METHOD OF CULTURING UNICELLULAR ORGANISMS

The invention provides a method of producing a culture of subterranean microorganisms, said method comprising: obtaining a sample of pressurized fluid from a subterranean reservoir; while maintaining said sample under pressure transferring it into a fermentation reactor; and incubating said sample in said reactor under elevated pressure.
This invention relates to a method of culturing unicellular organisms, in particular prokaryotes, e.g. eubacteria and archaea, to apparatus therefor, to uses and inhibition of such organisms in subterranean applications, to novel such organisms, and to compositions and libraries containing them.

Natural subterranean hydrocarbon reservoirs provide a finite source of oil and gas. It is thus important to optimize hydrocarbon recovery from such reservoirs. Many techniques are used for this purpose but there is a continuing need for new techniques.

To many people's surprise, the rock which is impregnated with the hydrocarbon in such reservoirs, even though it maybe thousands of metres below the surface and thus at very high temperatures and pressures, is not sterile. Unicellular organisms, in particular prokaryotes such as eubacteria and archaea, are present. It has even been proposed that such microorganisms may have been involved in the production of the hydrocarbon.

We have realised that promoting or inhibiting the growth of such endogenous microorganisms, or introducing cultures of microorganisms isolated from subterranean rock formations, provides a means of improving reservoir management. In order to do this however such microorganisms must be collected and cultured, and if appropriate challenged so that means for growth inhibition may be found.

While some microorganisms retrieved from subterranean rock formations have been cultured at ambient surface conditions, we have surprisingly found that the range of microorganisms that may be isolated and tested is significantly greater if culturing is effected under physicochemical conditions resembling those of the reservoir itself, and in particular if samples taken from a reservoir are maintained at elevated pressure before and during culturing.

This is particularly surprising since liquids, unlike gases, are only very poorly
compressible. Thus a bacterium in a liquid culture medium is essentially a liquid filled membrane within a surrounding liquid with negligible, if any, pressure differential across the membrane. It would therefore be expected that bacterial growth would be essentially unaffected by the pressure of the culture medium. This turns out not to be the case however as we have found that culturing hydrocarbon samples under high pressure and under ambient pressure results in growth of quite different organisms and in particular some organisms growing under high pressure conditions simply do not grow at ambient (i.e. earth surface) pressure. Moreover, certain of the enzymes used by these piezophilic organisms do not seem to function at ambient pressure, presumably due to differences in tertiary structure.

Thus while it has been known to culture individual subterranean microorganisms, it has not previously been known to culture a subterranean ecosystem consisting of a multiplicity of microorganisms some of which do not thrive under surface conditions. If the effects of a down-hole treatment are to be reproduced in laboratory conditions, then it is necessary to use the new method of this invention.

Thus viewed from one aspect the invention provides a method of producing a culture of subterranean microorganisms, said method comprising: obtaining a sample of pressurized fluid from a subterranean reservoir, e.g. from a fluid flow from such a reservoir; while maintaining said sample under pressure transferring it into a fermentation reactor; and incubating said sample in said reactor under elevated pressure, and, preferably also elevated temperature.

The sample will typically be incubated at a pressure of 100 to 900 bar, preferably 230 to 280 bar, especially preferably a pressure of 50 to 150% of that of the reservoir from which it was taken or of that of the formation which the cultured microorganism is intended to treat, more especially 80% to 120%, particularly 90 to 110%.

The sample is preferably a liquid hydrocarbon sample; however aqueous samples may also be used. Samples taken from reservoirs generally include gases, lipids,
organic acids, etc.

The incubation temperature will typically be 60 to 160°C, preferably 80 to 100°C, especially preferably within 20°C of that of the reservoir from which the sample was taken or that of the formation which the cultured microorganism is intended to treat, especially within 10°C.

Incubation is preferably effected by mixing the sample, before, during or after transfer to the fermentation reactor, with a nutrient. The nutrient may comprise minerals and/or vitamins but preferably comprises at least pulverulent rock, especially preferably of rock from the reservoir from which the sample was taken or from the formation that the cultured microorganism is intended to treat, or rock geologically comparable to such reservoir rock. If desired the nutrient will also comprise fluid from the reservoir from which the sample is taken. In general, the reservoir fluid will provide the carbon source for microorganism growth. Generally, as fermentation progresses, different nutrients or inhibitors may be added to determine their effects on microbial growth.

Incubation in the method of the invention will typically be for from 7 to 360 days, especially 180 to 240 days. If desired incubation may be monitored and controlled so as to be terminated if microorganism growth has not occurred or if it has reached a pre-set acceptable level. Monitoring may be done in situ or on material extracted from the fermentation reactor and may be effected in any suitable fashion. Thus, for example, sample turbidity, uptake of radioactive tracers in nutrient material, organic solids content, etc. may typically be used as parameters to be monitored. Monitoring may typically be effected by drawing off aliquots of the culture medium and subjecting these to analysis by GC, LC-MS, MS, etc., e.g. to show gas generation or consumption or lipid profiles.

Once sufficient microorganism growth has taken place, it will generally be desirable to identify the microorganisms that have proliferated in the culture. This can readily be done by cell lysis followed by nucleic acid fragmentation (e.g. using DNAses or...
RNAses), fragment replication (e.g. using PCR with primers universal to prokaryotes or groups thereof); and fragment separation (e.g. using gel electrophoresis). The fragment "signature" may then be compared against databases of prokaryote nucleic acid fragments in order to determine which microorganisms are present in the culture and whether any of these are novel. Where novel microorganisms are found, it will generally be desirable to isolate and possibly also sequence them. Isolation may typically be effected by dilution followed by incubation, i.e. using conventional techniques but still incubating under elevated pressure. Sequencing may be effected by conventional techniques.

Where a viable sample of an individual microorganism or the combination of microorganisms is desired, this maybe achieved by bringing the culture to ambient pressure and temperature very slowly, e.g. over at least 10 hours, preferably at least 24 hours. If desired, during the depressurization, the culture may be exposed to bursts of electrical field to cause electroporosis and hence relieve the pressure difference across the cellular membrane.

The microorganisms may then be separated from the culture by conventional means, e.g. centrifugation, resuspension in a sterile fluid, re-centrifugation, etc. The resulting material may then if desired be lyophilized for storage and/or transport. The fluids used will typically be mineral oil or glycerol.

More preferably, however, samples of the culture maybe stored in pressurized containers, e.g. at the pressure under which incubation took place. Such pressurized samples, and libraries thereof, form further aspects of the present invention.

Viewed from one such aspect the invention provides a pressurized container, e.g. having an internal pressure of 100 to 900 bar, especially 200 to 350 bar, containing therein a microorganism, optionally in a hydrocarbyl fluid (e.g. glycerol or mineral oil), and preferably provided with a valved sampling port. Viewed from a further aspect the invention provides a microorganism library comprising a plurality of such pressurized containers, e.g. at least 10, preferably at least 100.
Storing the culture under pressure but at ambient or sub-ambient temperature (e.g. down to liquid nitrogen temperature) is viable since subterranean microorganisms, under such conditions, may be essentially dormant.

In the method of the invention, the content of the mixture being incubated may be varied to mimic different subterranean conditions. Thus for example sulphate, salt, water, methane, nitrogen, and carbon dioxide contents may be varied to imitate conditions encountered as water penetration into the hydrocarbon bearing strata occurs, or those conditions when water, natural gas, nitrogen or carbon dioxide are injected into the reservoir to enhance hydrocarbon recovery, or those conditions encountered when a squeeze is applied to a producer well to effect a down-hole well treatment (e.g. to administer a scale inhibitor). In this way, from the culture proliferation during incubation, it is possible to determine conditions suited to microorganism growth; conditions suitable for suppression of microorganism growth; appropriate microorganisms for injection down-hole to produce biomass (e.g. to hinder water flow); etc.

With such factors determined, it is then possible to effect a down-hole treatment designed to enhance or suppress microorganism growth. This forms a further aspect of the present invention.

Viewed from a further aspect therefore the invention provides a process for subterranean reservoir treatment, e.g. a process for treatment of a hydrocarbon well, which process comprises injecting a culture of a microorganism into said reservoir, optionally together with nutrients for said microorganism, said culture having been produced by elevated pressure incubation.

Viewed from a still further aspect the invention provides a process for enhancing hydrocarbon recovery from a subterranean hydrocarbon reservoir which process involves injecting a fluid into said reservoir to drive the hydrocarbon to a producer well, wherein the composition of said fluid is preselected so as to inhibit or promote growth of microorganisms endogenous to said reservoir, e.g. on the basis of
performance of the method of the invention.

The driving fluid used in this regard will typically be water, nitrogen, methane or carbon dioxide, preferably carbon dioxide. The composition of the fluid may be selected for example to include nutrients which cause the endogenous microorganisms to proliferate under aqueous conditions, i.e. so that biomass build-up will occur in aqueous zones of the reservoir causing the pores in the rock to clog and so reduce water flow to the producer well(s). Such nutrients may typically include water-soluble mineral salts.

Such processes will typically involve the injection conditions conventional for squeezes to effect well treatment and for fluid injection to enhance hydrocarbon recovery and thus need not be described in further detail.

Where the method of the invention yields novel, i.e. previously unknown, microorganisms, these form a further aspect of the invention as do rock-free compositions containing them. Such compositions may be dried (e.g. hydrophilized, optionally with a cryoprotective agent, e.g. a sugar) or may be liquid and may be under pressure or at ambient pressure. Where liquid, the solvent is preferably an organic solvent other than the native hydrocarbon in which the microorganism was found, e.g. a mineral oil or glycerol.

The method of the invention is typically effected in a fermentation reactor capable of operating under down-hole pressures. This is quite different from conventional fermentation reactors which are generally glass vessels or thin metal-skinned vessels. Such high pressure reactors form a further aspect of the invention. Viewed from this aspect the invention provides a microorganism fermentation reactor comprising an incubation chamber having an inlet port, and preferably also a sample removal port, the walls of said chamber being of a material sufficiently strong to withstand a pressure differential of at least 180 bar, more preferably at least 200 bar, especially at least 300 bar, more particularly up to 900 bar.
The reactor of the invention is preferably provided with a fermentation monitor and a heat source, as well as with valves capable of operation to reduce the pressure in the chamber slowly, e.g. at a rate of less than 50 bar/hour, preferably less than 10 bar/hour, more preferably less than 1 bar/hour. The heat source may typically be integral to the incubation chamber; however on a laboratory scale the reactor may simply be placed in an oven.

The down-hole sample may be collected at or near the point within the well at which the hydrocarbon enters. Devices capable of sampling in this way are known already. Preferably however the sample is taken from the pressurized fluid flow after it leaves the ground using a pressurized displacement cell. The pressurized displacement cell is then preferably insulated or placed in a heated container to maintain the elevated temperature of the fluid sample before it is transferred to the fermentation reactor. The samples may if desired be taken from different flow-lines from the reservoir before they converge so that different microbial flora from different reservoir zones maybe detected.

Maintaining sample pressure from sample taking to transfer into the fermentation reactor is important as otherwise pressure-drop sensitive microorganisms will fragment and will not be grown in the reactor. Moreover maintaining pressure and temperature ensures the growth medium approximates to down-hole conditions in terms of dissolved gas content, etc.

The invention will now be described with reference to the accompanying drawings in which:

Figures 1, 5 and 6 are schematic drawings of reactors according to the invention;

Figures 2 and 3 are gel electrophoresis patterns for samples cultured using the reactor of Figure 1 and under control conditions; and

Figure 4 is a bar chart showing the numbers and types of microorganisms in cultures.
produced according to the method of the invention and in a control run.

Referring to Figure 1 there is shown a horizontal cross-section of an open-ended cylinder 1. This is machined from 316 steel or Hastelloy C and is capable of withstanding pressures of 350 bar.

The open end of cylinder 1 may be closed with a tapered plug 2 which incorporates an O-ring in groove 3 and has a central vent 4 for fluid addition or removal. Plug 2 is held in place by a hollow threaded bolt 5 which engages with the internal threading at the mouth of cylinder 1. Preferably a washer (not shown) is placed between plug 2 and bolt 5 to minimize rotation of plug 2 while bolt 5 is tightened or loosened.

Referring to Figures 2 and 3 there are shown DGGE patterns for 21 cultures. Those designated CHP were of samples taken and cultured under reservoir conditions, except where the designation includes the letter K which is a control sample cultured at 1 bar. It will be seen that bacteria in CHP samples grew that did not grow in the CHP-K sample.

Figure 2 relates to test samples amplified using primers universal for eubacteria. Figure 3 relates to test samples amplified using primers universal for Achaea.

Figure 4 shows the number of clones produced culturing four different samples taken from reservoirs either at high pressure (Pressure cells) or at ambient pressure (Controls), and then carrying out PCR amplification on the resultant cultures.

Figures 5 and 6 are, respectively, exploded and assembled schematics showing a cylinder 21 of 316 steel, a bolt 22 of JM7 (an aluminium bronze), a Teflon® plunger 23, a disk 24 of JM7, a plug 25 of 316 steel and Viton® O-rings 26.
Claims:

1. A method of producing a culture of subterranean microorganisms, said method comprising: obtaining a sample of pressurized fluid from a subterranean reservoir; while maintaining said sample under pressure transferring it into a fermentation reactor; and incubating said sample in said reactor under elevated pressure.

2. The method of claim 1 wherein said sample is incubated in said reactor at elevated temperature.

3. The method of either of claim 1 or 2 wherein said sample of pressurized fluid from a subterranean reservoir is obtained from a fluid flow from such a reservoir.

4. The method of any preceding claim wherein the sample is incubated at a pressure of 100 to 900 bar.

5. The method of any preceding claim wherein the sample is incubated at a pressure of 230 to 280 bar.

6. The method of any preceding claim wherein the sample is incubated at a temperature of 60 to 160°C.

7. The method of any preceding claim wherein the sample is incubated at a temperature of 80 to 100°C.

8. The method of any preceding claim wherein incubation is effected by mixing the sample with a nutrient comprising at least pulverulent rock.

9. The method of any preceding claim wherein incubation is for from 7 to 360 days.
10. The method of any preceding claim further comprising bringing the culture to ambient pressure and temperature over at least 10 hours.

11. A pressurized container provided with a valved sampling port, and containing therein a microorganism in a hydrocarbyl fluid.

12. The pressurized container of claim 11 having an internal pressure of 100 to 900 bar.

13. The pressurized container of either of claim 11 or 12 wherein said hydrocarbyl fluid is glycerol or mineral oil.

14. A microorganism library comprising a plurality of containers as defined in any of claims 11 to 13.

15. A process for subterranean reservoir treatment, which process comprises injecting a culture of a microorganism into said reservoir, optionally together with nutrients for said microorganism, said culture having been produced by elevated pressure incubation as defined in any one of claims 1 to 10.

16. The process of claim 15 wherein said subterranean reservoir is a hydrocarbon well.

17. A process for enhancing hydrocarbon recovery from a subterranean hydrocarbon reservoir which process involves injecting a fluid into said reservoir to drive the hydrocarbon to a producer well, wherein the composition of said fluid is preselected so as to inhibit or promote growth of microorganisms endogenous to said reservoir.

18. A microorganism fermentation reactor comprising an incubation chamber having an inlet port, and preferably also a sample removal port, the walls of said chamber being of a material sufficiently strong to withstand a pressure differential of at least 180 bar.
FIG. 4
## INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

**INV.** C12M1/34 C09K8/58

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)

C12M C09K

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**

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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**X** Further documents are listed in the continuation of Box C  

**X** See patent family annex

* Special categories of cited documents

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Date of the actual completion of the International search: 23 March 2007

Date of mailing of the international search report: 02/04/2007

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Cubas Alcaraz, Oose
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**International application No.**

PCT/GB2006/004443

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