SPORULATION-DEFICIENT THERMOPHILIC MICROORGANISMS FOR THE PRODUCTION OF ETHANOL

Field of the Invention

This invention relates to the production of microorganisms suitable for the production of ethanol. In particular, the invention relates to the modification of microorganisms to prevent sporulation.

Background to the Invention

Sporulation is a multi-stage developmental process that is responsible for the conversion of a growing cell into a dormant cell type, known as a spore or endospore. Spores are adapted for dispersal and survival for an extended period of time in unfavourable conditions and form part of the life cycle of many plants, algae and bacteria, such as the Bacillus species.

The primary regulator for entry into sporulation is the DNA-binding protein Spo0A (stage 0 sporulation protein A), which is a member of the response regulator family of transcription factors. Numerous other genes, including genes which encode five histidine autokinases (KinA, KinB, KinC, KinD and KinE) and two response proteins (Spo0B and Spo0F), are also involved in the control of the initiation of sporulation (Molle *et al.*; Mol. Microbiol.; 2003, 50(5):1683-1701). The activity of Spo0A is governed by a multi-component phosphorelay, which recognises and integrates environmental signals to initiate sporulation (Trach KA, *et al;* Mol. Microbiol. 1993; 8(1):69-79). Upon phosphorylation of its regulatory N-terminal domain, Spo0A-P binds to a DNA sequence element known as the "0A-box" which activates genes involved in sporulation. Deletion of the C-terminal domain of Spo0A, which is inactive until the N-terminus has been phosphorylated, has been shown to result in a sporulation-negative phenotype (Rowe-Magnus DA, *et al;* J. Bacteriol.; 2000; 182(15):4352-4355).

Spo0A has also been found to influence, directly or indirectly, the activation or repression of expression of over 500 genes in B. *subtilis*, and therefore indirectly mediates the global pattern of gene transcription via regulatory genes under its control (Molle *et al.*; Mol. Microbiol.; 2003, 50(5):1683-1701).

Sporulation is subject to catabolite repression, whereby the presence of glucose or other readily metabolized carbon sources inhibits sporulation by wild-type cells. In particular, glucose is known to repress the transcription of *spo0A* and *spo0F* (Myseliwiec, TH *et* al; J. Bacterial.; 1991; 173(6):1911-1919).

In a commercial fermentation process spores are undesirable for two main reasons:

1. Sporulation pauses active metabolism by an organism resulting in a reduction or cessation of the formation of a desired metabolic product; and
2. Sporulating microorganisms are more difficult to handle and control containment, therefore it is desirable to avoid the survival of commercial process microorganisms for environmental reasons, including health and safety, and also to prevent the uncontrolled release of the commercial strain.

The general process by which bacteria metabolise suitable substrates is glycolysis, which is a sequence of reactions that converts glucose into pyruvate with the generation of ATP. The fate of pyruvate in the generation of metabolic energy varies depending on the microorganism and the environmental conditions. The four principal reactions of pyruvate are illustrated in Figure 5.

First, under aerobic conditions, many microorganisms will generate energy using the citric acid cycle and the conversion of pyruvate into acetyl coenzyme A, catalysed by pyruvate dehydrogenase (PDH).

Second, under anaerobic conditions, certain ethanologenic organisms can carry out alcoholic fermentation by the decarboxylation of pyruvate into acetaldehyde, catalysed by pyruvate decarboxylase (PDC) and the subsequent reduction of acetaldehyde into ethanol by NADH, catalysed by alcohol dehydrogenase (ADH).

A third reaction, which also occurs in anaerobic conditions, is the conversion of pyruvate to acetyl CoA, catalysed by pyruvate formate lyase (PFL). Acetyl CoA is subsequently converted into acetaldehyde by the enzyme acetaldehyde dehydrogenase (AcDH) and ethanol is produced by the reduction of acetaldehyde catalysed by ADH.

A fourth process is the conversion of pyruvate into lactate which occurs through catalysis by lactate dehydrogenase (LDH).

There has been much interest in using microorganisms for the production of ethanol using either microorganisms that undergo anaerobic fermentation naturally or through the use of recombinant microorganisms which incorporate the pyruvate decarboxylase and alcohol dehydrogenase genes.

WO2008/038019 discloses microorganisms which comprise modifications to inactivate the native LDH and PFL genes and up-regulate the PDC, PDH and ADH genes in order to promote the formation of ethanol.

There is a need for further improvements to the production of ethanol from microorganisms.

Summary of the Invention

The present invention is based upon the surprising finding that inhibition of the *spo0A* gene in spore-forming thermophilic microorganisms results in increased ethanol tolerance of the microorganism, and also increased metabolism, which results in an increase in the rate of production of metabolic end-products such as ethanol.

According to a first aspect of the present invention, a thermophilic microorganism comprises a modification that decreases sporulation compared with wild-type, wherein a first modification inactivates the native *spo0A* gene.

The microorganism may be further modified to permit increased production of ethanol via inactivation of the native lactate dehydrogenase and, optionally, pyruvate formate lyase genes. Further modification can be made to upregulate the native pyruvate dehydrogenase gene or introducing an active pyruvate decarboxylase gene.

The microorganism may be further modified to permit increased production of ethanol from starch by increasing amylase gene expression.

The microorganism of the invention shows increased ethanol production and increased ethanol tolerance compared to wild-type.

According to a second aspect of the present invention, a method of producing ethanol comprises culturing a microorganism according to the definition provided above in suitable conditions in the presence of a C3, C5 or C6 sugar, or an oligomer thereof.

Description of the Drawings

The present invention is described with reference to the accompanying figures, wherein:

Figure 1 is the Spo0A nucleotide sequence (SEQ ID No.1);

Figure 2 is the Spo0A amino acid sequence (SEQ ID No.2);

Figure 3 illustrates the plasmid pTM014 (SEQ ID No.4);

Figure 4 illustrates the hypothetical promoter regions and genes of the PDH complex;

Figure 5 illustrates the four principal reactions of pyruvate;

Figure 6 illustrates the pGEM®-T Easy Vector;

Figure 7 illustrates the plasmid pTM031 (SEQ ID No.3);

Figure 8 illustrates schematically the organisation of *spo0A* and surrounding genes from a 4480bp sequence read of genomic DNA isolated from G.*thermoglucosidasius*;

Figure 9 outlines the two approaches to disrupting the *spo0A* gene;

Figure 10 illustrates the expected PCR product sizes of *spo0A* knock-out compared with the original *spo0A* gene;

Figure 11 is a graph showing alterations in the fermentation characteristics of TM242 in media comprising 8% w/v cellobiose and 2% yeast extract when ethanol vapour is partitioned from the fermentation broth ("gas-off") and when it is not partitioned from the broth; and

Figure 12a is a graph showing the fermentation characteristics of TM242 in media comprising 8% w/v cellobiose and 2% w/v yeast extract and Figure 12b shows the fermentation characteristics of TM444 in the same media.

Detailed Description of the Invention

The present invention relates to the modification of a thermophilic microorganism to prevent sporulation.

The invention is based upon the surprising finding that inhibition of sporulation is associated with increased ethanol tolerance, enabling larger yields of ethanol to be produced by microorganisms in batch fermentation processes. Non-sporulating microorganisms also have process advantages since they are easier to handle and control than spore-formers.

Furthermore, due to an increase in metabolism, it has been found that fermentation proceeds to completion at a faster rate when sporulation is prevented.

Sporulation can be prevented by modifying the microorganism to inactivate the native *spo0A* gene, preferably by deleting at least a portion of the *spo0A* gene or by targeted disruption of the gene. Preferably, as a result of the modification the microorganism is entirely sporulation-deficient.

The coding sequence of the *spo0A* gene (SEQ ID No.1) is shown in Figure 1. The amino acid sequence of the polypeptide encoded by the *spo0A* gene (SEQ ID No.2) is shown in Figure 2. Using this coding sequence, it is possible for the skilled person to target *spo0A* to achieve inactivation of the gene through different mechanisms. It is preferred if the *spo0A* gene is inactivated by the deletion of the gene sequence, or a portion thereof, preferably the C-terminal domain.

Methods to inactivate the gene will be apparent to the skilled person, based on the knowledge of the gene sequence, as disclosed herein

The gene sequence may be deleted or inactivated by insertion of additional DNA to the disrupt gene expression.

Methods of targeted gene disruption are well known in the art and include, for example, the integration of temperature-sensitive plasmids into the target gene on the chromosome. Integration of a plasmid may delete the target gene entirely, or may replace the complete gene with a portion of the gene that is non-functional. This can be achieved by isolating a sequence that includes the gene of interest, excising a portion of the gene, amplifying the remaining fragments, cloning these fragments into a temperature-sensitive plasmid and then transforming target microorganisms with the plasmid. The present invention is not limited to a specific method of inactivating the *spo0A* gene, however a detailed description of a suitable technique using the plasmid pTMO31 is provided in the 'Example' section.

The microorganism may be any thermophilic microorganism, but it is preferred if the microorganism is of the *Bacillus* species. In particular, it is preferred if the microorganism is a wild-type microorganism of the *Geobacillus* species, in particular *Geobacillus thermoglucosidasius*.

In a preferred embodiment, the microorganisms selected for modification are said to be "wild-type", i.e. they do not comprise any further laboratory-produced mutations in addition to the mutations described herein. The microorganisms may

be isolated from environmental samples expected to contain thermophiles. Isolated wild-type microorganisms will have the ability to sporulate. Furthermore, isolated wild-type microorganisms will have the ability to produce ethanol from pyruvate but, unmodified, lactate is likely to be the major fermentation product. The isolates are selected for their ability to grow on hexose and/or pentose sugars, and oligomers thereof, at thermophilic temperatures.

It is preferable that the microorganism of the invention has certain desirable characteristics which permit the microorganism to be used in a fermentation process. The microorganism should preferably have no restriction system, thereby avoiding the need for *in vivo* methylation. In addition, the microorganism should be stable to at least 3% w/v ethanol, preferably 5-10% w/v ethanol, and most preferably up to 20% w/v ethanol. The microorganisms should have the ability to utilise C_3, C_5 and C_6 sugars (or their oligomers) as a substrate, including cellulose, cellobiose, hemicellulose, starch and xylan. It is preferable if the microorganism is transformable at a high frequency. Furthermore, the microorganism should have a growth rate in continuous culture to support dilution rates of 0.3 h^{-1} and above.

The microorganism will be a thermophile and will grow in the temperature range of 40°C - 85°C. Preferably, the microorganism will grow within the temperature range 50°C - 70°C. In addition, it is desirable that the microorganism grows in conditions of pH 8 or below, in particular pH 4.5-pH 6.9.

Preferred microorganisms of the invention are identified herein as TM443 and TM444, each of which has been deposited at NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA with NCIMB Accession Nos. 41591 and 41588 respectively.

The thermophilic microorganism of the invention may be further modified to disrupt the expression of the lactate dehydrogenase gene (LDH).

Inactivating the lactate dehydrogenase gene helps to prevent the breakdown of pyruvate into lactate, and therefore promotes (under appropriate conditions) the breakdown of pyruvate into ethanol using pyruvate decarboxylase and alcohol dehydrogenase. It is preferable for the lactate dehydrogenase gene to be disrupted by a deletion within, or of, the gene.

The nucleic acid sequence for lactate dehydrogenase is now known. Using this sequence, it is possible for the skilled person to target the lactate

dehydrogenase gene to achieve inactivation of the gene through different mechanisms. It is possible to inactivate the lactate dehydrogenase gene by the insertion of a transposon. However, it is preferred if the lactate dehydrogenase gene is inactivated by the deletion of the gene sequence or a portion of the gene sequence. Deletion is preferred, as this avoids the difficulty of reactivation of the gene sequence which is often experienced when transposon inactivation is used. In a preferred embodiment, the lactate dehydrogenase gene is inactivated by the integration of a temperature-sensitive plasmid, which achieves natural homologous recombination or integration between the plasmid and the microorganism's chromosome. Preferably, the plasmid is pTM014 (SEQ ID No.4), which is illustrated in Figure 3. Chromosomal integrants can be selected for on the basis of their resistance to antibacterial agents. The integration into the lactate dehydrogenase gene may occur by a single cross-over recombination event or by a double (or more) cross-over recombination event.

The microorganism may also be modified to up-regulate the pyruvate dehydrogenase gene (PDH). Up-regulating the pyruvate dehydrogenase gene promotes the conversion of pyruvate into acetyl CoA, which can then be used, under appropriate conditions, to produce acetaldehyde and eventually ethanol using acetaldehyde dehydrogenase. A further advantage of up-regulating PDH is that pyruvate levels, which have an inhibitory effect on glucose uptake and glycolysis, are reduced. This further promotes ethanol production. PDH is a large enzyme complex, containing three units - E1: pyruvate decarboxylase (EC 1.2.4.1, not EC 4.1.1.1), E2: dihydrolipoamide transacetylase, and E3: dihydrolipoamide dehydrogenase. The complex requires several cofactors, including NAD, FAD, coenzyme A lipoic acid and thiamine pyrophosphate (TPP). Four genes code for the complex, as the E1 unit is a heterodimer of α and β subunits, and are often described as *pdh*A, *pdh*B, *pdh*C and *pdh*D (E1α, E1, E2β and E3 respectively). The E1 unit of PDH requires TPP in the same way that PDC (EC 4.1.1.1) requires TPP and catalyses a similar decarboxylation reaction, but in the presence of coenzyme A and lipoic acid - carried by other enzyme units - the product is acetyl CoA rather than acetaldehyde. However, PDC activity of the E1 unit has been measured when it has not been complexed with other units in PDH (Lessard & Perham; *The Journal of Biological Chemistry*; 1994, 269;14, 10378-10383; Tomar

et al; *Applied Microbiology and Biotechnology*; 2003, 62, 76-82; Frank *et al*; *Science*; 2004, 306; Oct 29, 872-876, supplementary data). Accordingly, PDC activity of EC 1.2.4.1 may be enhanced by the up-regulation of PDH so that acetaldehyde is produced over and above acetyl CoA. Enhanced PDH activity is also sought to remove the pyruvate bottleneck observed in LDH inactivated strains to allow more ethanol to be produced with less acetate and formate as side products.

To this end, the PDH genes and surrounding sequence were isolated using standard "genome walking" techniques. Approximately 8.8kb of DNA was isolated, sequenced and found to contain the following genes shown in Figure 4 and Table 1.

Table 1

Gene	Position (bp)	Proposed Function	Frame (aa's at 5' and 3')	Size (aa)
pdf2	746-192	Peptide deformylase	-3 (MIT - IER)	184
orf2	868-1497	Unknown - Hypothetical protein	+1 (MQR - IWK)	209
pdhA(α)	1875-2984	α - subunit of pyruvate hydrogenase	+3 (MGA - ESK)	369
pdhA(β)	3003-3965	β - subunit of pyruvate dehydrogenase	+3 (MIQ - INF)	320
pdhB	4058-5368	Dihydrolipoamide transacetylase	+2 (VAF - MEA)	436
lpd	5373-6785	Lipoamide dehydrogenase	+3 (MVV - ISK)	470
orf7	7432-6833	Unknown - Hypothetical protein	-1 (MNK - CTE)	199
orf8	7964-8647	Transposase	+2 (MDL - SPP)	227

The hypothetical promoter regions are shown in Figure 4 (arrow) - one upstream from the start of pdhA and a possible second promoter ahead of pdhB. A previous example of a secondary promoter in the PDH cluster was reported for *Bacillus subtilis* (Gao *et al*; *Journal of Bacteriology*, 2002, 184:10, 2780-2788), but most described PDH gene clusters have just one promoter upstream of the cluster (Neveling *et al*; *Biochimica Acta*; 1998 1385. 367-372). The upregulation can be carried out using techniques known in the art. In particular, upregulation can be carried out by introducing a suitable promoter or enhancer sequence upstream of the PDH complex.

The enzyme complex is known to work under both aerobic and anaerobic conditions (Carlsson *et al*; *Infection and Immunity*; 1985, 49(3):674-678) but it is generally considered to be an aerobic enzyme (Ch 15; *Principles of Biochemistry*; Lehninger, Nelson & Cox; 2nd Ed, Worth Publishers, New York, 1993, p447) with pyruvate formate lyase (PFL) its anaerobic counterpart. Both enzymes convert pyruvate, formed in glycolysis, to acetyl CoA to feed into the TCA cycle but the cycle only works completely under aerobic conditions. However, as it is desirable to use anaerobic conditions, promoters that operate in anaerobic conditions are preferred for use in the invention. Thus promoters for enzymes believed to work under anaerobic conditions - examples being the LDH promoter (P_ldh from G. *stearothermophilus* NCA1503), the PFL promoters (P_pfl from B.*cereus* ATCC14579, and G. *thermoglucosidasius* NCIMB11955) and ferredoxin promoters (P_ferrA from G. *stearothermophilus* DSM13240) – can be used, as in PCT/GB2007/03699 which is incorporated herein by reference.

In a preferred embodiment, a further modification is introduced to enhance the PDC activity, thereby promoting the conversion of pyruvate to acetaldehyde. This can be carried out by inactivating E2 (EC 2.3.1.12). Inactivation can be carried out in a manner similar to the inactivation of LDH, but with the E2 gene as the target for disruption.

In a further embodiment, a microorganism of the invention comprises a modification to inactivate the pyruvate formate lyase gene, thereby preventing/reducing the conversion of pyruvate to acetyl CoA and formate. Pyruvate formate lyase (PFL) is the "anaerobic counterpart" to pyruvate dehydrogenase (PDH) and converts pyruvate to acetyl CoA and formate (see Figure 6). While acetyl CoA can be converted to ethanol via acetaldehyde dehydrogenase (AcHD), formate is an undesired side-product which has the potential to inhibit growth in ethanolgenic organisms.

PFL was chosen as a target for knockout in order to promote the metabolic flux towards ethanol production and to improve the redox balance of the remaining pathway to ethanol synthesis. An additional advantage of this work was the elimination of formate production. PFL activity can be inactivated via transposon insertion, gene deletion or partial gene deletion to produce a mutant which does not rely on antibiotic selection for the continuation of the altered phenotype. However,

it is preferred if the pyruvate formate lyase gene is inactivated by the deletion of the gene sequence or a portion of the gene sequence. Deletion is preferred, as this avoids the difficulty of reactivation of the gene sequence which is often experienced when transposon inactivation is used. In this embodiment, it is preferred that the microorganism comprises both the lactate dehydrogenase inactivation and the up-regulation of the pyruvate dehydrogenase, so that, under anaerobic conditions, ethanol production is increased.

In a further preferred embodiment, the microorganism also comprises up-regulated pyruvate decarboxylase and/or alcohol dehydrogenase genes. The expression of these genes results in the production of enzymes which redirect the metabolism so that ethanol is the primary fermentation product. If the PDC gene is EC4.1.1.1, the gene will be heterologous and can be inserted in an expression cassette, as will be appreciated by the skilled person. If the PDC gene is EC1.2.4.1, it can be the homologous gene that is upregulated. The ADH gene may be heterologous or homologous. If the native gene is to be utilised, it may be upregulated by methods known in the art. Preferably, both PDC and ADH are expressed in the microorganism. The genes may be obtained from microorganisms that typically undergo anaerobic fermentation, including *Zymomonas* species, including *Zymomonas mobilis*.

Methods of the preparation and incorporation of a gene into microorganisms are known, for example in Ingram et al, Biotech & BioEng, 198; 58 (2 and 3): 204-214 and US5916787, the content of each being incorporated herein by reference. The gene may be introduced in a plasmid or integrated into the chromosome, as will be appreciated by the skilled person.

The thermophilic microorganism of the invention may be further modified to increase amylase gene expression compared to wild-type. Such modification is described in detail in WO2009/022158, the content of which is incorporated herein. This enables the microorganism to hydrolyse starch into glucose monomer units which can then be utilised as glycolytic substrates for the formation of pyruvate and subsequently ethanol. This modification therefore enables the increased production of ethanol from cheap, abundant, un-refined plant material.

Methods of increasing amylase expression and enzyme activity include the use of strong up-stream promoters to regulate transcription of the gene,

incorporation of additional amylase genes that are expressed at a higher frequency than the native amylase gene, or the expression of a more active amylase gene. The term "strong promoter" is defined herein as a promoter that expresses the corresponding protein to a level greater than 0.5% of the soluble protein in a cell.

In a preferred embodiment, a heterologous amylase gene encodes α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1). It is preferred that the amylase gene is derived from the *Geobacillus* species, in particular *Geobacillus stearothermophilus*.

The coding sequence of the α-amylase gene has been elucidated and the techniques enabling isolation and amplification of the gene are well known in the art. In order to enable the microorganism of the invention to exhibit increased amylase expression compared to wild-type, it is preferred that the amylase gene is placed under the control of a strong promoter, which operates in low-aeration or anaerobic conditions that favour ethanol production by thermophilic microorganisms. The promoter is preferably an ldh promoter and may be autologous, but is preferably heterologous, and is most preferably derived from the same species as the amylase gene. Examples of suitable promoters include, but are not limited to, P_ldh from *G.stearothermophilus* NCA1503, P_ferrA from G.*stearothermophilus* DSM13240 and P_pfl from *B.cereus* ATCC14579.

In another embodiment of the invention, a series of different strong promoters are placed upstream of the amylase gene in order to further enhance expression. Examples of suitable strong promoters include, but are not limited to, the glyceraldehyde-3-phosphate promoter (P_GAPDH) and amylase promoter from G. *stearothermophilus* NCA 1503.

The nucleic acid sequence of P_ldh is also known and techniques for cloning and assembling the promoter sequence upstream of the amylase gene are known to the skilled person.

The promoter/amylase sequence can be cloned into a suitable plasmid or expression vector containing multiple restriction sites. There are numerous suitable expression vectors which are commercially available, such as the pGEM®-T Easy Vector (Figure 6). Restriction enzymes can be used to excise the P_ldh/amylase construct as a specific fragment which can be ligated into the corresponding restriction site in a temperature-sensitive plasmid such as pTMO31 (Figure 7, SEQ

ID No. 3) able to use a pyruvate formate lyase knock-out plasmid. The plasmid construct comprising the amylase gene/ldh promoter can then be electroporated into the microorganism of the invention with subsequent homologous recombination with genomic DNA. Chromosomal integrants can be selected for on the basis of their resistance to antibacterial agents, such as ampicillin or kanamycin. Amylase activity can also be visualised as zones of starch clearing, for example on plate assays. The culture media may preferably comprise at least 1% w/v starch, preferably at least 10% w/v starch, and most preferably at least 20% w/v starch. The starch may be soluble or insoluble (e.g. grain starch).

An embodiment of the present invention will now be described, with reference to the accompanying drawings, in the following example. The present invention is exemplified but not limited, by the example.

Example

Two different Spo0A knock-out constructs were developed to take account of other sporulation genes adjacent to the target s*po0A* which could be transcriptionally affected upon out-of-frame *spo0A* disruption, as illustrated in Figure 8.

Thus an out-of-frame and an in-frame knockout cassette were produced as outlined in Figure 9. The out-of-frame cassette was generated by the removing of a 429bp region of the *spo0A* gene and replacing it with an engineered NotI restriction site to enable hybridisation of primers for PCR amplification of fragments comprising *spo0A* deletions, while the in-frame cassette was constructed by removing the naturally occurring 150bp MscI-MscI fragment.

The resulting fragments were cloned into pTM031, which is a 5.1kb plasmid derived from an EcoRI/SnaBI pUB110 fragment insert into pUC19. The plasmid map of pTM031 is illustrated in Figure 3 and the nucleic acid sequence of pTM031 corresponds to SEQ ID No.7. Nucleotides 1-239, 2634-2791 and 2848-5082 are derived from pUC19, nucleotides 240-2633 are derived from pUB110 and the remaining nucleotides (2792-2848) correspond to the multiple cloning site (MCS).

The resulting plasmids were then used to transform Geobacillus microorganisms which incorporated other modifications as set out below. Methods

of transformation, primary integration and stabilisation via selection of double cross-over mutants were employed in a variety of strain backgrounds, as detailed below.

Geobacillus thermoglucosidasius strain backgrounds

Strain name	Modification(s)
TM89	ldh^-
TM242	ldh^-, pdh_up, pfl^-
TM266	ldh^-, pdh_up, P_ldh(Ste), pfl^-
TM379	ldh^-, pdh_up, P_pfl(11955), pfl^-
TM333	ldh^-, pdh_up, pfl^-, P_ldh(NCA)/amyS(DSM22)

Results

Generation of Spo0A mutants in TM242

A total of 20 presumptive primary integrants (4 in-frame and 16 out-of-frame) of TM242 (NCIMB Accession No. 41589) were sub-cultured through two rounds of growth in 2TY medium at 60°C. Cells from each of these cultures were plated onto TGP medium and subsequently replicated onto TGP containing kanamycin at a final concentration of 12.5µg/ml. A total of 13 (5 in-frame, 8 out-of-frame) kanamycin-sensitive strains representing putative double cross-over mutants with a disrupted s*po0A* gene were identified.

Difco Sporulation Medium (DSM), made according to the following recipe, was used to demonstrated the ability of the mutants to sporulate. Testing was conducted before and after heat treatment to kill vegetative cells.

Difco Sporulation Medium (DSM)

	Per litre
Bacto nutrient broth (Difco)	8 g
10% (w/v) KCl	10 ml
1.2% (w/v) $MgSO_4 \cdot 7H_2O$	10 ml
1 M NaOH	~1.5 ml (pH to 7.6)

The volume is adjusted to 1 litre with ddH_20 and the pH is adjusted to 7.6. The solution is then autoclaved and allowed to cool to 50°C. The following sterile solutions (and antibiotics if required) are added prior to use:

1 M $Ca(NO_3)_2$	1 ml
0.01 M $MnCl_2$	1 ml
1 mM $FeSO_4$	1 ml

A dilution series for TM242 and one of its out-of-frame Spo0A-negative offspring, TM443, were plated on TGP both before and after both strains were heat treated at 90°C for 30 minutes. When not subjected to heat treatment, there was comparable growth between TM242 and TM443 at each dilution. However, after heat treatment there was a clear difference. While TM242 still showed growth at each dilution, albeit less than before heat treatment, there was no growth on the TM443 plate – even in the neat culture patch – indicating that TM242 can sporulate but TM443 cannot. To date, everything that has been done to make these strains sporulate has indicated that they are not capable of doing so.

In addition, genomic DNA was isolated from the TM242 double cross-over mutants and used as templates in PCR reactions together with the primers O/Spo0A1bF and O/Spo0A2R which flank the *spo0A* region, see Figure 10. PCR products were generated for each template and analysed by specific restriction enzyme digestion. The DNA fragments generated by these restriction digests are consistent with two of the strains representing out-of-frame mutants of the s*po0A* gene and one strain representing an in-frame deletion. Southern hybridisation analysis confirmed these results. Therefore, it can be concluded that a) the target s*po0A* gene has been knocked-out; and b) this has resulted in loss of sporulation in these strains.

Fermentation characteristics of Spo0A negative strains

The improved fermentation characteristics of Spo0A negative strains at lower sugar concentrations were demonstrated using urea salts media (USM) made according to the following recipe:

Urea Salts Media (USM)

	Final Concentration
$NaH_2PO_4.2H_2O$	10mM
K_2SO_4	10mM
Citric acid	2mM
$MgSO_4.7H_2O$	1.25mM
$CaCl^2.2H_2O$	0.02mM
$Na^2MoO^4.2H_2O$	1.65mM
Urea	50mM
$ZnSO_4.7H_2O$	25µM
$FeSO_4.7H_2O$	100µM
$MnSO_4.H_2O$	50µM
$CuSO_4.5H_2O$	5µM
$CuSO_4.7H_2O$	10µM
$NiSO_4.6H_2O$	16.85µM
H_3BO_3	6.5µM

The above components were added to deionised water and the following filter-sterilised reagents were added:

Biotin	12.5µM
Yeast extract	0.5% w/v (after autoclaving)

As shown in Tables 2A and 2B, it appears that under controlled fermentation conditions (1L batch, USM with 3% w/v glucose, 1% w/v yeast extract, pH 6.8, 60°C, aeration regime: 1 L/min and 600 rpm until OD > 5.0 then 0.2 L/min and 300 rpm) the out-of-frame mutant TM444 is able to consume sugar faster than TM242 and the in-frame mutants TM448 and TM450 perform less well.

Table 2A

Strain	Spo⁻	Aeration Switch		Complete sugar consumption/ hours	Max OD_{600}	Glucose / mM	Pyruvate/ mM
		Hrs	OD				
TM242		2.5	5.6	7.5	9.8	0.0	1.0
TM443	✓ o	2.3	5.8	6.7	8.6	0.0	0.0
TM444	✓ o	2.2	5.6	5.6	9.0	0.0	0.0
TM448	✓ i	3.0	5.3	9.8	7.2	0.0	1.0
TM450	✓ i	3.8	5.7	9.9	8.3	0.0	3.0

NB: 'i' denotes in-frame mutant, 'o' denotes out-of-frame mutant

Table 2B

Strain	Lactate/ mM	Formate/ mM	Actate/ mM	Ethanol/ mM	Ethanol yield post aeration switch/gg^{-1}	Overall ethanol yield/ gg^{-1}
TM242	10.0	0.0	13.0	314.0	0.44	0.42
TM443	16.0	0.0	15.0	300.0	0.44	0.40
TM444	13.0	0.0	15.0	303.0	0.46	0.42
TM448	6.0	0.0	21.0	243.0	0.42	0.33
TM450	5.0	0.0	28.0	252.0	0.43	0.35

As shown in Table 2B, the ethanol yield post-aeration switch of TM443 is equal to that of the parent strain TM242, whilst for TM444 is slightly improved. More importantly however, as shown in Table 2A, at these lower sugar concentrations (3% w/v glucose) TM443 and TM444 complete sugar consumption significantly faster than TM242. This is an advantageous characteristic in a commercial fermentation process. It is worth noting that in these fermentations, at 3% (w/v) sugar concentrations, TM444 and TM443 are able to utilise sugar more quickly. This is beneficial, as it allows more fermentation batches to be run over time, resulting in significant increases in overall ethanol production.

The enhanced ethanol tolerance of TM444 compared to TM242 is illustrated in Figure 11. It has been found that fermentation of 8% w/v cellobiose by TM242 could proceed to completion only if the ethanol produced during fermentation was

partitioned into the vapour phase and remove from the fermentation broth. As shown in Figure 11, when ethanol vapour was removed from the fermenter during fermentation (i.e. "gas-off") TM242 was able to utilise all of the cellobiose by the end of the fermentation, and the resulting total concentration of ethanol produced was increased, compared with fermentation without the removal of ethanol vapour from the broth. Interestingly, it was not necessary to portion off ethanol vapour in order for the same fermentation media to be fully fermented by TM444. This is because TM444 exhibits improved ethanol tolerance.

The improved fermentation characteristics of TM444 are further illustrated in Figures 12a and 12b, which show the fermentation curves for TM242 and TM444 respectively in media comprising 8% w/v cellobiose and 2% w/v yeast extract. By comparing the two graphs, it can be seen that, at elevated sugar concentrations, TM444 completed sugar consumption in approximately 10 hours, whilst some sugar still remained after 18 hours (i.e. the end of fermentation) when TM242 was used. Furthermore, the ethanol peak for TM242 shown in Figure 12a is significantly lower than the ethanol peak for TM444 shown in Figure 12b (527mM for TM242 compared with 729mM for TM444).

Therefore, it can be concluded from the data presented in Figures 11, 12a and 12b that, at elevated sugar concentrations, the sporulation-deficient mutant strains of the invention exhibit improved ethanol tolerance, an increased rate of sugar consumption and an increase in overall ethanol yield, compared to the parent strain.

Generation of Spo0A mutants in TM333

In further work, TM333 presumptive primary integrants of the Spo0A gene were sub-cultured through two successive rounds of growth in 2TY broth without antibiotic. Cells from the final round of sub-culturing were serially diluted and grown on TGP medium. Kanamycin-sensitive colonies representing potential double crossovers were identified by replica plating. Through a combination of PCR analysis and testing for sporulation, TM486, an out-of-frame sporulation-deficient derivative of TM333 has been identified as a useful strain for ethanol production. The TM486 strain has been deposited at NCIMB Ltd, Ferguson

Building, Craibstone Estate, Bucksburn, Aberdeen AB21 9YA and has the Accession No. NCIMB 41587.

Since this strain comprises the *amyS* gene present in the parent strain TM333, it provides the combined advantages of increased ethanol tolerance, rapid feedstock consumption and improved ethanol production that are associated with the *spo0A* mutation, together with the capacity to efficiently metabolise starch-based feedstock due to increased amylase activity.

The content of all of the publications referred to in the description is incorporated herein by reference.

SEQUENCE LISTING

<110> TMO Renewables Limited

<120> Sporulation-deficient thermophilic microorganisms for ethanol production

<130> JWJ01489WO

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<150> GB 0820262.4
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Claims

1. A thermophilic microorganism comprising a modification that decreases sporulation compared with wild-type.

2. A microorganism according to claim 1, wherein the modification inactivates the native *spo0A* gene.

3. A microorganism according to claim 2, wherein the modification comprises the deletion of at least a portion of the *spo0A* gene.

4. A microorganism according to claim 3, wherein the modification further comprises replacing the deleted portion of the *spo0A* gene with DNA encoding a restriction site.

5. A microorganism according to claim 4, wherein the restriction site is a NotI restriction site.

6. A microorganism according to any preceding claim, wherein the microorganism is *Geobacillus* species.

7. A microorganism according to any preceding claim, wherein the microorganism is *Geobacillus thermoglucosidasius*.

8. A microorganism according to any preceding claim, further comprising a modification that inactivates the native lactate dehydrogenase gene.

9. A microorganism according to claim 8, wherein the lactate dehydrogenase gene, or a portion thereof, has been deleted.

10. A microorganism according to claim 8 or claim 9, wherein the microorganism does not comprise an integration element in the lactate dehydrogenase gene.

11. A microorganism according to any preceding claim, further comprising a modification that inactivates the native pyruvate formate lyase gene.

12. A microorganism according to claim 11, wherein the pyruvate formate lyase gene, or a portion thereof, has been deleted.

13. A microorganism according to any preceding claim, further comprising a modification that up-regulates the pyruvate dehydrogenase gene.

14. A microorganism according to claim 13, wherein a gene promoter is inserted upstream of the pyruvate dehydrogenase gene, and wherein the promoter operates under anaerobic conditions.

15. A microorganism according to any preceding claim, further comprising a modification that enhances pyruvate decarboxylase activity.

16. A microorganism according to claim 15, wherein the modification inactivates the dihydrolipoamide transacetylase gene (EC 2.3.1.12).

17. A microorganism according to claim 15 or claim 16, wherein the dihydrolipoamide transacetylase gene, or a portion thereof, is deleted.

18. A microorganism according to any preceding claim, wherein the microorganism comprises a heterologous pyruvate decarboxylase gene.

19. A microorganism according to any preceding claim, wherein the microorganism comprises a heterologous alcohol dehydrogenase gene.

20. A microorganism according to any preceding claim, wherein the microorganism is stable in a culture medium comprising at least 3% w/v ethanol.

21. A microorganism according to any preceding claim wherein the microorganism is stable in a culture medium comprising at least 10% w/v ethanol.

22. A microorganism according to any proceeding claim, wherein the microorganism is stable in a culture medium comprising up to 20% w/v ethanol.

23. A microorganism according to any preceding claim, wherein the microorganism is transformable at high frequency.

24. A microorganism according to any preceding claim, wherein the microorganism grows at a temperature from 40°C - 85°C, preferably 50°C - 70°C.

25. A microorganism according to any preceding claim, wherein the microorganism is identified as TM443 (Accession Number 41591) or TM444 (Accession Number 41588).

26. A microorganism according to any preceding claim, wherein the microorganism comprises a heterologous amylase gene under the control of a promoter which operates in anaerobic conditions.

27. A microorganism according to claim 26, wherein the promoter is an ldh promoter.

28. A microorganism according to claim 27, wherein the ldh promoter is heterologous.

29. A microorganism according to claim 28, wherein the ldh promoter is derived from *Geobacillus stearothermophilus*.

30. A microorganism according to claim 26, wherein the amylase gene is under the control of a series of strong promoters.

31. A microorganism according to claim 30, wherein the strong promoters are heterologous.

32. A microorganism according to claim 31, wherein the strong promoters include an amylase promoter.

33. A microorganism according to claim 31 or claim 32, wherein the promoters are derived from G. *stearothermophilus*.

34. A microorganism according to any of claims 26 to 33, wherein the amylase gene is derived from *Geobacillus* species.

35. A microorganism according to any of claims 26 to 34, wherein the amylase gene is derived from *Geobacillus stearothermophilus*.

36. A microorganism according to any of claims 26 to 35, wherein the amylase gene encodes α amylase (EC 3.2.1.1).

37. A method of producing ethanol comprising culturing a microorganism according to any preceding claim under suitable conditions in the presence of a C_3, C_5 or C_6 sugar, or oligomer thereof.

38. A method according to claim 37, wherein the method is carried out at a temperature of between 40°C - 70°C.

39. A method according to claim 38, wherein the temperature is from 52°C - 65°C.

40. A method according to any of claims 37 to 39, wherein the microorganism is maintained in a culture at a pH of between 4.0 and 8.0.

41. A method of producing ethanol comprising culturing a microorganism according to any of claims 26 to 36 in culture media comprising at least 1% w/v starch.

42. A method according to claim 41, wherein the culture media comprises at least 10% w/v starch.

43. A method according to claim 42, wherein the culture media comprises at least 20% w/v starch.