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(54) Title: METHOD FOR PROCESSING LIGNOCELLULOSIC BIOMASS



Fig.1

(57) Abstract: The invention discloses novel methods for the synthesis of ethanol. In particular the invention provides methods for producing a biomass characterised by a reduced content in acetyl groups. The invention also discloses materials for use in said methods, in particular the invention provides a microalga comprising a heterologous nucleotide encoding an acetylesterase.

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Method for processing lignocellulosic biomass

Technical field

The present invention relates to biotechnology. More specifically, it relates to the composition and methods for using biological materials for producing useful products, such as ethanol. The invention permits improved production of ethanol from lignocellulosic biomass by obtaining a fermentation substrate characterised by a decreased acetyl-group content and/or increased biomass content using an alga.

Background

Ethanol is currently produced from lignocellulose-containing biomass such as corn stover, cereal straw and other agricultural and forest resources. Enzymatic digestion of lignocellulosic biomass containing plant cell wall components and other polysaccharide-containing materials produce saccharides in the process of saccharification, and these are useful as substrates for ethanol-producing microorganisms. The plant cell wall surrounds every cell in plants and supports growth, development and physical properties of plant tissues as it confers structural rigidity and acts both as a physical and a chemical barrier between cells. Agricultural and forest lignocellulosic biomass sources such as for example wheat straws and corn stover are mainly built up of a type of cell wall known as the secondary cell wall, wherein the main structural components are cellulose and hemicelluloses (e.g. acetylated xylan), which are cross-linked with lignins and structural proteins. Cellulose and xylan are attractive sources of feedstocks in the second generation (2G) biofuel production because they are polymers of glucose and xylose, respectively, which can be fermented by either conventional or newly developed yeast strains after saccharification.

However, due to elaborate crosslinking amongst the cell wall polymers, these lignocellulosic biomass materials are highly recalcitrant and pretreatments are often necessary to open-up the polymer networks to allow the efficient saccharification. Chemical and physical pretreatment of lignocellulose to disrupt plant cell wall components and permit improved access of cellulolytic enzymes is a common method of increasing saccharification yields. However, the harsh conditions of pretreatment may also generate functional groups within the lignocellulosic structure that result in undesirable interactions between cellulose and cellulolytic enzymes, rendering the degradation or conversion of lignocellulose suboptimal. A typical process of the 2G biofuel production involves washing and shredding of the transported raw biomass

materials, pretreatment at high temperature (~180 °C) alone or in the presence of mild acid, separation of the solids from the liquid fractions, overliming and neutralization of the liquid fraction for re-mixing with the solids, enzymatic saccharification of cellulose and xylan, and finally fermentation by yeast. Overliming permits the detoxification of several organic compounds such as furfural and hydroxymethylfurfural, but leaves the acetyl group content unchanged.

Summary

Lignocellulosic biomass is highly recalcitrant due to elaborate crosslinking amongst the cell wall polymers. This often requires the use of pretreatments to open-up the polymer networks to allow efficient saccharification to take place in preparation for ethanol production. Pretreatments cause the release of acetyl groups from biomass; however as disclosed herein acetyl groups may inhibit the digestibility of xylan by xylanases and reduce the efficiency of the enzymatic treatment. The survival of ethanol-producing microorganisms may also be hindered. Thus, it has been found that acetylesters may have a pronounced toxicity towards yeast, with the minimum inhibitory concentration of as low as 1%, while the concentration of acetyl groups in a typical hydrothermal pretreated biomass exceeds 3 %.

Methods of separating solid and liquid phases and diluting the liquid phase have been described. However such methods entail an increase in water and energy consumption and need for waste management.

Methods resulting in a reduction of acetyl groups 20% may lead to the reduction of bioethanol prices by 10%, which is considerable in commercial scale production.

The present invention describes a method and materials for using an engineered microalga for obtaining a biomass containing significantly less acetyl groups. The method typically involves incubation of lignocellulosic biomass with said microalgae in an aqueous suspension, resulting in a significant reduction of acetyl groups both within the solid lignocellulosic biomass and within the aqueous suspension. Biomass thus treated may be particularly useful for subsequent saccharification and fermentation and may allow improved saccharification and ethanol yields.

The microalga provided by the present invention is capable of utilizing acetyl groups as an organic carbon source, and has been engineered to secrete an acetylesterase that hydrolyses acetyl ester bonds present within the lignocellulosic biomass, thus releasing acetyl groups. It has been found that such microalga may be more efficient in reducing acetyl levels compared to use of purified enzymes. This process may also result in the disruption of interactions between acetyl-bound biopolymers and other components present in the lignocellulosic biomass and may facilitate the access of cellulolytic enzymes during saccharification, thereby improving the efficiency of saccharification.

In preferred embodiments of the invention, the said alga is maintained under conditions that promote the use of the said released acetyl groups as carbon source, thereby stimulating algal cell-proliferation and resulting in the depletion of acetyl groups in solution with a concomitant increase in total biomass. Finally, the newly-generated algal biomass may be saccharified and contacted with ethanol-producing microorganisms for the production of ethanol.

Thus, the implementation of the present invention represents a clear advantage over current strategies in the use of lignocellulosic biomass for ethanol production. This is achieved as a direct consequence of the alga of the invention providing an improved substrate for fermentation i.e. a substrate with reduced acetyl-group content. The employment of the methods of the invention may have several advantages e.g. reduction of starting material and/or production-time and/or increase of ethanol yield. The alga of the invention can preferably tolerate and grow on pre-treated lignocellulosic biomass, in particular in both hydrothermally and/or alkaline pretreated lignocellulosic biomass.

Thus, it is an aspect of the invention to provide a method for producing ethanol comprising steps of:

(a) providing a lignocellulosic biomass;
(b) providing a microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetylesterase under the control of a nucleic acid sequence directing expression of said acetylesterase in said microalga;
(c) contacting the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass with reduced levels of acetyl groups;

(d) contacting the deacetylated lignocellulosic biomass with a cellulolytic enzyme composition, thereby obtaining a deacetylated saccharified biomass material

(e) fermenting the deacetylated saccharified biomass material with one or more ethanol-producing microorganisms thereby producing ethanol; and

(f) optionally, recovering the ethanol from the processed sample,

wherein steps (a) and (b) may be performed simultaneously or sequentially in any order, and steps (d) and (e) may be performed simultaneously, partly simultaneously or sequentially in any order.

It is also an aspect of this invention to provide a method for producing a deacetylated biomass comprising steps of:

(a) providing a lignocellulosic biomass;

(b) providing a microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetylesterase under the control of a nucleic acid sequence directing expression of said acetylesterase in said microalga;

(c) contacting the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass with reduced levels of acetyl groups,

wherein steps (a) and (b) may be performed simultaneously or sequentially in any order.

Description of Drawings

Figure 1. Illustrates a construct for heterologous gene expression of a gene inserted into an algal nuclear genome.

Figure 2. Illustrates the expected band sizes of crAXE and Rack1 transcripts by reverse-transcriptase polymerase chain reaction. Expected products are marked by * for crAXE (876 bp) and ** for Rack 1 (651 bp).

Figure 3. Illustrates the expected band sizes of recombinant crAXE protein in two recombinant C. reinhardtii strains (crAXE03 and crAXE23) by immunoblotting. Cell lysates indicates amounts of crAXE present in cells while Media indicates amounts secreted to the surrounding growth media. WT denotes wildtype C. reinhardtii where no recombinant crAXE is detected in cell lysates or in the media.

Figure 4. Illustrates acetylesterase activity in the growth media from the wildtype and two recombinant crAXE strains from Figure 3 and compares it to that of 1, 10 and 50 mU commercial acetylxylan esterase.

Figure 5. Illustrates growth of two recombinant strains (crAXE03 and crAXE23) and WT C. reinhardtii in TAP, and the acetylesterase activities in the corresponding media as a function of time (days).

Figure 6. Illustrates growth of strain crAXE03 and WT C. reinhardtii in wheat AIR biomass supplemented with 10% TAP and also illustrates the amount of remaining acetylesters bound to the wheat biomass. The comparison to growth and acetylesterase activity of WT cells grown in the presence of 200 mU commercial acetylxylan esterase is also shown.

Figure 7. Illustrates growth of strain crAXE03 and WT C. reinhardtii in wheat AIR biomass with no addition of acetyl groups.

Figure 8. Is an alignment between different acetylxylan esterases of different sources. Conserved residues are marked by asterisk.

Definitions

The term “lignocellulosic biomass” as used herein refers to a plant material comprising lignin, hemicellulose and cellulose. Likewise, the term “lignocellulose” refers to the plant material comprising lignin, hemicellulose and cellulose.

“Feedstock” (also referred to as “substrate”) herein is used to refer to a substance composed of sugar-based biopolymers, e.g. carbohydrate polymers, which may be treated with enzymes so that the biopolymers are modified. In addition to carbohydrate polymers, the substrate may contain other components including but not limited to non-carbohydrate polymers, such as lignin.

“Biopolymers” herein refers to polymers produced by living organisms. Biopolymers contain monomeric units that are covalently bound to each other forming larger structures, which in turn may chemically bind to each other or to other types of polymers or components to form even larger structures, such as the plant cell wall.

Biopolymers, in a general manner, comprise polynucleotides, polypeptides, polysaccharides and phenolic polymers, such as lignin.

"Carbohydrate" herein includes all saccharides, for example polysaccharides, oligosaccharides, disaccharides or monosaccharides.

The term "host" as used herein, means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide.

The term "plant" as used herein refers to all eukaryotic organisms capable of photosynthesis. Thus, a plant may be a lower or a higher plant.

The term "aqueous suspension" as used herein refers to a liquid, usually water, which typically contains living organisms (e.g. algae) and optionally in addition may contain both organic and inorganic compounds.

The term "heterologous nucleic acid" as used herein refers to a nucleic acid sequence, which has been introduced into a host microalga, wherein said host does not endogenously comprise said nucleic acid. For example, said heterologous nucleic acid may be introduced into the host microalga by recombinant methods. Thus, the genome of the host microalga has been augmented by at least one incorporated heterologous nucleic acid sequence.

The term "recombinant" as used herein, refers to DNA or molecules synthesized from the information contained in such DNA molecules, that information being derived from multiple sources, creating nucleotide sequences that would not otherwise be found in the natural organisms. The DNA sequences used in the construction of recombinant DNA molecules can originate from any species. For example, viral, plant or fungal DNA may be joined to bacterial DNA to achieve the desired functionality.

The term "gene expression" as used herein, refers to the process by which information from a gene is used in the synthesis of a gene product. These products may be proteins or RNA.

The term “signal peptide” as used herein, refers to a short peptide sequence, present at either the N- or the C-terminus of newly synthesized proteins which thereby are destined towards the secretory pathway. At the end of the signal peptide there may in addition be a stretch of amino acids that is possibly recognized and may lead to the cleavage of the signal peptide from the protein by an enzyme signal peptidase. Signal peptidase may cleave either during or after completion of translocation throughout the secretory pathway to generate a free signal peptide and a mature protein. It is also possible that the signal peptide is not cleaved and remains as part of the protein. It is known by persons skilled in the art that signal peptides are heterogeneous, and that the efficiency of protein secretion is strongly determined by the signal peptide. Signal peptides may be encoded naturally by the cell, in which case it is known as a “native” peptide, or genetically grafted forming a recombinant protein.

The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

The term “tag peptide” as used herein, refers to a peptide sequence genetically grafted onto a recombinant protein and that may facilitate the purification and/or detection of said recombinant protein.

An “acetylesterase” is an enzyme that can hydrolyse carboxylic ester bonds. Likewise, an “acetylxylan esterase” catalysis the hydrolysis of acetyl groups from polymeric xylan and other compounds.

The term "cellulolytic enzyme" or "cellulase" refers to enzymes that hydrolyse a cellulosic material. Such enzymes include endoglucanase(s), cellobiohydrolase(s), beta-glucosidase(s), or combinations thereof.

The term "hemicellulolytic enzyme" or "hemicellulase" as used herein refer to enzymes that hydrolyse a hemicellulosic material. Examples of hemicellulases include, but are not limited to, an acetylmannan esterase, an acetylxylan esterase, an arabinanase, an arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

The term “ligninolytic enzyme” as used herein refers to enzymes that catalyse the breakdown of lignin by oxidation including, but not limited to peroxidases, such as lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16), and phenoloxidases of the laccase type.

The term "xylanase" means a 1,4-beta-D-xylan-xylohydrolase (E.C. 3.2.1.8) that catalyses the endohydrolysis of 1,4-beta-D-xylosidic linkages in xylans.

The term "endoglucanase" means an endo-1 ,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. 3.2.1.4) that catalyses endohydrolysis of 1 ,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components.

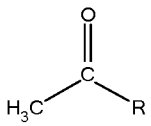
The term "cellobiohydrolase" means a 1,4-beta-D-glucan cellobiohydrolase (E.C. 3.2.1.91 and E.C. 3.2.1.176) that catalyses the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, cellooligosaccharides, or any beta-1,4-linked glucose containing polymer, releasing cellobiose from the reducing end (cellobiohydrolase I) or non-reducing end (cellobiohydrolase II) of the chain.

The term "alpha-L-arabinofuranosidase" means an alpha-L-arabinofuranoside arabinofuranohydrolase (EC 3.2.1.55) that catalyses the hydrolysis of terminal non-reducing alpha-L-arabinofuranoside residues in alpha-L-arabinosides. The enzyme acts on alpha-L-arabinofuranosides, alpha-L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. Alpha-L-arabinofuranosidase is also known as arabinosidase, alpha-arabinosidase, alpha-L-arabinosidase, alpha-arabinofuranosidase, polysaccharide alpha-L-arabinofuranosidase, alpha-L-arabinofuranoside hydrolase, L- arabinosidase, or alpha-L-arabinanase.

The term "alpha-glucuronidase" means an alpha-D- glucosiduronate glucuronohydrolase (EC 3.2.1.139) that catalyses the hydrolysis of an alpha-D-glucuronoside to D-glucuronate and an ethanol.

The term “expansin” as used herein, refers to the class of enzymes that are characterised by causing plant cell wall stress-relaxation and irreversible wall extension by loosening the linkages between cellulose microfibrils.

The term “acetyl groups” as used herein, refers to any chemical group of the general formula CH_3COR, illustrated in drawing A herein below, where R stands for a portion of a complete molecule, such as for example, in a non-limiting manner, a hydroxyl group, a polysaccharide, such as a xylan, or a polyphenol, such as lignin. Examples of acetyl groups include, but are not limited to esters of acetic acid, free acetate; the conjugate base of acetic acid with formula CH_3COO^- , or the carboxylic acid, acetic acid with formula CH_3OOH.

A. 

The term “Ethanol-producing microorganism” as used herein, refers to organisms that are capable of producing ethanol from a fermentable carbon source.

Detailed description

Method for producing ethanol

The present invention provides methods for producing ethanol from a lignocellulosic biomass. The methods of the invention generally comprise steps of producing a deacetylated lignocellulytic, e.g. they may comprise any of the methods described herein below in the section “Method for producing a deacetylated lignocellulosic biomass material”. Typically, methods for producing a deacetylated lignocellulosic biomass material comprises the steps of providing a lignocellulosic biomass and contacting it with a microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetylesterase. The said lignocellulosic biomass may be incubated with the said microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass with reduced levels of acetyl groups. The method of producing ethanol may further comprise the steps of:

(d) contacting the deacetylated lignocellulosic biomass material with a cellulolytic enzyme composition, e.g. a composition comprising one or more of the enzymes

described herein below in the section “Lignocellulolytic enzymes”, thereby obtaining a deacetylated saccharified biomass material

(e) fermenting the deacetylated saccharified biomass material with one or more ethanol-producing microorganisms thereby producing ethanol; and

(f) optionally, recovering the ethanol from the processed sample.

Step (e) may for example be performed as described herein below in the section “Fermentation of the deacetylated saccharified biomass with one or more Ethanol-producing microorganisms”.

Ethanol may be recovered from the processed sample by any useful method. Examples of useful methods for recovering ethanol are well known to the skilled person.

Method for producing a deacetylated lignocellulosic biomass material

The present invention provides methods for producing a deacetylated lignocellulosic biomass. Said methods may be performed separately, or they may be integrated into other methods, e.g. into methods of producing ethanol.

The methods of the invention generally comprise the steps of:

(a) providing a lignocellulosic biomass;

(b) providing a microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetylesterase under the control of a nucleic acid sequence directing expression of said acetylesterase in said microalga;

(c) contacting the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass with reduced levels of acetyl groups.

Said lignocellulosic biomass may be any one or more, or a combination thereof of lignocellulosic biomasses described herein above in the section “lignocellulosic biomass”.

Said microalga may be any one or more, or a combination thereof of microalgae described herein above in the section “Microalgae”.

Said aqueous suspension is best described herein above in the section “Contacting lignocellulosic biomass with the microalga”.

Said heterologous nucleic acid may be any one or more, or a combination thereof of heterologous nucleic acids described herein above in the section “Heterologous nucleic acid”.

Said acetylesterase capable of catalysing hydrolysis of carboxylic ester bonds may be any one or more, or a combination thereof of the enzymes described herein above in the section “Acetylesterase”.

As described in more detail herein below the “deacetylated lignocellulosic biomass” may comprise both solid deacetylated lignocellulosic biomass as well as said aqueous suspension and microalgae. Accordingly, it may be preferred that step (c) results in production of a deacetylated lignocellulosic biomass with reduced levels of total acetyl groups. As used herein the reduced levels of total acetyl groups refers to a reduced level of acetyl group in the deacetylated lignocellulosic biomass compared to the level of acetyl groups in the starting lignocellulosic biomass.

It may be preferred that the step (c) comprises or consists of incubating the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass with reduced levels of acetyl groups bound to biopolymers and a reduced level of acetyl groups in the aqueous suspension. The acetyl groups in the aqueous suspension may typically be free acetate group. Said reduced levels are preferable reduced compared to the starting level of acetyl groups in the lignocellulosic biomass and the aqueous suspension.

It may also be preferred that the step (c) comprises or consists of incubating the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass comprising reduced levels of total acetyl groups and an increased level of algal biomass.

As used herein an increased level of algal biomass refers to an increased level of algal biomass compared to the starting level of algal biomass, e.g. compared to the algal biomass provided in step b).

Incubating the lignocellulosic biomass with the microalgae in an aqueous suspension may be carried out in any one of the manners described herein above in the section "Contacting the lignocellulosic biomass", or a combination thereof.

Lignocellulosic biomass

Lignocellulose is the most abundantly available raw material on the Earth for the production of biofuels, such as ethanol. It is composed of carbohydrate polymers i.e. cellulose, hemicellulose, and an aromatic polymer i.e. lignin. These carbohydrate polymers contain different sugar monomers (six and five carbon sugars) which are bound to lignin. Lignocellulosic biomass can be broadly classified into virgin biomass, waste biomass and energy crops all of which can be used with the methods of the invention. Virgin biomass includes all naturally occurring terrestrial plants such as trees, bushes and grass. Waste biomass is produced as a byproduct of various industrial sectors such as agriculture (corn stover, sugarcane bagasse, straw etc.) and forestry (saw mill and paper mill discards). Energy crops are crops with high yield of lignocellulosic biomass produced to serve as a raw material for production of second generation biofuel; non-limiting examples of energy crops include switch grass (Panicum virgatum) and Elephant grass.

The lignocellulosic biomass preferably comprises or consists of plant parts obtained from one or several of the following: Poplar, willow, eucalyptus, locust, Miscanthus (silvergrass), switchgrass, elephant grass, reed canary grass, cereal straw, corn stover, coffee, cacao, sugar cane, sweet sorghum, sugar beet, canola, oil palm, maripa palm, and jatropha. The lignocellulosic biomass may also comprise or consist of plant parts obtained from one or several of the following: Treetops, branches, stumps, leaves, stems, seeds, husks, roots, inflorescences, cobs, bark, sawdust, palettes, furniture, demolition timber, forest residues, agricultural residues, municipal solid waste and wood chips and particles. Thus, the lignocellulosic biomass preferably comprises of consists of plant parts obtained from one or several of the following combinations: cereal straw (e.g. wheat straw), Corn stover, cacao shells, husks or cobs, coffee waste, sugar beet or sugar cane bagasse, presscake or pulp, or jatropha presscake, shells or fruit bunch.

The lignocellulosic biomass may be finely divided, e.g. it may be finely divided by a mechanical processing such as milling, shredding, chopping and/or compaction such as pelletizing, or bricking.

In a preferred embodiment, the lignocellulosic biomass does not undergo a pretreatment before being contacted with the microalga in the aqueous suspension. Thus, in preferred embodiments the lignocellulosic biomass consists of plant material, e.g. any of the above-mentioned plant materials, which optionally may be finely divided and optionally may have been washed in a aqueous liquid, but which otherwise has not undergone any further pretreatment.

In other embodiments, lignocellulosic biomass may be pretreated with mechanical and/or chemical modification or any combination of such methods before being contacted with the microalga in order to enhance the accessibility of the lignocellulose to enzymatic hydrolysis, in any way known in the art. Typically, pretreatment may comprise exposing the lignocellulosic material to (hot) water, steam (steam explosion), an acid, a base, a solvent, a peroxide, ozone, or a combination of any two or more thereof. A chemical pretreatment is often combined with heat pretreatment, e.g. between 150-220 °C for 1 to 30 minutes.

Microalga

The invention relates to a microalga and methods employing said microalga. The microalga may for example be any of the microalga described in this section.

In one embodiment the microalga may be any photosynthetic organism, such as a photosynthetic prokaryotic cyanobacteria or a eukaryotic algae. For example, the microalga may be a non-vascular alga, such as a unicellular or multicellular non-vascular alga. Multicellular microalgae may in particular to such algae that form colonies or cell aggregates during one or more phases of their life-cycle.

Said microalga may in one embodiment be selected from the group consisting of unicellular algae capable of using organic carbon as a carbon source. In a preferred embodiment, the microalga is a species particularly suited for polypeptide secretion. Thus, the microalga may be selected from the group consisting of members of the Chlamydomonadales order such as members of the Chlamydomonas, Haematococc us and Dunaliella genera e.g. Chlamydomonas reinhardtii, C. dysosmos,

Haematococcus pluvialis, Dunaliella salina, and Chlorella species including Chlorella vulgaris, or members of the Nannochloropsis genus, such as Nannochloropsis gaditana or Phaeodactylum tricornutum. In a preferred embodiment, the microalga is a cell wall deficient microalga, for example a cell wall deficient strain of Chlamydomonas reinhardtii, e.g. that strain known as UVM4. A cell wall deficient strain may offer several advantages over the wild type strain, such as its readiness in incorporating exogenous polynucleotides, its increased proliferation rate, and two safeguards against spreading of this strain in an uncontained environment, namely its poor ability to survive outside of a controlled environment and its inadequacy in carrying out sexual reproduction.

It is also comprised within the invention that the methods may employ more than one microalga, wherein each of the microalga may be selected from the microalga described above.

The microalga of the invention comprises a heterologous nucleic acid encoding a polypeptide comprising an acetylesterase, which may be any of the acetylesterases described in the section “Acetylesterase” herein below. However, in addition to this heterologous nucleic acid, the microalga may comprise one or more additional heterologous nucleic acids, for example any one or more of the following heterologous nucleic acids:

I. A heterologous nucleic acid encoding an enzyme selected from the group consisting of cellulases as described herein below.
II. A heterologous nucleic acid encoding an enzyme selected from the group consisting of hemicellulases as described herein below.
III. A heterologous nucleic acid encoding an enzyme selected from the group consisting of ligninolytic enzyme as described herein below.

The microalga may comprise one of more of the heterologous nucleic acids I., II., and III. in addition to the heterologous nucleic acid encoding acetylesterase, such as at least 1, for example at least 2, or such as all of heterologous nucleic acids I., II., and III.

Heterologous nucleic acid

It will be appreciated that the microalga described herein typically comprises one or more heterologous nucleic acids encoding one or more enzymes.

In general the heterologous nucleic acid encoding a polypeptide (also referred to as "coding sequence" in the following) is operably linked in sense orientation to one or more regulatory regions suitable for directing expression of the polypeptide. A coding sequence and a regulatory region are considered to be operably linked when the regulatory region and coding sequence are positioned so that the regulatory region is effective for regulating transcription or translation of the sequence.

"Regulatory region" refers to a nucleic acid having nucleotide sequences that influence transcription or translation initiation and rate, and stability and/or mobility of a transcription or translation product. Regulatory regions include, without limitation, promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, protein binding sequences, 5´ and 3´ untranslated regions (UTRs), transcriptional start sites, termination sequences, polyadenylation sequences, introns, and combinations thereof. A regulatory region typically comprises at least a core (basal) promoter. A regulatory region also may include at least one control element, such as an enhancer sequence, an upstream element or an upstream activation region (UAR). A regulatory region is operably linked to a coding sequence by positioning the regulatory region and the coding sequence so that the regulatory region is effective for regulating transcription or translation of the sequence.

The choice of regulatory regions to be included depends upon several factors, including the type of host organism. It is a routine matter for one of skill in the art to modulate the expression of a coding sequence by appropriately selecting and positioning regulatory regions relative to the coding sequence. It will be understood that more than one regulatory region may be present, e.g., introns, enhancers, upstream activation regions, transcription terminators, and inducible elements.

It will be appreciated that because of the degeneracy of the genetic code, a number of nucleic acids can encode a particular polypeptide; i.e., for many amino acids, there is more than one nucleotide triplet that serves as the codon for the amino acid. Thus, codons in the coding sequence for a given polypeptide can be modified such that optimal expression in a particular host organisms obtained, using appropriate codon bias tables for that host (e.g., microalgae). Nucleic acids may also be optimized to a GC-content preferable to a particular host, and/or to reduce the number of repeat sequences. As isolated nucleic acids, these modified sequences can exist as purified

molecules and can be incorporated into a vector or a virus for use in constructing modules for recombinant nucleic acid constructs.

Said aqueous suspension is described herein below in the section “Contacting lignocellulosic biomass with the microalga”.

Contacting lignocellulosic biomass with the microalga

The methods of the invention comprise a step of contacting lignocellulosic biomass with a microalga in an aqueous suspension. Preferably, said step (e.g. step (c) of the methods described herein) comprises or consists of incubating lignocellulosic biomass with microalgae in an aqueous suspension. In particular, the step can be performed as described herein in this section.

Incubating lignocellulosic biomass with the microalga may be carried out in several ways. In one embodiment, the microalga is provided in an aqueous suspension and said aqueous suspension may be added to the lignocellulosic biomass. In another embodiment the lignocellulosic biomass is incubated in an aqueous suspension to which the microalgae are then contacted.

The aqueous suspension thus in general comprises lignocellulosic biomass, water and microalgae. In addition, the aqueous suspension may comprise additional compounds, e.g. organic or inorganic compounds. For example, in some embodiments the aqueous suspension may comprise nutrients, salts or other compounds favourable for algal proliferation. In other embodiments, the aqueous suspension consists of lignocellulosic biomass, water, microalgae and products produced by incubating said biomass with microalgae.

In one embodiment, the microalgae may be incubated in an aqueous suspension under conditions that will promote cell proliferation and then contacted with the lignocellulosic biomass while optionally prolonging the conditions favouring cell proliferation.
In one embodiment, the aqueous suspension comprises acetyl groups that may support cell-proliferation of the microalgae in the early growth phase.

In one embodiment, the said aqueous suspension comprising acetyl groups that promote cell proliferation are provided by a method comprising the steps of:

(a) incubating microalgae with one batch of lignocellulosic biomass in an aqueous suspension,
(b) withdrawing a sample of aqueous suspension containing microalgae and hydrolysed acetyl groups ,
(c) contacting a second batch of lignocellulosic biomass with this sample.

Said sample may in particular be withdrawn at a time prior to that time at which the algal culture reaches saturation.
Thus, in a preferred embodiment, the said acetyl groups will be provided by contacting the lignocellulosic biomass with an aqueous suspension obtained from a previous lignocellulosic biomass incubation with microalgae.

In another embodiment, the acetyl groups are provided from another source, such as described in the examples, and by adding them to the aqueous suspension.

Aqueous suspension conditions that will promote cell proliferation may comprise physical and chemical conditions that combined will provide an environment within and around the aqueous suspension conducive to microalga cell division, protein synthesis and protein secretion over time. In a preferred embodiment, such conditions will provide an organic carbon source such as acetyl groups together with the optimal temperature and light conditions, as known in the art.

The time of incubation will typically be sufficiently long to obtain a deacetylated lignocellulosic biomass as described below.

Acetylesterase

The microalga to be used with the present invention comprises a heterologous nucleic acid encoding a polypeptide comprising an acetylesterase. Accordingly, said polypeptide preferably is capable of hydrolysing acetyl ester bonds between acetyl groups and polymeric saccharides and other compounds.
Enzymes capable of hydrolysing acetyl ester bonds between acetyl groups and polymeric saccharides and other compounds may also be referred to herein as an “acetylesterase”, and may be any of the enzymes described herein in this section.

In a preferred embodiment, the acetylesterase is an acetylxylan esterase having acetylxylan esterase activity. Acetylxylan esterase activity is the ability to catalyse the hydrolysis of acetyl groups from polymeric xylan and potentially other compounds, including but not limited to acetylated xylose, acetylated glucose, α-napthyl acetate and p-nitrophenyl acetate. Acetylesterase activity may result in at least 20%, preferably at least 90% reduction in acetyl-group content in the aqueous suspension comprising lignocellocellulosic biomass.

It is preferred that the acetylesterase capable of catalysing the hydrolysis of acetyl groups is an acetylxylan esterase (AXE) (also known as xylan acetylesterase), (EC 3.1.1.72).

Said acetylesterase may be derived from any suitable source, e.g. but in a preferred embodiment it is an aceteylesterase derived from a fungus or a bacterium, or a functional homologue thereof. Thus, for example the acetylesterase may be a acetylxylan esterase derived from Aspergillus nidulans, Trichoderma reesei, Talaromyces purpureogenus or Thermoanaerobacterium Sp.

AXEs are a structurally diverse groups of enzymes. An alignment of 4 acetylxylan esterases from different species is provided herein in figure 8. The AXE to be used with the invention may be any polypeptide, which when aligned with the polypeptides shown in figure 8 contains at least the amino acids marked by asterisk in figure 8.

Thus, for example the AXE may be the polypeptide of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, for example at least 80%, such as at least 90%, for example such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

Thus, for example the AXE may be the polypeptide of SEQ ID NO:2 or a functional homologue thereof sharing at least 70%, for example at least 80%, such as at least 90%, for example such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least

97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

For example the AXE may be the polypeptide of SEQ ID NO:6 or a functional homologue thereof sharing at least 70%, for example at least 80%, such as at least 90%, for example such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

For example the AXE may be the polypeptide of SEQ ID NO:7 or a functional homologue thereof sharing at least 70%, for example at least 80%, such as at least 90%, for example such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

For example the AXE may be the polypeptide of SEQ ID NO:8 or a functional homologue thereof sharing at least 70%, for example at least 80%, such as at least 90%, for example such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

The sequence identity is preferably calculated as described herein below in the section “Sequence identity”. A functional homologue of AXE, e.g. of a functional homologue of a polypeptide according to any one of SEQ ID NO:2, 4, 5, 6, 7, or 8 may be capable of catalysing reactions of hydrolysing acetyl groups from biopolymers or other compounds present in the lignocellulosic biomass as described above.

The AXE may also be a polypeptide sharing at least some sequence identity with the polypeptide of SEQ ID NO: 2, 4, 5, 6, 7, or 8, e.g. a polypeptide sharing at least 15%, such at least 25%, for example at least 40%, such as at least 50%, for example 60%, such as at least 70%, for example at least 80%, such as at least 90%, such as at least 95%, such as at least 98% sequence identity with the polypeptide of SEQ ID NO: 2, 4,

5, 6, 7, or 8, wherein the polypeptide contains at least the amino acids marked by "*" in figure 8, for example at least the amino acids marked by either ".", ":" or "*" in figure 8.

Functional homologues of AXE may be any polypeptide sharing aforementioned sequence identity with SEQ ID NO:2, 4, 5, 6, 7, or 8 and which also are capable of catalysing reaction(s) described in this section. In particular it is preferred that functional homologues of AXE also have acetylxylan esterase activity.

In one embodiment the polypeptide comprising an acetylesterase may comprise an acetylesterase as mentioned above as well as a signal peptide and/or a tag peptide. Thus, the polypeptide comprising an acetylesterase may comprise the polypeptide of SEQ ID NO:2, 6, 7, or 8 or any of the functional homologues thereof described above as well as a signal peptide and/or a tag peptide. A tag peptide may for example comprise an amino acid sequence that is useful for purifying and/or detecting said polypeptide. Examples of useful tag peptides are described below.

The signal peptide may be positioned either at the N-terminus or the C-terminus of the polypeptide of SEQ ID NO:2, 6, 7, or 8 or any of the functional homologues thereof described above, however preferably it is positioned at the N-terminus. The signal peptide may be any short peptide sequence directing a polypeptide for secretion or for other compartments in the secretory pathway. The signal peptide is usually 16-30 amino acids long and may comprise a core of hydrophobic amino acids as well as a cleavage site. In some embodiments the signal peptide may be a signal peptide derived from a polypeptide of algae origin. Multiple signal peptides are known to the skilled person and they can for example be identified using the UniProt database (www.uniprot.org), which contains annotations of signal peptides or the Signal Peptide Website (www.signalpeptide.de). A non-limiting example of a useful signal peptide is the gametolysin signal peptide provided herein as SEQ ID NO:9.

For example, the polypeptide comprising the acetylesterase enzyme may be the polypeptide of SEQ ID NO:4 or a functional homologue thereof sharing at least 70%, for example at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

For example, the polypeptide comprising the acetylesterase may be the polypeptide of SEQ ID NO:5 or a functional homologue thereof sharing at least 70%, for example at least 80%, such as at least 85%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% sequence identity therewith.

Functional homologues of polypeptides of SEQ ID NO:4 or 5 may be enzymes sharing aforementioned sequence identity with SEQ ID NO:4 or 5, and which also have acetylxylan esterase activity.

Tag peptides may be attached to proteins for various purposes, such as but not limited to: Affinity tags such as for example chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), Strep-tag or poly(His) tag, solubilization tags including thioredoxin (TRX) and poly(NANP), chromatography tags, such as FLAG-tag, epitope tags (short peptide sequences which are chosen because high-affinity antibodies can be reliably produced in many different species), such as V5-tag, Myc-tag, HA-tag or NE-tag. These tags are particularly useful for western blotting, immunofluorescence and immunoprecipitation experiments, although they also find use in antibody purification.

Fluorescence tags are used to give visual readout on a protein. GFP and its variants are the most commonly used fluorescence tags. More advanced applications of GFP include using it as a folding reporter (fluorescent if folded, colourless if not). Tag peptides may also allow specific enzymatic modification (such as biotinylation by biotin ligase) or chemical modification (such as reaction with FlAsH-EDT2 for fluorescence imaging).

The heterologous nucleic acid encoding the polypeptide comprising an acetylesterase may be any heterologous nucleic acid encoding any of the polypeptides described herein in this section. Thus, the heterologous nucleic acid may be any heterologous nucleic acid encoding any of the polypeptides of SEQ ID NO:2, 4, 5, 6, 7, or 8 or any of the functional homologues thereof described herein.

Thus, in one embodiment the heterologous nucleic acid may be any heterologous nucleic acid encoding a polypeptide comprising AXE of SEQ ID NO:2 or any functional homologue thereof. In one embodiment the heterologous nucleic acid may comprise or consist of SEQ ID NO:1.

In another embodiment, the heterologous nucleic acid encoding the enzyme capable of catalysing the reaction of hydrolysing acetyl groups from biopolymers or other compounds may be a heterologous nucleic acid comprising SEQ ID NO:1 in addition to nucleic acid sequences encoding one or more tag peptides. In a preferred embodiment, the tag peptide nucleic acid sequence encodes a streptomycin sequence at the C-terminus of the AXE protein. In one embodiment the heterologous nucleic acid may comprise or consist of SEQ ID NO:3.

In another embodiment, the heterologous nucleic acid encoding the enzyme capable of catalysing the reaction of hydrolysing acetyl groups from biopolymers or other compounds may be a heterologous nucleic acid comprising SEQ ID NO:1 or SEQ ID NO:3, in addition to nucleic acid sequences encoding a signal peptide. In a preferred embodiment the heterologous nucleic acid encoding a polypeptide comprising SEQ ID NO:5.

Lignocellulolytic enzymes

The methods of the invention may comprise a step of contacting deacetylated lignocellulosic biomass with a lignocellulolytic enzyme composition, which may comprise any of the lignocellulolytic enzymes described in this section.

In one aspect, the lignocellulolytic enzymes comprise or further comprise one or more (e.g., several) polypeptides selected from the group consisting of a cellulase, a hemicellulase, an esterase, an expansin, a ligninolytic enzyme, a pectinase, a peroxidase, a protease, and a swollenin.

In another aspect, the cellulase is preferably an enzyme selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase.

In another aspect, the hemicellulase is preferably an enzyme selected from the group consisting of an acetylmannan esterase, an acetylxylan esterase, an arabinanase, an

arabinofuranosidase, a coumaric acid esterase, a feruloyl esterase, a galactosidase, a glucuronidase, a glucuronoyl esterase, a mannanase, a mannosidase, a xylanase, and a xylosidase.

In another aspect, the lignocellulolytic enzyme composition may comprise one or more (e.g., several) lignocellulolytic enzymes.

In another aspect, the cellulolytic enzyme composition comprises an acetylmannan esterase. In another aspect, the cellulolytic enzyme composition comprises an arabinanase (e.g., alpha-L-arabinanase). In another aspect, the cellulolytic enzyme composition comprises an arabinofuranosidase (e.g., alpha-L- arabinofuranosidase). In another aspect, the cellulolytic enzyme composition comprises a coumaric acid esterase. In another aspect, the cellulolytic enzyme composition comprises a feruloyi esterase. In another aspect, the cellulolytic enzyme composition comprises a galactosidase (e.g., alpha-galactosidase and/or beta-galactosidase). In another aspect, the cellulolytic enzyme composition comprises a glucuronidase (e.g., alpha-D-glucuronidase). In another aspect, the cellulolytic enzyme composition comprises a glucuronoyl esterase. In another aspect, the cellulolytic enzyme composition comprises a mannanase. In another aspect, the cellulolytic enzyme composition comprises a mannosidase (e.g., beta-mannosidase). In another aspect, the cellulolytic enzyme composition comprises a xylanase. In a preferred aspect, the xylanase is a Family 10 xylanase. In another aspect, the cellulolytic enzyme composition comprises a xylosidase (e.g., beta-xylosidase).

In another aspect, the cellulolytic enzyme composition comprises an esterase. In another aspect, the cellulolytic enzyme composition comprises an expansin. In another aspect, the cellulolytic enzyme composition comprises a laccase. In another aspect, the cellulolytic enzyme composition comprises a ligninolytic enzyme. In a preferred aspect, the ligninolytic enzyme is a manganese peroxidase. In another preferred aspect, the ligninolytic enzyme is a lignin peroxidase. In another preferred aspect, the ligninolytic enzyme is a H_2O_2-producing enzyme. In another aspect, the cellulolytic enzyme composition comprises a pectinase. In another aspect, the cellulolytic enzyme composition comprises a peroxidase. In another aspect, the cellulolytic enzyme composition comprises a protease. In another aspect, the cellulolytic enzyme composition comprises a swollenin.

In the methods of the present invention, the enzyme(s) can be added prior to or during saccharification, saccharification and fermentation, or fermentation.

One or more (e.g., several) components of the cellulolytic enzyme composition may be wild-type proteins, recombinant proteins, or a combination of wild-type proteins and recombinant proteins. For example, one or more (e.g., several) components may be native proteins of a cell, which is used as a host cell to express recombinantly one or more (e.g., several) other components of the enzyme composition. One or more (e.g., several) components of the cellulolytic enzyme composition may be produced as monocomponents, which are then combined to form the enzyme composition. The cellulolytic enzyme composition may be a combination of multicomponent and monocomponent protein preparations.

Deacetylated lignocellulosic biomass

The term “deacetylated lignocellulosic biomass” as used herein refers to the material obtained after contacting a lignocellulosic biomass with a microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetyl esterase in an aqueous suspension.

Thus, the “deacetylated lignocellulosic biomass” may comprise solid deacetylated lignocellulosic biomass. Furthermore, the “deacetylated lignocellulosic biomass” may comprise said aqueous suspension and/or microalga.

The lignocellulosic biomass treated with the microalga of this invention is characterised by a reduced content in acetyl groups bound to biopolymers and other compounds within said lignocellulosic biomass.

It is understood that deacetylated lignocellulosic biomass may be partly or completely deacetylated. Thus the term “deacetylated lignocellulosic biomass” does not imply a complete absence of acetyl groups, but rather a reduction compared to untreated lignocellulosic biomass.

In a preferred embodiment of the invention, acetyl groups may be released into the aqueous suspension by the enzymatic activity of acetylesterase enzyme on said biopolymers and other compounds. In a preferred embodiment said acetylesterase

enzyme is secreted by the microalga of the invention. In another embodiment the acetyl groups are provided from other sources such as in the examples.
In a further preferred embodiment, the microalga of the invention uses the acetyl groups present within the aqueous suspension as carbon source under conditions that promote cell-proliferation, protein synthesis and protein secretion. The consumption of acetyl groups by the microalga results in a decrease of the acetyl group content in the aqueous suspension, thus producing a deacetylated biomass.

In one embodiment the final acetyl-groups concentration in the deacetylated lignocellulosic biomass material is reduced by at least 5% (w/w), at least 6% (w/w), at least 7% (w/w), at least 8% (w/w), at least 9% (w/w), at least 10% (w/w), at least 15% (w/w), at least 20% (w/w), at least 30% (w/w), at least 40% (w/w), or at least 50% (w/w) compared to the starting lignocellulosic biomass.

In one embodiment, the final concentration of acetyl bound to biopolymers in the deacetylated lignocellulosic biomass is reduced by at least 5% (w/w), at least 6% (w/w), at least 7% (w/w), at least 8% (w/w), at least 9% (w/w), at least 10% (w/w), at least 15% (w/w), at least 20% (w/w), at least 30% (w/w), at least 40% (w/w), or at least 50% (w/w) compared to the starting lignocellulosic biomass.

The content of acetyl groups in the deacetylated lignocellulosic biomass may thus be at the most 90%, such as at the most 80%, for example at the most 70%, such as at the most 60%, for example at the most 50%, such as at the most 40%, for example at the most 30%, such as at the most 20% of the content of acetyl groups in the starting lignocellulosic biomass.

In a preferred embodiment the content of acetyl groups in the deacetylated lignocellulosic biomass at the most 10% of the content of acetyl groups in the starting lignocellulosic biomass.

In particular, the deacetylated lignocellulosic biomass comprising solid deacetylated lignocellulosic biomass, aqueous suspension and microalga preferably comprise at the most 90%, such as at the most 80%, for example at the most 70%, such as at the most 60%, for example at the most 50%, such as at the most 40%, for example at the most

30%, such as at the most 20%, for example at the most 10% of the acetyl groups contained in the starting lignocellulosic biomass and aqueous suspension.

It is also preferred that the deacetylated lignocellulosic biomass comprises at the most 90%, such as at the most 80%, for example at the most 70%, such as at the most 60%, for example at the most 50%, such as at the most 40%, for example at the most 30%, such as at the most 20%, for example at the most 10% of the acetyl groups found in a lignocellulosic biomass treated with a similar microalga not comprising the heterologous nucleic acid encoding a polypeptide comprising an acetylesterase. A non-limiting example on how to determine acetyl groups is described in Example 6 herein below.

In one embodiment the deacetylated lignocellulosic biomass comprise at the most 3%, such as at the most 2%, for example at the most 1%, such as at the most 0.5% acetyl groups as determined by w/w. For example, the deacetylated lignocellulosic biomass may comprise at most 4% (w/w), preferably 0,1-3% (w/w), more preferably 0,1-2% (w/w), even more preferably 0,1-1% (w/w), or even more preferably 0,1-0,5% (w/w) acetyl groups.

As described herein elsewhere the deacetylated lignocellulosic biomass may comprise microalgae. It may be preferred that the deacetylated lignocellulosic biomass comprise at least twice, such as at least 4 times, for example at least 6 times, such as at least 10 times the amount of microalgal biomass compared to the amount of microalgal biomass provided in step (b) of the methods of the invention.

Fermentation of the deacetylated saccharified biomass with one or more ethanol-producing microorganisms

The methods according to the invention may comprise a step of fermenting a biomass material, e,g, a deacetylated saccharified biomass material with one or more ethanol-producing microorganisms thereby producing ethanol. This step may be performed simultaneously with, partly simultaneously with or subsequent to a step of incubating deacetylated biomass material with a cellulytic enzyme composition.

Thus, in some embodiments, the method for producing a product ethanol in a fermentation process includes (i) providing a microorganism that is capable of producing a product ethanol from a deacetylated saccharified biomass (referred to

hereafter as an ethanol-producing microorganism), and (ii) contacting the ethanol-producing microorganism with a deacetylated saccharified biomass whereby the ethanol-producing microorganism consumes the deacetylated saccharified biomass and produces ethanol. The ethanol-producing microorganism may be any ethanol-producing microorganism and the skilled person is well aware of microorganisms suitable for production of ethanol. As described above, one advantage of the present invention is the low levels of acetyl groups in aqueous suspension obtained after treatment of the lignocellulosic biomass with microalga according to the invention. Some ethanol-producing microorganisms – notably yeasts – may be inhibited by acetyl, e.g. by acetylesters. However, the methods of the present invention allows use of any ethanol-producing microorganism including microorganisms susceptible to acetylesters.

In a preferred embodiment, the said ethanol-producing organism is one falling within the Ascomycota phyla, such as for example within the Saccharomyces genus. In another embodiment the said ethanol-producing organism is one falling within the Proteobacteria phyla, such as within the Zymomonas or Escherichia genera, whether genetically modified or not.

After fermentation the ethanol produced may optionally be recovered from the remaining deacetylated saccharified biomass. In particular, it may be preferable that the produced ethanol is separated from the remaining deacetylated saccharified biomass and from the ethanol-producing microorganisms. Further, it may be preferable that the ethanol produced is at least partly purified from other components of the aqueous suspension, e.g. it may be at least partly separated from the water of the aqueous suspension. This may be done using any conventional method(s).

Separation of a liquid comprising ethanol from solid material, such as remains of the lignocellulosic biomass and the ethanol-producing microorganism may be done by mechanical methods such as filtration and/or settlement of solid materials.

The step of recovering ethanol may also comprise a step of distillation, which may be used to separate ethanol from the aqueous suspension or it may be used to separate ethanol from only the liquid phase of the aqueous suspension. Distillation involves evaporation of ethanol by application of heat, followed by collection of the evaporated ethanol. However, distillation typically results in ethanol of a purity of a maximum of

95–96%. Thus, the step of recovering ethanol may also comprise a step of dehydration allowing production of ethanol of higher purity. Dehydration may be done by any conventional method such as by azeotropic distillation, extractive distillation or by use of molecular sieves to remove water from ethanol.

Sequence identity

A high level of sequence identity indicates likelihood that the first sequence is derived from the second sequence. Amino acid sequence identity requires identical amino acid sequences between two aligned sequences. Thus, a candidate sequence sharing 80% amino acid identity with a reference sequence, requires that following alignment, 80% of the amino acids in the candidate sequence are identical to the corresponding amino acids in the reference sequence. Identity according to the present invention is determined by aid of computer analysis, such as, without limitations, the ClustalW computer alignment program (Higgins D., Thompson J., Gibson T., Thompson J.D., Higgins D.G., Gibson T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680), and the default parameters suggested therein. The ClustalW software is available from as a ClustalW WWW Service at the European Bioinformatics Institute http://www.ebi.ac.uk/clustalw. Using this program with its default settings, the mature (bioactive) part of a query and a reference polypeptide are aligned. The number of fully conserved residues are counted and divided by the length of the reference polypeptide.

The ClustalW algorithm may similary be used to align nucleotide sequences. Sequence identities may be calculated in a similar way as indicated for amino acid sequences.
In one important embodiment, the cell of the present invention comprises a nucleic acid sequence coding, as define herein.

Sequence listing

SEQ ID NO: 1	Nucleotide sequence of cDNA encoding *A. nidulans* acetylxylan esterase	GTGAAGCTCCAGTACCTGCTGTCCATCCTGCTCTACGCGTACTC CTGCACGGCCCTCATGCTGGACCGCCGCGACCCGACGCCCGGGC AGCTCTCCCAGGTGACCGACTTTGGGGATAACCCGACCAACGTC GGGTTTTACATTTACGTGCCCCAGAACCTGGCCTCGAACCCGGC CATCATTGTGGCTATTCATTACTGCACGGGCACGGCCCAGGCGT ACTACTCCGGGACCCCCTACGCGCAGTACGCTGAGACGTACGGG TTTATCGTCATCTACCCCGAGTCCCCCTACTCGGGGACGTGCTG GGACGTCTCCAGCCAGAGCACGCTCACGCACAACGGGGGGGGTA ACTCGAACTCGATTGCGAACATGGTCGACTGGACGATTAACCAG TACAACGCGGACGCCAGCCGGGTCTACGTCACGGGCACGTCGAG

		CGGCGCGATGATGACCAACGTCATGGCTGCTACCTACCCCAACC TGTTTGCCGCGGGGATCGCGTACGCGGGGGTGCCCGCTGGGTGC TTCTACAGCGAGGCGAACGTCGAGGACCAGTGGAACAGCACCTG CGCTCAGGGGCAGAGCATCTCCACGCCTGAGCATTGGGCCCAGA TCGCTCAGGCTATGTACTCCGGCTACGAGGGCTCCCGCCCCAAG ATGCAGATCTACCACGGGTCCGCGGACGCGACGCTCTACCCCCA GAACTACTACGAGACGTGCAAGCAGTGGGCTGGCGTCTTCGGCT ACAACTACGACTCGCCCCAGGAGGTCCAGAACGATACGCCTGTG GCTGGCTGGGCCAAGACCATTTGGGGTGAGAACCTCCAGGGTAT CCTGGCCGACGGCGTCGGGCACAACATCCAGATCCAGGGTGAGG AGGATCTGAAGTGGTTCGGTTTTACGAGC
SEQ ID NO: 2	Amino acid sequence of *A. nidulans* acetylxylan esterase Accesion number: Q5B037.2	VKLQYLLSILLYAYSCTALMLDRRDPTPGQLSQVTDFGDNPTNV GFYIYVPQNLASNPAIIVAIHYCTGTAQAYYSGTPYAQYAETYG FIVIYPESPYSGTCWDVSSQSTLTHNGGGNSNSIANMVDWTINQ YNADASRVYVTGTSSGAMMTNVMAATYPNLFAAGIAYAGVPAGC FYSEANVEDQWNSTCAQGQSISTPEHWAQIAQAMYSGYEGSRPK MQIYHGSADATLYPQNYYETCKQWAGVFGYNYDSPQEVQNDTPV AGWAKTIWGENLQGILADGVGHNIQIQGEEDLKWFGFTS
SEQ ID NO: 3	Nucleotide sequence of cDNA encoding strep tagged A. nidulans acetylxylan esterase	GTGAAGCTCCAGTACCTGCTGTCCATCCTGCTCTACGCGTACTC CTGCACGGCCCTCATGCTGGACCGCCGCGACCCGACGCCCGGGC AGCTCTCCCAGGTGACCGACTTTGGGGATAACCCGACCAACGTC GGGTTTTACATTTACGTGCCCCAGAACCTGGCCTCGAACCCGGC CATCATTGTGGCTATTCATTACTGCACGGGCACGGCCCAGGCGT ACTACTCCGGGACCCCCTACGCGCAGTACGCTGAGACGTACGGG TTTATCGTCATCTACCCCGAGTCCCCCTACTCGGGGACGTGCTG GGACGTCTCCAGCCAGAGCACGCTCACGCACAACGGGGGGGGTA ACTCGAACTCGATTGCGAACATGGTCGACTGGACGATTAACCAG TACAACGCGGACGCCAGCCGGGTCTACGTCACGGGCACGTCGAG CGGCGCGATGATGACCAACGTCATGGCTGCTACCTACCCCAACC TGTTTGCCGCGGGGATCGCGTACGCGGGGGTGCCCGCTGGGTGC TTCTACAGCGAGGCGAACGTCGAGGACCAGTGGAACAGCACCTG CGCTCAGGGGCAGAGCATCTCCACGCCTGAGCATTGGGCCCAGA TCGCTCAGGCTATGTACTCCGGCTACGAGGGCTCCCGCCCCAAG ATGCAGATCTACCACGGGTCCGCGGACGCGACGCTCTACCCCCA GAACTACTACGAGACGTGCAAGCAGTGGGCTGGCGTCTTCGGCT ACAACTACGACTCGCCCCAGGAGGTCCAGAACGATACGCCTGTG GCTGGCTGGGCCAAGACCATTTGGGGTGAGAACCTCCAGGGTAT CCTGGCCGACGGCGTCGGGCACAACATCCAGATCCAGGGTGAGG AGGATCTGAAGTGGTTCGGTTTTACGAGCTGGAGCCACCCGCAG TTCGAGAAGTAA
SEQ ID NO: 4	Amino acid sequence of strep tagged *A. nidulans* acetylxylan esterase	VKLQYLLSILLYAYSCTALMLDRRDPTPGQLSQVTDFGDNPTNV GFYIYVPQNLASNPAIIVAIHYCTGTAQAYYSGTPYAQYAETYG FIVIYPESPYSGTCWDVSSQSTLTHNGGGNSNSIANMVDWTINQ YNADASRVYVTGTSSGAMMTNVMAATYPNLFAAGIAYAGVPAGC FYSEANVEDQWNSTCAQGQSISTPEHWAQIAQAMYSGYEGSRPK MQIYHGSADATLYPQNYYETCKQWAGVFGYNYDSPQEVQNDTPV AGWAKTIWGENLQGILADGVGHNIQIQGEEDLKWFGFTSWSHPQ FEK
SEQ ID NO: 5	Nucleotide sequence of cDNA encoding strep tagged A. nidulans acetylxylan esterase comprising the gametolysin signal peptide	ATGTCGCTGGCGACGCGGCGCTTCGGCGCCGCAGCGGCGCTTCT AGTCGCCGCATGCGTGCTGTGCACAGCTCCTGCGTGGGCCGTGA AGCTCCAGTACCTGCTGTCCATCCTGCTCTACGCGTACTCCTGC ACGGCCCTCATGCTGGACCGCCGCGACCCGACGCCCGGGCAGCT CTCCCAGGTGACCGACTTTGGGGATAACCCGACCAACGTCGGGT TTTACATTTACGTGCCCCAGAACCTGGCCTCGAACCCGGCCATC ATTGTGGCTATTCATTACTGCACGGGCACGGCCCAGGCGTACTA CTCCGGGACCCCCTACGCGCAGTACGCTGAGACGTACGGGTTTA

		TCGTCATCTACCCCGAGTCCCCCTACTCGGGGACGTGCTGGGAC GTCTCCAGCCAGAGCACGCTCACGCACAACGGGGGGGGTAACTC GAACTCGATTGCGAACATGGTCGACTGGACGATTAACCAGTACA ACGCGGACGCCAGCCGGGTCTACGTCACGGGCACGTCGAGCGGC GCGATGATGACCAACGTCATGGCTGCTACCTACCCCAACCTGTT TGCCGCGGGGATCGCGTACGCGGGGGTGCCCGCTGGGTGCTTCT ACAGCGAGGCGAACGTCGAGGACCAGTGGAACAGCACCTGCGCT CAGGGGCAGAGCATCTCCACGCCTGAGCATTGGGCCCAGATCGC TCAGGCTATGTACTCCGGCTACGAGGGCTCCCGCCCCAAGATGC AGATCTACCACGGGTCCGCGGACGCGACGCTCTACCCCCAGAAC TACTACGAGACGTGCAAGCAGTGGGCTGGCGTCTTCGGCTACAA CTACGACTCGCCCCAGGAGGTCCAGAACGATACGCCTGTGGCTG GCTGGGCCAAGACCATTTGGGGTGAGAACCTCCAGGGTATCCTG GCCGACGGCGTCGGGCACAACATCCAGATCCAGGGTGAGGAGGA TCTGAAGTGGTTCGGTTTTACGAGCTGGAGCCACCCGCAGTTCG AGAAGTAA
SEQ ID NO: 6	Amino acid sequence of *Trichoderma reesei* acetylxylan esterase Accesion number: CAA93247.1	MPSVKETLTLLLLSQAFLATGSPVDGETVVKRQCPAIHVFGARET TVSQGYGSSATVVNLVIQAHPGTTSEAIVYPACGGQASCGGISY ANSVVNGTNAAAAAINNFHNSCPDTQLVLVGYSQGAQIFDNALC GGGDPGEGITNTAVPLTAGAVSAVKAAIFMGDPRNIHGLPYNVG TCTTQGFDARPAGFVCPSASKIKSYCDAADPYCCTGNDPNVHQG YGQEYGQQALAFINSQLSSGGSQPPGGGPTSTSRPTSTRTGSSP GPTQTHWGQCGGQGWTGPTQCESGTTCQVISQWYSQCL
SEQ ID NO: 7	Amino acid sequence of *Talaromyces purpureogenus* acetylxylan esterase Accesion number: Q8NJP6.1	MKSLSFSFLVTLFLYLTLSSARTLGKDVNKRVTAGSLQQVTGFG DNASGTLMYIYVPKNLATNPGIVVAIHYCTGTAQAYYTGSPYAQ LAEQYGFIVIYPQSPYSGTCWDVSSQAALTHNGGGDSNSIANMV TWTISQYNANTAKVFVTGSSSGAMMTNVMAATYPELFAAATVYS GVGAGCFYSSSNQADAWNSSCATGSVISTPAVWGGIAKNMYSGY SGSRPRMQIYHGSADTTLYPQNYYETCKQWAGVFGYNYDSPQST LANTPDANYQTTNWGPNLQGIYATGVGHTVPIHGAKDMEWFGFS GSGSSSTTTASATKTSTTSTTSTKTTSSTSSTTTSSTGVAAHWG QCGGSGWTGPTVCESGYTCTYSNAWYSQCL
SEQ ID NO: 8	Amino acid sequence of *Thermoanaerobacterium* Sp. acetylxylan esterase Accesion number: 3FCY_C Accesion number: Q8NJP6.1	MKHHHHHHPMSDYDIPTTENLYFQGAMGLFDMPLQKLREYTGTN PCPEDFDEYWNRALDEMRSVDPKIELKESSFQVSFAECYDLYFT GVRGARIHAKYIKPKTEGKHPALIRFHGYSSNSGDWNDKLNYVA AGFTVVAMDVRGQGGQSQDVGGVTGNTLNGHIIRGLDDDADNML FRHIFLDTAQLAGIVMNMPEVDEDRVGVMGPSQGGGLSLACAAL EPRVRKVVSEYPFLSDYKRVWDLDLAKNAYQEITDYFRLFDPRH ERENEVFTKLGYIDVKNLAKRIKGDVLMCVGLMDQVCPPSTVFA AYNNIQSKKDIKVYPDYGHEPMRGFGDLAMQFMLELYS
SEQ ID NO: 9	Gametolysin signal peptide	MSLATRRFGAAAALLVAACVLCTAPAWA

Examples

The invention is further illustrated by the following examples, which however are not intended as being limiting for the invention.

Materials and Methods

All standard chemicals and reagents were purchased from SigmaAldrich (http://www.sigmaaldrich.com). Restriction enzymes were purchased from New England Biolabs (https://www.neb.com). All the oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Strains and culture conditions.

C. reinhardtii cell wall-deficient strain UVM4 (Neupert et al., 2009) and the transgenic lines were cultivated in TAP media containing 1g/L acetic acid as the sole organic carbon source unless otherwise noted in examples [7 & 8]. Additionally all cultures throughout all examples [1-7] were supplemented with antibiotics to reduce the risk of contamination (50 $\mu g \cdot mL^{-1}$ ampicillin, 25 $\mu g \cdot mL^{-1}$ kasugamycin). Liquid cultures of C. reinhardtii were cultivated under constant illumination, using cool fluorescent white light at 120 rpm in an orbital shaker at 25°C. Cell concentration in the culture was determined by counting triplicate samples in a Neubauer haemocytometer.

Example 1

To obtain the expression cassette shown in figure 1, the nucleic acid sequence encoding Acetylxylan esterase (AXE) from Aspergillus nidulans SEQ ID NO:1 was codon optimized without its native signal peptide according to C. reinhardtii nuclear genome using the IDT Codon Optimization tool (https://eu.idtdna.com/CodonOpt). Codon optimized AnAXE was amplified using overlapping primers
(AXE-F: gctgtgcacagctcctgcgtgggccgtgaagctccagtacctgct,
AXE-R: cggttacttctcgaactgcgggtggctccagctcgtaaaaccgaa)
and cloned into pERC-SSVenus vector in frame with gametolysin signal peptide via Overlap extension PCR cloning as described by Bryksin and Matsumura (2010). The vector also contains the APHVIII resistance gene for selection on paromomycin, whose expression is controlled by the constitutively PSAD promoter. Transformation of E. coli strain TOP10, plasmid isolation and confirmation of the DNA sequence were according to standard protocols (Sambrook and Russell, 2001).

Example 2

C. reinhardtii cell wall deficient UVM4 mutant was transformed according to the glass bead method (Kindle, 1990), using 2 µg of transformation vector linearized with ScaI. Cells were incubated overnight then harvested at 1100 xg for 5 min and plated on TAP agar plates containing 15 $\mu g \cdot mL^{-1}$ paromomycin. After seven days of incubation a total

of 96 independent paromomycin resistant colonies were picked and cultivated under selective conditions to later be screened for gene integration by colony PCR as described by Cao et al. (2009) using gene specific oligonucleotides (CSP2 (also known as GSP3) 5´-CTAGAACTAGTGCTGAGGCTTG and GSP4 5'-CGAAGGATCCCGCTTCAAATAC).

In figure 2 shows the results of two lines, CrAXE03 and CrAXE23, grown in standard tris-acetate-phosphate (TAP) media, wherein the amount of acetic acid was $1g \cdot L^{-1}$, for 4 days and the presence of the crAXE transcript was analyzed by RT-PCR as follows: Total RNA was isolated from transformants grown for 4 days in TAP media ($1X10^7$ cells) using 1 ml TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Concentrations and qualities of purified RNAs were determined spectrophotometrically at 260 nm and by electrophoresis in on 1% (w/v) agarose gels, respectively. One microgram of each sample was reverse transcribed into cDNA with an iScript cDNA Synthesis kit (Bio-Rad) following the manufacturer's instructions. Based on the sequence of crAXE gene, primers designated as MR11 (CTCATGCTGGACCGCCGC) and MR12 (TTCTCGAACTGCGGGTGGCT) were designed for specific PCR amplification of a 879 bp crAXE fragment. PCR was performed with 2 µL of cDNA in a 50 µL reaction using HotMaster Taq DNA Polymerase (5PRIME, Germany) and gene specific primers. At the same time, PCRs were performed with primers Rack-F2 (TCCGTGGCTTTCTCGGTG) and Rack-R2 (GGTGTTGACGTAGCCGTG), which served to amplify housekeeping gene RACK, cDNA from non-transformed cells was used as a negative control. RT-PCR products were analyzed by gel electrophoresis.

Rack1 is a housekeeping gene and was used as a positive control. The crAXE transcript is detectable both in CrAXE03 and CrAXE23 lines while it was absent in WT. In contrast, all three strains showed the presence of the Rack1 indicating that the crAXE gene is expressed in CrAXE03 and CrAXE23 lines.

Example 3

In figure 3, CrAXE (SEQ ID NO:4) accumulation in the supernatant and cell lysates is demonstrated by immunoblotting using an anti-Strep antibody. Cell lysates obtained from two transgenic lines (CrAXE03 and CrAXE23) gave rise to a weak band at ca. 33 kD, which is expected based on the amino acid composition of the recombinant protein. In contrast, the cell-free media obtained from the same transgenic cultures gave rise to a stronger band at ca. 35 kDa. These results indicate that the recombinant crAXE

protein is expressed in CrAXE03 and CrAXE23 lines and secreted into the growth media. Larger apparent molecular size of the secreted crAXE protein detected in the media in comparison to crAXE detected in the cell lysates suggest that the secreted crAXE protein is glycosylated.

Immunoblotting was performed as follows: Cultures of transgenic lines were harvested by centrifugation at 5000 xg for 5 min at 25 °C. Pellets were resuspended in 4X Laemmli buffer (Bio-Rad, USA) and denatured at 90 °C for 5 min. Supernatants were either used directly for analysis or concentrated ten-fold by freeze drying and denatured in the presence of 4X Laemmli buffer as indicated above. Lysates and/or supernatants were subjected western blot for detection, samples were loaded on a 12% (w/v) Criterion™ TGX Stain-Free™ Protein Gel (BioRad, USA) and separated proteins were transferred to a polyvinylidene difluoride membrane (PVDF). Immunodetection of crAXE was carried out using Strep-tag Antibody (QIAGEN, Germany) in a 1:2000 dilution according to the manufacturer instructions. No cross reactivity with the proteins native to C. reinhardtii was detected by the anti-Strep tagantibody under the experimental conditions employed in this study. The secondary antibody (anti-mouse, lgG secondary antibody, horseradish peroxidase conjugate, SigmaAldrich) was used in a 1:2500 dilution.

Example 4

Figure 4 shows the results of growing the two transgenic lines in the TAP media until culture reached stationary phase. In order to determinate if the secreted recombinant crAXE protein (SEQ ID NO:4) has esterase activity, cell-free media were directly incubated in triplicates with 4-nitrophenyl acetate as the substrate and enzymatic activity was monitored every 10 min at 410 nm as detailed above. The esterase activity of the recombinant crAXE was compared to a commercial acetylxylan esterase from Orpinomyces sp. (Megazymes) and parental strain (the UVM strain) was use as a control. Both CrAXE03 and CrAXE23 lines showed strong activities while no activity was detectable for WT.

To determinate the enzymatic activity of the secreted acetylxylan esterase, transgenic lines were grown in liquid TAP media in conditions described above. Acetylesterase activity was monitored after 5 days of cultivation or every day. For each sample, cells were separated from the media by centrifugation at 3500·g for 5 min and 15 µL the cell-free media were transferred in triplicate in a 96-well plate. Substrate (35 µM 4-

nitrophenyl acetate in 100 mM potasium phosphate buffer, pH 7.0) was added to a final volume of 300 µL (Chung et al., 2002). Reactions were incubated at 37˚C and readings were taken at 410 nm in a SpectraMax 190 Microplate Reader (Molecular Devices, USA) every 5 minutes for 30–60 minutes. Product formation rates were calculated as µmol·min^{-1}·mL^{-1}.

Example 5

Wildtype (WT) and CrAXE-secreting (SEQ ID NO:4) lines were grown in TAP media and the growth kinetics were monitored. The AnAXE transgenic lines grew similarly to WT at the later growth phase albeit with slight delay during early growth phase, as compared to WT. These results indicate that there is a minimal metabolic cost as a result of the transgene expression in the CrAXE strains. Concomitant to the increasing cell numbers, increasing acetylesterase activities were recorded in CrAXE transgenic line media. The results obtained are shown in figure 5.

Example 6

Cell-proliferation rates of WT and CrAXE03 strains were compared in a modified TAP media, designated as TAP0.1, which contained 0.1 mL·L^{-1} acetic acid and was supplemented with two concentrations of wheat ethanol-insoluble residue (AIR) biomass (8 and 15 mg·L^{-1}). AIR was obtained by reducing wheat biomass to small pieces with a ball miller and then subjecting it to several washes with 96% (v/v) ethanol, 70% (v/v) ethanol, 100% (v/v) acetone and allowing to dry. This modified TAP media was used to improve the initial growth of the algal cells. Final cell numbers and remaining acetate in the medium supernatants and in the wheat lignocellulosic biomass were measured after 12 days by filtering the saponified samples through a 0.22 µm membrane and transferring them to a HPLC vial. Acetic acid in the samples was separated with a Synergi Hydro-RP (Phenomenex) column, detected with a prominence DAD SPD-M20A Shimadzu and quantified based on an external calibration curve. The samples were run isocratically at 20 mM K_3PO_4 (pH 2.9), at 0.6ml·min^{-1}. Results are shown in figure 6; the parental strain showed limited growth when cultivated in the TAP0.1 media supplemented with 8 and 15 mg·L^{-1} wheat biomass AIR, demonstrating that the parental strain is not capable of utilizing the wheat biomass AIR as a carbon source for growth. When a commercial xylan acetylesterase (200 mU) was added to the culture of the parental strain, significant enhancement of the growth was discernible, indicating that indeed acetic acid released by a xylan acetylesterase can

support the growth of the microalga. Remarkably, CrAXE03 was able to grow in the TAP0.1 medium supplemented with 8mg·L^{-1} wheat biomass AIR without addition of the commercial xylan acetylesterase. Even more surprisingly, crAXE03 was able to grow better than WT with the addition of 200mU commercial acetylesterase, thus demonstrating the increased efficiency of the microalga of the present invention over the use of algae in the presence of a commercially purified enzyme. Further increase in the growth was observed with an increasing amount of the wheat biomass to 15 mg·mL^{-1}.

The acetic acid contents in the culture supernatants were measured by reverse-phase high-pressure liquid chromatography (RP-HPLC). No detectable level of free acetic acid was measured in any of the sample (data not shown), which is consistent with the ability of C. reinhardtii to use acetic acid in the media. The levels of acetylesters bound to the wheat biomass AIR were determined by collecting the wheat biomass AIR from the culture supernatants by centrifugation and hydrolysing polymer-bound acetylesters by sodium hydroxide treatment as detailed above. The resulting hydrolysates, containing free acetic acid, were analysed by RP-HPLC. Alkaline-labile acetylester content in the wheat biomass AIR obtained from wheat biomass AIR derived from the parental strain was almost the same as that the untreated wheat biomass AIR, corroborating with the growth data that the parental strain is not capable of hydrolysing polymer-bound acetylester. In contrast, the level of acetylester found in the biomass co-cultivated with the parental strain in the presence of a commercial xylan acetylesterase was significantly lower as compared to the afore-mentioned samples, and exhibited approximately 20-25% of the untreated wheat biomass AIR. Surprisingly, co-cultivation with the transgenic CrAXE03 line reduced the content of alkaline-labile acetylester even further, to below 5% of that in the untreated wheat biomass AIR. These results demonstrate that CrAXE03 secreting crAXE (SEQ ID NO:4) can effectively hydrolyse xylan acetyl esters and sequester acetic acid released in to the media and use it as a carbon source for cell-proliferation.

Example 7

Cultures of CrAXE03 were monitored for 12 days in the TAP0.1 media supplemented with 15 mg mL-1 of wheat straw AIR. The cultures were let stand without mixing for 8 days after which the CrAXE03 culture had significant growth enhancement over WT as result of CrAXE (SEQ ID NO:4) activity released by the cells. Results are shown in figure 7.

Example 8

In-silico sequence alignment of acetylxylan esterases polypeptide sequences listed in the section “Sequence listing” herein above SEQ ID NO:1 (Aspergillus nidulans Q5B037.2), SEQ ID NO:6 (Trichoderma reesei CAA93247.1), SEQ ID NO:7 (Talaromyces purpureogenus Q8NJP6.1), SEQ ID NO:8 (Thermoanaerobacterium Sp. 3FCY_C) are shown in figure 8. Identical amino acids with all other sequences are indicated by asterisks, conserved substitutions are indicated by colons, semi-conserved substitutions are indicated by periods. The alignment was performed with Clustal W.

Claims

1. A method for producing ethanol comprising steps of:
(a) providing a lignocellulosic biomass;
(b) providing a microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetylesterase under the control of a nucleic acid sequence directing expression of said acetyl esterase in said microalga;
(c) contacting the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass with reduced levels of acetyl groups ;
(d) contacting the deacetylated lignocellulosic biomass with one or more lignocellulolytic enzymes, thereby obtaining a deacetylated saccharified biomass.
(e) fermenting the deacetylated saccharified biomass with one or more ethanol-producing microorganisms thereby producing ethanol; and
(f) optionally, recovering the ethanol from the remaining deacetylated saccharified biomass

wherein steps (a) and (b) may be performed simultaneously or sequentially in any order, and steps (d) and (e) may be performed simultaneously, partly simultaneously or sequentially in any order.

2. A method for producing a deacetylated lignocellulosic biomass comprising steps of:
(a) providing a lignocellulosic biomass;
(b) providing a microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetyl esterase under the control of a nucleic acid sequence directing expression of said acetyl esterase in said microalga;
(c) contacting the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass with reduced levels of acetyl groups ;

wherein steps (a) and (b) may be performed simultaneously or sequentially in any order.

3. The method according to any one of the preceding claims, wherein step (c) comprises or consists of incubating the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic

biomass comprising reduced levels of acetyl groups bound to biopolymers and a reduced level of acetyl groups in the aqueous suspension.

4. The method according to any one of the preceding claims, wherein step (c) comprises or consists of incubating the lignocellulosic biomass with the microalga in an aqueous suspension, thereby producing a deacetylated lignocellulosic biomass comprising reduced levels of acetyl groups and an increased level of algal biomass.

5. The method according to any one of the preceding claims, wherein said levels are reduced compared to the starting level of acetyl groups in the lignocellulosic biomass provided in step (a).

6. The method according to any one of the preceding claims, wherein the lignocellulosic biomass contacted with the microalga in step (c) has not been pretreated.

7. The method according to any one of the preceding claims, wherein the aqueous suspension contains aqueous suspension and microalgae from a previously-treated aqueous suspension batch.

8. A microalga comprising at least one heterologous nucleic acid encoding a polypeptide comprising an acetylesterase under the control of a nucleic acid sequence directing expression of said acetylesterase in said microalga.

9. The microalga according to claim 8, wherein said microalga secretes one or more lignocellulolytic enzymes.

10. The microalga according to any one of claims 8 to 9, wherein the microalga is a chlorophyte, such as of the genus Chlamydomonas, e.g. C. reinhardtii.

11. The microalga according to any one of claims 8 to 10, wherein the microalga is capable of utilising free acetyl groups produced by acetyl esterase as carbon source.

12. The microalga according any one of claims 8 to 11, wherein the acetylesterase is a fungal enzyme, preferably from a filamentous fungus such as the genus Aspergillus e.g. A. nidulans.

13. The microalga according to any one of claims 8 to 12, wherein the acetyl esterase is an acetylxylan esterase classified under EC 3.1.1.72.

14. The microalga according to any one of claims 8 to 13, wherein the acetylesterase is a polypeptide of SEQ ID NO: 2 or a functional homologue thereof having a sequence identity with the sequence set out in SEQ ID NO: 2 of at least 70 %, such as at least 85%, for example at least 90%, such as at least 95%.

15. The microalga according to any one of claims 8 to 13, wherein the acetylesterase is a polypeptide of SEQ ID NO: 6, 7 or 8 or a functional homologue thereof having a sequence identity with the sequence set out in SEQ ID NO: 6, 7 or 8 of at least 70 %, such as at least 85%, for example at least 90%, such as at least 95%.

16. The microalga according to any one of claims 8 to 15, wherein the polypeptide comprising the acetyl esterase further comprises a signal peptide directing secretion of the polypeptide.

17. The microalga according to any one of claims 8 to 16, wherein the polypeptide comprising the acetyl esterase further comprises a tag peptide.

18. The microalga according to any one of claims 8 to 17, wherein the polypeptide comprising the acetyl esterase is a polypeptide of SEQ ID NO: 4 or 5 or a functional homologue thereof having a sequence identity with the sequence set out in SEQ ID NO: 4 or 5 of at least 80 %, at least 85%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, or at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

19. The microalga according to any one of claims 8 to 18, wherein the polypeptide comprises or consists of a polypeptide sharing at least 15% sequence identity with one of the polypeptides of SEQ ID NO: 2, 4, 5, 6, 7, or 8, wherein the polypeptide contains as least the amino acids marked by “*” in figure 8.

20. The microalga according to any one of claims 8 to 19, wherein the heterologous nucleic acid comprises or consists of a nucleotide sequence that has a sequence identity to the coding sequence of SEQ ID NO: 1 or 3 of at least 80%, at least

85%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%.

21. The microalga according to any one of claims 8 to 20, wherein the polypeptide comprising the acetyl esterases is secreted by the microalga.

22. The method according to any one of claims 1 to 7, wherein the microalga is a microalga according to any one of claims 8 to 21.

23. The method according to any one of claims 1 to 7 and 22, wherein the lignocellulosic biomass material comprises or consists of plant parts.

24. The method according to claim 23, wherein the plant parts are virgin biomass, waste biomass, energy crops or any combination thereof.

25. The method according to any one of claims 1 to 7 and 22 to 24, wherein the lignocellulosic biomass material is selected from the group consisting of cereal straw, wood, leaves, stems, seeds, roots, inflorescences corn stower, switchgrass, forest residues, agricultural residues, industrial residues, wood chips, and municipal solid waste.

26. The method according to any one of claims 1 to 7 and 22 to 25, wherein the content of acetyl groups in the aqueous suspension is at most 4% (w/w), preferably at the most 3% (w/w), more preferably at the most 2% (w/w), even more preferably at the most 1% (w/w), or even more preferably at the most 0,5% (w/w).

27. The method according to any one of claims 1 to 7 and 22 to 26, wherein the final acetyl-groups concentration in the deacetylated lignocellulosic biomass material is reduced by at least 5% (w/w), at least 6% (w/w), at least 7% (w/w), at least 8% (w/w), at least 9% (w/w), at least 10% (w/w), at least 15% (w/w), at least 20% (w/w), at least 30% (w/w), at least 40% (w/w), or at least 50% (w/w) compared to the starting lignocellulosic biomass.

28. The method according to any one of claims 1 to 7 and 22 to 27, wherein the final concentration of acetyl bound to biopolymers in the deacetylated lignocellulosic biomass is reduced by at least 5% (w/w), at least 6% (w/w), at least 7% (w/w), at

least 8% (w/w), at least 9% (w/w), at least 10% (w/w), at least 15% (w/w), at least 20% (w/w), at least 30% (w/w), at least 40% (w/w), or at least 50% (w/w) compared to the starting lignocellulosic biomass.

29. The method according to any one of claims 1 to 7 and 22 to 28, wherein free acetyl groups produced by acetyl esterase activity in step (c) are used as carbon source promoting algal cell-proliferation.

30. The method according to any one of claims 1 to 7 and 22 to 29, wherein the lignocellulosic biomass comprises more algae than provided in step (b).

31. The method according to any one of claims 1 to 7 and 22 to 30, wherein the deacetylated lignocellulosic biomass comprise at least twice, such as at least 4 times, for example at least 6 times, such as at least 10 times the amount of microalgal biomass compared to the amount of microalgal biomass provided in step (b).



Fig.1

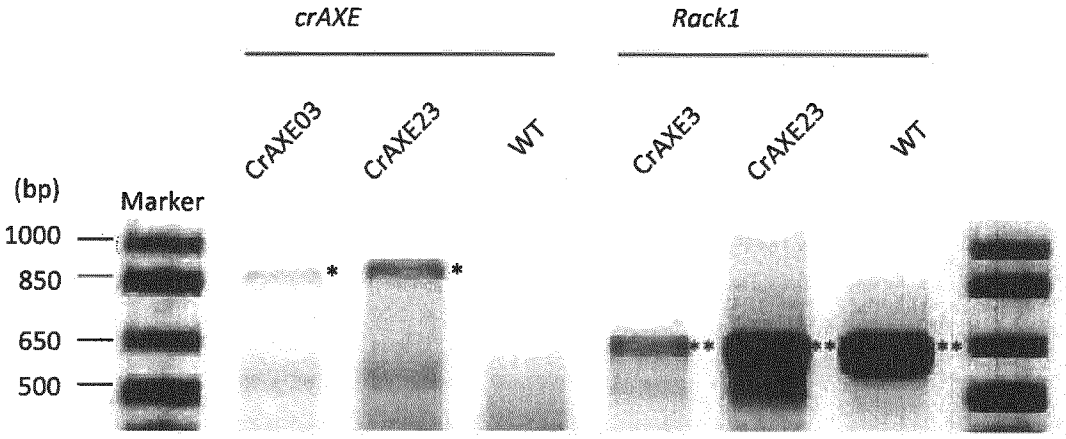


Fig.2

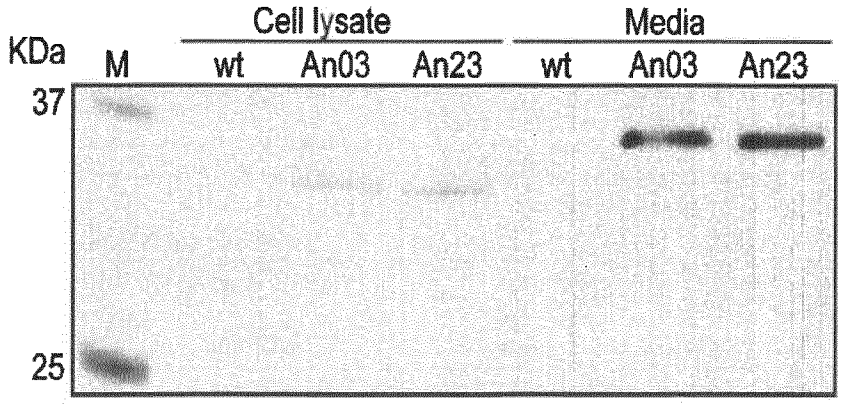


Fig.3

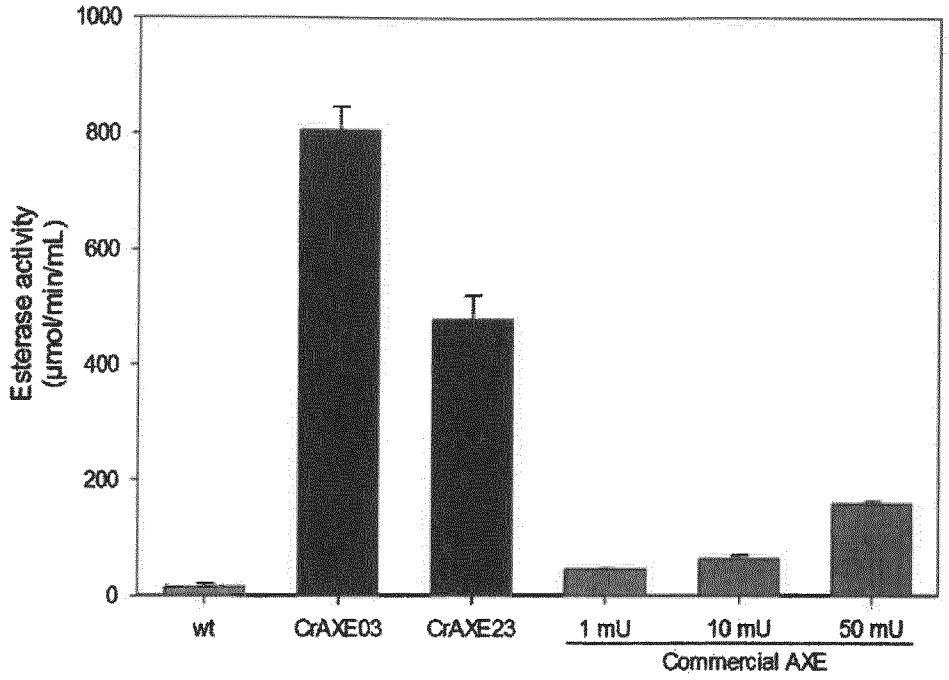

1000
800
600
400
200
0
Esterase activity
(µmol/min/mL)
wt
CrAXE03
CrAXE23
1 mU
10 mU
50 mU
Commercial AXE

Fig.4

Growth curves of the CrAXE strains and WT

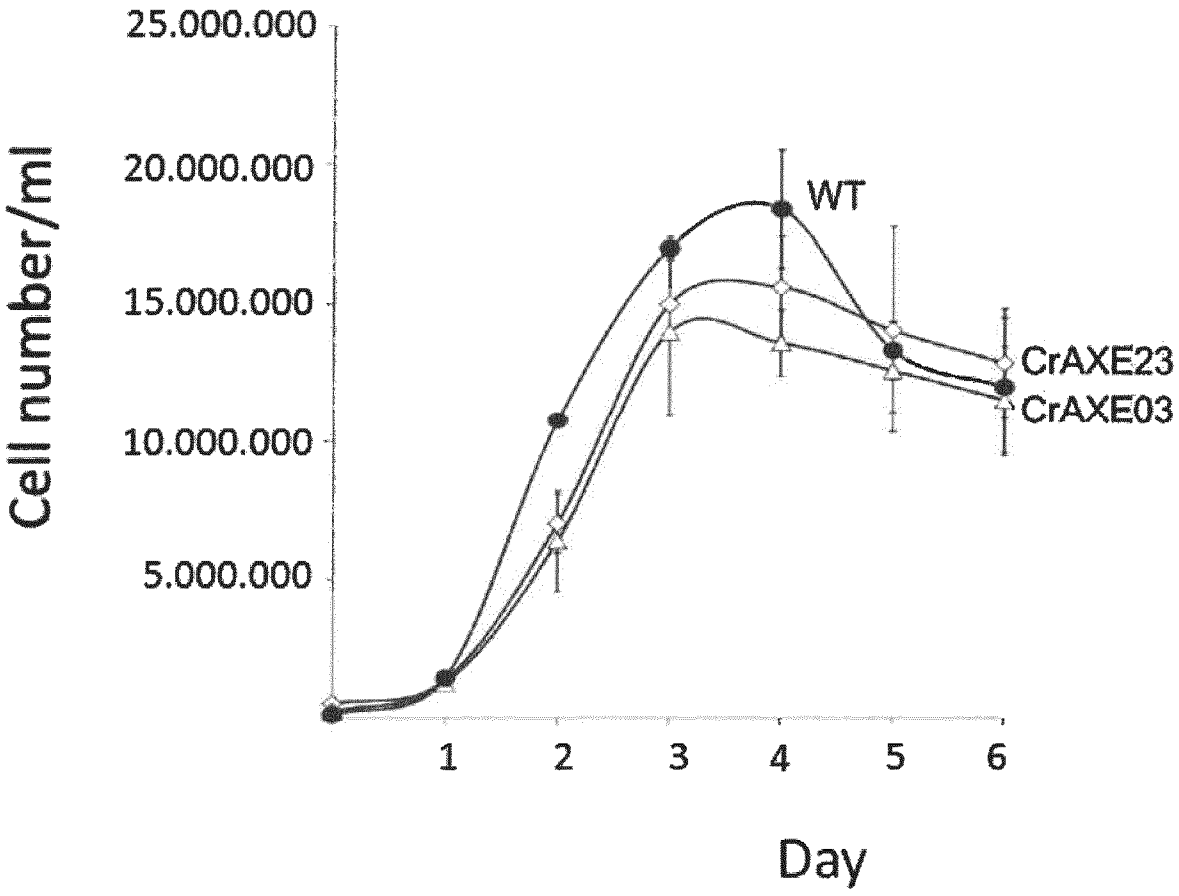


Fig. 5A

Acetylesterase activities secreted by the CrAXE strains and WT

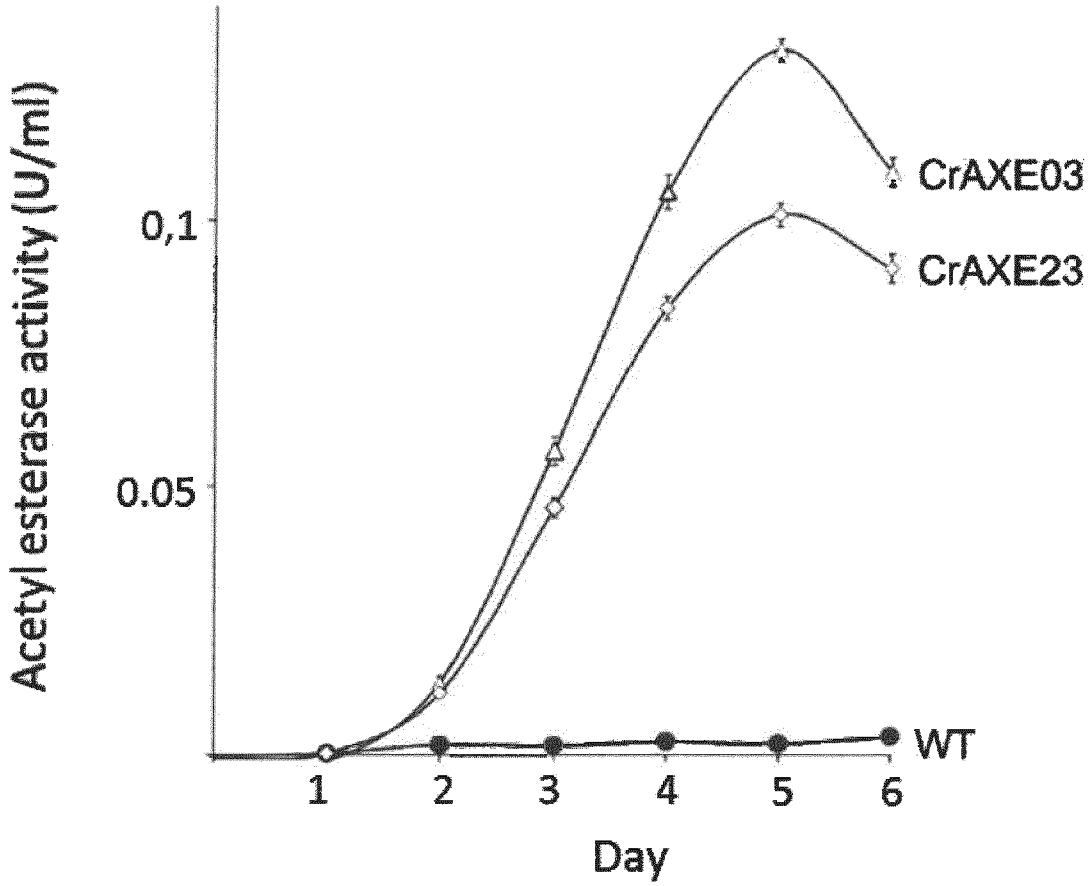


Fig.5B

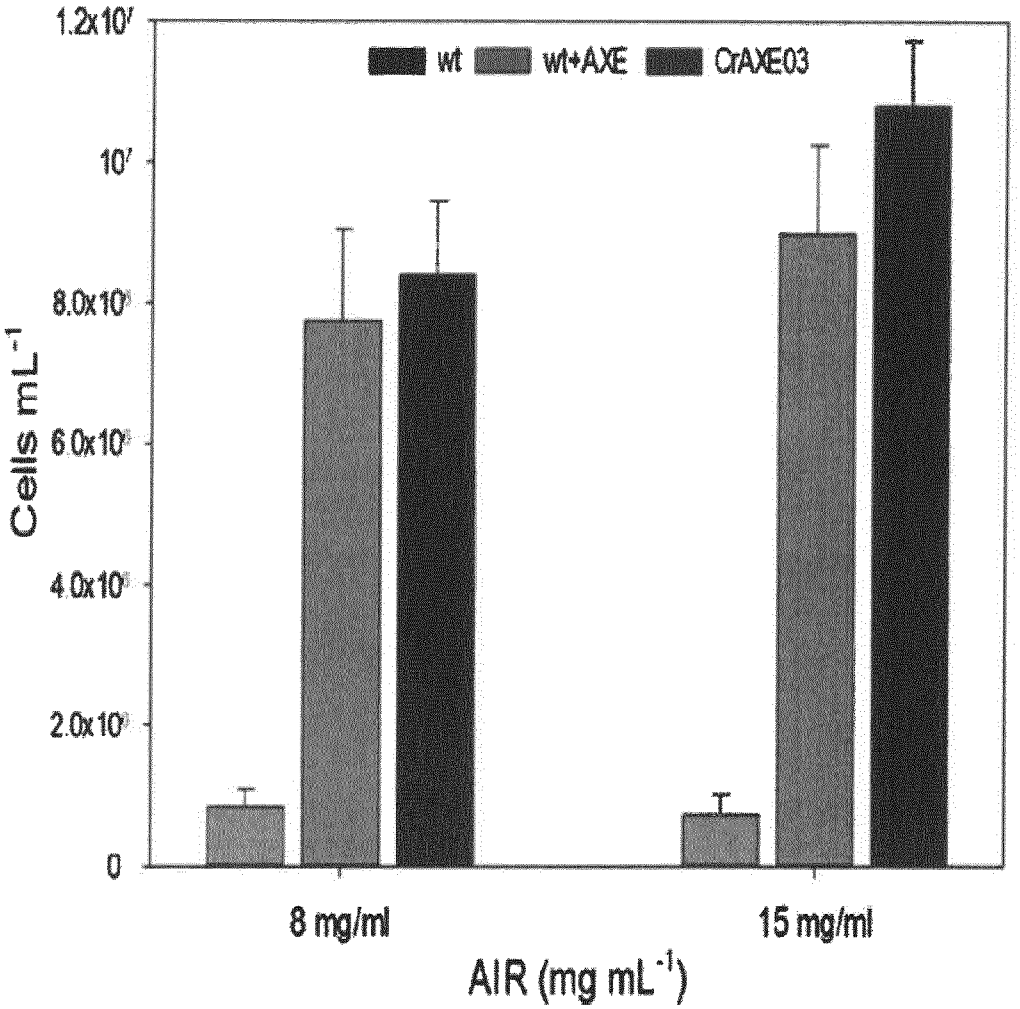


Fig. 6A

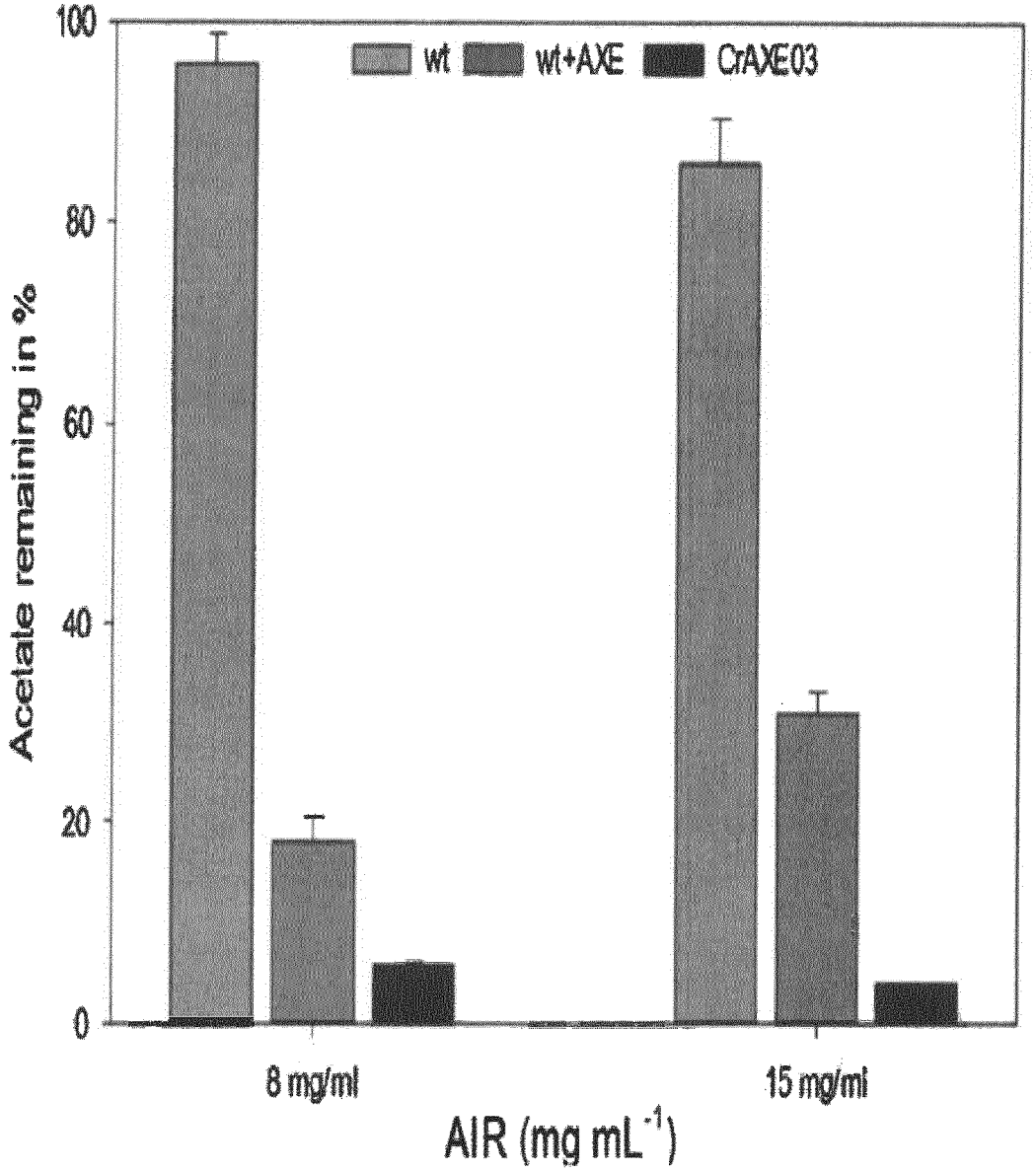

100
80
60
40
20
0
wt
wt+AXE
CrAXE03
Acetate remaining in %
8 mg/ml
15 mg/ml
AIR (mg mL-1)

Fig.6B

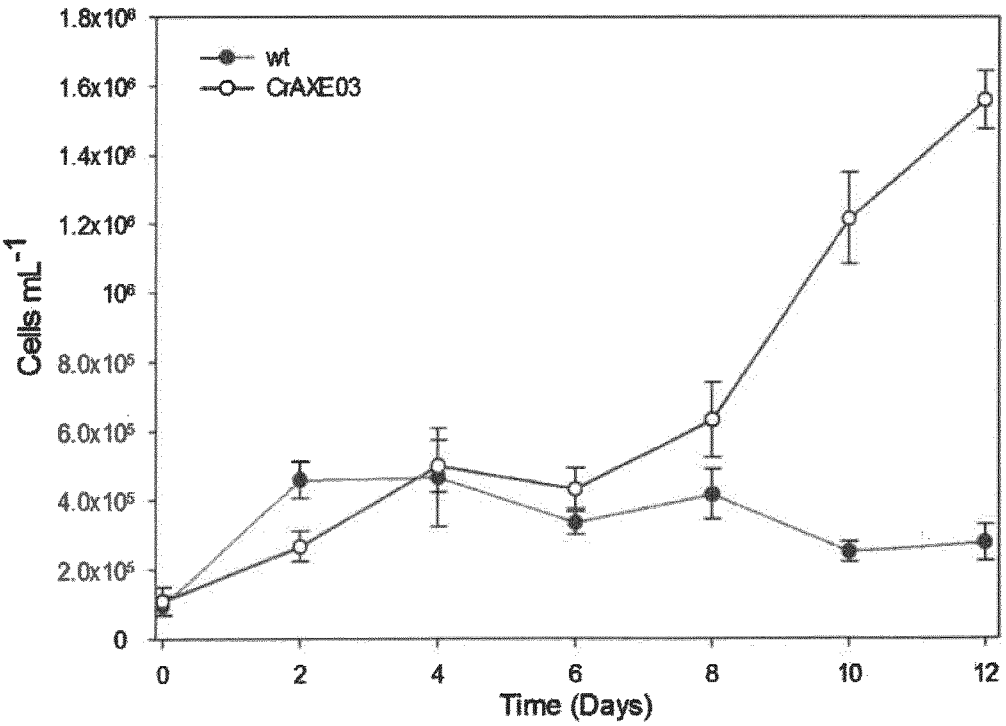

1.8x10⁶
1.6x10⁶
1.4x10⁶
1.2x10⁶
10⁶
8.0x10⁵
6.0x10⁵
4.0x10⁵
2.0x10⁵
0
Cells mL⁻¹
wt
CrAXE03
0
2
4
6
8
10
12
Time (Days)

Fig.7

```
3FCY_C          MKHHHHHHPMSDYDIPTTENLYFQGAMGLFDMPLQK---LREYTGTNPCPEDFDEYWNRA
CAA93247.1      -------MPSVK-ETLTLLLSQAFLATGS---PVDG---ETVVKRQCPAIHVF-------
Q5B037.2        ---------MVKLQYLLSILLYAYSCTAL---MLDR---RDPTPGQLSQVTDF-------
Q8NJP6.1        -------MKSLSFSFLVTLFLYLTLSSAR---TLGKDVNKRVTAGSLQQVTGF-------
                           . .           . .    :                    *

3FCY_C          LDEMRSVDPKIELKESSFQVSFAECYDLYFTGVRGARIHAKYIKPKTEGKHPALI--RFH
CAA93247.1      -------------------------------GARETTV------SQGYGSSATVVNLVIQ
Q5B037.2        -------------------------------GDNPTNVGFYIYVPQNLASNPAII-VAIH
Q8NJP6.1        -------------------------------GDNASGTLMYIYVPKNLATNPGIV-VAIH
                                              * . :        .:  .. . ::   ::

3FCY_C          GYSSNSGDWNDKLNYV----AAGFTVVAMDVRGQGGQSQDVGGVTGNTLNGHIIRGLDDD
CAA93247.1      AHPGTTSE----------------AIVYPACGGQAS----CGGISYAN---SVVNGTNAA
Q5B037.2        YCTGTAQAYYSGTPYAQYAETYGFIVIYPESPYSGT----CWDVSSQS---TLTHNGGGN
Q8NJP6.1        YCTGTAQAYYTGSPYAQLAEQYGFIVIYPQSPYSGT----CWDVSSQA---ALTHNGGGD
                  ...:                    ::      ..      .::       : .. .

3FCY_C          ADNMLFRHIFLDTAQLAGIVMNMPEVDEDRVGVMGPSQGGGL--SLACAALEPRVRKVVS
CAA93247.1      AAAI---------NNFHN------SCPDTQLVLVGYSQGAQIFDNALCGGGDPGEGITNT
Q5B037.2        SNSI---------ANMVDWTINQYNADASRVYVTGTSSGAMM--TNVMAATYPNLFAAGI
Q8NJP6.1        SNSI---------ANMVTWTISQYNANTAKVFVTGSSSGAMM--TNVMAATYPELFAAAT
                :  :          ::        .    .: : * *.*. :  .   ..  *    .

3FCY_C          EYP------FLSDYKR--VWDLDLAKNA-------YQEITDYFRLFDPRHERENEVFTKL
CAA93247.1      AVP-------------------LTAGA-------VSAVKAAIFMGDPRNIHGLPYN--V
Q5B037.2        AYAGVPAGCFYSEANVEDQWNSTCAQGQSISTPEHWAQIAQAMYSGYEGSRPKMQIY--H
Q8NJP6.1        VYSGVGAGCFYSSSNQADAWNSSCATGSVISTPAVWGGIAKNMYSGYSGSRPRMQIY--H
                  .                    : .          :   :

3FCY_C          GYIDVKNLAKRIKGDVLMCVGLMDQVCP---------PSTVFAAYNNIQSKKDI------
CAA93247.1      GTCTTQGFDARPAGFVCPSASKIKSYCD---------AADPYCCTGNDPNVHQGYGQEYG
Q5B037.2        GSADATLYPQNYYETCKQWAGVFGYNYDSPQEVQNDTPVAGWAKTIWGENLQGILADGVG
Q8NJP6.1        GSADTTLYPQNYYETCKQWAGVFGYNYDSPQSTLANTPDANYQTTNWGPNLQGIYATGVG
                *   .    .        .. :              .   :    . . :

3FCY_C          KVYPDYGHEPMRGFGDLA-----------------------------------------
CAA93247.1      QQALAFINSQLSSGGSQPPGGGPTSTSRPTSTRTGSSPGPTQT--------------HW
Q5B037.2        HNIQIQGEEDLKWFGFTS-----------------------------------------
Q8NJP6.1        HTVPIHGAKDMEWFGFSGSGSSSTTTASATKTSTTSTTSTKTTSSTSSTTTSSTGVAAHW
                :       . :   *

3FCY_C          ------------------MQFMLELYS---
CAA93247.1      GQCGGQGWTGPTQCESGTTCQVISQWYSQCL
Q5B037.2        -----------------------------
Q8NJP6.1        GQCGGSGWTGPTVCESGYTCTYSNAWYSQCL
```

Fig.8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/076185

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/06 C12N1/12
ADD.

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)
C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, COMPENDEX, FSTA, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FIYINFOLUWA A. ADESIOYE ET AL: "Phylogeny, classification and metagenomic bioprospecting of microbial acetyl xylan esterases", ENZYME AND MICROBIAL TECHNOLOGY, vol. 93-94, 29 July 2016 (2016-07-29), pages 79-91, XP029756625, * See page 79 (Abstract), page 80 (Introduction) and page 87 (Table 3); early online publication *	1-31

A	PETER BIELY ET AL: "Recent progress in understanding the mode of action of acetylxylan esterases", JOURNAL OF APPLIED GLYCOSCIENCE, vol. 61, 2014, pages 35-44, XP002777300, * See pages 41-42 (Concluding remarks) *	1-31

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] 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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2018	29/01/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Korsner, Sven-Erik

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JUNHUA ZHANG ET AL: "The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed", BIOTECHNOLOGY FOR BIOFUELS, vol. 4, 2011, pages 1-9, XP055050350, * See page 1 (Abstract) *	1-31
A	MELISSA A. SCRANTON ET AL: "Chlamydomonas as a model for biofuels and bio-products production", THE PLANT JOURNAL, vol. 82, 2015, pages 523-531, XP002777304, * See page 526 (Biodiesel) *	1-31
A	US 2011/165635 A1 (COPENHAVER ET AL) 7 July 2011 (2011-07-07) * See claims 78, 81, 84, 91, 94 and 96 *	1-31
A,P	TAMILVENDAN MANAVALAN ET AL: "Optimization of acetyl xylan esterase gene expression in Trichoderma reesei and its application to improve the saccharification efficiency on different biomasses", PROCESS BIOCHEMISTRY, vol. 58, 13 May 2017 (2017-05-13), pages 160-166, XP002777302, * See page 160 (Abstract); early online publication *	1-31

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/076185

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2011165635 A1	07-07-2011
		WO 2009132008 A2	29-10-2009