

METHOD FOR CULTURING MICROORGANISMS

Field of the Invention

This invention relates to the production of microorganisms suitable for the production of ethanol as a product of bacterial fermentation.

5 Background to the Invention

Bacterial metabolism can occur through various different mechanisms depending on the bacterial species and environmental conditions. Heterotrophic bacteria, which include all pathogens, obtain energy from oxidation of organic compounds, with carbohydrates (particularly glucose), lipids and protein being the most commonly oxidised
10 compounds. Biological oxidation of these organic compounds by bacteria results in synthesis of ATP as the chemical energy source. The process also permits generation of more simple organic compounds (precursor molecules) which are required by the bacterial cell for biosynthetic reactions. The general process by which bacteria metabolise suitable substrates is glycolysis, which is a sequence of reactions that
15 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. There are three principle reactions of pyruvate.

First, under aerobic conditions, many micro-organisms will generate energy using the citric acid cycle and the conversion of pyruvate into acetyl coenzyme A, catalysed by
20 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).

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

There has been much interest in using micro-organisms for the production of ethanol using either micro-organisms that undergo anaerobic fermentation naturally or through the use of recombinant micro-organisms which incorporate genes involved in the
30 production of ethanol. Although there has been some success in producing ethanol by using these micro-organisms, fermentation is often compromised by the increased concentration of the ethanol, especially where the micro-organism has a low level of ethanol tolerance.

Thermophilic bacteria have been proposed for ethanol production, and their use
35 has the advantage that fermentation can be carried out at elevated temperatures which allows the ethanol produced to be removed as vapour at temperatures above 50°C; this

also permits fermentation to be carried out using high sugar concentrations. However, finding suitable thermophilic bacteria which can produce ethanol efficiently is problematic.

WO01/49865 discloses a Gram-positive bacterium which has been transformed with a heterologous gene encoding pyruvate decarboxylase and which has native alcohol
5 dehydrogenase function, for the production of ethanol. The bacterium is a thermophilic *Bacillus* and the bacterium may be modified by the inactivation of the lactate dehydrogenase gene using transposon insertion. The bacteria disclosed in WO01/49865 are all derived from *Bacillus* Strain LLD-R, a sporulation-deficient strain that arose spontaneously from culture, and in which the *ldh* gene has been inactivated by
10 spontaneous mutation or by chemical mutagenesis. Strains LN and TN are disclosed as improved derivatives of strain LLD-R. However, all strains contain a *Hae* III type restriction systems that impedes plasmid transformation and therefore prevents the transformation within un-methylated DNA.

WO01/85966 discloses microorganisms that are prepared by *in vivo* methylation
15 to overcome the restriction problems. This requires transformation with *Hae* III methyltransferase from *Haemophilus aegyptius* into strains LLD-R, LN and TN. However, strains LLD-R, LN and TN are unstable mutants and spontaneously revert to lactate-producing wild-type strains, particularly at low pH and in high sugar concentrations. This results in fermentation product changes from ethanol to lactate,
20 making the strains unsuitable for ethanol production.

WO02/29030 discloses that strain LLD-R and its derivatives include a naturally-occurring insertion element (IE) in the coding region of the *ldh* gene. Transposition of this into (and out of) the *ldh* gene and subsequent gene inactivation is unstable, resulting in reversion. The proposed solution to this was to integrate plasmid DNA into the IE
25 sequence.

Therefore, in the art, the production of microorganisms for ethanol production relies on modifying laboratory-produced chemically mutated *Bacillus* microorganisms, treating these with *in vivo* methylation procedures and further modifying the microorganisms to integrate plasmid DNA into the IE sequence. The procedure is
30 complex, uncertain and there are also regulatory issues on how the strains can be used.

There is therefore a need for improved microorganisms for ethanol production.

Summary of the Invention

According to a first aspect of the present invention, a method for the production of thermophilic microorganisms suitable for the production of ethanol comprises:

- 35 (i) culturing a thermophilic microorganism under aerobic or anaerobic conditions in a suitable culture media; and

- (ii) incorporating increasing amounts of ethanol into the culture media to induce ethanol tolerance.

Description of the Invention

5 The present invention is based on a treatment of a thermophilic microorganism to make the microorganism more tolerant to ethanol, and therefore, better able to produce ethanol. Increasing the ethanol tolerance of the microorganisms allows the microorganisms to be more resistant to the ethanol produced during their fermentation. This allows improvement in fermentation.

10 The method for the production of the thermophilic microorganisms involves culturing the thermophilic microorganisms under aerobic or anaerobic conditions in a suitable culture media and incorporating amounts of ethanol into the culture media to induce ethanol tolerance. In one embodiment increasing the ethanol into the culture media is carried out over time and usually in increments, to allow the microorganisms to adapt to the increased ethanol in the media. It is preferable to incorporate ethanol up to
15 a final concentration of 3% w/v and then increase the ethanol concentration by 0.5% w/v, or less, until the concentration of ethanol in the media is at least 6% w/v more preferably, at least 7.5% w/v. In one embodiment, the initial culture media comprises 3% w/v ethanol and this is then increased by 0.5% increments to 6% w/v and then by 0.25% increments to 7.5% w/v.

20 During this procedure, the cell density can be monitored to ensure that cell growth is continuing. Preferably, if the cell density (determined by OD 600nm) falls by more than 25% and continues to decline as the ethanol concentration is increased, then the concentration of ethanol is allowed to fall to the previous highest level and the culture re-established before continuing with the ethanol treatment.

25 An alternative method for the production of thermophilic organisms with higher ethanol tolerance involves continuous culturing the thermophilic microorganisms under aerobic or anaerobic conditions in appropriate culture medium. Once the culture has reached a steady state, where the growth of the organism has reached a constant rate (as determined by OD 600nm), an amount of ethanol is added, in one addition, to the
30 culture in order to bring the ethanol to a specific desired concentration, for example 10 or 20% w/v of the set working volume of the culture. The continuous culture is continued at a low dilution rate, preferably $0.08-0.15\text{ h}^{-1}$, allowing slow reduction of the ethanol concentration, until the original growth rate of the thermophilic organism is restored (as determine by OD 600nm). At this point a second amount of ethanol is added, the same
35 quantity as the first and in one addition, to the culture, again in order to bring the ethanol to a specific desired concentration. The process of allowing the culture to recover to the

original growth rate is repeated and further batches of ethanol are added until the culture is found to recover quickly, this being taken as less than twenty-four hours. At this point one of two outcomes will occur. Either ethanol tolerant strains are selected from this culture by sub-culturing at the desired ethanol concentration, preferably over 7.5% w/v, or
5 larger quantities of ethanol are added to the culture and the process ethanol addition followed by growth rate recovery is repeated.

The microorganisms may be grown in defined media at 55°C - 65°C with a carbon limited substrate and a pH of 6.0 to 7.5 (preferably 6.3 to 7.2) at dilution rates of 0.08 to 0.5.

10 In batch culture, a process similar to the process described above can be performed, with growth in any suitable media with excess carbon and ethanol being added in early log phase growth. Cells from this initial culture can then be used at the end of the log phase growth to inoculate a fresh flask with an incremental amount of ethanol being added again at early log phase. This procedure may be repeated with
15 incremental amounts of ethanol being added to the culture media at early log phase.

The thermophilic microorganisms to be used in the present invention may be modified to disrupt or enhance expression of the genes involved in the relevant biochemical pathways for ethanol biosynthesis, for example the disruption of the lactate-dehydrogenase gene. This results in pyruvate metabolism being channelled away from
20 lactate production and towards ethanol production, with enhanced levels of ethanol observed in lactate-negative mutants.

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
25 dehydrogenase.

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

30 The microorganisms may be "wild-type", i.e. they are not laboratory-produced mutants. The microorganisms may be isolated from environmental samples expected to contain thermophiles. Isolated wild-type microorganisms will have the ability to produce ethanol but, unmodified, lactate is likely to be the major fermentation product. The isolates are also selected for their ability to grow on hexose and/or pentose sugars at
35 thermophilic temperatures. Non-wild-type, mutant, microorganisms may also be used.

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 have the ability to
5 utilise C5 and C6 sugars as a substrate, including cellulose and starch. It is preferable if the microorganism is transformable at a high frequency. Furthermore, the microorganism should have a growth rate in continuous culture of above 0.3hr^{-1} .

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
10 50°C - 70°C . In addition, it is desirable that the microorganism grows in conditions of pH 6.5 or below, in particular pH6.5-pH4.5.

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 preferred if the
15 lactate dehydrogenase gene is inactivated either by the insertion of a transposon, or, preferably, 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
20 temperature-sensitive plasmid (plasmid pUB190-ldh), which achieves natural homologous recombination or integration between the plasmid and the microorganism's chromosome. Chromosomal integrants can be selected for on the basis of their resistance to an antibacterial agent (kanamycin). The integration into the lactate dehydrogenase gene may occur by a single cross-over recombination event or by a
25 double (or more) cross-over recombination event.

In a preferred embodiment, the micro-organism comprises a heterologous alcohol dehydrogenase gene and a heterologous pyruvate decarboxylase gene. The expression of these heterologous genes results in the production of enzymes which redirect the metabolism so that ethanol is the primary fermentation product. These genes may be
30 obtained from micro-organisms that typically undergo an aerobic fermentation, including *zymomonas* species, including *zymomonas mobilis*.

Methods for the preparation and incorporation of these genes into microorganisms are known, for example in Ingram et al, Biotech & BioEng, 1998; 58 (2+3): 204-214 and US 5916787, the content of each being incorporated herein by
35 reference. The genes may be introduced in a plasmid or integrated into the chromosome, as will be appreciated by the skilled person.

The microorganisms of the invention may be cultured under conventional culture conditions, depending on the thermophilic microorganism chosen. The choice of substrates, temperature, pH and other growth conditions can be selected based on known culture requirements, for example see WO01/49865 and WO01/85966. Suitable culture and fermentation conditions are indicated in Tables 1, 2 and 3:

Table 1

Chemical	Vol./L	Final Conc.
NaH ₂ PO ₄ ·2H ₂ O		25 mM
K ₂ SO ₄		10 mM
Citric acid. H ₂ O		2 mM
Mg SO ₄ ·7H ₂ O		1.25 mM
CaCl ₂ ·2H ₂ O		0.02 mM
Sulphate TE Solution	5 ml	See below
Na ₂ MoO ₄ ·2H ₂ O		1.65 µM
Yeast Extract	10g	
Antifoam	0.5ml	
Post-auto addns :		
4M Urea	25 ml	100 mM
1% Biotin	300 µl	12 µM
20% Glucose ²	50ml	1%

Table 2

10 Sulphate Trace Elements Stock Solution

Chemical	g l ⁻¹ (ml)	g l ⁻¹ (ml)	Medium Conc.
Conc. H ₂ SO ₄	5 ml	50 ml	
Zn SO ₄ ·7H ₂ O	1.44	14.4	25 µM
Fe SO ₄ ·7H ₂ O	5.56	55.6	100 µM
Mn SO ₄ ·H ₂ O	1.69	16.9	50 µM
Cu SO ₄ ·5H ₂ O	0.25	2.5	5 µM
Co SO ₄ ·7H ₂ O	0.562	5.62	10 µM
Ni SO ₄ ·6H ₂ O	0.886	8.86	16.85 µM
H ₃ BO ₃	0.08		
Del- H ₂ O (final)	1000 ml	10 litres	

Table 3

15 Fermenter Conditions

Inoculum	10% v/v
Volume	1000 ml
Temperature	60°C

PH	7.0 controlled with NaOH
Aeration	0.4 vvm
N ₂ flow	0.05 lpm
Agitation	400 rpm
Media	Urea Sulphates CDM for Fermenters
Sugar feed	100 ml 50% glucose
Antifoam	

The invention is illustrated in the following Example, with reference to the accompanying drawings.

5

Example

The following media were prepared:

SAM2 – perL

10

Yeast extract	1.0g
Tryptone	0.5g
NH ₄ Cl	1.0g
NaH ₂ PO ₄	0.5g
15 MgSO ₄ ·7H ₂ O	0.2g
KCl	0.2g
MnCl ₂ ·4H ₂ O	3mg (add 100µL of a 30mg/mL stock)
CaCl ₂ ·2H ₂ O	5mg (add 100µL of a 50mg/mL stock)
20 PIPES buffer	12.096g

Total volume in distilled water: 950mL, adjust to pH 7.0 with NaOH or H₂SO₄. Autoclave.

25 After cooling 2.5mL of Sulphates Trace Elements stock solution was added (see Table 2), together with 50mL 20% w/v filter sterilized sugar solution.

Modified US (Urea Salts) Media (USM)

30 Glucose	10.0 g/L
Yeast extract	0.8 g/L
Citric acid	0.42 g/L
MgSO ₄	0.31 g/L
NaH ₂ PO ₄	3.1 g/L
K ₂ SO ₄	3.5 g/L
35 Urea	3.0 g/L
CaCl ₂	2 mg/L
Na ₂ MoO ₄	4 mg/L
Trace element solution (Table 2)	5.0 ml/L

40 For solid media 20.0 g/L of bacto-agar was added.
Corrected to pH 7 prior to sterilisation with 3M NaOH

TGP medium

	Bacto tryptone	17.0 g/L
	Soy peptone	3.0 g/L
5	NaCl	5.0 g/L
	K ₂ HPO ₄	2.5 g/L
	Sodium pyruvate	4.0 g/L
	Glycerol	4.0 mL/L

- 10 For solid media 20.0 g/L of bacto-agar was added. The medium was corrected to pH 7 prior to sterilisation with 3M NaOH.

Ethanol tolerance of a wild-type organism (NCIMB 11955) was tested in order to determine the starting point. The organism was grown overnight (LB agar plate, 60°C) and a colony used to inoculate an overnight culture (100mL USM, 1% glucose, 60°C, 250rpm). This culture was then used to inoculate a triplicate series of flasks containing 0, 1, 2, 3, or 4% ethanol which were then grown for 36 hours before the growth was measured (50mL USM, 1% glucose, 60°C, 250rpm). The results are shown in Figure 1 and suggest that NCIMB 11955 will not tolerate more than 4% ethanol.

Using this result as a basis for comparison, experiments were performed to increase the ethanol tolerance of mutant TM89 to 8% v/v ethanol.

Fermentation Method

25	Microorganism:	Gt TM-89
	Inoculum:	50ml 2xYT culture (7% v/v)
	Apparatus:	LH glass fermenter (700ml working volume) Temperature and pH control with the Anglicon control system Mixed with a magnetic stirrer.
30	Set values:	Temperature: 60°C pH: 6.80 air: 0.2-0.4vvm stirrer speed: 225rpm flow rate controlled with Watson Marlow Pump (0-100 ml/hr)
35	Medium:	SAM2 2% glucose, 0.05% organic antifoam (see media sheet). PH controlled using 10% NaOH
	Ethanol spike:	75ml or 150ml ethanol (10-20% spike)

The strategy adopted was to:

- 40 1) Achieve steady states and measure product yields/sugar utilization; and
- 2) Spike culture with ethanol, allow culture to recover (ethanol will wash out over time), remove samples and prepare glycerol stocks, repeating step (2) as necessary.

In order to evaluate ethanol tolerance, the following protocol was developed.

1. Add 5ml of TGP broth to two sterile universals.
2. Add 100µl of TM-89 and TM89-1 glycerol stock to each tube respectively.
3. Culture for 5-6 hours (therefore active cells, as they should be in log phase) at 60°C with shaking.
4. Measure the OD₆₀₀ of each broth (so you know the starting of OD₆₀₀).
5. Prepare 11 sterile universal tubes with a final volume of 10ml TGP broth containing 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% 10% (v/v) concentrations of ethanol, in duplicate for each TM-89 strain (ie. 44 tubes).
6. Inoculate each tube with 100µl of cells from the corresponding 5ml TGP broths from stages 1 + 2.
7. Culture overnight at 60°C with shaking.
8. Remove 1ml from each tube, dilute 1:5 and measure the OD₆₀₀ against H₂O blank.

15

The results were analysed as follows:

- (1) Optical density of culture measured using a Jencons Spectrophotometer [cell concentration (g/L) was calculated from $A_{600} \times 0.3$].
- (2) Glucose concentration was measured using a blood glucose meter (Roche).
- (3) Ethanol concentration measured by using an enzyme based assay kit (supplied by R-Biopharm).

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The results are illustrated in Figure 2. The strain isolated at the end of the fermentation displayed consistently higher OD values than the starting TM89 strain in TGP and in TGP with a range of ethanol concentrations. There was a significant difference in growth at 5% ethanol indicating improved ethanol tolerance.

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CLAIMS

1. A method for the production of thermophilic microorganisms suitable for the production of ethanol, comprising:
 - i. culturing a thermophilic microorganism under aerobic or anaerobic conditions in a suitable culture media; and
 - ii. incorporating amounts of ethanol into the culture media to induce ethanol tolerance;wherein the wild-type thermophilic microorganism is modified by inactivation of the native lactate dehydrogenase gene,
wherein the native lactate dehydrogenase gene, or a portion thereof, has been deleted; and
wherein the microorganism does not comprise a restriction system.
2. A method according to claim 1, wherein an increasing amount of ethanol is added into the culture media, with the microorganisms allowed to adapt to the added ethanol prior to the next incremental addition of ethanol.
3. A method according to claim 1 or claim 2, wherein the ethanol is incorporated to a final concentration of at least 3% w/v.
4. A method according to any preceding claim, wherein the ethanol is incorporated to a final concentration of at least 6% w/v.
5. A method according to any preceding claim, wherein the ethanol is incorporated to a final concentration of at least 7.5% w/v.
6. A method according to any preceding claim, wherein the ethanol is incorporated in increments of 0.5% w/v or less.
7. A method according to any preceding claim, wherein the ethanol is incorporated to the culture media at early log phase.
8. A method according to any preceding claim, wherein the microorganism is

Geobacillus thermoglucosidasius.

9. A method according to any preceding claim, wherein the microorganism comprises a heterologous *pdc* gene.
10. A method according to any preceding claim, wherein the microorganism comprises a heterologous *adh* gene.
11. A method according to any preceding claim, wherein the microorganism can metabolise cellobiose and/or starch.
12. A method according to any preceding claim, wherein the microorganism is transformable at high frequency.
13. A method according to any preceding claim, wherein the microorganism grows at a temperature from 40°C - 85°C, preferably 50°C - 70°C.