Title: THERMOANAEROBACTER MATHRANII STRAIN BGI

Abstract: Strict anaerobic thermophilic bacterium belonging to the group of Thermoanaerobacter mathranii and mutants and derivatives thereof. The bacterium is particularly suitable for the production of fermentation products such as ethanol, lactic acid, acetic acid and hydrogen from lignocellulosic biomass.
THERMOANAEROBACTER MATHRANII STRAIN BG1

TECHNICAL FIELD

The present invention relates to a novel thermophilic bacterium belonging to the group of Thermoanaerobacter mathranii.

BACKGROUND OF THE INVENTION

The industry of producing fermentation products such as ethanol and lactic acid, is facing the challenge of redirecting the production process from fermentation of relatively easily convertible but expensive starchy materials, to the complex but inexpensive lignocellulosic biomass such as wood and residues from agricultural crops, e.g. straw. Unlike starch, which contains homogenous and easily hydrolysed polymers, lignocellulosic biomass contains cellulose (25-53%), hemicellulose (20-35%), polyphenolic lignin (10-25%) and other extractable components. Typically, the first step in utilisation of lignocellulosic biomass is a pre-treatment step, in order to fractionate the components of lignocellulosic material and increase their surface area. The pre-treatment method most often used is acid hydrolysis, where the lignocellulosic material is subjected to an acid such as sulphuric acid whereby the sugar polymers cellulose and hemicellulose are partly or completely hydrolysed to their constituent sugar monomers. Another type of lignocellulose hydrolysis is steam explosion, a process comprising heating of the lignocellulosic material by steam injection to a temperature of 190-230°C. A third method is wet oxidation wherein the material is treated with oxygen at 150-185°C. The pre-treatments can be followed by enzymatic hydrolysis to complete the release of sugar monomers. This pre-treatment step results in the hydrolysis of cellulose into glucose while hemicellulose is transformed into the pentoses xylene and arabinose and the hexoses glucose, galactose and mannose. Thus, in contrast to starch, the hydrolysis of lignocellulosic biomass results in the release of pentose sugars in addition to hexose sugars. This implies that useful fermenting organisms need to be able to convert both hexose and pentose sugars to a desired fermentation products such as ethanol.

Traditional microorganisms used for e.g. ethanol fermentation, Saccharomyces cerevisiae and Zymomonas mobilis, do not metabolize pentoses such as xylose and arabinose, and extensive metabolic engineering is thus necessary to improve performance on lignocellulosic substrates. Gram-positive thermophilic bacteria have unique advantages over the conventional ethanol production strains. The primary advantages are their broad substrate specificities and high natural production of ethanol. Moreover, ethanol fermentation at high temperatures (55-70°C) has many advantages over mesophilic fermentation. One advantage of thermophilic fermentation is the minimisation of the problem of contamination in
continuous cultures, since only a few microorganisms are able to grow at such high
temperatures in un-detoxified lignocellulose hydrolysate.

Presently, dependent on the pre-treatment method, cellulases and hemicellulases often have
to be added to the pre-treated lignocellulosic hydrolysate in order to release sugar-
monomers. These enzymes contribute significantly to the production costs of the
fermentation products. However, many thermophilic gram-positive strains possess a range of
the relevant enzymes and supplementary additions could become less expensive if a
thermophilic gram-positive strain is used. Fermentation at high temperature also has the
additional advantages of high productivities and substrate conversions and facilitated product
recovery.

Lignocellulose hydrolysates contain inhibitors such as furfural, phenols and carboxylic acids,
which can potentially inhibit the fermenting organism. Therefore, the organism must also be
tolerant to these inhibitors. The inhibitory effect of the hydrolysates can be reduced by
applying a detoxification process prior to fermentation. However, the inclusion of this extra
process step increases significantly the total cost of the fermentation product and should
preferably be avoided. For example, it has been estimated that overliming of willow
hydrolysate increase the cost of ethanol production using *Escherichia coli* by 22 % (Von
Sivers et al., 1994). It is therefore preferred that the microorganism is capable of producing
fermentation products from undetoxified hemicellulose or holocellulose hydrolysates to make
it usable in an industrial lignocellulosic-based fermentation process due to the high cost of
detoxification process.

It is also particularly advantageous if the potential microorganism is capable of growing on
high concentrations of lignocellulosic hydrolysates, i.e. lignocellulosic hydrolysates with high
dry-matter content. This is of particular importance when the microorganism is for alcohol
production such as ethanol production, since distillation costs increase with decreasing
concentrations of alcohol.

US 6,555,350 describes a *Thermoanaerobacter* strain which is capable of converting pentoses
to ethanol. However, this strain has a significant side production of lactate and has only been
tested in lignocellulosic hydrolysate having a dry-matter concentration of less than 6% wt/wt.

Larsen et al., 1997 describes a *Thermoanaerobacter mathranii* strain A3 which could only
grow in wheat straw hydrolysate with a 6% dry-matter concentration (60 g/l dry weight of
wheat straw supplemented with xylose) and is reported not to be able to grow on galactose.
It is therefore one object of the present invention to provide a microorganism which is capable of overcoming the above mentioned obstacles, in particular for the production of ethanol.

**SUMMARY OF THE INVENTION**

Accordingly, the present invention pertains to a *Thermoanaerobacter mathranii* bacterial strain selected from BG1 (DSMZ Accession number 18280) and mutants thereof. The invention is based on the isolation of the bacterial strain BG1 which is capable of growing and producing fermentation products on very high dry-matter concentrations of lignocellulosic hydrolysates. Furthermore, BG1 has broad substrate specificity, and is capable of utilising pentoses such as xylose and arabinose and hexoses. The strain further has the advantage of being thermophilic and thus is capable of growing at high temperatures resulting in high productivities and substrate conversion rates, low risk of contamination and facilitated product recovery.

The invention further relates to a method of producing a fermentation product by culturing a strain according to the invention under suitable conditions.

**DETAILED DESCRIPTION OF THE INVENTION**

As mentioned above, the present invention pertains to a *Thermoanaerobacter mathranii* strain selected from BG1 and mutants thereof.

The invention is based on the isolated bacterial strain BG1 which has been deposited in accordance with the terms of the Budapest Treaty on 17 May 2006 with DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany under DSMZ accession number 18280.

As is apparent from the following, the preferred bacteria of the present invention have been deposited. Other bacteria of the present invention can therefore be obtained by mutating the deposited bacteria and selecting derived mutants having enhanced characteristics. Desirable characteristics include an increased range of sugars that can be utilised, increased growth rate, ability to produce higher amounts of fermentation products such as ethanol, etc. Suitable methods for mutating bacteria and selecting desired mutants are described in Functional analysis of Bacterial genes: A practical Manual, edited by W. Schumann, S.D. Ehrlich & N. Ogasawara, 2001.
The base strain BG1 is capable of growing and producing fermentation products on very high dry-matter concentrations of lignocellulosic hydrolysates. In the present context the term "lignocellulosic hydrolysate" is intended to designate a lignocellulosic biomass which has been subjected to a pre-treatment step whereby lignocellulosic material has been at least partially separated into cellulose, hemicellulose and lignin thereby having increased the surface area of the material. The lignocellulosic material may typically be derived from plant material, such as straw, hay, garden refuse, comminuted wood, fruit hulls and seed hulls.

The pre-treatment method most often used is acid hydrolysis, where the lignocellulosic material is subjected to an acid such as sulphuric acid whereby the sugar polymers cellulose and hemicellulose are partly or completely hydrolysed to their constituent sugar monomers. Another type of lignocellulose hydrolysis is steam explosion, a process comprising heating of the lignocellulosic material by steam injection to a temperature of 190-230°C. A third method is wet oxidation wherein the material is treated with oxygen at 150-185°C. The pre-treatments can be followed by enzymatic hydrolysis to complete the release of sugar monomers. This pre-treatment step results in the hydrolysis of cellulose into glucose while hemicellulose is transformed into the pentoses xylose and arabinose and the hexoses glucose, galactose and mannose. The pre-treatment step may in certain embodiments be supplemented with treatment resulting in further hydrolysis of the cellulose and hemicellulose. The purpose of such an additional hydrolysis treatment is to hydrolyse oligosaccharide and possibly polysaccharide species produced during the acid hydrolysis, wet oxidation, or steam explosion of cellulose and/or hemicellulose origin to form fermentable sugars (e.g. glucose, xylose and possibly other monosaccharides). Such further treatments may be either chemical or enzymatic. Chemical hydrolysis is typically achieved by treatment with an acid, such as treatment with aqueous sulphuric acid, at a temperature in the range of about 100-150°C. Enzymatic hydrolysis is typically performed by treatment with one or more appropriate carbohydrate enzymes such as cellulases, glucosidases and hemicellulases including xylanases.

It was surprisingly found that the bacterial strain according to invention is capable of growing in a medium comprising a hydrolysed lignocellulosic biomass material having a dry-matter content (or as also used herein "total solids", TS) of at least 10% wt/wt, such as at least 15% wt/wt, including at least 20% wt/wt, and even as high as at least 25% wt/wt. As mentioned previously, this has the great advantage that it may not be necessary to dilute the hydrolysate before the fermentation process, and thereby it is possible to obtain higher concentrations of fermentation products such as ethanol, and thereby the costs for subsequently recovering the fermentation products may be decreased. For example the distillation costs for ethanol will increase with decreasing concentrations of alcohol.
The bacterial strain according to the invention is an anaerobic thermophilic bacterium, and it is capable of growing at high temperatures even at or above 70°C. The fact that the strain is capable of operating at this high temperature is of high importance in the conversion of the lignocellulosic material into fermentation products. The conversion rate of carbohydrates into e.g. ethanol is much faster when conducted at high temperatures. For example, ethanol productivity in a thermophilic *Bacillus* is up to ten-fold faster than a conventional yeast fermentation process which operates at 30°C. Consequently, a smaller production plant is required for a given volumetric productivity, thereby reducing plant construction costs. As also mentioned previously, at high temperature, there is a reduced risk of contamination from other microorganisms, resulting in less downtime, increased plant productivity and a lower energy requirement for feedstock sterilisation. The high operation temperature may also facilitate the subsequent recovery of the resulting fermentation products.

Numerous fermentation products are valuable commodities which are utilised in various technological areas, including the food industry and the chemical industry. Presently, the increasing global energy requirements have resulted in increasing focus on alternatives to fossil fuels as energy sources, and ethanol derived from plant materials (bioethanol) has received particular attention as a potential replacement for or supplement to petroleum-derived liquid hydrocarbon products.

Lactic acid, which is another fermentation product, is extensively used in the cosmetics industry as an anti-aging chemical, and the food industry use lactic acid in a variety of food stuffs to act as an acidity regulator. Recently, lactic acid has also attracted much attention for its potential use in biodegradable polyesters.

The strain according to invention has the potential to be capable of producing a number of different fermentation products, including acids, alcohols, ketones and hydrogen. In one embodiment, the alcohol is selected from ethanol, butanol, propanol, methanol, propanediol and butanediol. In a further embodiment the acid is lactic acid, propionate, acetate, succinate, butyrate or formate and the ketone is acetone.

As mentioned above, BG1 is a wild type strain isolated from an Icelandic hot-spring, and it has several highly advantageous characteristics needed for the conversion of lignocellulosic biomass material. Thus, this base strain possesses all the genetic machinery for the conversion of both pentose and hexose sugars to various fermentation products such as lactic acid and ethanol.

As will be apparent from the below examples, the examination of the complete 16S rDNA sequence (SEQ ID NO:14) showed that strain BG1 is closely related to *Thermoanaerobacter*
*matrhanii* strain A3 (Larsen et al., 1997). This places BG1 in cluster V of the Clostridia. Although the strains are closely related, they are very different when it comes to tolerance to hemicellulosic hydrolysates. A3 could only grow in up to 40% wheat straw hydrolysate (of 60 g/l dry weight of wheat straw supplemented with xylose, i.e. a dry-matter concentration of 6% wt/wt) while BG1 can grow and produce ethanol from undiluted hydrolysate at high dry-matter concentrations (up to at least 25%) with no addition of sugar or enzymes. Furthermore, strain A3 is reported not to be able to grow on galactose (Larsen et al., 1997), whereas BG1 grows nicely and produces primarily ethanol with galactose as the sole carbon source. Thus, the strain according to the invention is capable of growing on galactose as the sole carbon source.

As shown in the accompanying examples the first hours of BG1 growth at pH=7 and 70°C in xylose minimal medium, ethanol and acetate are produced in equimolar amounts. When the culture then enters late exponential phase, the specific ethanol production exceeds that of acetate significantly. The pH after fermentation was unchanged, and it is therefore not expect the effect to be caused by pH. Likewise, it was shown that by replacing the headspace N\textsubscript{2}/CO\textsubscript{2} with pure hydrogen or adding acetate had no effect on the fermentation, and the mechanisms behind the shift to ethanol formation in late exponential phase are therefore still elusive.

The examples also illustrate that the pH of the medium has a strong effect on the product profile of BG1. Above pH 6.5, the production of ethanol was dominant, and almost no lactate was produced. Below pH 6.0, lactate production increased at the expense of ethanol, and at pH 5.0, no growth was observed. The same effect of pH on lactate production has been observed for the closely related thermophilic bacterium *Thermoanaerobacter wiegelli*. Crude cell extracts were found to contain a fructose-1,6-diphosphate (FDP) activated lactate dehydrogenase (LDH). The affinity of FDP was dependent on extracellular pH. Maximal activation was observed at pH 6.2, and no activation was observed at pH 8.2 (Cook, 2000). Since this effect is common in Thermoanaerobacter species, it is also likely to be the cause in BG1 (Lamed and Zeikus, 1980; Carreira et al., 1982; Bryant, 1991).

The following examples also illustrate the effect of addition of ethanol on the growth rate and metabolite distribution of BG1. Low ethanol tolerance is a major obstacle for the commercial exploitation of thermophilic bacteria and selection for ethanol resistant strains is therefore of great importance. However, an ethanol resistant mutant of *Clostridium thermohydrodsulfuricum*, 39EA, could grow at ethanol concentrations up to 8% (w/v) at 45°C, and up to 3.3% (w/v) at 68°C (Lovitt et al., 1984), and a mutant strain of *Thermoanaerobacter ethanolicus*, 39E-H8, displayed an ethanol tolerance of 8% at 60°C (Burdette et al., 2002). BG1 is very tolerant to ethanol at 70°C, when compared to other
thermophilic anaerobic strains (Herrero and Gomez, 1980; Larsen et al., 1997; Lovitt et al., 1988; Wiegel and Ljungdahl, 1981; Rani et al., 1996). At 2.8% of exogenous ethanol, the growth rate was still 27% of the rate without ethanol added, in an unadapted culture.

*Thermoanaerobacter ethanolicus* E39 has been reported to consume exogenously added ethanol using the primary alcolhol dehydrogenase (Burdette et al., 2002). This is the case even in the ethanol tolerant mutant E39-H8, which has no (or decreased) primary alcohol dehydrogenase activity. In contrast, BG1 was found to produce ethanol even at high concentrations of exogenous ethanol. The lactate production was found to increase in BG1 when ethanol was added. Ethanol production at high ethanol concentrations is pivotal for ethanol producing microorganisms.

As opposed to starchy or cellulosic substrates, which are almost exclusively broken down to glucose, lignocellulosic biomass contains several different sugar monomers, including both hexoses and pentoses. If these are to be converted into a fermentation product such as ethanol in a continuous process, it is necessary for all the sugars to be taken up and metabolized simultaneously. Co-fermentation has proven to be problematic in many of the traditional ethanol producing microorganisms. *Saccharomyces cerevisiae* and Zymomonas mobilis have been successfully engineered for cofermentation of glucose and xylose, but cofermentations with arabinose seem to be more problematic (Dien et al., 2000; Lawford and Rousseau, 2002; Ho et al., 1998). In gram positive bacteria in which carbon metabolism was studied at the molecular level, glucose was shown to inhibit transcription of the xylAB operon encoding enzymes responsible for the initial metabolism of xylose, by catabolite repression (Hueck and Hillen, 1995). In contrast, transcription of the xylAB operon of *Thermoanaerobacter ethanolicus* is not repressed by glucose, and a simultaneous degradation of glucose and xylose is seen (Erbezni et al., 1998). As shown in the Examples, BG1 degraded the sugar mixture of glucose, xylose, arabinose and galactose simultaneously as well as the mixture of glucose, xylose, galactose and mannose.

The hydrolysis of lignocellulosic biomass results in release of microbial inhibitors (Klinke et al., 2004), and washing of the hydrolysate might therefore increase BG1 ethanol productivity. However, in the experiments shown in the Examples, washing significantly decreased the ethanol productivity of BG1. This effect is likely to be caused by washing out of readily fermentable pentosans, thereby lowering the initial sugar concentration. In a similar study on pretreated aspen wood, it was shown that washing efficiently removed inhibitors, but also resulted in a drastic 75% loss of available pentosans (Saddler and Chan, 1984). The presence of inhibitors in the hydrolysate does not seem to be a major obstacle for BG1 fermentations, and BG1 seems to have a great advantage over traditional ethanol producing microorganisms in concentrated non-detoxified lignocellulosic hydrolysates.
It is demonstrated in the following examples, that the base strain BG1 in advantageous embodiments may be modified in order to obtain mutants or derivatives of BG1, with improved characteristics. Thus, in one embodiment there is provided a bacterial strain according to the invention which is a variant or mutant of BG1 wherein one or more genes have been inserted, deleted or substantially inactivated. Genes may be inserted, deleted or substantially inactivated using suitable gene manipulation tools and genetic engineering procedures which are well known in the art, e.g. gene cloning systems, homologous recombination and techniques described in Sambrook & Russell "Molecular Cloning: A Laboratory Manual" (Third Edition), Cold Spring Harbor Laboratory Press.

In particular, it has surprisingly been found by the present inventors, that the ethanol producing capability of BG1 may be significantly increased by inactivating the gene encoding lactate dehydrogenase (LDH) (EC 1.1.1.27). Thus it was found, that the ethanol production, when grown on glucose, was increased from about 51-56% of the theoretical maximum yield in the wild type BG1 to about 84-91% in the lactate dehydrogenase deficient strain BG1L1.

Therefore, it is contemplated that the strain in accordance with the invention may be a modified version of BG1 wherein the gene encoding lactate dehydrogenase (LDH) (EC 1.1.1.27) has been inactivated by the deletion of said gene, or wherein the gene has been substantially inactivated by the mutation, deletion or insertion of one or more amino acids in gene.

In one embodiment there is provided a lactate dehydrogenase deficient mutant strain BG1L1 which has been deposited in accordance with the terms of the Budapest Treaty on 17 May 2006 with DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany under DSMZ accession number 18283.

As mentioned above, lactic acid (or lactate) is a widely used e.g. in the food industry and is therefore a valuable fermentation product. It was found by the present inventors, that the wild type strain BG1 can be modified to produce increased amounts of lactate as compared to the wild type by inactivating the gene encoding pyruvate ferredoxin oxidoreductase (EC 1.2.7.1). Thus, it was found that the metabolism of wild type BG1 could be completely shifted from the production of ethanol to the production of lactate. Hence, it is one object of the invention to provide a strain derived from BG1 wherein a gene encoding pyruvate ferredoxin oxidoreductase (EC 1.2.7.1) has been down-regulated or substantially inactivated, e.g. inactivated by the mutation, deletion or insertion of one or more amino acids in the gene. More specifically, there is provided a derivative of BG1 having increased lactate producing characteristics which is designated BG1PF1 and has been deposited in accordance with the terms of the Budapest Treaty on 17 May 2006 with DSMZ - Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany under DSMZ accession number 18282.

Hydrogen is widely used in the petroleum and chemical industries, i.a. for the processing of fossil fuels, for hydroalkylation, hydridesulfurization and hydrocracking, and it is used for the hydrogenation of fats and oils (found in items such as margarine), and in the production of methanol. Additionally, hydrogen can be used as an energy source, and can be burned in e.g. combustion engines.

Acetic acid is a valuable product which is widely used in industry, mainly for the production of vinyl acetate monomer, ester production, vinegar, and for use as a solvent. The global demand of acetic acid is around 6.5 million tonnes per year.

The present inventors have constructed an improved BG1 mutant strain, which is capable of producing both more hydrogen and acetic acid in the form of acetate than the wild type strain BG1. This was performed by down-regulation of the HydABCD gene encoding hydrogenase of BG1.

Thus in accordance with the invention, there is provided a mutant strain or derivative of BG1, wherein a gene encoding a hydrogenase or a hydrogenase subunit has been down-regulated or substantially inactivated. The inactivation of the gene may be performed by mutation, deletion or insertion of one or more amino acids in the gene. More specifically, the gene encoding hydrogenase or a hydrogenase subunit may be selected from [Fe]-hydrogenases and [NiFe]-hydrogenases (EC 1.6.5.3, EC 1.12.7.2, EC 1.12.99.6) such as NuoE, NuoF, NuoG, EchB, EchC, EchD, EchE and EchF.

Accordingly, in one embodiment there is provided a strain according to the invention having improved hydrogen and acetic acid production capabilities which is designated BG1H1. BG1H1 has been deposited in accordance with the terms of the Budapest Treaty on 17 May 2006 with DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany under DSMZ accession number 18281.

It is further contemplated, that in certain embodiments, it may be useful to down-regulate or substantially inactivate a gene encoding an acetate kinase (EC 2.7.2.1) and/or phosphate acetyltransferase (EC 2.3.1.8) in BG1 or mutants thereof. The inactivation may be performed by mutation, deletion or insertion of one or more amino acids in said genes.

As mentioned above, BG1 posses the genetic machinery to enable it to convert both hexose sugars and pentose sugars to a range of desired fermentation products, including ethanol.
However, it may for certain embodiments be desired to insert one or more additional genes into the strain according to the invention. Thus, in order to improve the yield of the specific fermentation product, it may be beneficial to insert one or more genes encoding a polysaccharase into the strain according to the invention. Hence, in specific embodiments there is provided a strain according to the invention wherein one or more genes encoding a polysaccharase which is selected from cellulases (such as EC 3.2.1.4); beta-glucanases, including glucan-1,3 beta-glucosidases (exo-1,3 beta-glucanases, such as EC 3.2.1.58), 1,4-beta-cellobiohydrolase (such as EC 3.2.1.91) and endo-1,3(4)-beta-glucanases (such as EC 3.2.1.6); xylanases, including endo-1,4-beta-xylanases (such as EC 3.2.1.8) and xylan 1,4-beta-xylosidase (such as EC 3.2.1.37); pectinases (such as EC 3.2.1.15); alpha-glucuronidase, alpha-L-arabinofuranosidase (such as EC 3.2.1.55), acetylesterase (such as EC 3.1.1.-), acetylxylanesterase (such as EC 3.1.1.72), alpha amylase (such as EC 3.2.1.1), beta-amylase (such as EC 3.2.1.2), glucoamylase (such as EC 3.2.1.3), pullulanase (such as EC 3.2.1.41), beta-glucanase (such as EC 3.2.1.73), hemicellulase, arabinosidase, mannanases including mannan endo-1,4-beta-mannosidase (such as EC 3.2.1.78) and mannan endo-1,6-alpha-mannosidase (such as EC 3.2.1.101), pectin hydrolase, polygalacturonase (such as EC 3.2.1.15), exopolygalacturonase (such as EC 3.2.1.67) and pectate lyase (such as EC 4.2.2.10).

Depending on the desired fermentation product, it is contemplated that in certain embodiments it is useful to insert heterologous genes encoding a pyruvate decarboxylase (such as EC 4.1.1.1) or to insert a heterologous gene encoding an alcohol dehydrogenase (such as EC 1.1.1.1, EC 1.1.1.2, EC 1.1.1.71, or EC 1.1.99.8) or to up-regulate an already existing gene encoding alcohol dehydrogenase.

In accordance with the invention a method of producing a fermentation product comprising culturing a strain according to the invention under suitable conditions is also provided.

The strain according to the invention is a strict anaerobic microorganism, and hence it is preferred that the fermentation product is produced by a fermentation process performed under strict anaerobic conditions. Additionally, the strain according to invention is a thermophilic microorganism, and therefore the process may perform optimally when it is operated at temperature in the range of about 40-95°C, such as the range of about 50-90°C, including the range of about 60-85°C, such as the range of about 65-75°C.

For the production of certain fermentation products, it may be useful to select a specific fermentation process, such as batch fermentation process, including a fed batch process or a continuous fermentation process.
In accordance with the invention, the method is useful for the production of a wide range of fermentation products including acids, alcohols, ketones and hydrogen. Thus fermentation products such as ethanol, butanol, propanol, methanol, propanediol, butanediol, lactic acid, propionate, acetate, succinate, butyrate, formate and acetone may be produced in accordance with the invention.

The invention will know be further described in the following non-limiting examples and figures.

**SHORT DESCRIPTION OF THE FIGURES**

Figure 1. Phylogenetic tree based on 16S rDNA sequence analysis showing the position of strain BG1 among related thermophilic Clostridia. T. is a Thermoanaerobacter species, Tm. is a Thermoanaerobacterium. The bar shows 2% nucleotide substitutions.

Figure 2: Product distribution from a 24 hour batch fermentation of BG1. ▲: xylose (mM), △: lactate (mM), o: acetate (mM), ●: ethanol (mM), ♦: hydrogen (mM), □: cell density (OD578).

Figure 3. Effect of temperature on the specific growth rate of strain BG1 grown anaerobically in batch with 5 g/l xylose. Standard deviations from 3 independent measurements are shown with bars.

Figure 4. Product formation from BG1 batch fermentations at different pH. △: lactate (mM), o: acetate (mM), ●: ethanol (mM).

Figure 5. Batch fermentations with strain BG1 grown on BA medium with 5 g/l xylose and varying concentrations of ethanol added to the medium. The product yield in mM product per g xylose consumed is shown as a function of the initial ethanol concentration in the medium. o: acetate, △: lactate, ●: ethanol.

Figure 6. BG1 sugar consumption in batch fermentation with mixed sugars as carbon source. The sugar concentration of two different sugar mixture experiments (each in duplicate) is shown as a function of time after inoculation with strain BG1. ■: glucose, ▲: xylose, o: arabinose, x: galactose, □: mannose

Figure 7. BG1 (open symbols) and BG1L1 (closed symbols) grown in batch at varying concentrations of glucose. A: Ethanol (squares), lactate (circles) and acetate (triangles) as a function of ethanol concentration. B: Carbon recoveries of the experiments shown in A.
Figure 8. BG1 (open symbols) and BG1L1 (closed symbols) grown in batch at varying concentrations of xylose. A: Ethanol (squares), lactate (circles) and acetate (triangles) as a function of ethanol concentration. B: Carbon recoveries of the experiments shown in A.

Figure 9. BG1 (open symbols) and BG1L1 (closed symbols) grown in batch at varying concentrations of exogenously added ethanol. A: Ethanol (squares), lactate (circles) and acetate (triangles) as a function of ethanol concentration. B: Carbon recoveries of the experiments shown in A.

Figure 10. Abbreviations in Table: HRT: hydraulic retention times, Glu: glucose, Xyl: xylose, Ace: acetate, CGlu: consumed glucose, CXyl: consumed xylose, CS: total sugar consumed, YAce/TS: acetate yield (g/g initial sugars), YEtOH/TS: ethanol yield (g/g initial sugars), YEtoH/TS: ethanol yield of initial sugars corrected for ethanol evaporation, QEtoH: volumetric ethanol productivity, CR: carbon recovery.

Figure 11. Influent sugar concentrations of various wheat straw hydrolysate suspensions. Undiluted wheat straw hydrolysate (23% DM; 77 g/l soluble sugars; 57 g/l glucose and 20 g/l xylose).

Figure 12. Product yields obtained with a BG1L1 in a FBR at 70°C from various wheat straw hydrolysate suspensions.

Figure 13. Influent sugar concentrations (A) and sugar conversions (B) for various acid hydrolyzed corn stover hydrolysate suspensions from a continuous fluidized bed reactor with immobilized thermophilic anaerobic bacterium BG1L1 at 70°C.

Figure 14. Effluent product concentrations (acetate and ethanol) and influent acetate concentration for various acid hydrolyzed corn stover hydrolysate suspensions from continuous fluidized bed reactor with immobilized thermophilic anaerobic bacterium BG1L1 at 70°C.

Figure 15. Ethanol yield and carbon recovery for various acid hydrolyzed corn stover hydrolysate suspensions from continuous fluidized bed reactor with immobilized thermophilic anaerobic bacterium BG1L1 at 70°C.

Figure 16. Ethanol and acetate yield for various wet exploded wheat straw hydrolysate suspensions from a continuous fluidized bed reactor with the immobilized thermophilic anaerobic bacterium BG1L1 at 70°C.
Figure 17. Construct used for deletion of a 7762 bp region, containing pyruvate ferredoxin oxidoreductase subunit encoding genes, from the chromosome of BG1. up: a region upstream of the BG11 pyruvate ferredoxin oxidoreductase. down: a region downstream of the BG1 pyruvate ferredoxin oxidoreductase. HTK: a gene encoding a highly thermostable kanaycin resistance cassette.

Figure 18. End-product distribution after batch fermentation at 70°C with five independent clones of BG1PF.

Figure 19. Construct used for introduction of anti-sense cassettes into the chromosome of BG1. up: a region upstream of the BG1 lactate dehydrogenase. down: a region downstream of the lactate dehydrogenase gene. HTK: a gene encoding a highly thermostable kanaycin resistance cassette. prom: promoter. ter: terminator. a 335 bp fragment from hydA was cloned in the anti-sense direction into a cloning site between the promoter and terminator.

Figure 20. Northern blot analysis of total RNA isolated from BG1, BG1L1 and BG1H1 grown on glucose or xylose. The RNA was isolated from exponentially growing cells. Upper panel: a probe directed towards the hydA antisense was used. Lower panel: a probe directed towards the hydA part of the hyd mRNA. RNA size marker bands are shown to the right.

Figure 21. Growth, xylose consumption, acetate, lactate, ethanol and hydrogen production of BG1 (open and closed squares), BG1L1 (triangles) and BG1H1 (open and closed circles), grown in batch.
EXAMPLES

Materials and methods

The following materials and methods were applied in the below Examples:

Strains and growth conditions

Strain BG1 was isolated anaerobically from an Icelandic hot-spring at 70°C. All strains were cultured at 70°C anaerobically in minimal medium (BA) with 2 g/l yeast extract as in (Larsen et al., 1997) unless otherwise stated. For solid medium, roll tubes (Hungate RE, 1969; Bryant MP, 1972) containing BA medium with 11 g/l phytagel and additional 3.8 g/l MgCl₂6H₂O was used. For cloning purposes, *Escherichia coli* Top10 (Invitrogen, USA) was used. Top10 was routinely cultivated at 37°C in Luria-Bertani medium (Ausubel et al., 1997) supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin when needed.

Continuous reactors

Fermentation medium used for continuous cultivation was prepared and supplemented with the same minerals, trace metals, and yeast extract as described above. The initial pH of the medium was adjusted to 7.4-7.7 and it was autoclaved at 120°C for 30 min. To insure anaerobic conditions, medium was flushed for 45 minutes with a mixture of N₂/CO₂ (4:1), and finally Na₂S was injected into the bottle to give a final concentration of 0.25 g/l.

The reactor was a water-jacketed glass column with 4.2 cm inner diameter and 20 cm height. The working volume of the reactor was 200 ml. The influent entered from the bottom of the reactor and the feeding was controlled by a peristaltic pump (Model 503S-10rpm, Watson Marlow, Falmouth, UK). Recirculation flow was achieved by using an identical peristaltic pump (Model 503-50rpm, Watson Marlow, Falmouth, UK), with a degree of recirculation to ensure up-flow velocities in the reactor of 1 m/h. The pH was maintained at 7.0 by addition of NaOH (1-2M), unless otherwise stated. The reactor was loaded with 75 ml carrier material and finally the entire reactor system, including tubing and recirculation reservoir, was autoclaved at 120°C for 30 min. Liquid samples were taken from sampling ports located on the top of the reactor, close to the reactor outlet. The experiments were performed at 70°C by external heating and recirculation of hot water in the glass jacket.

During the experiments, whenever steady state was achieved, HRT or sugar concentrations were changed. The criteria for steady-state conditions were that all parameters must be held
constant for at least five residence times. The reactor performance at different steady state
was monitored by measuring the sugar and end-fermentation product concentrations. During
the experiment, sterile syringes and needles were used to take the samples from the influent
and effluent, and the samples were stored at −20°C until analyzing. Effluent gas samples
were taken to determine the carbon dioxide and hydrogen content.

Test for contamination

A 1 ml sample was taken from the reactor and chromosomal DNA was purified using the DNA
purification kit from A&A Biotech (Poland). PCR reactions were setup using the Pfu
polymerase (MBI Fermentas, Germany) and the primers B-all 27F (SEQ ID NO:1; GAG TTT
GAT CCT GGC TCA G) and B-all 1492R (SEQ ID NO:2; ACG GCT ACC TTA CGA CT),
which anneal to bacterial rDNA. The fragments were purified using the Qiaex II kit from
Qiagen, treated with PNK (MBI Fermentas), cloned into pBluescript SK+ (Stratagene) treated
with CIAP (MBI Fermentas), and transformed into Escherichia coli Top10 (Invitrogen). 50
clones were picked and the inserts were amplified using B-all 27F and B-all 1492R primers.
The resulting fragments were digested with AluI and MboI restriction enzymes (MBI
Fermentas) and were run on a 3% agarose gel. Only one digestion pattern was found. Two
fragments were sent for sequencing (MWG Biotech, Germany) and were identified as strain
A10. PCR reactions were also run using primers ldhcw1 and ldhcw2 annealing to regions
upstream and downstream of the lactate dehydrogenase respectively. Otherwise, the same
reaction conditions as for the B-all primers, were used. The obtained fragments were cloned
(as above), 26 were analysed by restriction fragment length polymorphism. Again, this
resulted in only one pattern. Two fragments were sequenced.

Analytical methods

The strains were grown in BA medium without antibiotics in batch for 24-48 hours as stated.
The culture supernatants were analyzed for glucose, xylose, acetate, lactate and ethanol
using an organic acid analysis column (Aminex HPX-87H column (Bio-Rad)) on a Hewlett
Packard series 1100 HPLC at 65°C with 4 mM H₂SO₄ as eluent.

Enzymes and reagents

If not stated otherwise enzymes were supplied by MBI Fermentas (Germany) and used
according to the suppliers’ recommendations. PCR reactions were performed with a 1 unit : 1
unit mixture of Taq polymerase and Pfu polymerase. Chemicals were of molecular grade and
were purchased from Sigma-Aldrich Sweden AB.
16S rRNA analysis

200 µl of BG1 overnight culture was harvested, treated for 2 minutes by microwave at maximum effect and used as a PCR template. A 1500 bp 16S rDNA was amplified by PCR using primers B1 (GAG TTT GAT CCT GGC TCA G) (SEQ ID NO:3) and B2 (ACG GCT ACC TTG TTA CGA CTT) (SEQ ID NO:4). The blunt ended PCR fragment was treated with polynucleotide kinase and cloned into SmaI digested and CIP (Calf Intestinal Phosphatase) treated pBluescript SK+ vector (Stratagene). 24 clones from the resulting DNA library were analysed by restriction enzyme fragment analyses using AluI, MboI and Hin6I restriction enzymes. 6 fragments were sent for sequencing at MWG-Biotech (Germany). The alignment was made using VectorNTi and the tree was drawn using the MEGA2 program (Kumar et al., 2001).

Analytical techniques

The culture supernatants were analyzed for cellobiose, glucose, xylose, acetate, lactate and ethanol using an organic acid analysis column (Aminex HPX-87H column (Bio-Rad Laboratories, CA USA)) on HPLC at 65°C with 4 mM H₂SO₄ as eluent. The ethanol and acetate measurements were validated using gas chromatography with flame ionization detection. Mixed sugars were measured on HPLC using a Phenomenex, RCM Monosaccharide (00H-0130-K0) column at 80°C with water as eluent. Mannose and arabinose could not be distinguished using this setup and were therefore tested in separate cultures. Hydrogen was measured using a GC82 Gas chromatograph (MikroLab Aarhus, Denmark).

Construction of ldh knock-out cassette

The final knock-out construct, p3CH contains 1) a DNA fragment upstream of the l-ldh gene of BG1, amplified using primers Idhup1F (SEQ ID NO:5; 5'-TTCCATATCTGTGATCCCGTAAAG) and Idhup2R (SEQ ID NO:6; 5'-ATTAATACAATAGTTTGGCACAATCC), 2) a gene encoding a highly thermostable kanamycin resistance amplified from plasmid pUC18HTK (Hoseki et al., 1999), and 3) a DNA fragment downstream of the l-ldh gene of BG1, amplified using primers ldhdown3F (SEQ ID NO:7; 5'-ATATAAAAAATCGACGTGTGAA) and ldhdown4R (SEQ ID NO:8; 5'-CACCTATTTTGCACTTTTTTTC). The plasmid p3CH was linearised and electroporated into BG1.

Construction of hydrogenase antisense construct

The DNA fragment SEQ ID NO:9 containing the hydrogenase A antisense cassette was inserted into p3CH.
Construction of pfor knock-out cassette

The final knock-out construct, pPF contains 1) a DNA fragment upstream of the l-ldh gene of BG1, amplified using primers pforup1F (SEQ ID NO:10; 5'-GAGGATTTAAGAAGGGGAGTTGG) and pforup2R (SEQ ID NO:11; 5'-ATTTCACTCCCTGCTTAAAG), 2) a gene encoding a highly thermostable kanamycin resistance amplified from plasmid pUC18HTK (Hoseki et al., 1999), and 3) a DNA fragment downstream of the l-ldh gene of BG1, amplified using primers pfardown3F (SEQ ID NO:12; 5'-CGAGAAGCTGATCCCCAGAAGA) and pfardown4R (SEQ ID NO:13; 5'-CAGACTACTACAACTGGATCTAGC). The plasmid p3PF was linearised and electroporated into BG1.

Electroporation of BG1.

All handling, except for the electroporation event, was performed under anaerobic conditions. For preparation of competent cells, 150 ml BA amended with 2 g/l yeast extract and 5 g/l xylose was inoculated with a fresh ON culture of BG1 to an OD578 of 0.1 and incubated at 70°C. At OD578 = 0.5 the cells were chilled on ice, harvested (3500 rpm, 35 min, 4°C) and resuspended in 10 ml cold EP buffer (0.3M sucrose, 10% glycerol, 3.20 mM Na₂S, pH 7, flushed with sterile N₂ gas). The wash was repeated and finally the pellet was re-suspend in 4 ml EP-buffer without Na₂S. The cells were stored at -80°C in aliquots. 0.2 ml of competent cells were transferred to electroporation cuvettes (0.1 cm electrode gap) containing 2 μg of linearized plasmid DNA and subjected to a pulse of 25 μF, 500 ohm, and 2.0 kV. The cells were transferred to anaerobic serum bottles containing 10 ml BA medium with 2 g/l yeast extract. The electroporated cells were allowed to recover at 70°C for 16 hours. They were then diluted 10 fold into BA medium containing 35 μg/ml kanamycin. After 24 hours, the transfer into kanamycin containing medium was repeated and the culture was grown for 24 hours at 70°C. Using the Hungate technique (Bryant MP, 1972; Hungate RE, 1969) 0.5 ml of culture was transferred to roll tubes with 35 μg/ml of kanamycin. After 2 days of incubation at 70°C, colonies were picked and inoculated in BA medium without antibiotics. Chromosomal DNA was purified from 4 individual recombinant clones and from wt BG1. A 2.1 kb fragment was amplified from the chromosomal DNA using primers IdhCw1 and IdhCw2 annealing just upstream and just downstream of the l-dh gene respectively. These fragments were analyzed by restriction enzyme digests and were sequenced. PCR reactions using internal l-ldh primers only yielded products when wt BG1 was used as template.

Northern blot analysis

RNA was purified from 50 ml of exponentially growing Thermoanaerobacter cells using the Total RNA kit from A&A Biotechnology (Poland) as recommended by the supplier. 5 μg of total
RNA was run in 1% agarose gels containing 80 mM Guanidine thiocyanate (mRNA blots) or 5% denaturing acrylamide gels (antisense blots). Blotting was performed using the TurboBlotter system (Scheicher & Schuell BioScience GmbH, Germany) as recommended. Hybridization was performed using the UltraHyb solution from Ambion, Inc. (TX, USA) and washing was according to standard procedures (Ausubel et al., 1997). The sense and antisense probes were transcribed from pSKPhyD (The same hydD fragment used in the antisense construct, but without promoter and terminator sequences, inserted into the MCS of pBluescript SK+ (Invitrogen)) using labelled α-P³²CTP.

Yield and carbon recovery

Theoretical maximum yields and carbon recoveries were calculated based on the following reactions (ATP and NAD(P)+ conversions are not included):

1 M glucose → 2 M lactate,

1 M glucose → 2 M acetate + 2 M CO₂,

1 M Glucose → 2 M Ethanol + 2 M CO₂

3 M Xylose → 5 M Lactate,

3 M Xylose → 5 M Acetate + 5 M CO₂,

3 M Xylose → 5 M Ethanol + 5 M CO₂

The theoretical maximum yields of ethanol from glucose and xylose are therefore 2 and 1.67 moles per mole respectively.

Carbon recoveries were calculated as:

\[
\frac{3 \times (\text{mM lactate} + \text{mM acetate} + \text{mM ethanol produced})}{n \times (\text{mM substrate consumed})} \times 100\%
\]

where n is 5 for xylose and 6 for glucose.
EXAMPLE 1

16S analysis of BG1

A library of 1500 bp 16S rDNA fragments from strain BG1 was constructed and analysed by restriction fragment length polymorphism analysis for different sequences. Only one digestion pattern was found and 6 clones were sequenced. The six sequences were identical and DNA homology search at www.ncbi.nlm.nih.gov placed strain BG1 in the group of *Thermoanaerobacter mathranii*, which now consist of strains A3 and BG1. The 16S rDNA sequence of BG1 is shown in the sequence list as SEQ ID NO:14. Except for seven unsequenced positions in the GenBank (Benson et al., 2005) sequence of strain A3, the two strains have identical 16S rDNA sequences. As figure 1 shows, the closest other relatives are *Thermoanaerobacter thermocopiae* (98% identity), *Thermoanaerobacter acetoethylicus* (95%) and *Thermoanaerobacter italicus* (95%). BG1 is closely related to *Thermoanaerobacter mathranii* strain A3 (Larsen et al., 1997). This places BG1 in cluster V of the *Clostridia* as previously described (Collins et al., 1994). Although the strains are closely related, they are very different when it comes to tolerance to hemicellulosic hydrolysates. A3 could only grow in up to 40% wheat straw hydrolysate (of 60 g/l dry weight of wheat straw supplemented with xylose) while BG1 can grow and produce ethanol from undiluted hydrolysate with no addition of sugar or enzymes. Furthermore, strain A3 is reported not to be able to grow on galactose (Larsen et al., 1997), whereas BG1 grows nicely and produces primarily ethanol with galactose as the sole carbon source (data not shown).

EXAMPLE 2

Fermentation Products of BG1

BG1 was grown anaerobically in batch for 24 hours with 27 mM xylose as the sole carbon source (figure 2). In the first four hours of fermentation, almost equal amounts of acetate and ethanol were produced. Between 4 and 6 hours of growth, ethanol production increased relative to acetate, and in the late exponential phase or in stationary phase, almost exclusively ethanol was produced. Only a minor amount of lactate was produced throughout the experiment. Hydrogen production seemed to follow the production of acetate with approximately equimolar amounts of the two compounds produced.
EXAMPLE 3

Temperature, pH and ethanol tolerance of BG1

As can be seen from figure 3, the temperature optimum of strain BG1 is around 70°C, which is the same as the temperature of the hot spring from which BG1 was isolated. BG1 was found to grow in the pH range from pH = 5.0 to 7.5. The product formation at different pH values of the culture medium was tested in batch experiments. As figure 4 shows, ethanol production was favoured in the pH range from 6.5 to 7.5, whereas lactate production was predominant at a lower pH. The optimal yield of ethanol was 1.15 mM ethanol / mM xylose at pH = 6.5 and pH = 7.5. At pH 5.5 the ethanol yield was 0.44 mM / mM. This corresponds to 69% and 26% of the theoretical maximum yield respectively.

Ethanol tolerance is of major importance for ethanol production. The product formation was investigated at elevated exogenous ethanol concentrations. As shown in figure 5, ethanol yields dropped dramatically at increased ethanol concentrations. At 0% initial ethanol in the medium, the yield was 1.05 mM ethanol / mM xylose consumed, whereas at 2.8% ethanol it was 0.31 mM/mM. This decrease in ethanol production was primarily due to increased lactate production (6.4 fold increase), but acetate production also increased by 40%. Apparently no other major products are formed, since carbon recoveries calculated from acetate, ethanol, lactate yields are in the range of 92-99% at all concentrations (data not shown).

EXAMPLE 4

BG1 co-fermentation of hemicellulose sugars

Simultaneous uptake and metabolism of different sugars is a desired but not very common trait for microorganisms used for ethanol production. BG1 was tested in batch on two different sugar mixtures, each containing four different hemicellulose sugar monomers, to study if the mixed monomers can be metabolized simultaneously (figure 6). Mannose and arabinose could not be distinguished in our HPLC setup and were therefore tested in separate mixtures. As can be seen from figure 6, a simultaneous degradation of all added sugars was observed between 6 and 8 hours of growth. The degradation rate of xylose was as high as that of glucose, although the onset of xylose degradation was slightly delayed. Galactose and arabinose were the last to be degraded, and the rate of degradation was slower than both the glucose and xylose rates. The rate of mannose degradation was the fastest observed, but again, the onset of degradation was delayed compared to glucose.
EXAMPLE 5

BG1L1 and BGL2: deletion of the l-Ldh gene from BG1

To prevent the formation of lactate, the L-LDH of BG1 was deleted. The purified pKHFr3rev plasmid, containing the thermostable kanamycin resistance gene flanked by the regions 5 upstream and downstream from ldh, was linearized and electroporated into BG1, and positive recombinants were selected using the thermostable kanamycin resistance gene. Several independent clones were isolated and verified by PCR. PCR products from two clones were subsequently sequenced and were found to contain the kanamycin resistance cassette instead of the lactate dehydrogenase gene as expected. The two mutant strains were named 10 BG1L1 and BG1L2.

The wt BG1 strain and the two strains BG1L1 and BG1L2 were grown on different concentrations of glucose and xylose to test their ethanol production performance (Figure 7 and 8). For simplicity only BG1L1 is shown, but the parallel strain, BG1L2, showed very similar results and even had a slightly higher maximum yield on xylose than BG1L1.

15 As can be seen from figure 7 and 8, the product distribution is greatly changed in the mutant. No detectable lactate is produced using either glucose or xylose as substrate, confirming the deletion of the lactate dehydrogenase gene. It also shows that the ldh gene described here is either the only ldh gene in BG1 or the primary one. The wild type BG1 strain responds to increased substrate concentration by increasing lactate production, especially when grown on xylose. A corresponding lower ethanol and acetate production is seen. The acetate production is constant or lower at higher sugar concentrations and, since it cannot produce lactate, a constant high or increasing ethanol production is seen. The ethanol yields are significantly improved in the mutant: On glucose, yields of 84 - 91% of the theoretical maximum are seen in BG1L1 as compared to 51 - 56% in the wt strain. On xylose the ethanol yields of BG1L1 and BG1wt are 76 - 80% and 40 - 63% respectively. Carbon recoveries were between 91 and 106% (figures 7B and 8B). Recoveries above 100% probably arise from metabolism of yeast extract components.

30 Figure 9 shows the ethanol production of BG1 compared to BG1L at increasing concentrations of ethanol in the medium. The wt BG1 strain responds to increased ethanol concentrations in the medium by increasing lactate production dramatically. Acetate production increases around two fold. The ethanol yield decreases to only 20 % of the maximum theoretical yield when 2.8% (v/v) ethanol is added. In BG1L, there is no lactate production and the production of acetate does not increase significantly at higher ethanol concentrations. As a result, the ethanol yield is high at all concentrations of ethanol in the medium. In fact, the
highest yield measured is 84% of the theoretical maximum at 1.95% of ethanol added and at 2.8% of ethanol the yield is still 72%. Carbon recoveries were between 84 and 99 % (figure 9B). The experiment was repeated with the independent clone BG1L2 with similar results.

EXAMPLE 6

Continuous fermentation of sugars using BG1L1.

The potential of using immobilized thermophilic anaerobic bacteria for continuous ethanol fermentation was investigated in a lab-scale fluidized bed reactor operated at 70°C (Figure 10). The effect of hydraulic retention time (HRT) on ethanol production and productivity was examined at a feed stream with 10 g/l xylose. Product concentrations and xylose conversion were almost unaffected by gradually decreased HRT from 8 to 1 hour. Sugar conversion was higher than 97.8% yielding 0.33 g-ethanol/g-initial sugars and ethanol productivity gradually increased from 0.43 to 3.34 g/l/h. The second experiment was performed to investigate the co-fermentation of glucose and xylose. Both sugars were simultaneously and effectively converted to ethanol with sugar utilization higher than 90.6% at sugar mixtures up to 54 g/l. At these sugar concentrations, the ethanol production increased gradually and the maximum ethanol concentration achieved was 15.35 g/l. Ethanol yields were 0.28-0.40 g-ethanol /g-initial sugars. The maximum ethanol productivity obtained was 1.1 g/l/h at HRT of 8 hours and 30 g/l sugars. This study demonstrated that active immobilized cell culture of thermophilic anaerobic bacteria was possible. The reactor was operated continuously for 140 days with no contamination and showed good long-term performance.

EXAMPLE 7

Continuous fermentation of steam exploded wheat straw using BG1L1

Steam exploded wheat straw hydrolysate (SEWS) was prepared by steam explosion followed by enzymatic hydrolysis (using Celluclast and Novozyme188 provided by Novozymes A/S) to release the constituent sugars, glucose and xylose. SEWS was provided by ELSAM, DK. The hydrolysate had dry matter content of 23% (DM), and glucose and xylose were, 57 g/l and 30 g/l, respectively. To counteract bacterial contamination, the SEWS hydrolysate medium was heated up to 121°C for 1 min. Two SEWS suspensions were prepared by addition of respective volume of water given the desired concentrations of 7.5% and 15% DM corresponding to glucose-xylose mixtures of 12 and 43 g/l, respectively (Fig.11). Despite, the SEWS medium was both sterilized and undetoxified, strain BG1L1 was capable as well of co-fermenting glucose and xylose efficiently with relatively high ethanol yield of 0.39-0.4 g/g (Fig.12). Glucose was completely utilized (>98%) for both tested SEWS suspensions,
whereas at xylose conversion decrease from 99% to 80% at 15% (DM) SEWS, however, overall sugar conversion was higher than 90%. Acetate was the main by-product and remained relatively low during the entire fermentation (0.07-0.08 g/g) (Fig.12).

In all these fermentations, only minor amounts of lactate were produced, as expected since the strain is a lactate dehydrogenase deficient mutant.

During both experiments lasting for approximately 140 days, the reactor was checked regularly for contamination by purifying chromosomal DNA from reactor samples, and no other species than BG1L1 were found. The deletion of the lactate dehydrogenase was also found to be stable as shown by sequencing of the lactate dehydrogenase region.

**EXAMPLE 8**

*Continuous fermentation of acid pre-treated corn stovers using BG1L1*

Corn stover hydrolysate (PCS), prepared by dilute sulfuric acid hydrolysis, was provided by the National Renewable Energy Laboratory (Golden, CO, USA). The hydrolysate had a total solids (TS) content of 30% (wt), and xylose, glucose and acetic acid concentrations were 67 g/l, 15 g/l and 14 g/l, respectively. Corn stover hydrolysate in concentrations of 2.5% - 15% TS was used. Because of the low total sugar concentration in the hydrolysate suspensions of 2.5% and 5% TS, extra 5 g/l xylose was added to these suspensions to prevent eventual process problems caused by the relatively low sugar content.

With PCS hydrolysate concentrations in the range of 2.5-10% TS, ethanol production increased gradually and relatively high and stable ethanol yields in a range of 0.41-0.43 g/g were obtained (Fig. 15). Almost complete sugar utilization (higher than 95%) was achieved for PCS of 2.5-7.5% TS, whereas at 10% (TS), the sugar conversion decreased to appr. 85% (Fig. 16). At a PCS concentration of 15% TS, sugar conversion was 70% and relatively high ethanol yields of close to 0.35 g/g was obtained. The lower sugar conversion at 15% (TS) PCS (Fig. 13) compared to other hydrolysate concentrations might be attributed to the growth and product inhibition caused by negative combination effect of high concentrations of acetate, other inhibitors present in the hydrolysate and salt accumulation resulted from based added for pH control (Lynd et al, 2001; Palmqvist and Hahn-Hägerdal 2000; Zalvidar et al. 2001). However, the low ethanol yield at PCS of 15% TS (Fig. 15) was probably due to higher ethanol evaporation than expected, since the carbon recovery was low (CR<0.9).

Acetate production increased from approximately 1 to 3.5 g/l (Fig.14). However, because of high initial acetate concentrations (appr. 1-7 g/l) in the feed stream, a rather high concentration of nearly 10 g/l acetate was present in the effluent, which is significant with
regard to the inhibitory effect of acetic acid to the fermentation. These results clearly show the high tolerance of the organism towards metabolic inhibitors present in undetoxified PCS.

EXAMPLE 9

BG1PF: a lactate producing mutant of strain BG1

A strain derived from BG1 was made by integration of the DNA fragment shown in figure 20 into the chromosome of BG1. The purified ppta32K plasmid, containing the thermostable kanamycin resistance gene flanked by the regions upstream and downstream from pfor, was linearized and electroporated into BG1, and positive recombinants were selected using the thermostable kanamycin resistance gene. Several independent clones were isolated and verified by PCR. PCR products from two clones were subsequently sequenced and were found to contain the kanamycin resistance cassette instead of the pfor genes as expected.

Five independent clones of BG1PF were grown in batch for two days with 5 g/l xylose as carbon source. As can be seen from figure 21, a complete shift in metabolism is seen, and production of lactate is now the major product of fermentation.

EXAMPLE 10

BG1H1: a BG1 mutant with increased hydrogen production constructed by down-regulation of uptake hydrogenase

Hydrogenases can be involved in both production and in uptake of hydrogen. In BG1, two sequences with similarity known hydrogenases were found. One (hydA BG1) was 80.4% identical on the DNA level to the *Thermoanaerobacter tengcongensis* hydA gene and 91.8% identical on the amino acid level to the corresponding HydA protein. The HydA protein is one out of 4 subunits in a cytosolic NAD(H) dependent Fe-only hydrogenase of *Ta. Tengcongensis* (1015). The NADH dependent hydrogenase activity is inhibited by high hydrogen partial pressure, whereas the the aldehyde dehydrogenase and alcohol dehydrogenase activities are higher in *T. tengcongensis* grown at elevated p(H₂). Similarly the ethanol production is almost abolished in fermenter cultures with low p(H₂).

The second sequence in BG1 with similarity to hydrogenases was found to be 73% identical to the echE gene of *Thermoanaerobacter tengcongensis* and 76% identical to the EchE protein subunit of the Ech hydrogenase of *Thermoanaerobacter tengcongensis*. The Ech hydrogenase is a ferredoxin dependent [NiFe] hydrogenase found in the membrane fraction
of *Thermoanaerobacter tengcongensis* extracts. Both *Thermoanaerobacter tengcongensis*
hydrogenases are primarily hydrogen evolving enzymes.

In *Thermoanaerobacter* BG1, the primary product is ethanol both under high and low \( p(H_2) \).
It is therefore likely that it employs a different strategy for hydrogen and ethanol production.

The effect of downregulation of the two hydrogenases was studied by introducing cassettes
for downregulation of echE and hydA expression into the chromosome of BG1. The basic
construction of the cassettes is shown in figure 22. When the cassette is introduced into BG1,
the lactate dehydrogenase gene is removed, and the promoter is activated. This results in the
expression of a small transcript complementary to the hydA and echE containing mRNA
respectively. This expression will in most cases result in lower levels of mRNA due to
digestion of double stranded RNA.

The expression of the hydA antisense RNA and the corresponding downregulation of hydA
mRNA was validated by Northern blots (figure 23). Total RNA was purified from BG1,
transferred to a membrane and probed with probes complementary to hydA anti-sense RNA
and mRNA respectively. The promoter used to express the anti-sense RNA has been shown to
be repressed by glucose and induced in the presence of xylose. In accordance with this, the
anti-sense is only seen during growth on xylose. The wild-type BG1 strain and the BG1L1
strain, in which ldh has been deleted, but no anti-sense is inserted, are also shown as
controls. As expected, no hydA anti-sense RNA is seen in these strains. The transcript from
the hydA operon is expected to be around 500 b in length. When a probe directed against the
hydA containing mRNA is used, a band which migrates as approximately 6000-7000 b is
seen. One to two smaller and more intense bands of approx 3000-4000 b are also seen, in
particular when the cells are grown with xylose as the carbon source. The smallest band is
probably a cross-hybridization to 16S rRNA. The RNA corresponding to the 3-4000 b RNA
varies greatly in intensity. The level of this species is much higher during growth on xylose as
compared to glucose and higher in the strain deleted for the lactate dehydrogenase as
compared to the wild-type. When grown on glucose, the levels of this band is equal in BG1L1
and BG1H1, while during growth on xylose it is much more intense in the strain where no
anti-sense is expressed. This corresponds nicely to the expression of the anti-sense RNA
expression in the upper panel.

As figure 24 shows, the BG1H1 produce more acetate, more hydrogen, and less ethanol than
the control strains. This indicates that the HydABCD hydrogenase of BG1 is involved in
hydrogen uptake, rather than production as it was shown for *Ta. tengcongensis*. As NAD(P)H
is needed for the reduction of acetylCoA to acetaldehyde and acetaldehyde to ethanol, it is
likely that the HydABCD hydrogenase is involved in the reduction of these cofactors using
hydrogen. Both acetate and hydrogen are valuable products and strain BG1H1 may therefore be preferred if a higher hydrogen and acetate production is a target.
REFERENCES


CLAIMS

1. A *Thermoanaerobacter mathranii* strain selected from BG1 (DSMZ Accession number 18280) and mutants thereof.

2. A strain according to claim 1, wherein the strain is capable of growing in a medium comprising a hydrolysed lignocellulosic biomass material having a dry-matter content of at least 10% wt/wt.

3. A strain according to claim 2, wherein the dry-matter content is at least 15% wt/wt.

4. A strain according to claim 2, wherein the dry-matter content is at least 20% wt/wt.

5. A strain according to claim 2, wherein the dry-matter content is at least 25% wt/wt.

6. A strain according to claim 2, wherein said hydrolysed lignocellulosic biomass material is selected from the group consisting of garden refuse, comminuted wood, straw, hay, fruit hulls and seed hulls.

7. A strain according to claim 1, which is capable of growing at or above 70°C.

8. A strain according to claim 1, which is capable of growing on galactose as the sole carbon source.

9. A strain according to claim 1, which is capable of producing a fermentation product selected from the group consisting of an acid, an alcohol, a ketone and hydrogen.

10. A strain according to claim 9, wherein the alcohol is selected from the group consisting of ethanol, butanol, propanol, methanol, propanediol and butanediol.

11. A strain according to claim 9, wherein the acid is selected from the group consisting of lactic acid, propionate, acetate, succinate, butyrate and formate.

12. A strain according to claim 9, wherein the ketone is acetone.

13. A strain according to claim 1, wherein one or more genes have been inserted, deleted or substantially inactivated.
14. A strain according to claim 1, wherein a gene encoding lactate dehydrogenase (LDH) (EC 1.1.1.27) has been down-regulated or substantially inactivated.

15. A strain according to claim 14, wherein the gene encoding lactate dehydrogenase (LDH) (EC 1.1.1.27) has been inactivated by the deletion of said gene.

16. A strain according to claim 14, wherein the gene encoding lactate dehydrogenase (LDH) (EC 1.1.1.27) has been substantially inactivated by the mutation, deletion or insertion of one or more amino acids in said gene.

17. A strain according to claim 14, which is BG1L1 (DSMZ Accession number 18283).

18. A strain according to claim 1, wherein a gene encoding pyruvate ferredoxin oxidoreductase (EC 1.2.7.1) has been down-regulated or substantially inactivated.

19. A strain according to claim 1, wherein a gene encoding pyruvate ferredoxin oxidoreductase (EC 1.2.7.1) has been substantially inactivated by the mutation, deletion or insertion of one or more amino acids in said gene.

20. A strain according to claim 18 which is BG1PF1 (DSMZ Accession number 18282).

21. A strain according to claim 1, wherein a gene encoding a hydrogenase or a hydrogenase subunit has been down-regulated or substantially inactivated.

22. A strain according to claim 21, wherein the gene encoding hydrogenase or a hydrogenase subunit has been substantially inactivated by the mutation, deletion or insertion of one or more amino acids in said gene.

23. A strain according to claim 21, wherein a gene encoding hydrogenase or a hydrogenase subunit is selected from the group consisting of [Fe]-hydrogenases and [NiFe]-hydrogenases (EC 1.6.5.3, EC 1.12.7.2, 1.12.99.6) such as NuoE, NuoF, NuoG, EchB, EchC, EchD, EchE, EchF

24. A strain according to claim 21 which is BG1H1 (DSMZ Accession number 18281).

25. A strain according to claim 1, wherein a gene encoding an acetate kinase (EC 2.7.2.1) has been substantially inactivated by the mutation, deletion or insertion of one or more amino acids in said gene.
26. A strain according to claim 1, wherein a gene encoding a phosphate acetyltransferase (EC 2.3.1.8) has been substantially inactivated by the mutation, deletion or insertion of one or more amino acids in said gene.

27. A strain according to claim 1, wherein one or more genes have been inserted.

28. A strain according to claim 27, wherein one or more genes encoding a polysaccharase has been inserted.

29. A strain according to claim 28, wherein the polysaccharase is selected from the group consisting of cellulases (EC 3.2.1.4); beta-glucanases, including glucan-1,3 beta-glucosidases (exo-1,3 beta-glucanases, EC 3.2.1.58), 1,4-beta-cellobiohydrolase (EC 3.2.1.91) and endo-1,3(4)-beta-glucanases (EC 3.2.1.6); xylanases, including endo-1,4-beta-xylanases (EC 3.2.1.8) and xylan 1,4-beta-xylosidase (EC 3.2.1.37); pectinases (EC 3.2.1.15); alpha-glucuronidase, alpha-L-arabinofuranosidase (EC 3.2.1.55), acetylesterase (EC 3.1.1.--), acetylxylanesterase (EC 3.1.1.72), alpha amylase (EC 3.2.1.1), beta-amylase (EC 3.2.1.2), glucoamylase (EC 3.2.1.3), pullulanase (EC 3.2.1.41), beta-glucanase (EC 3.2.1.73), hemicellulase, arabinosidase, mannanases including mannan endo-1,4-beta-mannosidase (EC 3.2.1.78) and mannan endo-1,6-alpha-mannosidase (EC 3.2.1.101), pectin hydrolase, polygalacturonase (EC 3.2.1.15), exopolygalacturonase (EC 3.2.1.67) and pectate lyase (EC 4.2.2.10).

30. A strain according to claim 27, wherein one or more genes encoding a pyruvate decarboxylase (EC 4.1.1.1) has been inserted.

31. A strain according to claim 27, wherein one or more genes encoding an alcohol dehydrogenase (EC 1.1.1.1, EC 1.1.1.2, EC 1.1.1.71, EC 1.1.99.8) has been inserted.

32. A strain according to claim 1, wherein the expression of one or more genes encoding an alcohol dehydrogenase has been increased.

33. A method of producing a fermentation product comprising culturing a strain according to any of claims 1-32 under suitable conditions.

34. A method according to claim 33, which is a fermentation process performed under strict anaerobic conditions.
35. A method according to claim 33, which is operated at temperature in the range of about 40-95°C, such as the range of about 50-90°C, including the range of about 60-85°C, such as the range of about 65-75°C.

36. A method according to claim 34, wherein the fermentation process is a batch fermentation process.

37. A method according to claim 34, wherein the fermentation process is a continuous fermentation process.

38. A method according to claim 33, wherein the fermentation product is selected from the group consisting of an acid, an alcohol, a ketone and hydrogen.

39. A method according to claim 38, wherein the alcohol is selected from the group consisting of ethanol, butanol, propanol, methanol, propanediol and butanediol

40. A method according to claim 38, wherein the acid is selected from the group consisting of lactic acid, propionate, acetate, succinate, butyrate and formate.

41. A method according to claim 38, wherein the ketone is acetone.
Fig. 4

![Graph showing concentration vs pH](image-url)
Fig. 6

Mix A

Mix B

SUBSTITUTE SHEET (RULE 26)
Fig. 7

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<th>Strain</th>
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<th>BGIL1</th>
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<tr>
<td>2.21</td>
<td>4.48</td>
<td>3.89</td>
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<td>2.02</td>
<td>4.67</td>
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<tr>
<td>C rec</td>
<td>1.04</td>
<td>0.97</td>
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8/21

Fig. 8

A

![Graph showing the relationship between xylitol yield (mM/min) and xylitol (g/L).]

B

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<tr>
<td>C rea</td>
<td>0.97</td>
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Fig. 9

A

B

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<td>C reco</td>
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<td>8</td>
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<td>40.35</td>
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Fig. 13

A

![Graph A showing the concentration of glucose, xylose, and total sugar against PCS (TS, %)]

B

![Graph B showing the conversion of glucose, xylose, and total sugar against PCS (TS, %)]
Fig. 15

The graph illustrates the relationship between PCS (TS, %) and ethanol yield (g-ethanol/g-consumed sugars) along with carbon recovery. The graph shows two distinct lines:

- Black squares represent the yield of ethanol.
- Black circles represent the carbon recovery.

The x-axis represents the PCS (TS, %) ranging from 3 to 15, while the y-axis represents the ethanol yield and carbon recovery ranging from 0.00 to 1.00.

Substitute Sheet (Rule 26)
Fig. 20

Xylose  Glucose
BG1  BG1L1  BG1H1  BG1  BG1L1  BG1H1

exp: 590

exp: 5200
BioGasol ApS
Søeltofs plads, bygning 227
2800 Lyngby
Denmark

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITORY AUTHORITY identified at the bottom of this page:

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:
BG1

Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY:
DSM 18280

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
( x ) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I. above, which was received by it on 2006-05-17 (Date of the original deposit).

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I. above was received by this International Depository Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITORY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH
Address: Mascheroder Weg 16
D-38124 Braunschweig

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized official(s):

Date: 2006-05-24

1 Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001
**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure**

**International Form**

**BioGasol ApS**  
Soeltofts plads, bygning 227  
2800 Lyngby  
Denmark

**Receipt in the case of an original deposit**  
Based pursuant to Rule 7.3 by the International Depository Authority identified at the bottom of this page.

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| Address: Mecklenburger Weg 1b  
D-38124 Braunschweig |
| Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized official(s): |
| V. Wehler |
| Date: 2006-05-24 |

1 Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP4 (gloss page) 12/2001
**INTERNATIONAL FORM**

BioGasol ApS  
Søetofis plads, bygning 227  
2800 Lyngby  
Denmark  

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| Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH  
| Address: Mascheroder Weg 1b  
| D-38124 Braunschweig  
| Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): |
| V. Wes-ns  
| Date: 2006-05-24 |

1 Where Rule 6.4 (d) applies, such date is the date on which the status of International depositary authority was acquired.

Form: DSMZ-BPv4 (sole page) 12/2001
BioGasol ApS
Slettofts plads, bygning 227
2800 Lyngby
Denmark

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR: BG1L1
Accession number given by the INTERNATIONAL DEPOSITORIAL AUTHORITY: DSM 18283

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
( x ) a proposed taxonomic designation

(Mark with a cross where applicable.

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I. above, which was received by it on 2006-05-17 (Date of the original deposit)

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on for conversion.

V. INTERNATIONAL DEPOSITORIAL AUTHORITY

| Name: DSMZ-DIETISCHE SAMMLUNG VON MICROORGANISMEN UND ZELLKULTUREN GmbH |
| Address: Max-Eyth-Weg 1b |
| D-38124 Braunschweig |

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized official(s):

V. Weis
Date: 2006-05-24

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP4 (sole page) 12/2001
INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 3, line 19.

B. IDENTIFICATION OF DEPOSIT

Name of depositary institution
DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

Address of depositary institution (including postal code and country)
Mascheroder Weg 1b
D-38124 Braunschweig

Date of deposit 24 May 2006
Accession Number DSM 18280

C. ADDITIONAL INDICATIONS (leave blank if not applicable)

This information is continued on an additional sheet

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

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INDICATIONS RELATING TO DEPOSITED MICROORGANISM
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(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 9, line 22.

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet [X]

Name of depositary institution
DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

Address of depositary institution (including postal code and country)
Mascheroder Weg 1b
D-38124 Braunschweig

Date of deposit
24 May 2006

Accession Number
DSM 18281

C. ADDITIONAL INDICATIONS (leave blank if not applicable)

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E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

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Form PCT/RO/134 (July 1998; reprint January 2004)
INDICATIONS RELATING TO DEPOSITED MICROORGANISM 
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 8, line 33.

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depositary institution
DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)

Address of depositary institution (including postal code and country)
Mascheroder Weg 1b
D-38124 Braunschweig

Date of deposit
24 May 2006

Accession Number
DSM 18282

C. ADDITIONAL INDICATIONS (leave blank if not applicable)

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E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

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INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 8, line 20.

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This information is continued on an additional sheet

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)
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Form PCT/RO/134 (July 1998; reprint January 2004)
A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N1/20 C12P7/06 C12P7/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C12P

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>A</td>
<td>DESAI S G ET AL: &quot;Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in Thermoanaerobacterium saccharolyticum JW/SL-YS485&quot; APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER VERLAG, BERLIN, DE, vol. 65, no. 5, 6 March 2004 (2004-03-06), pages 600-605, XP002393736 ISSN: 0175-7598 page 604, left-hand column, line 1 - right-hand column, last line</td>
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1,13-15, 33-39

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

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

Date of the actual completion of the international search: 5 September 2007

Date of mailing of the international search report: 20/09/2007

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tx: 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Huber, Angelika
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<td>AHRING B K ET AL: &quot;Production of ethanol from wet oxidised wheat straw by Thermoanaerobacter mathranii&quot; BIORESOURCE TECHNOLOGY, vol. 68, no. 1, April 1999 (1999-04), pages 3-9, XP002449341 ISSN: 0960-8524 abstract</td>
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