranosidase, an acetyl-xylan esterase, an α -D-glucuronidase, an cellobiohydrolase, a feruloyl esterase, a coumaroyl esterase, an α -galactosidase, a β -galactosidase, a β -mannanase or a β -mannosidase.

Herein, an endoxylanase (EC 3.2.1.8) is any polypeptide which is capable of catalyzing the endohydrolysis of 1,4- β -D-xylosidic linkages in xylans. This enzyme may also be referred to as endo-1,4- β -xylanase or 1,4- β -D-xylan xylanohydrolase. An alternative endoxylanase is EC 3.2.1.136, a glucuronoarabinoxylan endoxylanase, an enzyme that is able to hydrolyse 1,4 xylosidic linkages in glucuronoarabinoxylans.

Herein, a β -xylosidase (EC 3.2.1.37) is any polypeptide which is capable of catalyzing the hydrolysis of 1,4- β -D-xylans, to remove successive D-xylose residues from the non-reducing termini. Such enzymes may also hydrolyze xylobiose. This enzyme may also be referred to as xylan 1,4- β -xylosidase, 1,4- β -D-xylan xylohydrolase, exo-1,4- β -xylosidase or xylobiase.

Herein, an α -L-arabinofuranosidase (EC 3.2.1.55) is any polypeptide which is capable of acting on α -L-arabinofuranosides, α -L-arabinans containing (1,2) and/or (1,3)- and/or (1,5)-linkages, arabinoxylans and arabinogalactans. This enzyme may also be referred to as α -N-arabinofuranosidase, arabinofuranosidase or arabinosidase.

Herein, an α -D-glucuronidase (EC 3.2.1.139) is any polypeptide which is capable of catalyzing a reaction of the following form: α -D-glucuronoside + $H_2O \rightarrow$ alcohol + D-glucuronate. This enzyme may also be referred to as α -glucuronidase or α -glucosiduronase. These enzymes may also hydrolyse 4-O-methylated glucuronic acid, which can also be present as a substituent in xylans. Alternative is EC 3.2.1.131: xylan α -1,2-glucuronosidase, which catalyses the hydrolysis of α -1,2-(4-O-methyl)glucuronosyl links.

Herein, an acetyl-xylan esterase (EC 3.1.1.72) is any polypeptide which is capable of hydrolysis of specifically the ester linkages of the acetyl groups in positions 2 and/or 3 of the xylose moieties of natural xylan.

Herein, a feruloyl esterase (EC 3.1.1.73) is any polypeptide which is capable of catalyzing a reaction of the form: feruloyl-saccharide + $H_2O \rightarrow$ ferulate + saccharide. The saccharide may be, for example, an oligosaccharide or a polysaccharide. It may typically catalyze the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in 'natural' substrates. p-nitrophenol acetate and methyl ferulate are typically poorer substrates. This enzyme may also be referred to as cinnamoyl ester hydrolase, ferulic acid esterase or hydroxycinnamoyl esterase. It may also be referred to as a

hemicellulase accessory enzyme, since it may help xylanases and pectinases to break down plant cell wall hemicellulose and pectin.

Herein, a coumaroyl esterase (EC 3.1.1.73) is any polypeptide which is capable of catalyzing a reaction of the form: coumaroyl-saccharide + H₂O → coumarate + saccharide. The saccharide may be, for example, an oligosaccharide or a polysaccharide. This enzyme may also be referred to as trans-4-coumaroyl esterase, trans-p-coumaroyl esterase, p-coumaroyl esterase or p-coumaric acid esterase. This enzyme also falls within EC 3.1.1.73 so may also be referred to as a feruloyl esterase.

Herein, an α-galactosidase (EC 3.2.1.22) is any polypeptide which is capable of catalyzing the hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides, including galactose oligosaccharides, galactomannans, galactans and arabinogalactans. Such a polypeptide may also be capable of hydrolyzing α-D-fucosides. This enzyme may also be referred to as melibiase.

Herein, a β-galactosidase (EC 3.2.1.23) is any polypeptide which is capable of catalyzing the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. Such a polypeptide may also be capable of hydrolyzing α-L-arabinosides. This enzyme may also be referred to as exo-(1→4)-β-D-galactanase or lactase. β-galactosidase is an enzyme that may accept as its substrate both hemicellulose and pectine, therefore it is classified as a hemicellulase and as a pectinase.

Herein, a β-mannanase (EC 3.2.1.78) is any polypeptide which is capable of catalyzing the random hydrolysis of 1,4-β-D-mannosidic linkages in mannans, galactomannans and glucomannans. This enzyme may also be referred to as mannan endo-1,4-β-mannosidase or endo-1,4-mannanase.

Herein, a β-mannosidase (EC 3.2.1.25) is any polypeptide which is capable of catalyzing the hydrolysis of terminal, non-reducing β-D-mannose residues in β-D-mannosides. This enzyme may also be referred to as mannanase or mannase.

Herein, a pectinase is any polypeptide which is capable of degrading or modifying pectin. A polypeptide which is capable of degrading pectin is one which is capable of catalysing the process of breaking down pectin into smaller units, either partially, for example into oligosaccharides, or completely into sugar monomers. A pectinase according to the invention may give rise to a mixed population of oligosaccharides and sugar monomers when contacted with the pectinase. Such degradation will typically take place by way of a hydrolysis reaction.

A host cell according to the invention may comprise a heterologous polynucleotide encoding any pectinase, for example an endo polygalacturonase, a pectin methyl esterase, an endo-galactanase, a β -galactosidase, a pectin acetyl esterase, an endo-pectin lyase, pectate lyase, alpha rhamnosidase, an exo-galacturonase, an exo-polygalacturonate lyase, a rhamnogalacturonan hydrolase, a rhamnogalacturonan lyase, a rhamnogalacturonan acetyl esterase, a rhamnogalacturonan galacturonohydrolase or a xylogalacturonase.

Herein, an endo-polygalacturonase (EC 3.2.1.15) is any polypeptide which is capable of catalyzing the random hydrolysis of 1,4- α -D-galactosiduronic linkages in pectate and other galacturonans. This enzyme may also be referred to as polygalacturonase pectin depolymerase, endopolygalacturonase, pectolase, pectin hydrolase, pectin polygalacturonase, poly- α -1,4-galacturonide glycanohydrolase, endogalacturonase; endo-D-galacturonase or poly(1,4- α -D-galacturonide) glycanohydrolase.

Herein, a pectin methyl esterase (EC 3.1.1.11) is any enzyme which is capable of catalyzing the reaction: $\text{pectin} + n \text{ H}_2\text{O} \rightarrow n \text{ methanol} + \text{pectate}$. The enzyme may also be known as pectinesterase, pectin demethoxylase, pectin methoxylase, pectin methylesterase, pectase, pectinoesterase or pectin pectylhydrolase.

Herein, an endo-galactanase (EC 3.2.1.89) is any enzyme capable of catalyzing the endohydrolysis of 1,4- β -D-galactosidic linkages in arabinogalactans. The enzyme may also be known as arabinogalactan endo-1,4- β -galactosidase, endo-1,4- β -galactanase, galactanase, arabinogalactanase or arabinogalactan 4- β -D-galactanohydrolase.

Herein, a pectin acetyl esterase is defined herein as any enzyme which has an acetyl esterase activity which catalyzes the deacetylation of the acetyl groups at the hydroxyl groups of GalUA residues of pectin

Herein, an endo-pectin lyase (EC 4.2.2.10) is any enzyme capable of catalyzing the eliminative cleavage of (1 $\rightarrow$ 4)- α -D-galacturonan methyl ester to give oligosaccharides with 4-deoxy-6-O-methyl- α -D-galact-4-enuronosyl groups at their non-reducing ends. The enzyme may also be known as pectin lyase, pectin *trans*-eliminase; endo-pectin lyase, polymethylgalacturonic transeliminase, pectin methyltranseliminase, pectolyase, PL, PNL or PMGL or (1 $\rightarrow$ 4)-6-O-methyl- α -D-galacturonan lyase.

Herein, a pectate lyase (EC 4.2.2.2) is any enzyme capable of catalyzing the eliminative cleavage of (1→4)-α-D-galacturonan to give oligosaccharides with 4-deoxy-α-D-galact-4-enuronosyl groups at their non-reducing ends. The enzyme may also be known polygalacturonic transeliminase, pectic acid transeliminase, polygalacturonate lyase, endopectin methyltranseliminase, pectate transeliminase, endogalacturonate transeliminase, pectic acid lyase, pectic lyase, α-1,4-D-endopolygalacturonic acid lyase, PGA lyase, PPase-N, endo-α-1,4-polygalacturonic acid lyase, polygalacturonic acid lyase, pectin *trans*-eliminase, polygalacturonic acid *trans*-eliminase or (1→4)-α-D-galacturonan lyase.

Herein, an alpha rhamnosidase (EC 3.2.1.40) is any polypeptide which is capable of catalyzing the hydrolysis of terminal non-reducing α-L-rhamnose residues in α-L-rhamnosides or alternatively in rhamnogalacturonan. This enzyme may also be known as α-L-rhamnosidase T, α-L-rhamnosidase N or α-L-rhamnoside rhamnohydrolase.

Herein, exo-galacturonase (EC 3.2.1.82) is any polypeptide capable of hydrolysis of pectic acid from the non-reducing end, releasing digalacturonate. The enzyme may also be known as exo-poly-α-galacturonosidase, exopolygalacturonosidase or exopolygalacturanosidase.

Herein, exo-galacturonase (EC 3.2.1.67) is any polypeptide capable of catalyzing: $(1,4\text{-}\alpha\text{-D-galacturonide})_n + \text{H}_2\text{O} \rightarrow (1,4\text{-}\alpha\text{-D-galacturonide})_{n-1} + \text{D-galacturonate}$. The enzyme may also be known as galacturan 1,4-α-galacturonidase, exopolygalacturonase, poly(galacturonate) hydrolase, exo-D-galacturonase, exo-D-galacturonanase, exopoly-D-galacturonase or poly(1,4-α-D-galacturonide) galacturonohydrolase.

Herein, exopolygalacturonate lyase (EC 4.2.2.9) is any polypeptide capable of catalyzing eliminative cleavage of 4-(4-deoxy-α-D-galact-4-enuronosyl)-D-galacturonate from the reducing end of pectate, i.e. de-esterified pectin. This enzyme may be known as pectate disaccharide-lyase, pectate exo-lyase, exopectic acid transeliminase, exopectate lyase, exopolygalacturonic acid-*trans*-eliminase, PATE, exo-PATE, exo-PGL or (1→4)-α-D-galacturonan reducing-end-disaccharide-lyase.

Herein, rhamnogalacturonan hydrolase is any polypeptide which is capable of hydrolyzing the linkage between galactosyluronic acid and rhamnopyranosyl in an endo-fashion in strictly alternating rhamnogalacturonan structures, consisting of the disaccharide [(1,2-α-L-rhamnoyl-(1,4)-α-galactosyluronic acid)].

Herein, rhamnogalacturonan lyase is any polypeptide which is any polypeptide which is capable of cleaving α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalpA linkages in an endo-fashion in rhamnogalacturonan by beta-elimination.

Herein, rhamnogalacturonan acetyl esterase is any polypeptide which catalyzes the deacetylation of the backbone of alternating rhamnose and galacturonic acid residues in rhamnogalacturonan.

Herein, rhamnogalacturonan galacturonohydrolase is any polypeptide which is capable of hydrolyzing galacturonic acid from the non-reducing end of strictly alternating rhamnogalacturonan structures in an exo-fashion.

Herein, xylogalacturonase is any polypeptide which acts on xylogalacturonan by cleaving the β -xylose substituted galacturonic acid backbone in an *endo*-manner. This enzyme may also be known as xylogalacturonan hydrolase.

An α -L-arabinofuranosidase may accept as its substrate both hemicellulose and pectine, therefore it is classified as both a hemicellulase and a pectinase.

Herein, an α -L-arabinofuranosidase (EC 3.2.1.55) is any polypeptide which is capable of acting on α -L-arabinofuranosides, α -L-arabinans containing (1,2) and/or (1,3)- and/or (1,5)-linkages, arabinoxylans and arabinogalactans. This enzyme may also be referred to as α -N-arabinofuranosidase, arabinofuranosidase or arabinosidase.

Herein, endo-arabinanase (EC 3.2.1.99) is any polypeptide which is capable of catalyzing endohydrolysis of 1,5- α -arabinofuranosidic linkages in 1,5-arabinans. The enzyme may also be known as endo-arabinase, arabinan endo-1,5- α -L-arabinosidase, endo-1,5- α -L-arabinanase, endo- α -1,5-arabanase; endo-arabanase or 1,5- α -L-arabinan 1,5- α -L-arabinanohydrolase.

In a special embodiment of the invention, the at least four different enzymes are at least two different cellobiohydrolases, for example a cellobiohydrolase I and/or a cellobiohydrolase II, optionally at least an endoglucanase, for example β -glucanase CEA and at least a β -glucosidase.

Preferably, one of said different cellobiohydrolases has at least 70% identity with SEQ ID No. 1 and the other of said different cellobiohydrolases has at least 70% identity with SEQ ID No. 7, wherein the endoglucanase has at least 70% identity with SEQ ID No. 3 and wherein the β -glucosidase has at least 70% identity with SEQ ID No. 5.

For example, one cellobiohydrolase may have at least 75%, for example at least 80%, for example at least 85%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 97%, for example at least 98%, for example at least 99% identity with SEQ ID NO. 1.

For example, the other cellobiohydrolase may have at least 75%, for example at least 80%, for example at least 85%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 97%, for example at least 98%, for example at least 99% identity with SEQ ID NO. 7.

For example, the endoglucanase may have at least 75%, for example at least 80%, for example at least 85%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 97%, for example at least 98%, for example at least 99% identity with SEQ ID NO. 3.

For example, the β -glucosidase may have at least 75%, for example at least 80%, for example at least 85%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 97%, for example at least 98%, for example at least 99% identity with SEQ ID NO. 5.

Preferably, the enzymes produced by the host cell of the invention have a cellulase activity of at least 2 WSU in 16 times or more diluted supernatant or broth, for example a cellulase activity of at least 3 WSU or a cellulase activity of at least 5 WSU or more in 16 times diluted supernatant or broth. The broth or supernatant may for example be 10000 times diluted, 5000 times diluted or 2500 times diluted.

With 1 WSU is meant 0.119 mg/ml glucose released from 2.1 w/v % washed pre-treated wheat straw by 200 μ l of enzyme mix in 20 hours at 65°C at pH 4.50.

When the heterologous cellulase, hemicellulase and/or pectinase is to be secreted from the host cell into the cultivation medium, an appropriate signal sequence can be added to the polypeptide in order to direct the *de novo* synthesized polypeptide to the secretion route of the host cell. The person skilled in the art knows to select an appropriate signal sequence for a specific host. The signal sequence may be native to the host cell, or may be foreign to the host cell. As an example, a signal sequence from a protein native to the host cell can be used. Preferably, said native protein is a highly secreted protein, i.e. a protein that is secreted in amounts higher than 10% of the total amount of protein being secreted.

As an alternative for a signal sequence, the polypeptide of the invention can be fused to a secreted carrier protein, or part thereof. Such chimeric

construct is directed to the secretion route by means of the signal sequence of the carrier protein, or part thereof. In addition, the carrier protein will provide a stabilizing effect to the polypeptide according to the invention and or may enhance solubility. Such carrier protein may be any protein. Preferably, a highly secreted protein is used as a carrier protein. The carrier protein may be native or foreign to the polypeptide according to the invention. The carrier protein may be native or may be foreign to the host cell. Examples of such carrier proteins are glucoamylase, prepro sequence of alpha-Mating factor, cellulose binding domain of *Clostridium cellulovorans* cellulose binding protein A, glutathione S-transferase, chitin binding domain of *Bacillus circulans* chitinase A1, maltose binding domain encoded by the *malE* gene of *E. coli* K12, beta-galactosidase, and alkaline phosphatase. A preferred carrier protein for expression of such chimeric construct in *Aspergillus* cells is glucoamylase.

The polynucleotides

The term "polynucleotide" is identical to the term "nucleic acid molecule" and can herein be read interchangeably. The term refers to a polynucleotide molecule, which is a ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecule, either single stranded or double stranded. A polynucleotide may either be present in isolated form, or be comprised in recombinant nucleic acid molecules or vectors, or be comprised in a host cell. The polynucleotide may be synthetic or may be isolated from chromosomal DNA.

As used herein, the term "gene" refers to nucleic acid molecules, which may be isolated from chromosomal DNA or may be synthesized based on a sequence listing, which includes an open reading frame encoding a polypeptide.

The polynucleotide according to the invention may be a synthetic polynucleotide. The synthetic polynucleotide may be optimized in its codon use, preferably according to the methods described in WO2006/077258 and/or PCT/EP2007/055943, which are herein incorporated by reference. PCT/EP2007/055943 addresses codon-pair optimization. Codon-pair optimisation is a method wherein the nucleotide sequences encoding a polypeptide have been modified with respect to their codon-usage, in particular the codon-pairs that are used, to obtain improved expression of the nucleotide sequence encoding the polypeptide and/or improved production of the encoded polypeptide. Codon pairs are defined as a set of two subsequent triplets (codons) in a coding sequence.

The term "coding sequence" as defined herein is a sequence, which is transcribed into mRNA and translated into a polypeptide according to the invention. The

boundaries of the coding sequence are generally determined by the ATG or other start codon at the 5'-end of the mRNA and a translation stop codon sequence terminating the open reading frame at the 3'-end of the mRNA. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

The at least four different heterologous polynucleotides encoding the enzymes chosen from the group of cellulases, hemicellulases and pectinases can be chosen from any of the known polynucleotides.

Preferably, the polynucleotide is chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases, is chosen from the group of polynucleotides encoding cellulases and hemicellulases, more preferably from the group of polynucleotides encoding cellulases.

In an embodiment, a polynucleotide according to the invention is chosen from the polynucleotides that encode a thermostable enzyme. Herein, this means that the enzyme has a temperature optimum of about 60°C or higher, for example about 70°C or higher, such as about 75°C or higher, for example about 80°C or higher such as 85°C or higher. The skilled person may select suitable polynucleotides using common knowledge. They may for example be isolated from thermophilic microorganisms, or may be designed by the skilled person and artificially synthesized. In one embodiment the polynucleotides may be isolated from thermophilic filamentous fungi. Examples of thermophilic filamentous fungi from which polynucleotides encoding thermostable enzymes may be isolated are: *Acremonium*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, and *Tolypocladium*.

In a special aspect of the invention the at least four different polynucleotides are at least the polynucleotides encoding a cellobiohydrolase I and/or a cellobiohydrolase II, an endoglucanase and a β -glucosidase.

Preferably, the polynucleotide encoding the cellobiohydrolase I has at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 87%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 96%, for example at least 97%, for example at least 98%, for example at least 99% or 100% % identity with SEQ ID No. 2 (polynucleotide encoding cellobiohydrolase I from *Talaromyces emersonii* CBS 39364).

Preferably, the polynucleotide encoding the cellobiohydrolase II has at least at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 87%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 96%, for example at least 97%, for example at least 98%, for example at least 99% or 100% % identity with SEQ ID No. 8 (polynucleotide encoding cellobiohydrolase II from *Talaromyces emersonii* CBS 39364).

Preferably, the polynucleotide encoding the endoglucanase has at least at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 87%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 96%, for example at least 97%, for example at least 98%, for example at least 99% or 100% % identity with SEQ ID No. 4 (polynucleotide encoding endoglucanase from *Talaromyces emersonii* CBS 39364).

Preferably, the polynucleotide encoding the β -glucosidase has at least at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 87%, for example at least 90%, for example at least 93%, for example at least 95%, for example at least 96%, for example at least 97%, for example at least 98%, for example at least 99% or 100% % identity with SEQ ID No. 6 (polynucleotide encoding β -glucosidase from *Talaromyces emersonii*).

In a special embodiment of the invention, the polynucleotide encoding cellobiohydrolase I having at least 70% identity with SEQ ID No. 2 (polynucleotide encoding cellobiohydrolase I from *Talaromyces emersonii* CBS 39364), the polynucleotide having at least 70% identity with SEQ ID No. 8 (polynucleotide encoding cellobiohydrolase II from *Talaromyces emersonii* CBS 39364), the polynucleotide encoding having at least 70% identity with SEQ ID No. 4 (the polynucleotide encoding endoglucanase from *Talaromyces emersonii* CBS 39364) and the polynucleotide having at least 70% identity with SEQ ID No. 6 (the polynucleotide encoding β -glucosidase from *Talaromyces emersonii*) are present in the host cell, preferably an *Aspergillus* host cell.

Aspergillus is a very good host cell for the polynucleotides of *Talaromyces emersonii*: cellobiohydrolase I of SEQ ID No. 2, cellobiohydrolase II of SEQ ID No. 8, endogluconase of SEQ ID No. 4 and β -glucosidase of SEQ ID No. 6) and those polynucleotides having at least 70% identity therewith. Surprisingly, it has

been found that cellobiohydrolase I of SEQ ID No. 2, cellobiohydrolase II of SEQ ID No. 8, endoglucanase of SEQ ID No. 4 and β -glucosidase of SEQ ID No. 6) are expressed in and secreted by *Aspergillus* without any adaptation of these sequences to the preferred codon usage and signal sequences of *Aspergillus*.

Preferably, the polynucleotides encoding the at least four different enzymes chosen from the group of cellulases, hemicellulases and pectinases are original or codon optimized polynucleotides originating from a *Talaromyces* strain, more preferably from a *Talaromyces emersonii* strain, more preferably from the *Talaromyces emersonii* strain deposited at CENTRAAL BUREAU VOOR SCHIMMELCULTURES, Uppsalalaan 8, P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands on 1st July 2009 having the Accession Number CBS 39364, or a functionally equivalent mutant thereof. This latter strain is indicated herein as *Talaromyces emersonii* CBS 39364.

Amino acid or nucleotide sequences are said to be homologous when exhibiting a certain level of similarity. Whether two homologous sequences are closely related or more distantly related is indicated by "percent identity" or "% identity", which is high or low respectively.

For the purpose of this invention, it is defined here that in order to determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the complete sequences are aligned for optimal comparison purposes. In order to optimize the alignment between the two sequences gaps may be introduced in any of the two sequences that are compared. Such alignment is carried out over the full length of the sequences being compared. The identity is the percentage of identical matches between the two sequences over the reported aligned region.

A comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. The skilled person will be aware of the fact that several different computer programs are available to align two sequences and determine the homology between two sequences (Kruskal, J. B. (1983) An overview of sequence comparison In D. Sankoff and J. B. Kruskal, (ed.), Time warps, string edits and macromolecules: the theory and practice of sequence comparison, pp. 1-44 Addison Wesley). The percent identity between two amino acid sequences can be determined using the Needleman and Wunsch algorithm for the alignment of two sequences. (Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453). The algorithm aligns amino acid sequences as well as nucleotide sequences. The Needleman-Wunsch algorithm has been implemented in

the computer program NEEDLE. For the purpose of this invention the NEEDLE program from the EMBOSS package was used (version 2.8.0 or higher, *EMBOSS: The European Molecular Biology Open Software Suite* (2000) Rice, P. Longden, I. and Bleasby, A. Trends in Genetics 16, (6) pp276—277, <http://emboss.bioinformatics.nl/>). For protein sequences, EBLOSUM62 is used for the substitution matrix. For nucleotide sequences, EDNAFULL is used. Other matrices can be specified. For purpose of the invention, the parameters used for alignment of amino acid sequences are a gap-open penalty of 10 and a gap extension penalty of 0.5. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

The protein sequences mentioned herein can further be used as a “query sequence” to perform a search against sequence databases, for example to identify other family members or related sequences. Such searches can be performed using the BLAST programs. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). BLASTP is used for amino acid sequences and BLASTN for nucleotide sequences. In the BLAST program, the default settings may be used:

- Cost to open gap: default = 5 for nucleotides/ 11 for proteins
- Cost to extend gap: default = 2 for nucleotides/ 1 for proteins
- Penalty for nucleotide mismatch: default = -3
- Reward for nucleotide match: default = 1
- Expect value: default = 10
- Wordsize: default = 11 for nucleotides/ 28 for megablast/ 3 for proteins

The nucleic acid sequences as mentioned herein can further be used as a “query sequence” to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) J. Mol. Biol. 215:403—10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention.

Homologous polynucleotide sequences can be isolated, for example, by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of nucleotide sequences as mentioned herein.

The template for the reaction can be cDNA obtained by reverse transcription of mRNA prepared from strains known or suspected to express a polynucleotide according to the invention. The PCR product can be subcloned and sequenced to ensure that the amplified sequences represent the sequences of a new nucleic acid sequence, or a functional equivalent thereof.

The PCR fragment can then be used to isolate a full-length cDNA clone by a variety of known methods. For example, the amplified fragment can be labeled and used to screen a bacteriophage or cosmid cDNA library. Alternatively, the labeled fragment can be used to screen a genomic library.

PCR technology also can be used to isolate full-length cDNA sequences from other organisms. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source. A reverse transcription reaction can be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis.

The resulting RNA/DNA hybrid can then be "tailed" (e.g., with guanines) using a standard terminal transferase reaction, the hybrid can be digested with RNase H, and second strand synthesis can then be primed (e.g., with a poly-C primer). Thus, cDNA sequences upstream of the amplified fragment can easily be isolated. For a review of useful cloning strategies, see e.g., Sambrook *et al.*, *infra*.

For example, with the term 'at least 70% identity with a sequence listing of an amino acid sequence is meant that the percentage obtained from following the method described above, the percentage obtained is 70% or higher.

Process for the preparation of the host cell

In another aspect, the invention relates to a process for the preparation of a host cell according to the invention comprising the steps of:

- (a) Providing one or more expression cassettes comprising at least four different heterologous polynucleotides chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases and the control sequences required for expression of these polynucleotides
- (b) Providing a selectable marker
- (c) Transforming cells with the one or more expression cassettes and the selectable marker from step (b)

- (d) Selecting the thus formed host cells that produce the at least four different cellulases, hemicellulases and/or pectinases encoded by the at least four different heterologous polynucleotides.

The term "nucleic acid construct" is herein referred to as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains all the control sequences required for expression of a coding sequence, wherein said control sequences are operably linked to said coding sequence.

The term "operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence directs the production of a polypeptide.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of mRNA and / or a polypeptide, either *in vitro* or in a host cell. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, Shine-Delgarno sequence, optimal translation initiation sequences (as described in Kozak, 1991, J. Biol. Chem. 266:19867-19870), a polyadenylation sequence, a pro-peptide sequence, a pre-pro-peptide sequence, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. Control sequences may be optimized to their specific purpose. Preferred optimized control sequences used in the present invention are those described in WO2006/077258, which is herein incorporated by reference.

The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

The control sequence may be an appropriate promoter sequence (promoter).

The control sequence may also be a suitable transcription terminator (terminator) sequence, a sequence recognized by a filamentous fungal cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the

nucleic acid sequence encoding the polypeptide. Any terminator, which is functional in the cell, may be used in the present invention.

Preferred terminator sequences for filamentous fungal cells are obtained from the polynucleotides encoding *A. oryzae* TAKA amylase, *A. niger* glucoamylase (glaA), *A. nidulans* anthranilate synthase, *A. niger* alpha-glucosidase, trpC and *Fusarium oxysporum* trypsin-like protease.

The control sequence may also be a suitable leader sequence (leaders), a non-translated region of an mRNA which is important for translation by the filamentous fungal cell. The leader sequence is operably linked to the 5'-terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence, which is functional in the cell, may be used in the present invention.

Preferred leaders for filamentous fungal cells are obtained from the polynucleotides encoding *A. oryzae* TAKA amylase and *A. nidulans* triose phosphate isomerase and *A. niger* glaA and phytase.

Other control sequences may be isolated from the *Penicillium* IPNS gene, or pcbC gene, the beta tubulin gene. All the control sequences cited in WO 01/21779 are herewith incorporated by reference.

The control sequence may also be a polyadenylation sequence, a sequence which is operably linked to the 3'-terminus of the nucleic acid sequence and which, when transcribed, is recognized by the filamentous fungal cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence, which is functional in the cell, may be used in the present invention.

Preferred polyadenylation sequences for filamentous fungal cells are obtained from the polynucleotides encoding *A. oryzae* TAKA amylase, *A. niger* glucoamylase, *A. nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease and *A. niger* alpha-glucosidase.

The term "promoter" is defined herein as a DNA sequence that binds RNA polymerase and directs the polymerase to the correct downstream transcriptional start site of a nucleic acid sequence encoding a biological compound to initiate transcription. RNA polymerase effectively catalyzes the assembly of messenger RNA complementary to the appropriate DNA strand of a coding region. The term "promoter" will also be understood to include the 5'-non-coding region (between promoter and translation start) for translation after transcription into mRNA, cis-acting transcription control elements such as enhancers, and other nucleotide sequences capable of interacting with transcription factors. The promoter may be any appropriate promoter

sequence suitable for a eukaryotic or prokaryotic host cell, which shows transcriptional activity, including mutant, truncated, and hybrid promoters, and may be obtained from polynucleotides encoding extra-cellular or intracellular polypeptides either homologous (native) or heterologous (foreign) to the cell. The promoter may be a constitutive or inducible promoter.

Examples of inducible promoters that can be used are a starch-, copper-, oleic acid- inducible promoters. The promoter may be selected from the group, which includes but is not limited to promoters obtained from the polynucleotides encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral alpha-amylase, *A. niger* acid stable alpha-amylase, *A. niger* or *A. awamori* glucoamylase (glaA), *R. miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase, *A. nidulans* acetamidase, the NA2-tpi promoter (a hybrid of the promoters from the polynucleotides encoding *A. niger* neutral alpha-amylase and *A. oryzae* triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof.

Preferably, the expression of the at least four polynucleotides encoding cellulase, hemicellulase and/or pectinase is driven by a promoter that is active in glucose containing medium, for example a glucoamylase promoter, preferably the glaA promoter as this makes that the presence of a cellulase inducer is not required.

Therefore, the invention also relates to a host cell capable of producing cellulase in the absence of cellulase inducer in a glucose medium.

Other preferred promoters are the promoters described in WO2006/092396 and WO2005/100573, which are herein incorporated by reference.

In order to facilitate expression and/or translation, the polynucleotide or the nucleic acid construct according to the invention may be comprised in an expression vector such that the polynucleotide(s) encoding the enzyme(s) of interest is operably linked to the appropriate control sequences for expression and/or translation *in vitro*, or in prokaryotic or eukaryotic host cells.

The expression vector may be any vector (e.g., a plasmid or virus), which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence encoding the polypeptide(s) of interest. The choice of the vector will typically depend on the compatibility of the vector with the cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, i. e., a vector, which

exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extra-chromosomal element, a mini-chromosome, or an artificial chromosome. An autonomously maintained cloning vector may comprise the AMA1-sequence (see e.g. Aleksenko and Clutterbuck (1997), Fungal Genet. Biol. 21: 373-397).

Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The integrative cloning vector may integrate at random or at a predetermined target locus in the chromosomes of the host cell. In a preferred embodiment of the invention, the integrative cloning vector comprises a DNA fragment, which is homologous to a DNA sequence in a predetermined target locus in the genome of host cell for targeting the integration of the cloning vector to this predetermined locus. In order to promote targeted integration, the cloning vector is preferably linearized prior to transformation of the cell. Linearization is preferably performed such that at least one but preferably either end of the cloning vector is flanked by sequences homologous to the target locus. The length of the homologous sequences flanking the target locus is preferably at least 30 bp, preferably at least 50 bp, preferably at least 0.1 kb, even preferably at least 0.2 kb, more preferably at least 0.5 kb, even more preferably at least 1 kb, most preferably at least 2 kb. Preferably, the efficiency of targeted integration into the genome of the host cell, i.e. integration in a predetermined target locus, is increased by augmented homologous recombination abilities of the host cell.

Preferably, the homologous flanking DNA sequences in the cloning vector, which are homologous to the target locus, are derived from a highly expressed locus meaning that they are derived from a gene, which is capable of high expression level in the host cell. A gene capable of high expression level, i.e. a highly expressed gene, is herein defined as a gene whose mRNA can make up at least 0.5% (w/w) of the total cellular mRNA, e.g. under induced conditions, or alternatively, a gene whose gene product can make up at least 1% (w/w) of the total cellular protein, or, in case of a secreted gene product, can be secreted to a level of at least 0.1 g/l (as described in EP 357 127 B1).

More than one copy of a nucleic acid sequence may be inserted into the cell to increase production of the product encoded by said sequence. This can be done, preferably by integrating into its genome copies of the DNA sequence, more preferably by targeting the integration of the DNA sequence at one of the highly expressed locus defined in the former paragraph. Alternatively, this can be done by including an

amplifiable selectable marker gene with the nucleic acid sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleic acid sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent. To increase even more the number of copies of the DNA sequence to be over expressed the technique of gene conversion as described in WO98/46772 may be used.

The vector system may be a single vector or plasmid or two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon.

The vectors preferably contain one or more selectable markers, which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. The selectable marker may be introduced into the cell on the expression vector as the expression cassette or may be introduced on a separate expression vector.

A selectable marker for use in a filamentous fungal cell may be selected from the group including, but not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricinacetyltransferase), *bleA* (phleomycin binding), *hygB* (hygromycinphosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents from other species. Preferred for use in an *Aspergillus* and *Penicillium* cell are the *amdS* (see for example EP 635574 B1, EP0758020A2, EP1799821A2, WO 97/06261A2) and *pyrG* genes of *A. nidulans* or *A. oryzae* and the *bar* gene of *Streptomyces hygroscopicus*. More preferably an *amdS* gene is used, even more preferably an *amdS* gene from *A. nidulans* or *A. niger*. A most preferred selectable marker gene is the *A.nidulans amdS* coding sequence fused to the *A.nidulans gpdA* promoter (see EP 635574 B1). Other preferred *AmdS* markers are those described in WO2006/040358. *AmdS* genes from other filamentous fungi may also be used (WO 97/06261).

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g. Sambrook & Russell, *Molecular Cloning: A Laboratory Manual, 3rd Ed.*, CSHL Press, Cold Spring Harbor, NY, 2001; and Ausubel *et al.*, *Current Protocols in Molecular Biology*, Wiley InterScience, NY, 1995).

Furthermore, standard molecular cloning techniques such as DNA isolation, gel electrophoresis, enzymatic restriction modifications of nucleic acids, Southern analyses, transformation of cells, etc., are known to the skilled person and are for example described by Sambrook et al. (1989) "Molecular Cloning: a laboratory manual", Cold Spring Harbor Laboratories, Cold Spring Harbor, New York and Innis et al. (1990) "PCR protocols, a guide to methods and applications" Academic Press, San Diego.

Using the desired polynucleotide sequence as a hybridization probe, nucleic acid molecules used in the invention can be isolated using standard hybridization and cloning techniques (e. g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

A nucleic acid may be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

Furthermore, oligonucleotides corresponding to or hybridizable to a nucleotide sequence according to the invention can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

Preferably, the host cell, for example *Aspergillus* is engineered to improve the expression of the polynucleotides of interest.

Preferably, the efficiency of targeted integration into the genome of the host cell, i.e. integration in a predetermined target locus, is increased by augmented homologous recombination abilities of the host cell. Such phenotype of the cell preferably involves a deficient *hdfA* or *hdfB* as described in WO2005/095624A2. WO2005/095624A2 discloses a preferred method to obtain a filamentous fungal cell comprising increased efficiency of targeted integration.

Optionally, the host cell comprises an elevated unfolded protein response (UPR) compared to the wild type cell to enhance production abilities of a polypeptide of interest. UPR may be increased by techniques described in US2004/0186070A1 and/or US2001/0034045A1 and/or WO01/72783A2 and/or WO2005/123763A1. More specifically, the protein level of HAC1 and/or IRE1 and/or PTC2 has been modulated, and/or the SEC61 protein has been engineered in order to obtain a host cell having an elevated UPR.

Alternatively, or in combination with an elevated UPR, the host cell is genetically modified to obtain a phenotype displaying lower protease expression and/or protease secretion compared to the wild-type cell in order to enhance production abilities of a polypeptide of interest. Such phenotype may be obtained by deletion and/or modification and/or inactivation of a transcriptional regulator of expression of proteases. Such a transcriptional regulator is e.g. prtT. Lowering expression of proteases by modulation of prtT may be performed by techniques described in US2004/0191864A1.

Alternatively, or in combination with an elevated UPR and/or a phenotype displaying lower protease expression and/or protease secretion, the host cell displays an oxalate deficient phenotype in order to enhance the yield of production of a polypeptide of interest. An oxalate deficient phenotype may be obtained by techniques described in WO2004/070022A2.

Alternatively, or in combination with an elevated UPR and/or a phenotype displaying lower protease expression and/or protease secretion and/or oxalate deficiency, the host cell displays a combination of phenotypic differences compared to the wild cell to enhance the yield of production of the polypeptide of interest. These differences may include, but are not limited to, lowered expression of glucoamylase and/or neutral alpha-amylase A and/or neutral alpha-amylase B, protease, and oxalic acid hydrolase. Said phenotypic differences displayed by the host cell may be obtained by genetic modification according to the techniques described in US2004/0191864A1.

Alternatively, or in combination with an elevated UPR and/or a phenotype displaying lower protease expression and/or protease secretion and/or oxalate deficiency and a combination of phenotypic differences compared to the wild cell to enhance the yield of production of the polypeptide of interest, the host cell displays a deficiency in toxin genes, disabling the ability of the filamentous fungal host cell to express toxins. Such toxins include, but are not limited to, ochratoxins, fumonisins, cyclapiazonic acid, 3-nitropropionic acid, emodin, malformin, aflatoxins and secalonc acids. Such deficiency is preferably such as described in WO2000/039322A1.

The person skilled in the art knows how to transform cells with the one or more expression cassettes and the selectable marker. For example, the skilled person may use one or more expression vectors, wherein the one or more cloning vectors comprise the expression cassettes and the selectable marker.

Transformation of the cells may be conducted by any suitable known methods, including e.g. electroporation methods, particle bombardment or microprojectile bombardment, protoplast methods and *Agrobacterium* mediated

transformation (AMT). Preferably the protoplast method is used. Procedures for transformation are described by J.R.S. Fincham, Transformation in fungi. 1989, microbiological reviews. 53, 148-170.

Transformation of the host cell by introduction of a polynucleotide an expression vector or a nucleic acid construct into the cell is preferably performed by techniques well known in the art (see Sambrook & Russell; Ausubel, *supra*). Transformation may involve a process consisting of protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* cells are described in EP 238 023A2 and Yelton *et al.*, 1984, Proceedings of the National Academy of Sciences USA 81:1470-1474. Suitable procedures for transformation of *Aspergillus* and other filamentous fungal host cells using *Agrobacterium tumefaciens* are described in e.g. De Groot *et al.*, *Agrobacterium tumefaciens-mediated transformation of filamentous fungi*. Nat Biotechnol. 1998, 16:839-842. Erratum in: Nat Biotechnol 1998 16:1074. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, Gene 78:147156 or in WO 96/00787A2. Other methods can be applied such as a method using biolistic transformation as described in: Christiansen *et al.*, *Biolistic transformation of the obligate plant pathogenic fungus, Erysiphe graminis f.sp. hordei*. 1995, Curr Genet. 29:100-102. Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, Journal of Bacteriology 153: 163; and Hinnen *et al.*, 1978, Proceedings of the National Academy of Sciences USA 75: 1920.

In order to enhance the amount of copies of the polynucleotide of interest in the host cell, multiple transformations of the host cell may be required. In this way, the ratios of the different enzymes produced by the host cell may be influenced. Also, an expression vector may comprise multiple expression cassettes to increase the amount of copies of the polynucleotide(s) to be transformed.

Another way could be to choose different control sequences for the different polynucleotides, which – depending on the choice - may cause a higher or a lower production of the desired enzyme(s).

An advantage of the host cell of the invention is that the host cell of the invention can be used for the preparation of an enzyme composition having a consistent composition.

The cells transformed with the selectable marker can be selected based on the presence of the selectable marker. In case of transformation of

(*Aspergillus*) cells, usually when the cell is transformed with all nucleic acid material at the same time, when the selectable marker is present also the polynucleotide(s) encoding the desired enzyme(s) are present.

However, in order to ensure that the desired enzymes are produced by the host cell, the host cell can be selected based on the presence of the enzymes in the fermentation broth. The presence of the desired enzyme activity (WSU) can be detected in the fermentation broth as described in the experimental section below. Alternatively, the presence of the desired enzymes can be determined using SDS-PAGE analysis as described in the experimental section below.

The enzyme composition

In another aspect, the invention relates to the enzyme composition comprising at least four different cellulases, hemicellulases and/or pectinases produced by the host cell according to the invention.

The enzyme composition may be the unpurified fermentation broth, wherein the host cell has produced and secreted the at least four different cellulases, hemicellulases and/or pectinases, which fermentation broth contains the host cells or it may be a further purified form of the fermentation broth. Methods for purification of enzymes are known to the person skilled in the art (downstream processing as described below).

In one embodiment, CBHI is provided in an enzyme composition that comprises BG, EG and CBHII. In an embodiment thereof, the amounts of enzymes are chosen so that BG is present at 2-12%, CBHI at 10-65%, CBHII at 10-40% and EG at 12-70%, or in an embodiment thereof BG at 4-12%, EG at 18-50%, CBHII at 10-35% and CBHI at 10-60% of the total protein dose (w/w).

A further aspect of the present invention concerns the option of downstream processing of the cultivation broth. The batch process applied according to the invention facilitates downstream processing, especially because of the high yield of the valuable compound and the low amount of by-products. Downstream processing may include recovery as well as formulation steps.

After the cultivation process is ended, the valuable product may be recovered from the cultivation broth, using standard technology developed for recovery of the valuable compound of interest. The relevant downstream processing technology to be applied thereby depends on the nature and cellular localization of the valuable compound and on the desired purity level of the product of interest. In a typical recovery process, the

biomass is separated from the cultivation fluid using e.g. centrifugation or filtration. The valuable compound then is recovered from the biomass in the case that the valuable product is accumulated inside or is associated with the microbial cells. The term "recovering" includes isolating, extracting, harvesting, separating or purifying the compound from culture media. Isolating the compound can be performed according to any conventional isolation or purification methodology known in the art including, but not limited to, alteration of pH, solvent extraction, dialysis, cell filtration and ultrafiltration, concentration, lyophilisation and the like.

In yet another aspect, the invention relates to a process for the production of an enzyme composition according to the invention comprising at least four different cellulases, hemicellulases and/or pectinases comprising the steps of:

- (a) providing the host cell of the invention
- (b) allowing production and secretion of the at least four different cellulases, hemicellulases and/or pectinases by the host cell and
- (c) optional recovering of the thus obtained enzyme composition.

The invention also relates to the enzyme composition obtainable by this process.

For example, a host cell of the invention may be cultured in a suitable medium and under conditions allowing the cellulase mixture to be expressed and/or isolated.

The culture takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e. g., Bennett, J. W. and LaSure, L., eds., *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared using published compositions (e. g., in catalogues of the American Type Culture Collection).

The medium, also mentioned herein as cultivation medium, is not critical to the invention. Nutrients may be added to the process according to the needs of the host cell in question, provided that the nutrients are supplied in excess. The cultivation medium conveniently contains a carbon source, a nitrogen source as well as additional compounds required for growth of the microorganism and/or the formation of the product. For instance, additional compounds may be present for inducing the production of the product. Examples of suitable carbon sources known in the art include glucose, maltose, maltodextrins, sucrose, hydrolysed starch, starch, molasses and oils. Examples of nitrogen sources known in the art include soy bean meal, corn steep liquor, yeast extract,

ammonia, ammonium salts and nitrate salts. Examples of additional compounds include phosphate, sulphate, trace elements and vitamins. The total amount of carbon and nitrogen source to be added may vary depending on e.g. the needs of the host cell and/or the time allowed for expression and secretion of the enzymes. The ratio between carbon and nitrogen source may vary considerably, whereby one determinant for an optimal ratio between carbon and nitrogen source may be the elemental composition of the product to be formed. Additional compounds required for growth of the host cell and/or for product formation, like phosphate, sulphate or trace elements, may be added in amounts that may vary between different classes of the host cell, i.e. between fungi, yeasts and bacteria. In addition, the amount of additional compound to be added may be determined by the type of product formed.

With 'recovering of the thus obtained enzyme composition' is meant that the enzyme composition may be separated from the host cell and other components that are not the desired enzymes (see downstream processing as described above).

Preferably, the host cell is allowed to produce and secrete the at least four different cellulases, hemicellulases and/or pectinases in a glucose containing medium, which glucose containing medium preferably does not contain a cellulase inducer for reasons as described herein.

Of course, the enzyme composition of the present invention may also comprise further components, for example proteins, such as enzymes; or other components.

In case of proteins, these proteins may have been produced and secreted by the host cell, but they may have also been separately added to the enzyme composition.

For example, the enzyme composition may comprise an auxiliary enzyme activity. Such additional activities may be derived from classical sources and/or produced by a genetically modified organism.

Therefore, in addition, one or more (for example two, three, four or all) enzymes chosen from the group of amylases, proteases, preferably from the group of proteases that do not degrade the cellulases, hemicellulases and/or pectinases present in the enzyme composition; lipases, ligninases, hexosyltransferases, glucuronidases, expansins, cellulose induced proteins, cellulose integrating proteins and other proteins may be present in the enzyme composition of the invention.

"Protease" includes enzymes that hydrolyze peptide bonds (peptidases), as well as enzymes that hydrolyze bonds between peptides and other moieties, such as sugars (glycopeptidases). Many proteases are characterized under EC 3.4, and are suitable for use in the invention incorporated herein by reference. Some specific types of proteases include, cysteine proteases including pepsin, papain and serine proteases including chymotrypsins, carboxypeptidases and metalloendopeptidases.

"Lipase" includes enzymes that hydrolyze lipids, fatty acids, and acylglycerides, including phosphoglycerides, lipoproteins, diacylglycerols, and the like. In plants, lipids are used as structural components to limit water loss and pathogen infection. These lipids include waxes derived from fatty acids, as well as cutin and suberin.

"Ligninase" includes enzymes that can hydrolyze or break down the structure of lignin polymers. Enzymes that can break down lignin include lignin peroxidases, manganese peroxidases, laccases and feruloyl esterases, and other enzymes described in the art known to depolymerize or otherwise break lignin polymers. Also included are enzymes capable of hydrolyzing bonds formed between hemicellulosic sugars (notably arabinose) and lignin. Ligninases include but are not limited to the following group of enzymes: lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), laccases (EC 1.10.3.2) and feruloyl esterases (EC 3.1.1.73).

"Hexosyltransferase" (2.4.1-) includes enzymes which are capable of catalyzing a transferase reaction, but which can also catalyze a hydrolysis reaction, for example of cellulose and/or cellulose degradation products. An example of a hexosyltransferase which may be used in the invention is a β -glucanosyltransferase. Such an enzyme may be able to catalyze degradation of (1,3)(1,4)glucan and/or cellulose and/or a cellulose degradation product.

"Glucuronidase" includes enzymes that catalyze the hydrolysis of a glucuronoside, for example β -glucuronoside to yield an alcohol. Many glucuronidases have been characterized and may be suitable for use in the invention, for example β -glucuronidase (EC 3.2.1.31), hyaluronoglucuronidase (EC 3.2.1.36), glucuronosyl-disulfoglucosamine glucuronidase (3.2.1.56), glycyrrhizinate β -glucuronidase (3.2.1.128) or α -D-glucuronidase (EC 3.2.1.139).

The enzyme composition may comprise an expansin or expansin-like protein, such as a swollenin (see Salheimo *et al.*, Eur. J. Biochem. 269, 4202-4211, 2002) or a swollenin-like protein.

Expansins are implicated in loosening of the cell wall structure during plant cell growth. Expansins have been proposed to disrupt hydrogen bonding between cellulose and other cell wall polysaccharides without having hydrolytic activity. In this way, they are thought to allow the sliding of cellulose fibers and enlargement of the cell wall. Swollenin, an expansin-like protein contains an N-terminal Carbohydrate Binding Module Family 1 domain (CBD) and a C-terminal expansin-like domain. For the purposes of this invention, an expansin-like protein or swollenin-like protein may comprise one or both of such domains and/or may disrupt the structure of cell walls (such as disrupting cellulose structure), optionally without producing detectable amounts of reducing sugars.

A composition of the invention may comprise the polypeptide product of a cellulose integrating protein, scaffoldin or a scaffoldin-like protein, for example CipA or CipC from *Clostridium thermocellum* or *Clostridium cellulolyticum* respectively.

Scaffoldins and cellulose integrating proteins are multi-functional integrating subunits which may organize cellulolytic subunits into a multi-enzyme complex. This is accomplished by the interaction of two complementary classes of domain, i.e. a cohesion domain on scaffoldin and a dockerin domain on each enzymatic unit. The scaffoldin subunit also bears a cellulose-binding module (CBM) that mediates attachment of the cellulosome to its substrate. A scaffoldin or cellulose integrating protein for the purposes of this invention may comprise one or both of such domains.

A composition of the invention may comprise a cellulose induced protein or modulating protein, for example as encoded by *cip1* or *cip2* gene or similar genes from *Trichoderma reesei* / *Hypocrea jecorina* (see Foreman *et al.*, J. Biol. Chem. 278(34), 31988-31997, 2003). The polypeptide products of these genes are bimodular proteins, which contain a cellulose binding module and a domain which function or activity can not be related to known glycosyl hydrolase families. Yet, the presence of a cellulose binding module and the coregulation of the expression of these genes with cellulases components indicates previously unrecognised activities with potential role in biomass degradation.

A composition of the invention may be composed of a member of each of the classes of the polypeptides mentioned above, several members of one polypeptide class, or any combination of these polypeptide classes.

A composition of the invention may be composed of polypeptides, for example enzymes, from (1) commercial suppliers; (2) cloned genes expressing polypeptides, for example enzymes; (3) complex broth (such as that resulting from growth of a microbial strain in media, wherein the strains secrete proteins and enzymes into the media; (4) cell lysates of strains grown as in (3); and/or (5) plant material expressing polypeptides, for example enzymes. Different polypeptides, for example enzymes in a composition of the invention may be obtained from different sources.

The activities in the enzyme composition may be thermostable. Herein, this means that the activity has a temperature optimum of about 60°C or higher, for example about 70°C or higher, such as about 75°C or higher, for example about 80°C or higher such as 85°C or higher. Activities in the enzyme composition will typically not have the same temperature optima, but preferably will, nevertheless, be thermostable.

In addition, enzyme activities in the enzyme composition may be able to work at low pH. For the purposes of this invention, low pH indicates a pH of about 5.5 or lower, about 5 or lower, about 4.5 or lower, about 4.0 or lower or about 3.8 or lower or about 3.5 or lower.

Activities in the enzyme composition may be defined by a combination of any of the above temperature optima and pH values.

Industrial application of the enzyme composition

In principle, an enzyme composition of the invention may be used in any process which requires the treatment of a material which comprises non-starch polysaccharide. Thus, a polypeptide or enzyme composition of the invention may be used in the treatment of non-starch polysaccharide material. Herein, non-starch polysaccharide material is a material which comprises or consists essential of one or, more typically, more than one non-starch polysaccharide.

Typically, plants and fungi and material derived therefrom comprise significant quantities of non-starch polysaccharide material. Accordingly, a polypeptide of the invention may be used in the treatment of a plant or fungal material or a material derived therefrom.

An important component of plant non-starch polysaccharide material is lignocellulose (also referred to herein as lignocellulolytic biomass). Lignocellulose is plant material that is composed of cellulose and hemicellulose and lignin. The carbohydrate polymers (cellulose and hemicelluloses) are tightly bound to the lignin by hydrogen and covalent bonds. Accordingly, a polypeptide of the invention may be used in the treatment of lignocellulolytic material. Herein, lignocellulolytic material is a material which comprises or consists essentially of lignocellulose. Thus, in a method of the invention for the treatment of a non-starch polysaccharide, the non-starch polysaccharide may be a lignocellulosic material/biomass.

Accordingly, the invention provides a method of treating a non-starch polysaccharide in which the treatment comprises the degradation and/or modification of cellulose and/or hemicellulose.

Degradation in this context indicates that the treatment results in the generation of hydrolysis products of cellulose and/or hemicellulose and/or a pectic substance, i.e. saccharides of shorter length are present as result of the treatment than are present in a similar untreated non-starch polysaccharide. Thus, degradation in this context may result in the liberation of oligosaccharides and/or sugar monomers.

All plants and fungi contain non-starch polysaccharide as do virtually all plant- and fungal-derived polysaccharide materials. Accordingly, in a method of the invention for the treatment of a non-starch polysaccharide, said non-starch polysaccharide may be provided in the form of a plant or a plant derived material or a material comprising a plant or plant derived material, for example a plant pulp, a plant extract, a foodstuff or ingredient therefore, a fabric, a textile or an item of clothing.

The enzyme compositions of the invention can extremely effectively hydrolyze lignocellulolytic material, for example corn stover or wheat straw, into monomeric sugars (the hydrolysis of lignocellulosic material is also referred to herein as saccharification of lignocellulosic material) which can then be further converted into a useful product, such as ethanol.

Therefore, in another aspect, the invention relates to a process for the saccharification of lignocellulosic material comprising the steps of

- optional pretreatment of lignocellulosic material and
- contacting the lignocellulosic material with an enzyme composition according to the invention to produce one or more sugars.

The enzyme compositions of the invention can be used to carry out highly effective hydrolysis of a lignocellulosic substrate. This is highly significant in the

context of commercially viable fuel ethanol production from lignocellulosic biomass since lower amounts of enzyme will be required (as compared with currently available products).

In addition, currently available enzymes having cellulase activity, typically derived from *Trichoderma*, function at mesophilic temperatures, such as from 45°C to 50°C and at pH 5.0. This, however, may lead to bacterial infection reducing product yield, so it is desirable to carry out saccharification at a temperature of 65°C or higher. In addition, the use of mesophilic temperatures increases the viscosity of the biomass being used such that the dry matter content used is limited. Also, when acid pretreated biomass is used as a substrate, the pH must be raised so that the enzyme can saccharify the sugars in the biomass. In the context of a commercially viable fuel ethanol industry, this implies a requirement for, for example, sodium hydroxide or calcium sulphate and the production of huge quantities of the corresponding salts, for example gypsum in the case of sodium hydroxide. Accordingly, it is desirable to carry out saccharification using an enzyme which can operate at a pH of pH 4.0 or lower.

Moreover, this hydrolysis may be carried out at a high temperature especially, if the polynucleotides encoding the at least four different enzymes chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases are polynucleotides originating from a *Talaromyces* strain, for example from a *Talaromyces emersonii* strain.

Hydrolysis of a lignocellulosic substrate at a higher temperature, for example at a temperature of 65°C or higher, is favorable as it (i) reduces the risk of bacterial infection and (ii) results in a less viscous biomass pulp. The effect of the latter is significant since it enables the better blending of enzymes, resulting in a higher operational dry matter in the plant and allows a consequent higher ethanol concentration to be achieved. Thus, less energy need be used improving sustainability and a smaller fermentation process will be required requiring lower investment.

A pretreatment of the lignocellulosic material usually occurs at high temperature; therefore if the polynucleotides encoding the at least four different enzymes are chosen from the group of polynucleotides encoding cellulases, hemicellulases or pectinases are polynucleotides originating from a *Talaromyces* strain, for example from a *Talaromyces emersonii* strain, there is no need to completely cool the lignocellulosic material to low temperatures, such as below 40°C before the enzyme composition is added. Instead, the enzyme composition may for example be added to the lignocellulosic material having a temperature of up to 80°C, for example to

lignocellulosic material having a temperature in the range of from 60 to 80°C, for instance at about 65°C.

This shortens the process for the saccharification of lignocellulosic material considerably, as well as provides for a greener route. The reason for this is that addition of enzymes at higher temperatures requires less cooling of the pre-treated lignocellulosic materials, which saves energy, and enzymes can be added earlier to the lignocellulosic material, which saves time.

For example, the hydrolysis of the lignocellulosic material by the enzyme composition of the invention may be performed at a temperature of at least 30°C, for example of at least 37°C, for example of at least 40°C, for example of at least 50°C, for example of at least 56°C, for example of at least 60°C, for example of at least 65°C, for example of at least 70°C.

For example, the hydrolysis of the lignocellulosic material by the enzyme composition of the invention may be performed at a temperature of at most 100°C, for example of at most 90°C, for example of at most 80°C, for example of at most 70°C.

Also, this hydrolysis may be carried out at low pH, for example at a pH of pH 4.0 or lower. This is desirable since biomass is often pretreated with acid. Biomass treated in this way does not have to be pH adjusted if the enzymes subsequently used for saccharification are capable of acting at low pH. This implies a lower requirement of, for example, sodium hydroxide or calcium sulphate and a process in which there is no waste salt. This is significant in a process in which, for example, fuel ethanol is to be produced since huge quantities of material are consumed in such processes. This allows the process according to the invention to be carried out with no or little pH adjustment is required, i.e. there is no or a reduced requirement for the addition of acids or bases. The process may thus be carried out as a zero or low waste process (no or low salt production) and/or as a process in which no or little inorganic chemical input is required.

In addition, it has been shown that the enzyme composition can effectively hydrolyze biomass when high dry matter contents are used. It is highly desirable that enzymes used in the production of, for example, fuel ethanol are able to operate on substrates having high viscosity (i.e. high dry weight composition) since this allows higher amounts of the final product, for example, fuel ethanol, to be achieved.

Significantly, a method of the invention may be carried out using high levels of dry matter (of the lignocellulosic material) in the hydrolysis reaction. Thus, the

invention may be carried out with a dry matter content of about 5% or higher, about 8% or higher, about 10% or higher, about 15% or higher, about 20% or higher, about 25% or higher, about 30% or higher, about 35% or higher or about 40% or higher.

Preferably in the method, the lignocellulosic material is orchard primings, chaparral, mill waste, urban wood waste, municipal waste, logging waste, forest thinnings, short- rotation woody crops, industrial waste, wheat straw, oat straw, rice straw, barley straw, rye straw, flax straw, soy hulls, rice hulls, rice straw, corn gluten feed, sugar cane, corn stover, corn stalks, corn cobs, corn husks, miscanthus, sweet sorghum, canola stems, soybean stems, prairie grass, gamagrass, foxtail; sugar beet pulp, citrus fruit pulp, seed hulls, cellulosic animal wastes, lawn clippings, cotton, seaweed, softwood, poplar, pine, shrubs, grasses, wheat, sugar cane bagasse, corn, corn hobs, corn kernel, fiber from kernels, products and by-products from wet or dry milling of grains, municipal solid waste, waste paper, yard waste, herbaceous material, agricultural residues, forestry residues, waste paper, pulp, paper mill residues, branches, bushes, canes, an energy crop, forest, a fruit, a flower, a grain, a grass, a herbaceous crop, a leaf, bark, a needle, a log, a root, a sapling, a shrub, switch grass, a tree, a vegetable, fruit peel, a vine, sugar beet pulp, wheat midlings, oat hulls, hard or soft wood, organic waste material generated from an agricultural process, forestry wood waste, or a combination of any two or more thereof.

The composition is reacted with substrate under any appropriate conditions. For example, enzymes can be incubated at about 25°C, about 30 °C, about 35 °C, about 37 °C, about 40 °C, about 45 °C, about 50 °C, about 55 °C, about 60°C, about 65°C, about 70°C, about 75°C, about 80°C, about 85°C, about 90°C or higher. That is, they can be incubated at a temperature of from about 20°C to about 95 °C, for example in buffers of low to medium ionic strength and/or from low to neutral pH. By "medium ionic strength" is intended that the buffer has an ion concentration of about 200 millimolar (mM) or less for any single ion component. The pH may range from about pH 2.5, about pH 3.0, about pH 3.5, about pH 4.0, about pH 4.5, about pH 5, about pH 5.5, about pH 6, about pH 6.5, about pH 7, about pH 7.5, about pH 8.0, to about pH 8.5. Generally, the pH range will be from about pH 3.0 to about pH 9.

Typically, the reaction may be carried out under low pH conditions as defined above. Thus, a method of the invention may be carried out such that no pH adjustment (i.e. to a more neutral pH is required). That is to say, an acid pretreated feedstock may be used as is with no requirement to addition of, for example, sodium hydroxide, prior to addition of an enzyme composition of the invention.

The feedstock may be washed prior to liquefaction/hydrolysis. Such washing may be with, for example, water.

Incubation of a composition under these conditions results in release or liberation of substantial amounts of the sugar from the lignocellulosic material. By substantial amount is intended at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95% or more of available sugar.

A liquefaction/hydrolysis or presaccharification step involving incubation with an enzyme or enzyme mixture can be utilized. This step can be performed at many different temperatures but it is preferred that the presaccharification occur at the temperature best suited to the enzyme mix being tested, or the predicted enzyme optimum of the enzymes to be tested. The temperature of the pretreatment may range from about 10 °C to about 100 °C, about 30 °C to about 80 °C, about 40 °C to about 70 °C, about 50 °C to about 70 °C, preferably about 60 °C to about 70 °C, more preferably about 65 °C. In the absence of data on the temperature optimum, it is preferable to perform the pretreatment reactions at 37 °C first, then at a higher temperature such as 65 °C. The pH of the presaccharification mixture may range from about 2.0 to about 10.0, but is preferably about 3.0 to about 5.0. Again, it may not be necessary to adjust the pH prior to saccharification since the enzyme composition is typically suitable for use at low pH as defined herein.

The liquefaction/hydrolysis or presaccharification step reaction may occur from several minutes to several hours, such as from about 1 hour to about 120 hours, preferably from about 2 hours to about 48 hours, more preferably from about 2 to about 24 hours, most preferably for from about 2 to about 6 hours. The liquification/hydrolysis may occur from several minutes to several hours, such as from about 6 hours to about 168 hours, preferably about 12 hours to about 96 hours, more preferably about 24 hours to about 72 hours, even more preferably from about 24 hours to about 48 hours.

Depending on the application, e.g. when the enzymes or enzyme composition are used in a process where liquifaction/hydrolysis is combined with fermentation, the process may be carried out as separate hydrolysis and fermentation (SHF) and simultaneous hydrolysis and fermentation (SSF). In SSF a presaccharification may be appropriate. SHF and SSF will be described in more detail below.

The invention further relates to a method for the conversion of lignocellulosic material into useful product comprising the following steps: a)

pretreatment of one or more lignocellulosic material to produce pretreated lignocellulosic material; b) enzymatic treatment of the pretreated lignocellulosic material to produce one or more sugar; c) converting the sugar into one or more useful product and d) separating the one or more useful products, wherein in step a), b) or c) an enzyme composition of the invention is used or added.

Therefore, the invention also relates to a process for the preparation of a fermentation product, including amino acids, vitamins, pharmaceuticals, animal feed supplements, specialty chemicals, chemical feedstocks, plastics, solvents, fuels, or other organic polymers, lactic acid, ethanol, fuel ethanol or chemicals, plastics, dicarboxylic acids, such as for example succinic acid, itaconic acid, adipic acid; (bio)fuels, including ethanol, methanol, butanol, synthetic liquid fuels and biogases, wherein the one or more sugars that is or are produced according to the process of the invention, is fermented with a fermenting microorganism, preferably yeast to produce the fermentation product.

With 'fermenting microorganism' is meant a microorganism having the ability to convert the one or more sugars into the fermentation product.

Such a process may be carried out without any requirement to adjust the pH during the process. That is to say, the process is one which may be carried out without the addition of any acid(s) or base(s). However, this excludes a pretreatment step, where acid may be added. The point is that the enzyme composition of the invention is capable of acting at low pH and, therefore, there is no need to adjust the pH of acid of an acid pretreated feedstock in order that saccharification may take place. Accordingly, a method of the invention may be a zero waste method using only organic products with no requirement for inorganic chemical input.

Preferably, in the method according to the invention, the useful product is one or more of ethanol, butanol, lactic acid, a plastic, an organic acid, a solvent, an animal feed supplement, a pharmaceutical, a vitamin, an amino acid, an enzyme or a chemical feedstock. The present invention provides relates to a composition which comprises cellulolytic and/or hemicellulolytic enzyme activity and which has the ability to modify, for example degrade, a non-starch carbohydrate material. A non-starch carbohydrate material is a material which comprises, consists of or substantially consists of one or more non-starch carbohydrates. Carbohydrate in this context includes all saccharides, for example polysaccharides, oligosaccharides, disaccharides or monosaccharides.

A composition as described herein typically modifies a non-starch carbohydrate material by chemical modification of such material. Chemical modification of the carbohydrate material may result in the degradation of such material, for example by hydrolysis, oxidation or other chemical modification such as by the action of a lyase.

A non-starch carbohydrate suitable for modification by a composition as described herein is lignocellulose. The major polysaccharides comprising different lignocellulosic residues, which may be considered as a potential renewable feedstock, are cellulose (glucans), hemicelluloses (xylans, heteroxylans and xyloglucans). In addition, some hemicellulose may be present as glucomannans, for example in wood-derived feedstocks. The enzymatic hydrolysis of these polysaccharides to soluble sugars, including both monomers and multimers, for example glucose, cellobiose, xylose, arabinose, galactose, fructose, mannose, rhamnose, ribose, galacturonic acid, glucuronic acid and other hexoses and pentoses occurs under the action of different enzymes acting in concert.

In addition, pectins and other pectic substances such as arabinans may make up considerably proportion of the dry mass of typically cell walls from non-woody plant tissues (about a quarter to half of dry mass may be pectins).

Cellulose is a linear polysaccharide composed of glucose residues linked by β -1,4 bonds. The linear nature of the cellulose fibers, as well as the stoichiometry of the β -linked glucose (relative to α) generates structures more prone to interstrand hydrogen bonding than the highly branched α -linked structures of starch. Thus, cellulose polymers are generally less soluble, and form more tightly bound fibers than the fibers found in starch.

A method for the preparation of a fermentation product may optionally comprise recovery of the fermentation product.

Such a process may be carried out under aerobic or anaerobic conditions. Preferably, the process is carried out under micro-aerophilic or oxygen limited conditions.

An anaerobic fermentation process is herein defined as a fermentation process run in the absence of oxygen or in which substantially no oxygen is consumed, preferably about 5 or less, about 2.5 or less or about 1 mmol/L/h or less, and wherein organic molecules serve as both electron donor and electron acceptors.

An oxygen-limited fermentation process is a process in which the oxygen consumption is limited by the oxygen transfer from the gas to the liquid. The

degree of oxygen limitation is determined by the amount and composition of the ingoing gasflow as well as the actual mixing/mass transfer properties of the fermentation equipment used. Preferably, in a process under oxygen-limited conditions, the rate of oxygen consumption is at least about 5.5, more preferably at least about 6 and even more preferably at least about 7 mmol/L/h. A method for the preparation of a fermentation product may optionally comprise recovery of the fermentation product.

In the absence of oxygen, NADH produced in glycolysis and biomass formation, cannot be oxidised by oxidative phosphorylation. To solve this problem many microorganisms use pyruvate or one of its derivatives as an electron and hydrogen acceptor thereby regenerating NAD⁺.

Thus, in a preferred anaerobic fermentation process pyruvate is used as an electron (and hydrogen acceptor) and is reduced to fermentation products such as ethanol, butanol, lactic acid, 3 -hydroxy-propionic acid, acrylic acid, acetic acid, succinic acid, citric acid, malic acid, fumaric acid, an amino acid, 1,3-propane-diol, ethylene, glycerol, a β -lactam antibiotic and a cephalosporin.

The fermentation process is preferably run at a temperature that is optimal for the cell. Thus, for most yeasts or fungal host cells, the fermentation process is performed at a temperature which is less than about 42°C, preferably less than about 38°C. For yeast or filamentous fungal host cells, the fermentation process is preferably performed at a temperature which is lower than about 35, about 33, about 30 or about 28°C and at a temperature which is higher than about 20, about 22, or about 25°C.

The ethanol yield on xylose and/or glucose in the process preferably is at least about 50, about 60, about 70, about 80, about 90, about 95 or about 98%. The ethanol yield is herein defined as a percentage of the theoretical maximum yield.

The invention also relates to a process for producing a fermentation product.

In a preferred process the cell ferments both the xylose and glucose, preferably simultaneously in which case preferably a cell is used which is insensitive to glucose repression to prevent diauxic growth. In addition to a source of xylose (and glucose) as carbon source, the fermentation medium will further comprise the appropriate ingredient required for growth of the cell. Compositions of fermentation media for growth of microorganisms such as yeasts are well known in the art

The fermentation processes may be carried out in batch, fed-batch or continuous mode. A separate hydrolysis and fermentation (SHF) process or a simultaneous saccharification and fermentation (SSF) process may also be applied. A

combination of these fermentation process modes may also be possible for optimal productivity. These processes are described hereafter in more detail.

SSF mode

For Simultaneous Saccharification and Fermentation (SSF) mode, the reaction time for liquefaction/hydrolysis or presaccharification step is dependent on the time to realize a desired yield, i.e. cellulose to glucose conversion yield. Such yield is preferably as high as possible, preferably 60% or more, 65% or more, 70% or more, 75% or more 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more, even 99.5% or more or 99.9% or more.

According to the invention very high sugar concentrations in SHF mode and very high product concentrations (e.g. ethanol) in SSF mode are realized. In SHF operation the glucose concentration is 25g/L or more, 30 g/L or more, 35g/L or more, 40 g/L or more, 45 g/L or more, 50 g/L or more, 55 g/L or more, 60 g/L or more, 65 g/L or more, 70 g/L or more, 75 g/L or more, 80 g/L or more, 85 g/L or more, 90 g/L or more, 95 g/L or more, 100 g/L or more, 110 g/L or more, 120g/L or more or may e.g. be 25g/L-250 g/L, 30g/L-200g/L, 40g/L-200 g/L, 50g/L-200g/L, 60g/L-200g/L, 70g/L-200g/L, 80g/L-200g/L, 90 g/L, 80g/L-200g/L.

Product concentration in SSF mode

In SSF operation, the product concentration (g/L) is dependent on the amount of glucose produced, but this is not visible since sugars are converted to product in the SSF, and product concentrations can be related to underlying glucose concentration by multiplication with the theoretical maximum yield (Y_{ps} max in gr product per gram glucose)

The theoretical maximum yield (Y_{ps} max in gr product per gram glucose) of a fermentation product can be derived from textbook biochemistry. For ethanol, 1 mole of glucose (180 gr) yields according to normal glycolysis fermentation pathway in yeast 2 moles of ethanol (=2x46 = 92 gr ethanol. The theoretical maximum yield of ethanol on glucose is therefore $92/180 = 0.511$ gr ethanol/gr glucose.

For Butanol (MW 74 gr/mole) or iso butanol, the theoretical maximum yield is 1 mole of butanol per mole of glucose. So Y_{ps} max for (iso-)butanol = $74/180 = 0.411$ gr (iso-)butanol/gr glucose.

For lactic acid the fermentation yield for homolactic fermentation is 2 moles of lactic acid (MW = 90 gr/mole) per mole of glucose. According to this stoichiometry, the Yps max = 1 gr lactic acid/gr glucose.

For other fermentation products a similar calculation may be made.

SSF mode

In SSF operation the product concentration is 25g * Yps g/L /L or more, 30 * Yps g/L or more, 35g * Yps /L or more, 40 * Yps g/L or more, 45 * Yps g/L or more, 50 * Yps g/L or more, 55 * Yps g/L or more, 60 * Yps g/L or more, 65 * Yps g/L or more, 70 * Yps g/L or more , 75 * Yps g/L or more, 80 * Yps g/L or more, 85 * Yps g/L or more, 90 * Yps g/L or more, 95 * Yps g/L or more, 100 * Yps g/L or more, 110 * Yps g/L or more, 120g/L * Yps or more or may e.g. be 25 * Yps g/L-250 * Yps g/L, 30 * Yps g/L-200 * Yps g/L, 40 * Yps g/L-200 * Yps g/L, 50 * Yps g/L-200 * Yps g/L, 60 * Yps g/L-200 * Yps g/L, 70 * Yps g/L-200 * Yps g/L, 80 * Yps g/L-200 * Yps g/L, 90 * Yps g/L , 80 * Yps g/L-200 * Yps g/L

Accordingly, the invention provides a method for the preparation of a fermentation product, which method comprises:

- a. degrading lignocellulose using a method as described herein; and
- b. fermenting the resulting material,

thereby to prepare a fermentation product.

Fermentation product

The fermentation product of the invention may be any useful product. In one embodiment, it is a product selected from the group consisting of ethanol, n-butanol, isobutanol, lactic acid, 3-hydroxy-propionic acid, acrylic acid, acetic acid, succinic acid, fumaric acid, malic acid, itaconic acid, maleic acid, citric acid, adipic acid, an amino acid, such as lysine, methionine, tryptophan, threonine, and aspartic acid, 1,3-propanediol, ethylene, glycerol, a β -lactam antibiotic and a cephalosporin, vitamins, pharmaceuticals, animal feed supplements, specialty chemicals, chemical feedstocks, plastics, solvents, fuels, including biofuels and biogas or organic polymers, and an industrial enzyme, such as a protease, a cellulase, an amylase, a glucanase, a lactase, a lipase, a lyase, an oxidoreductases, a transferase or a xylanase.

Recovery of the fermentation product

For the recovery of the fermentation product existing technologies are used. For different fermentation products different recovery processes are appropriate. Existing methods of recovering ethanol from aqueous mixtures commonly use fractionation and adsorption techniques. For example, a beer still can be used to process a fermented product, which contains ethanol in an aqueous mixture, to produce an enriched ethanol-containing mixture that is then subjected to fractionation (e.g., fractional distillation or other like techniques). Next, the fractions containing the highest concentrations of ethanol can be passed through an adsorber to remove most, if not all, of the remaining water from the ethanol.

The invention will now be elucidated by way of the following examples, without however being limited thereto.

EXAMPLES

Example 1

Expression of multiple *Talaromyces Emersonii* cellulases in *Aspergillus Niger*

This example describes the cloning and expression of *T. emersonii* CBS 39364 cellobiohydrolase-I (CBHI), *T. emersonii* CBS 39364 cellobiohydrolase-II (CBHII), *T. emersonii* β -glucanase CEA CBS 39364 (EG), and *T. emersonii* β -glucosidase (BG) as presented in the GenBank database with accession number AAL69548 in *A. niger* WT-1. In addition, cellulase activity of transformants is compared with cellulase activity of an empty strain after growing the strains in shake flasks.

Cloning of *T. emersonii* coding regions in expression vectors

The polynucleotides (DNA-sequences) encoding *T. emersonii* cellobiohydrolase-I (CBHI), *T. emersonii* beta-glucanase CEA (EG), and *T. emersonii* β -glucosidase (BG) were synthesised by DNA2.0 (Menlo Park, USA) and cloned as *EcoRI/SnaBI* fragment into the pGBTOP-8 vector (EP 0635574A1), comprising the glucoamylase (*glaA*) promoter and terminator sequence, resulting in vector pGBTOPEBA205, pGBTOPEBA8 and pGBTOPEBA4, respectively. For cloning purposes, 198 nucleotides of the 3' part of the glucoamylase promoter was also synthesised with the coding sequences. The amino acid sequences of the *T. emersonii*

cellobiohydrolase-I (CBHI), *T. emersonii* beta-glucanase CEA (EG), and *T. emersonii* β -glucosidase (BG) are represented by SEQ ID NO: 1, 3, and 5, respectively. The DNA sequences are represented by SEQ ID NO: 2, 4 and 6, respectively. Figure 1 represents a map of a pGBTOPEBA205 containing the DNA-sequence encoding the *T. emersonii* CBHI under control of the *glaA* promoter within vector pGBTOP12. pGBTOPEBA205 is representative for pGBTOPEBA8, which comprises the DNA-sequence encoding the *T. emersonii* EG, and pGBTOPEBA4, which comprises DNA-sequence encoding the *T. emersonii* BG.

The polynucleotide encoding *T. emersonii* cellobiohydrolase-II (CBHII), was obtained from a *T. emersonii* cDNA library as described in patent WO2001/070998 A1. Figure 2 represents a map of pGBFINEBA176 containing the DNA-sequence encoding the *T. emersonii* CBHII under control of the *glaA* promoter within vector pGBFIN11 (WO 9932617 (A2)). The pGBFIN11 vector also contains the selection marker, AmdS (EP0758020 (A2), which selects for the ability to use acetamide as sole nitrogen source. The amino acid sequence and nucleotide sequence are represented by SEQ ID NO: 7 and 8, respectively.

Transformation of *Aspergillus niger* with multiple cellulases

Aspergillus niger WT-1 strain was used for transformations. The *Aspergillus niger* WT-1 strain is derived from the *A.niger* strain deposited at the CBS Institute under the deposit number CBS 513.88 (Pel *et al.*, *Genome sequencing and analysis of the versatile cell factory Aspergillus niger* CBS 513.88. 2007, Nat Biotechnol. 25:189-90). It comprises a deletion of the gene encoding glucoamylase (*glaA*), which was constructed by using the "MARKER-GENE FREE" approach as described in EP 0635574 (A1). In this patent application it is extensively described how to delete *glaA* specific DNA sequences in the genome of CBS 513.88. The procedure resulted in a MARKER-GENE FREE Δ *glaA* recombinant *A. niger* CBS513.88 strain, possessing finally no foreign DNA sequences at all.

In order to introduce the pGBTOPEBA vectors and pGBFINEBA176 in *Aspergillus niger* WT-1, transformation and subsequent transformant selection was carried out essentially as described in WO 9846772 (A2) and WO 9932617 (A2). In brief, linear DNA of the vector was isolated after digestion with *NotI*, to remove the *E.coli* sequences from the vector. After transformation of the cells with the linear DNA, transformants were selected on media comprising acetamide as sole nitrogen source and colony purified.

Transformants were cultured in shake flasks in 100 ml of CSM-MES medium as described in EP 635 574 A1 at 34°C at 170 rpm in an incubator shaker using a 500 ml baffled shake flask. After different time-points of fermentation, supernatant samples were harvested to determine expression by SDS-PAGE analysis. Protein samples were separated under reducing conditions on NuPAGE 4-12% Bis-Tris gel (Invitrogen, Breda, The Netherlands) and stained with Sypro Ruby (Invitrogen, Breda, The Netherlands)) according to manufacturer's instructions.

Figure 3A shows the SDS-PAGE gel of a subset of tested transformants expressing multiple cellulases. The theoretical molecular weights of the proteins encoded by pGBTOPEBA4, pGBTOPEBA8, pGBFINEBA176 and pGBTOPEBA205 are shown in Table 1.

Table 1: Theoretical molecular weight of the proteins encoded by pGBTOPEBA4, pGBTOPEBA8, pGBFINEBA176 and pGBTOPEBA205

Construct	Protein encoded by construct	Theoretical molecular weight
pGBTOPEBA4	BG	92 kDa
pGBTOPEBA8	EG	37 kDa
pGBFINEBA176	CBHII	48 kDa
pGBTOPEBA205	CBHI	49 kDa

As expected, different combinations of overexpressed cellulases were observed. From the SDS-PAGE analysis a host cell capable of expressing and secreting the at least four different cellulases, such as for example transformant 19, can be selected.

To determine whether the transformants were able to degrade pre-treated wheat straw a WSU assay was performed to determine cellulase activity.

Wheat Straw assay (WSU assay)

In order to measure cellulase activity a WSU activity assay was performed. WSU activity was measured in supernatants (the liquid part of the broth wherein the cells were cultured) of an empty strain and the transformant:

Preparation of pre-treated, washed wheat straw substrate.

Dilute-acid pre-treated wheat straw which was washed with water until the solution with wheat straw was pH 6.5 or higher and the mass was

homogenised using an ultra-turrax, lyophilized and grinded prior to analysis. To obtain pre-treated wheat straw a dilute acid pre-treatment as described in Linde, M. et al, Biomass and Bioenergy 32 (2008), 326-332 and equipment as described in Schell, D.J., Applied Biochemistry and Biotechnology (2003), vol. 105-108, pp 69-85, may be used.

With 1 WSU is meant 0.119 mg/ml glucose released from 2.1 w/v % washed pre-treated wheat straw by 200 µl of enzyme mix in 20 hours at 65°C at pH 4.50.

The glucose release is not a linear function of the quantity of enzyme in the composition. In other words, twice the amount of enzyme does not automatically result in twice the amount of glucose in equal time. Therefore, it is preferred to choose the dilution of the composition to be tested for WSU activity such that a WSU does not exceed 40.

Measurement of cellulase activity in WSU/ml

400 µl of supernatants harvested from shake flask experiments were diluted 16-fold. Diluted sample was used to perform two measurements in which 200 µl of diluted sample was analysed. In the first measurement, 200 µl diluted sample was transferred to a vial containing 700 µL water containing 3% (w/v) dry matter of the pretreated washed wheat straw substrate and 100 µl of 250 mM citrate buffer, the final pH was adjusted to pH 4.5. In the second measurement, the blank sample, 200 µl of diluted sample was transferred to a vial that contained 700 µl of water instead of pretreated washed wheat straw substrate, and 100 µl of 250 mM citrate buffer, the final pH was adjusted to pH 4.5. The assay samples were incubated for 20 and /or 60 hr at 65 °C. After incubation of the assay samples, 100 µl of internal standard solution (20 g/L maleic acid, 40 g/L EDTA in D₂O) was added. The amount of glucose released, was based on the signal at 5.20 ppm, relative to Dimethyl-sila-pentane-sulfonate determined by means of 1D ¹H NMR operating at a proton frequency of 500 MHz, using a pulse program with water suppression, at a temperature of 27°C. The WSU number was calculated from the data by subtracting by the amount of glucose that was detected in the blank sample from the amount of glucose that was measured in the sample incubated with wheat straw.

The results of the WSU assay are shown in Figure 3B. In supernatants of strains expressing a single cellulase or in empty strains, no WSU activity could be

measured. In *A.niger* transformants expressing all four cellulases, e.g. transformant 18 and 19, up to 17 WSU/ml was measured.

The experiment indicates that pGBTOPEBA4, pGBTOPEBA8, pGBFINEBA176 and pGBTOPEBA205 are expressed in *A.niger* and that *A.niger* strains overexpressing multiple *T. emersonii* cellulases in maltose-containing medium are able to degrade pre-treated wheat straw.

Materials and methods (Example 2)

Fermentation Medium:

Talaromyces medium 1

Glucose	20 g/L
Yeast extract (Difco)	20 g/L
Clerol FBA3107 (AF)	4 drops/L
pH	6.0
Sterilize	20 min at 120°C

Talaromyces medium 2

Salt fraction	15 g
Cellulose	30 g
Bacto peptone	7.5 g
Grain flour	15 g
KH ₂ PO ₄	10 g
CaCl ₂ .2H ₂ O	0.5 g
Clerol FBA3107 (AF)	0.4 ml
pH	5
Water	Adjust to one liter
Sterilize	20 min at 120°C

Talaromyces medium 3

Salt fraction	15 g
Glucose	50 g
Bacto peptone	7.5 g
KH ₂ PO ₄	10 g

CaCl ₂ .2H ₂ O	0.5 g
Clerol FBA3107 (AF)	0.4 ml
pH	5
Water	Adjust to one liter
Sterilize	20 min at 120°C

Spore batch preparation

Strains were grown from stocks on *Talaromyces* agar medium in 10 cm diameter Petri dishes for 5-7 days at 40°C. Strain stocks were stored at -80°C in 10% glycerol.

Shake flask growth protocol

Spores were directly inoculated into 500 ml shake flasks containing 100 ml of either *Talaromyces* medium 1 or 2 and incubated at 45°C at 250 rpm in an incubator shaker for 3-4 days.

Sample preparation

For shake flask cultures, 3 ml of culture broth was transferred to a 12 ml disposable tube and centrifuged for 10 min at 5200 g. At least 1 ml of supernatant was harvested.

Protein analysis

Protein samples were separated under reducing conditions on NuPAGE 4-12% Bis-Tris gel (Invitrogen, Breda, The Netherlands) and stained as indicated. Gels were stained with either InstantBlue (Expedeon, Cambridge, United Kingdom), SimplyBlue safestain (Invitrogen, Breda, The Netherlands) or Sypro Ruby (Invitrogen, Breda, The Netherlands)) according to manufacturer's instructions.

For Western blotting, proteins were transferred to nitrocellulose. The nitrocellulose filter was blocked with TBST (Tris buffered saline containing 0,1% Tween 40) containing 3% skim-milk and incubated for 16 hours with anti-FLAG M2 antibody (Sigma, Zwijndrecht, The Netherlands). Blots were washed twice with TBST for 10 minutes and stained with Horse-radish-peroxidase conjugated rabbit-anti-mouse antibody (DAKO, Glostrup, Denmark) for 1 hour. After washing the blots five times with TBST for 10 minutes, proteins were visualized using SuperSignal (Pierce, Rockford, U.S.A).

Wheat Straw assay (WSU assay).

Preparation of pre-treated, washed wheat straw substrate.

The washed wheat straw substrate was homogenised using an ultra-turrax, washed, lyophilized and grinded prior to analysis.

Measurement of cellulase activity in WSU/ml

Cellulase activity was herein measured in terms of "Wheat Straw Units" (WSU) per milliliter in a Wheat Straw assay (WSU assay). The washed wheat straw substrate was ultraturaxed, washed, lyophilized and grinded prior to analysis.

400 µl of supernatants harvested from shake flask experiments were diluted 16-fold. Duplicate, 200 µl samples were transferred to two suitable vials: one vial containing 700 µL 3% (^w/_w) dry matter of the pretreated, washed wheat straw substrate and 100 µl 250 mM citrate buffer, buffered at pH 4.5. The other vial consisted of a blank, where the 700 µl 3% (^w/_w) dry matter pretreated, washed wheat straw substrate was replaced by 700 µl water, with 100 µl 250 mM citrate buffer, buffered at pH 4.5. The assay samples are incubated for 20 and /or 60 hr at 65 °C. After incubation of the assay samples, a fixed volume of D₂O containing an internal standard, maleic acid is added. The amount of sugar released, is based on the signal between 5.25 – 5.20 ppm, relative to Dimethyl-sila-pentane-sulfonate determined by means of 1D ¹H NMR operating at a proton frequency of 500 MHz, using a pulse program with water suppression, at a temperature of 27°C. The cellulase enzyme solution may contain residual sugars. Therefore, the results of the assay are corrected for the sugar content of the enzyme solution.

Example 2

Overexpression of multiple *Talaromyces emersonii* cellulases in *Talaromyces emersonii*

Cloning of *T. emersonii* genes in expression vectors

The genes encoding *T. emersonii* cellobiohydrolase-I (CBHI), *T. emersonii* beta-glucanase CEA (EG), and *T. emersonii* β-glucosidase (BG) were synthesised by DNA2.0 (Menlo Park, USA) and cloned as *EcoRI/SnaBI* fragment into the pGBTOP12 vector, comprising the glucoamylase promoter and terminator sequence, resulting in vector pGBTOPEBA205, pGBTOPEBA8 and pGBTOPEBA4, respectively. For cloning purposes, 198 nucleotides of the 3' part of the glucoamylase promoter was also synthesised with the genes. The amino acid sequences of the *T. emersonii*

cellobiohydrolase-I (CBHI), *T. emersonii* beta-glucanase CEA (EG), and *T. emersonii* β -glucosidase (BG) are represented by SEQ ID NO: 1, 3, and 5, respectively. The DNA sequences of the genes are represented by SEQ ID NO: 2, 4 and 6, respectively. Figure 7 represents a map of a pGBTOPEBA205 containing the *T. emersonii* CBHI protein under control of the glaA promoter within vector pGBTOP12. pGBTOPEBA205 is representative for pGBTOPEBA8, which comprises *T. emersonii* EG, and pGBTOPEBA4, which comprises *T. emersonii* BG.

The gene encoding *T. emersonii* cellobiohydrolase-II (CBHII), was obtained from a *T. emersonii* cDNA library described in patent WO/2001/070998. Figure 4 represents a map of pGBFINEBA176 containing the *T. emersonii* CBHII protein under control of the glaA promoter within vector pGBFIN11. The amino acid sequence and nucleotide sequence are represented by SEQ ID NO: 7 and 8, respectively.

Transformation of *T. emersonii* with constructs encoding cellulases

Transformation of *T. emersonii* with constructs encoding cellulases was performed as described in EXAMPLE 1 of PCT/EP2010/066796. In total, 10 μ g of DNA was used to co-transform *T. emersonii*: 1 μ g of pAN8-1 and 2 μ g of each of the vectors pGBTOPEBA4, pGBTOPEBA8, pGBTOPEBA205 and pGBFINEBA176.

Screening for transformants expressing all 4 cellulases

Transformants were picked from plates and further grown into 96 wells microtiter plates (MTP) containing *Talaromyces* agar medium for 5 days at 40°C. The plates were replica plated using a 96-pin replicator into 96-well MTPs containing PDA medium. The MTP plates were incubated for 3 days at 40°C and used to harvest spores for shake flask analysis. To do this, 100 μ l of *Talaromyces* medium 1 was added to each well and after resuspending the mixture, 30 μ l of spore suspension was used to inoculate 170 μ l of *Talaromyces* medium 1 in MTP plates. The 96-well plates were incubated in humidity shakers (Infors) for 44°C at 550 rpm, and 80% humidity for 96 hours. Plungerplates were used to push down the mycelium and, subsequently, approximately 100 μ l of supernatant was harvested per well.

Approximately 10 μ l of supernatant was analysed for protein expression using the E-PAGE 96 Protein electrophoresis system (Invitrogen, Breda, The Netherlands). Gels were stained with SimplyBlue protein staining and transformants expressing multiple cellulases were selected. Spores of interesting transformants were harvested from MTP master plates and used for spore batch preparations.

T. emersonii shake flask fermentations and sample analysis

T. emersonii transformants expressing one or more cellulases were used for shake flask fermentations in *Talaromyces* medium 2 containing 5% of glucose. Analysis of protein expression by SDS-PAGE analysis was performed. Proteins were visualised using SYPRO Ruby protein staining.

The results of the SYPRO Ruby stained SDS-PAGE gel is presented in Figure 4A. The different transformants expressed different combinations and expression levels of cellulases. The supernatant of transformant 20 (strain 20), contained all 4 cellulases, while, in contrast, no cellulase proteins were observed in the empty strain (FBG142). Therefore, multiple cellulases can simultaneously be overexpressed in *T. emersonii* in the presence of glucose.

In order to test cellulase activity in *T. emersonii* transformants expressing one or more cellulases, WSU activity was measured in supernatants of an empty strain and the transformants. The results of the WSU assay is shown in Figure 4B. In supernatants harvested after 72 hours from cultures of the empty strain grown in medium containing glucose no WSU activity could be measured. In contrast, in transformants a range of activities could be observed.

Transformant 20 expressing all 4 cellulases showed highest activity: almost 6 WSU/ml, or 5 WSU/ml or more. Transformants 20 and 28 had an activity of 4 WSU/ml or more, Transformants 20, 28, 6, 64, 33, 11 and 48 had an activity of 3 WSU/ml or more, Transformants 20, 28, 6, 64, 33, 11, 48, 36, 35 and 1 had an activity of 2,5 WSU/ml or more, and Transformants 20, 28, 6, 64, 33, 11, 48, 36, 35, 1, 43, 53 and 7 had an activity of 2 WSU/ml or more. All other transformants had an activity well below 1,5 WSU/ml.

To test whether transformant 20 also produced cellulase activity in the absence of an inducer, a shake flask fermentation was performed using *Talaromyces* medium 3. Supernatants were harvested at day 3, 4 and 5 and analysed for WSU activity. The results of the WSU assay are shown in Table 2.

Table 2. Results of WSU activity measurement in supernatants of an empty strain and *T. emersonii* transformant 20 in Talaromyces medium 3.

Strain	cellulase activity (WSU/ml)		
	Day 3	Day 4	Day 5
Transformant 20 (multiple recombinant cellulases)	6.1	7.7	8.1
Empty strain	0.0	0.4	0.9

No cellulase activity was observed in day 3 sups of an empty strain, while some activity was observed at later time-points. In contrast, the transformant overexpressing multiple cellulases under control of the glaA promoter showed WSU activity at day 3 (6.1 WSU/ml), and the activity further increased over time.

This experiment shows that *T. emersonii* transformants comprising multiple cellulases under control of the glaA promoter are able to produce cellulase activity in glucose containing medium with and without cellulose. The transformant can be obtained by screening a pool of transformants that have been transformed with 4 cellulase constructs.

CLAIMS

1. Host cell comprising at least four different heterologous polynucleotides chosen from the group of polynucleotides encoding cellulases, hemicellulases and pectinases, wherein the host cell is capable of producing the at least four different enzymes chosen from the group of cellulases, hemicellulases and pectinases, wherein the host cell is a filamentous fungus and is capable of secretion of the at least four different enzymes.
2. Host cell according to claim 1, wherein the host cell is an *Aspergillus* strain.
3. Host cell according to claim 1 or claim 2, wherein the host cell is an *Aspergillus* strain chosen from CBS 513.88 and derivatives thereof.
4. Host cell according to any one of claims 1-3, wherein the at least four different enzymes are chosen from the group of cellulases and hemicellulases, preferably from the group of cellulases.
5. Host cell according to any one of claims 1-4, wherein the at least four different enzymes are at least two different cellobiohydrolases, optionally at least an endoglucanase and at least a β -glucosidase.
6. Host cell according to any one of claims 1-5, wherein one of said different cellobiohydrolases has at least 70% identity with SEQ ID No. 1 and the other of said different cellobiohydrolases has at least 70% identity with SEQ ID No. 7, wherein the endoglucanase has at least 70% identity with SEQ ID No. 3 and wherein the β -glucosidase has at least 70% identity with SEQ ID No. 5.
7. Host cell according to any one of claims 1-6, wherein the at least four different heterologous polynucleotides originate from a *Talaromyces* strain, preferably a *Talaromyces emersonii* strain, more preferably from *Talaromyces emersonii* CBS 39364.
8. Host cell according to any one of claims 1-7, having a cellulase activity of 2 WSU or more in 16 times or more diluted supernatant or broth.
9. Host cell according to any one of claims 1-8, capable of producing cellulase in the absence of cellulase inducer in a glucose containing medium.
10. Process for the preparation of a host cell according to any one of claims 1-9 comprising the steps of:
 - (a) Providing one or more expression cassettes comprising at least four different heterologous polynucleotides chosen from the group of

- (b) polynucleotides encoding cellulases, hemicellulases and pectinases and the control sequences required for expression of these polynucleotides
 - (c) Providing a selectable marker
 - (d) Transforming cells with the one or more expression cassettes and the selectable marker from step (b)
 - (e) Selecting the thus formed host cells that produce the at least four different cellulases, hemicellulases and/or pectinases encoded by the at least four different heterologous polynucleotides.
11. Enzyme composition comprising at least four different cellulases, hemicellulases and/or pectinases produced by the host cell according to any one of claims 1-9.
12. Process for the production of an enzyme composition comprising at least four different cellulases, hemicellulases and/or pectinases comprising the steps of:
- (a) providing the host cell of any one of claims 1-9
 - (b) allowing production and secretion of the at least four different cellulases, hemicellulases and/or pectinases by the host cell and
 - (c) optional recovering of the thus obtained enzyme composition.
13. Enzyme composition obtainable by the process of claim 12.
14. Process for the saccharification of lignocellulosic material comprising the steps of:
- optional pretreatment of lignocellulosic material and
 - contacting the lignocellulosic material with an enzyme composition of claim 11 or claim 13 to produce one or more sugars.
15. Process for the preparation of a fermentation product, including amino acids, vitamins, pharmaceuticals, animal feed supplements, specialty chemicals, chemical feedstocks, plastics, solvents, fuels, or other organic polymers, lactic acid, ethanol, fuel ethanol or chemicals, plastics, succinic acid, itaconic acid, adipic acid and (bio)fuels, including ethanol, methanol, butanol, synthetic liquid fuels and biogas, wherein the one or more sugars that is or are produced according to the process of claim 14, is fermented with a fermenting microorganism, preferably yeast to produce the fermentation product.

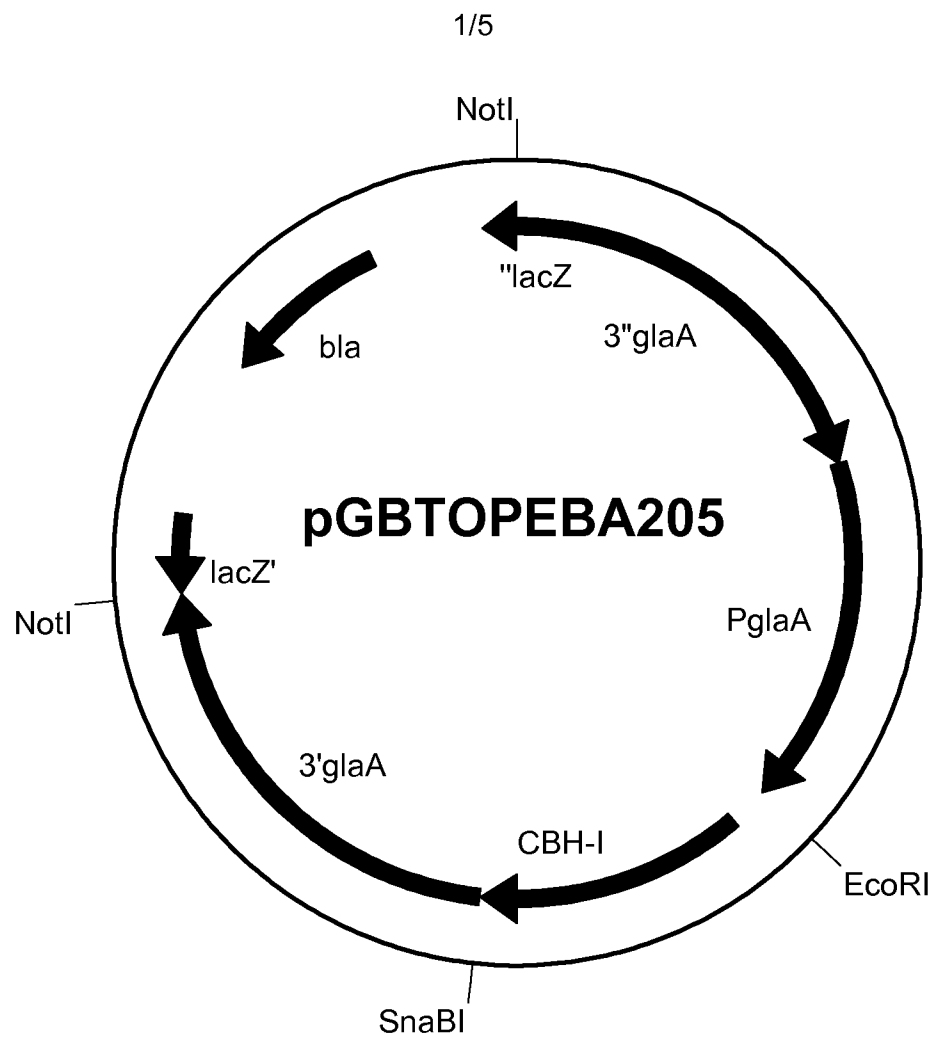


Fig. 1

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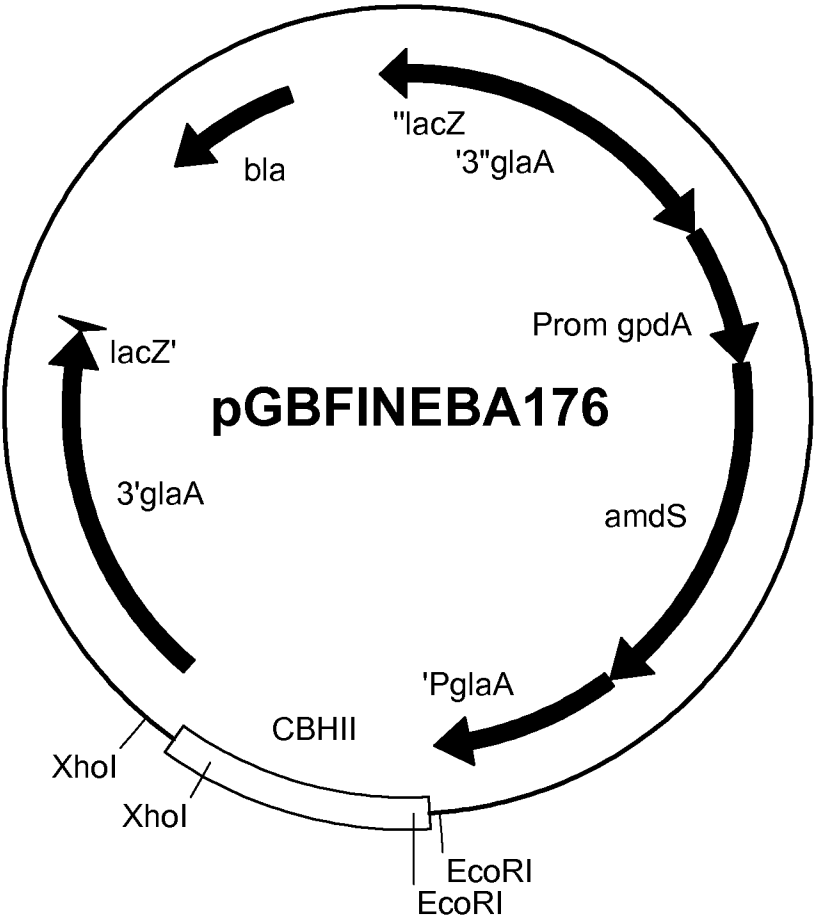


Fig. 2

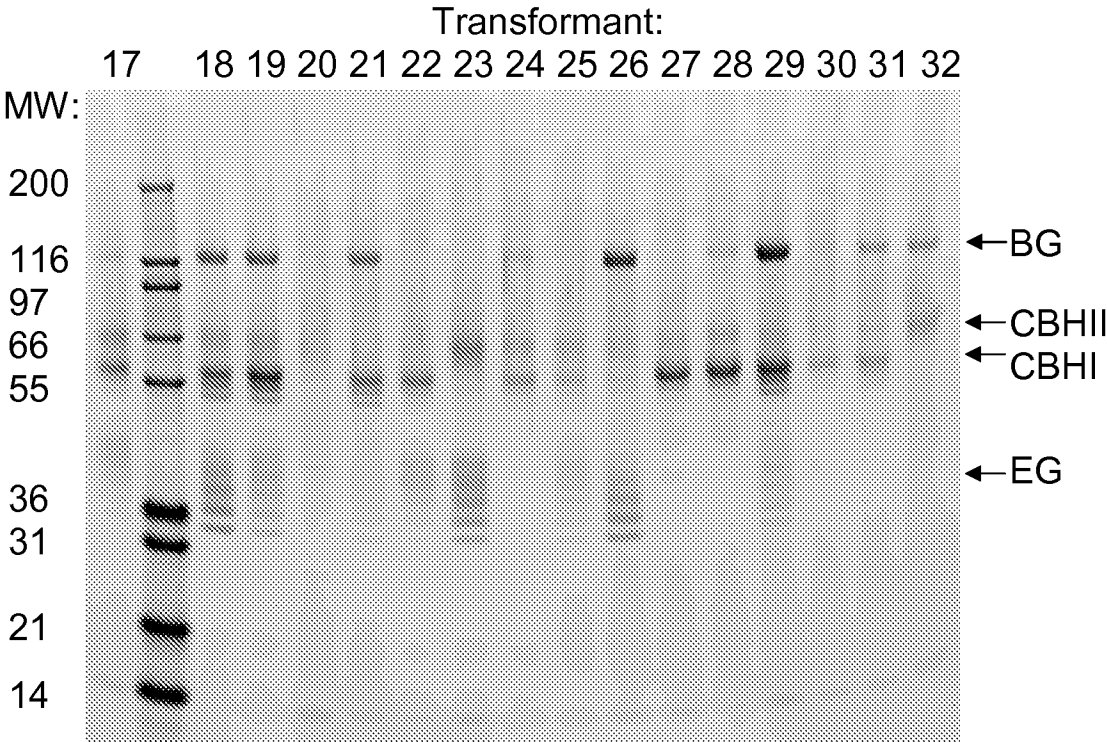


Fig. 3A

Fig3B/5

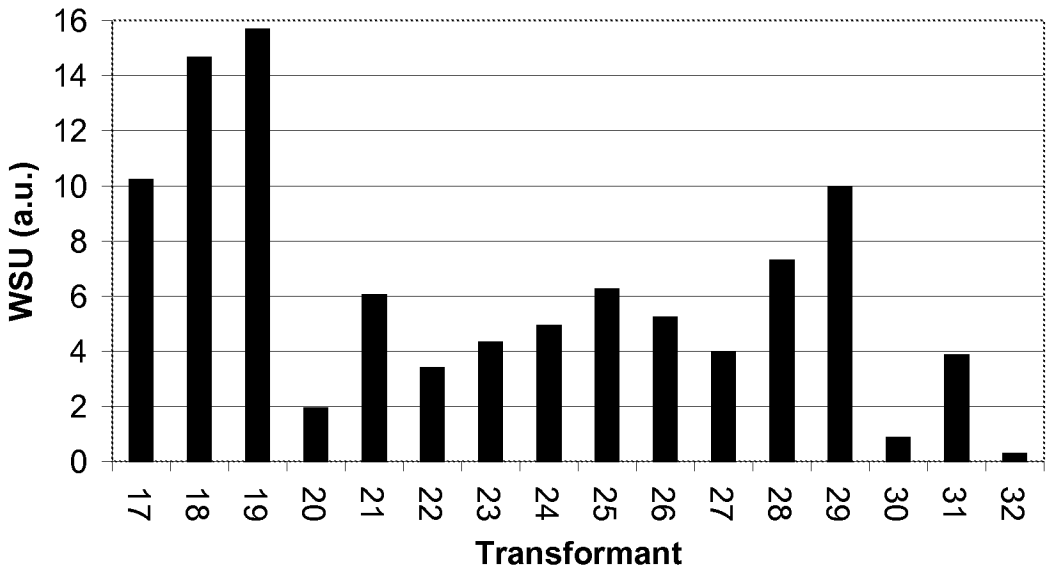


Fig. 3B

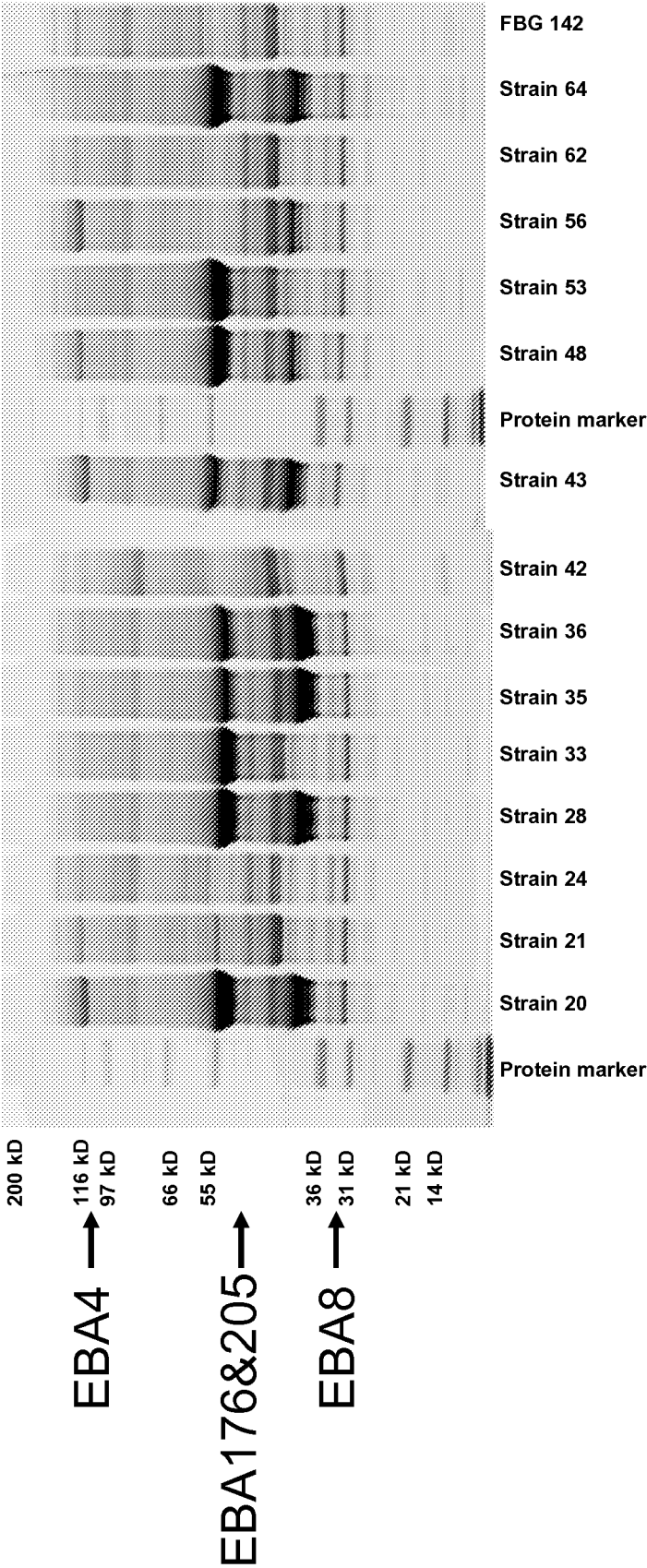


Fig 4A

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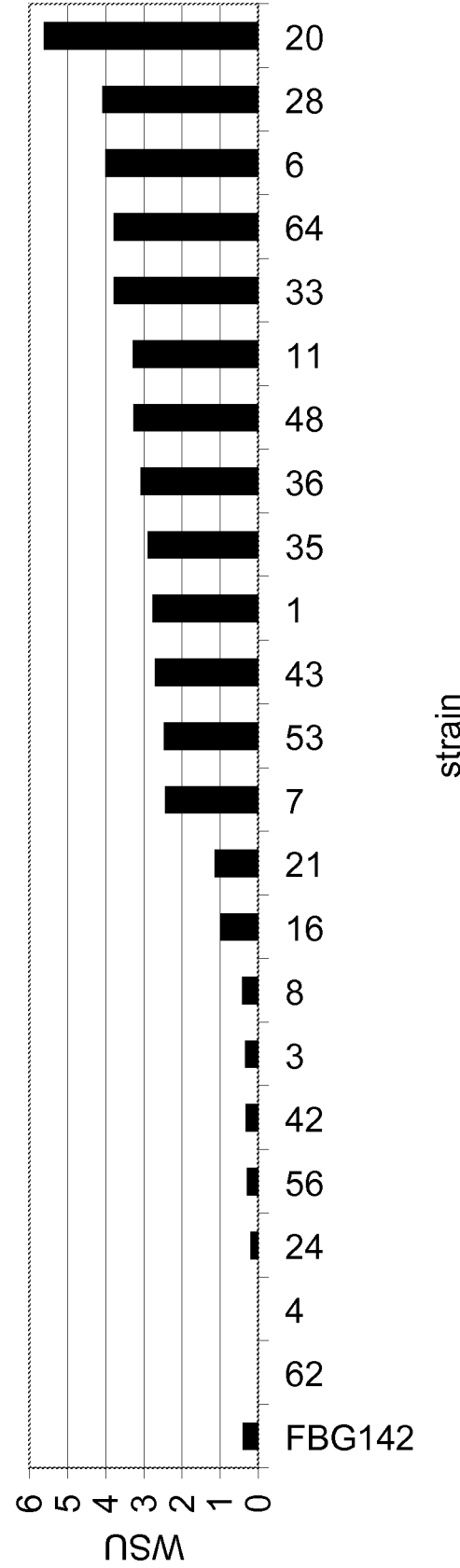


Fig 4B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/052059

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N9/42 C12P19/14 ADD. C12P21/02 C12N1/15		
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) C12N C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, COMPENDEX, FSTA, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1987, PENTTILA M: "CONSTRUCTION AND CHARACTERIZATION OF CELLULOLYTIC YEASTS", XP002592485, Database accession no. PREV198885067522 abstract & TECHNICAL RESEARCH CENTRE OF FINLAND PUBLICATIONS, no. 39, 1987, pages 1-54, ISSN: 0358-5069 ----- -/--	1-15
☒ 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 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 15 April 2011		Date of mailing of the international search report 29/04/2011
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Lejeune, Robert

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/052059

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PETERSEN S H ET AL: "Development of a polysaccharide degrading strain of <i>Saccharomyces cerevisiae</i> ", BIOTECHNOLOGY TECHNIQUES, vol. 12, no. 8, August 1998 (1998-08), pages 615-619, XP002591487, ISSN: 0951-208X the whole document -----	1-15
X	GAO D ET AL: "Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover", BIORESOURCE TECHNOLOGY, vol. 101, no. 8, 30 November 2009 (2009-11-30), pages 2770-2781, XP002591486, ISSN: 0960-8524 the whole document -----	11,13-15
X	WO 2007/091231 A1 (NAT UNIV IRELAND [IE]; TUOHY MARIA GERARDINE [IE]; MURRAY PATRICK GERA) 16 August 2007 (2007-08-16) the whole document -----	11,13-15
A	WO 2004/070022 A2 (DSM IP ASSETS BV [NL]; WENZEL THIBAUT JOSE [NL]; MEULENBERG ROGIER [NL]) 19 August 2004 (2004-08-19) cited in the application page 5 -----	1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2011/052059

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)

☐

 on paper
 - ☒

 in electronic form
 - b. (time)

☒

 in the international application as filed
 - ☐

 together with the international application in electronic form
 - ☐

 subsequently to this Authority for the purpose of search
2.

☐

 In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2011/052059

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007091231 A1	16-08-2007	AU 2007213401 A1	16-08-2007
		CA 2642133 A1	16-08-2007
		CN 101460614 A	17-06-2009
		EP 1989300 A1	12-11-2008
		IE 20060090 A2	13-06-2007
		JP 2009525747 T	16-07-2009
		US 2010028485 A1	04-02-2010

WO 2004070022 A2	19-08-2004	AU 2004209618 A1	19-08-2004
		CA 2513833 A1	19-08-2004
		CN 1836033 A	20-09-2006
		EP 1590444 A2	02-11-2005
		JP 2006516399 T	06-07-2006
		US 2006127976 A1	15-06-2006
