

PATENT SPECIFICATION

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(54) PROCESS FOR THE PRODUCTION OF
SINGLE CELL PROTEINS AND MICROORGANISMS
FOR USE THEREIN

(71) We, PHILLIPS PETROLEUM COMPANY, a corporation organised and existing under the laws of the State of Delaware, United States of America, of Bartlesville, Oklahoma, United States of America, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The invention relates to a fermentation process for the production of single cell protein and to novel microorganisms for use therein.

Efforts to relieve the worldwide shortages of protein have included various biosynthesis processes wherein biologically produced single cell proteins (SCP) are obtained by the growth of a variety of microorganisms on a variety of carbon-containing substrates. The carbon and energy sources used as substrates should be available widely, relatively cheap, uniform, and safe in that they do not leave harmful residues in the protein product ultimately obtained by microbial conversion. Petroleum hydrocarbons have been employed as a carbon and energy source, but have faced practical difficulties in the lack of water solubility, in the high consumption of oxygen to assist in the microbial conversion, and allegedly in traces of potentially carcinogenic agents from the petroleum feedstocks entering or adhering to the protein product.

Other processes have centered on the use of oxygenated hydrocarbon derivatives as feedstocks, due to their water solubility and hence ease of handling since microbial conversion processes are essentially conducted under aqueous conditions. Such feedstocks are readily available either from petroleum sources, natural gas sources, various waste/garbage processing and conversion of methane and the like, from fermentation of various grains and the like, destructive distillation of wood, and so on. Such oxygenated hydrocarbons, whatever their source, are widely available and relatively cheap feedstocks for fermentation processes. Advantages accrue in that these feedstocks are partially oxygenated, so that substantially reduced molecular oxygen requirements are involved for the microbial conversion-growth process.

However, another difficult and limiting factor in the commercialization of the single cell protein processes has been the necessity to function at relatively moderate temperatures of about 20 to 50°C., and more preferably not over about 35°C. The microbial conversion is a highly exothermic oxidation reaction with large quantities of heat being produced, which heat must be removed continuously and consistently or risk the overheating of the system and death of the microorganisms, or at least the severe limitation and growth encountered as temperature rise, and hence severe reductions in efficiencies.

Many processes have concentrated on employment on one or other of the many available yeasts as the microorganism. Many yeasts are available, and the yeast cells generally are slightly larger than a bacteria cell, and sometimes provide easier separation from the fermentation process.

However, the bacteria offer advantages, since higher crude protein contents of the cell are obtained from bacteria as compared to production obtainable from the yeasts in general, the yeasts having higher proportions of nonprotein structural material in their cells. Bacteria usually have a significantly higher true protein content, frequently being nutritionally higher in the important sulfur amino acids and lysine.

Discovery of bacteria with the capability of rapid growth and high productivity rates at relatively high fermentation process temperatures would be distinctly advantageous. High temperature growth operation means less heat to be removed, less cooling

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apparatus involved, and ultimately relatively smaller amounts of heat needed for sterilization, coagulation, and separation processes. Danger of contamination with other microbes is also greatly reduced. Thermophilic or thermotolerant bacteria are needed for commercialization of the single cell protein process.

The present invention is based on the discovery of two very unique thermophilic species of bacteria, division *Protophyta*, class *Schizomycetes*, order *Eubacteriales*, family *Bacillaceae*, genus *Bacillus*, with highly desirable and useful properties. These unique thermophilic species grow better at higher temperatures than at conventional temperatures.

These two unique species are thermophilic, grow effectively with high productivity on oxygenated hydrocarbon feedstocks, particularly lower alcohols, most preferably methanol or ethanol, at temperatures wherein other known *Bacillus* species either are relatively unproductive, or simply cannot tolerate, or are unproductive and intolerant of an oxygenated hydrocarbon feedstock. These unique species identified by us as *Bacillus* sp. strain 72 and *Bacillus* sp. strain 47 have been deposited with the official depository United States Department of Agriculture, Agricultural Research Service, Northern Region Research Laboratory, Peoria, Illinois 61604, NRRL strain designations NRRL B-8066 and NRRL B-8065 respectively. The *Bacillus* strains are highly productive at relatively high fermentation temperatures, producing desirable and valuable single cell protein products with a high protein content of desirable amino acid type and balance. These unique high temperature preferring *Bacillus* species mean improved rates of single cell protein production, with reduced cooling requirements when grown on a carbon and energy substrate of an oxygenated hydrocarbon, preferably a lower alcohol, more preferably methanol or ethanol, and presently preferred is methanol or a substantially methanol-containing substrate. Further, it is anticipated that these high temperature preferring novel *Bacillus* species will find particular application in foam-filled fermentation means.

Culture No. 72 NRRL B-8066 is a *Bacillus* species, gram positive, spore-forming microbe of narrow rod-shaped appearance. No colored pigments have been observed in the cells.

Culture No. 47 NRRL B-8065 is a *Bacillus* species, gram positive, spore-forming microbe of thick rod-shaped appearance. No colored pigments have been observed in the cells.

The present invention, providing as it does the process for culturing oxygenated hydrocarbon assimilating microbial cells belonging to two new species of microorganisms, is directed toward aerobic culturing in a medium containing oxygenated hydrocarbon as carbon and energy source, at relatively elevated fermentation temperatures, resulting in rapid propagation of the cells.

The newly isolated novel and unique microorganisms can be characterized by properties as shown in the following tabulation along with properties of several known *Bacillus* cultures:

Culture Property or Test Result	Bacillus Cultures				
	<i>B. licheniformis</i> NRRL B-1001	<i>B. coagulans</i> NRRL B-1103	<i>B. subtilis</i> ATCC 10774	<i>B. stearothermophilus</i> ATCC 12987	
Gram Staining	Positive	Positive	Positive	Variable	
Spore Forming	Yes	Yes	Yes	Yes	
Aerobic	Yes	Yes	Yes	Yes	
Approx. Size, μ	0.5 x 1-2.5	0.6 x 1-5	0.7-0.8 x 2-3	0.6-1 x 2-5	
Motility	Yes	Yes	Yes	Yes	
Optimum temp., °C	55	55	28-40	30	
Max. temp., °C	60-65	60-65	50-55	50-55	
pH Range	5-9	5-9	5-8.6	4.5-6.6	
Optimum pH (IM2 medium)	6.2-6.8	6.2-6.5	NG	NG	
Growth factors	none required	none required	thiamine, biotin	none required	
Pigments n IM2 medium+1.5% CH ₃ OH	tan, H ₂ O sol., insol. in organic solvents	yellow, H ₂ O NG sol., insol. in organic solvents	NG	NG	
Cell appearance	rods to long chains	rods	rods, no chains	rods, no chains	rods, sometimes filamentous
Colony appearance on IM2 medium+ 1.5% CH ₃ OH	circular raised, pale tan	circular raised, off white	NG	NG	
Growth @ 55°C on:					
Nutrient broth	-	-	+	+	
Nutrient broth +1% CH ₃ OH	+	+	-	+	
IM2+0.5% CH ₃ OH	+	+	-	-	
IM2+1.5% CH ₃ OH	+	+	-	-	
Glucose	-	-	-	-	
CH ₃ OH	+	+	+	+	
CH ₃ CH ₂ OH	+	+	-	-	
HCHO	+	+	-	-	
BHM+1.5% CH ₃ OH	+	+	-	-	
+0.1% NaCl	+	+	-	-	
BHM+5% CH ₃ OH	+	+	-	-	
Plate count+ 1.5% CH ₃ OH	translucent spreading edges	translucent glistening small	-	-	

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+ = visible evidence of growth as determined by increase in turbidity of the starting mixture.

- = not growing; no visible evidence of growth.

± = maintaining itself; but no visible evidence of additional growth.

NG = did not grow on this media.

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These comparisons were all made so as to have as direct a comparison between species as possible. Of course, as with all microorganisms, some of the characteristics may be subject to some variation depending on the medium and particular conditions.

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The carbon and energy source material or substrate for the fermentation process of this invention employing the novel and unique species of bacteria is a carbon-oxygen-hydrogen-containing water-soluble compound or compounds. The term oxygenated hydrocarbon is designed to be a generic term descriptive of the compounds

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employable, and not necessarily a limiting term referring to the source of the substrate. The oxygenated hydrocarbons can include alcohols, ketones, esters, ethers, acids, and aldehydes, which are substantially water-soluble in character, and should be limited, because of this characteristic, to up to about 10 carbon atoms per molecule.

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Illustrative examples include: methanol, ethanol, propanol, butanol, pentanol, hexanol, 1,7 - heptanediol, 2 - heptanol, 2 - methyl - 4 - pentanol, pentanoic acid, 2 - methylbutanoic acid, 2 - pentanol, 2 - methyl - 4 - butanol, 2 - methyl - 3 - butanol, 2 - butanol, 2 - methyl - 1 - propanol, 2 - methyl - 2 - propanol, 2 - propanol, formic acid, acetic acid, propanoic acid, formaldehyde, acetaldehyde, propanal, butanal, 2 - methylpropanal, butanoic acid, 2 - methylpropanoic acid, pentanoic acid, glutaric acid, hexanoic acid, 2 - methylpentanoic acid, heptanedioic acid, heptanoic acid, 4 - heptenone, 2 - heptanone, octanoic acid, 2 - ethylhexanoic acid, glycerine, ethylene glycol, propylene glycol, 2 - propanone, 2 - butanone, diethyl ether, methyl ethyl ether, dimethyl ether, di - n - propyl ether, n - propyl isopropyl ether, and mixtures of two or more thereof.

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A preferred group of such carbon and energy source materials are the water-soluble aliphatic monohydric hydrocarbyl alcohols due to their water solubility, still more preferred are the lower alcohols of 1 to 4 carbon atoms per molecule for commercial availability, still more preferred are ethanol and methanol, presently with methanol being most preferred, due to the low relative cost of these feedstocks.

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It is feasible to employ mixtures of any of these oxygenated hydrocarbons if desired or convenient. For example, a commercially available material sometimes termed "methyl fuel" (C. & E.N., September 17, 1973, page 23) is a mixture of methanol and controlled percentages of higher alcohols containing up to 4 carbon atoms per molecule, and is a suitable substrate.

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Petroleum gases can be oxidized and employed, such as methane, and ethane which provide mixtures of predominantly the corresponding alcohol, as well as varieties of ketones, aldehydes, ethers and acids and hydrocarbon fractions from various petroleum sources can be utilized for this purpose.

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Culturing of the novel species of bacteria with oxygenated hydrocarbon feedstocks can be advantageously carried out at a temperature in the range of 45—65°C., more preferably 50 to 60°C., and most preferably, for optimum growth rate, at about 55°C.

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Culturing is accomplished in a growth media comprising an aqueous mineral salt medium, the carbon and energy source material, molecular oxygen, and, of course, starting inoculum of the particular species to be employed.

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High concentrations of some of the described carbon and energy substrates, such as methanol or formaldehyde may be inhibitory to satisfactory microbial growth or even toxic to the microorganisms in the fermentations employing the novel *Bacillus* species. Relatively high concentrations of substrates thus should be avoided, so that it is desirable to maintain the substrate concentration in the fermentation means at a level of from 0.01 to 5 volume/volume percent, preferably from 0.01 to 0.5 volume/volume percent so as to neither starve nor inhibit the growth rates.

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Oxygen can be supplied to the fermentation media or broth in any form capable of being assimilated readily by the inoculant microorganism. While molecular oxygen supplying compounds can be utilized, these are not normally commercially practical. Thus, molecular oxygen conventionally is supplied as the molecular oxygen-containing gas, such as air at atmospheric or elevated pressure, or oxygen-enriched air where convenient and available from sources depending on particular location of the SCP process operation. In effect, using the oxygenated hydrocarbon substrate, a part of the oxygen needed for growth of the microorganism is supplied by the oxygen content of the substrate. Nevertheless, additional quantities of molecular oxygen must be

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supplied for growth since the assimilation of the substrate and corresponding growth of the microorganism is, in effect, a combustion process. Preferably, from 0.1 to 10, more preferably from 0.7 to 2.5, volumes per minute of air of normal oxygen content are supplied to the reactor per volume of liquid in the fermentor, or in terms of oxygen, the respective ranges are from 0.02 to 2.1, and 0.14 to 0.55.

The pressure employed for the microbiological conversion process can range widely, and pressures of about 0.1 to 100 atmospheres, preferably 1 to 30 atmospheres, and more preferably slightly over atmospheric pressure, are employed as a balance of equipment cost vs. O_2 solubility achieved. Greater than atmospheric pressures are advantageous in that such pressures tend to increase the dissolved oxygen concentration in the aqueous fermentation admixture, which in turn can increase cellular growth rate. Higher than atmospheric pressures are preferred at higher fermentation temperatures where oxygen solubilities tend to decrease. Foam-filled fermentation means tend to assist oxygen transfer necessary for high cell densities and rapid growth rates.

The two unique *Bacillus* species used in this invention require mineral nutrients and a source of assimilable nitrogen, in addition to the oxygen and carbon and energy sources as described. The source of nitrogen can be any nitrogen-containing compound which is capable of releasing nitrogen in a form suitable for metabolic utilization by the organism. While a variety of organic nitrogen source compounds such as other proteins or urea can be employed, usually inorganic nitrogen source materials are more economical and practical. Suitable inorganic nitrogen-containing compounds include such as ammonia, ammonium hydroxide, various ammonium salts such as ammonium citrate, ammonium phosphate, ammonium sulfate, ammonium pyrophosphate, or various other individual compounds can be utilized. Ammonia gas is convenient and can be employed by bubbling through the aqueous fermentation media in suitable amounts.

The pH range in the aqueous microbial fermentation admixture will generally be in the exemplary range of from 5 to 9, more preferably from 6 to 8, and most preferably from 6 to 7.5, although exact preferences depend on the microorganism used and the medium employed. In IM2 media, see *infra*, the optimum pH range for NRRL B-8065 is 6.2—6.8 and for NRRL B-8066 from 6.2—6.5.

When the carbon and energy source is or contains an aldehyde, in amounts potentially deleterious to the microorganism, the deleterious aldehyde effects can be avoided by first treating the substrate with a suitable amount of a nitrogen-containing compound, preferably ammonia, ammonium hydroxide, or other ammonium compound, in a ratio of about 0.01 to 10 mol equivalents of said nitrogen-containing compound for each mol of aldehyde. Such a treated substrate then is not only the carbon and energy source but also contains the necessary nitrogen source in whole or part.

In addition to the oxygen, nitrogen, and carbon and energy sources, it is necessary to supply necessary amounts in proper proportions of selected mineral nutrients in the feed media in order to assure proper microorganism growth, and maximize the assimilation of the oxidized hydrocarbons by the cells in the microbial conversion process.

A source of phosphate or other phosphorus, magnesium, calcium, sodium, manganese, molybdenum, copper, ions appear to provide the essential minerals. The recipe shown below can be used to culture the novel *Bacillus* species of used in this invention, though they will grow on other than methanol-containing substrates. The following is given for guidance to those skilled in the art.

Medium IM2 (solid)

Component	Amount
KH_2PO_4	2.0 g
K_2HPO_4	3.0 g
$MgSO_4 \cdot 7H_2O$	0.4 g
$CaCl_2 \cdot 2H_2O$	0.04 g
NaCl	0.1 g
$(NH_4)_2SO_4$	2.0 g
Agar	15 g
Trace mineral solution ^(a)	0.5 ml
Distilled water	1,000 ml
Sterile methanol ^(b) to give	1.5 vol %

(a) See recipe below.

(b) Added just prior to use.

For a liquid media, simply omit the agar above.

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Trace Mineral Solution

Component	Amount
CuSO ₄ · 5H ₂ O	0.06 g
KI	0.08 g
MnSO ₄ · H ₂ O	0.3 g
Na ₂ MoO ₄ · 2H ₂ O	0.2 g
H ₃ BO ₃	0.02 g
ZnSO ₄ · 7H ₂ O	2.0 g
FeCl ₃ · 6H ₂ O	4.8 g
Distilled water	1,000 g
H ₂ SO ₄ (conc.)	3 ml

Other materials such as yeast extracts, vitamins and biotin or other growth factors, can be added typically in the trace amounts known to the fermentation art.

The culturing of the novel *Bacillus* species according to this invention can be conducted as a batch, though preferably as a continuous process, by methods known in the fermentation arts, but most preferably in a foam-filled fermentation reactor. Certainly, the continuous process possesses a number of advantages in terms of commercial operations for the production of large quantities of microbial cells, and thus is a preferred mode of performing the invention.

In either a batch, or the preferred continuous operation, all equipment, reactor, or fermentation means, vessel or container, piping, attendant circulating or cooling devices, are sterilized, usually by employing steam such as at about 250°F. for several minutes, such as about 15 minutes. The sterilized reactor is inoculated with a culture of the specified microorganism in the presence of all the required nutrients, and including the oxygen, and the oxygenated hydrocarbon feed.

In continuous process, as the culture begins to grow, the continuous introduction of air, nutrient medium, nitrogen source if added separately, and oxygenated feedstock such as alcohol, is maintained. The addition rate of the various streams can be varied so as to obtain as rapid a cell growth as possible consistent with the efficient utilization of the oxygenated hydrocarbon input, so that the objective of a maximized high yield of cell weight per weight of carbon and energy source material charged is obtained. The feed rate of the carbon and energy source material should be adjusted so that the amounts being fed to the fermenter is substantially the same as the rate of consumption by the organism, to avoid overfeeding, particularly of toxic materials, such as an alcohol or aldehyde, which might inhibit the growth or even kill the microorganisms. A satisfactory condition usually is exhibited by there being little or no carbon and energy source material in the effluent being withdrawn from the fermenter, though a satisfactory check can be obtained by watching the carbon and energy source material content of the fermenter effluent and maintaining it at a desirable low level such as about 0.1 to 0.5 volume/volume percent. Of course, any of the feed streams can be added either incrementally or continuously as desired or convenient.

Instrumentation should be maintained to measure cell density, pH, dissolved oxygen content, alcohol or other feedstock concentration in the fermenter, temperature, feed rates of input and output streams. It is preferred that material fed to the fermenter be sterilized prior to introduction into the fermenter. Where the oxygenated hydrocarbon feedstock is a material capable of sterilizing other materials, such as the methanol, ethanol, or formaldehyde, in some instances, it may be convenient to add this component to other streams, such as the mineral media, in sterilizing amounts, and thus accomplish several purposes without the necessity for a separate sterilization of the mineral media such as by heat.

The type of fermenter employed is not critical in the practice of the fermentation process of the invention, though presently preferred is operation in a foam-filled fermenter. High productivity with oxygenated hydrocarbon feed is best achieved in a continuous process when the process occurs in a foam-filled system. At the recommended fermentation temperatures *supra* high growth rates very stable foam conditions are obtained. Of course, watch must be maintained to control growth rates to avoid foam out of the fermenter which could lower the liquid volume and cause some loss of the fermenter contents. Addition of antifoam is to be avoided, if at all possible, since antifoams such as the silicones may be detrimental to the dissolved oxygen content at the recommended high temperatures, and may cause the organism to grow at a slower rate, cut productivity, or even to die. The foam produced is not harmful to growth and is definitely beneficial in maintaining the organisms in a system of high dissolved oxygen. The encouragement of such foam process in a fermenter designed

to encourage and maintain the produced foam is beneficial to the process of achieving the increased O₂ transfer necessary to maintain the high cell density and rapid growth rate which these thermophilic organisms require. The process of using a foam-filled fermenter with the present microorganisms, which produce substantial foam, results in the SCP being most efficiently produced.

Foam-filled operation of the fermenter is particularly suited for carrying out fermentation processes in which large quantities of gases are to be maintained in intimate contact with the liquid phase, so as to obtain a reaction along relatively large areas of contacting interface. Thus, fermentation is improved, and heat transfer is improved as to control, uniformity, and avoidance of hot spots.

One presently preferred type of fermenter can be observed in Figure 1 on page 37 of *Process Biochemistry*, June, 1972, to which can be added a conduit for introducing a molecular oxygen-containing gas into the vessel at any convenient point, preferably within the draft tube and just above the mixing device since the aspirating action of the mixing device then can be advantageously employed in aiding the introduction of air into the contents of the vessel. This type of fermenter operates efficiently with its contents substantially completely converted to a foam or low density emulsion with very high consequent oxygen transfer rates being achieved.

Another type of fermenter that can be employed is the airlift fermenter which is described in the article by Wang et al in the *Proceedings Eighth World Petroleum Congress*, Vol. 5, pp. 149-156 (1971) published by Applied Science Publishers Ltd., London, England.

Still another type fermenter that can be utilized is the pressure cycle fermenter which is described on page 63 of *Chemical Engineering*, January 7, 1974.

Finally, another type of fermenter which is suitable is the well known tank equipped with a blade stirrer and a submerged aeration device. One fermenter of this type is shown in U.S. Patent 2,983,652.

Where a foam-filled fermentation mode is not employed, it may be necessary to use an antifoam agent in carrying out the fermentation process of the instant invention. Suitable antifoam agents and methods of applying the same are well known in the fermentation art, though such methods of operation are much less preferred.

Both the cellular and extracellular products of culturing the novel *Bacillus* species on the substrates according to this invention can be recovered by conventional means. The cells can be separated from the fermenter effluent by centrifugation or filtration. The cell-free effluent can then be treated with acetone or a lower alcohol such as methanol or ethanol to precipitate any polymeric material produced extracellularly. The cell-free effluent also can be treated by solvent extraction and/or base extraction to recover if desired other extracellular products such as pigments, vitamins, or organic acids produced during the culturing process. The microbial cells usually are killed by heat or chemical means and this can be done before or after separation of the cells from the fermenter effluent. The bacterial cells are a valuable source of protein for man as well animals. For human consumption the cells can be treated to reduce the nucleic acid content, but for animal feed purposes such treatment does not appear necessary.

The following examples are illustrative of the present invention. Particular amounts of materials, or particular types of oxygenated hydrocarbon-containing feedstocks employed should be considered as illustrative and not as limiting. Example I is by way of comparison.

EXAMPLE I

Several cultures of known thermophilic microorganisms were obtained from two culture depositories, The United States Department of Agriculture, Northern Regional Research Laboratories, Peoria, Illinois, and The American Type Culture Collection ATCC, Washington, D.C. These cultures were received in lyophilized condition. The cultures were tested for growth at 55°C. in a variety of different media. The results of these tests are shown in Table I below. The cultures employed were as follows:

Species	Deposit No.
<i>Bacillus subtilis</i>	ATCC 10774
<i>Bacillus stearothermophilis</i>	ATCC 12987
<i>Bacillus stearothermophilis</i>	NRRL B1102
<i>Bacillus coagulans</i>	NRRL B1103
<i>Bacillus coagulans</i>	NRRL B1168
<i>Bacillus licheniformis</i>	NRRL B1001

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TABLE I

Culture No.	1 ^(a)	2 ^(b)	3 ^(c)	4 ^(d)	5 ^(e)
NRRL B1001	+	+	+	+	—
NRRL B1103	+	+	+	+	—
ATCC 12987	+	+	+	+	—
NRRL B1168	+	+	+	+	—
NRRL B1102	—	—	—	—	—
ATCC 10774	+	—	—	—	—

(a) Nutrient Broth (NB)

(b) NB+1% methanol

(c) NB (1/2 strength)+1% methanol

(d) NB (1/4 strength)+1% methanol

(e) IM2 medium+0.5% methanol

The nutrient broth used above is a conventional culture medium having 3 g/l of beef extract and 5 g/l of peptone. Medium IM2 is the medium as described hereinbefore except for the absence of agar in these run using liquid media. NRRL B1102 would not grow even on nutrient broth. ATCC10744 would not grow in the presence of methanol (Medium 2). Although the other cultures would grow in the presence of methanol, they evidently were unable to utilize methanol as the sole source of carbon and energy for their growth. These results are in dramatic contrast to the desirable results obtained with the unique *Bacillus* species employed in the process of this invention since the unique *Bacillus* species were able to efficiently utilize methanol as their sole carbon and energy source.

EXAMPLE II

A continuous fermentation run was carried out according to the process of this invention. The inoculum for the run was 500 ml of an aqueous dispersion of cells of Culture No. 72, NRRL B-8066, which had been grown for 24 hours on the medium BH-M with 1.5% by volume methanol:

Medium BH-M

Component	Amount
KH ₂ PO ₄	2.0 g/l
K ₂ HPO ₄	3.0 g/l
MgSO ₄ · 7H ₂ O	0.4 g/l
CaCl ₂ · 2H ₂ O	0.04 g/l
(NH ₄) ₂ SO ₄	2 g/l
Trace mineral solution*	10 ml/l

*The trace mineral solution was composed of the following materials:

Component	Amount
FeSO ₄ · 7H ₂ O	0.11 g/l
ZnSO ₄ · 7H ₂ O	0.03 g/l
CuSO ₄ · 5H ₂ O	0.02 g/l
MnSO ₄ · H ₂ O	0.02 g/l
H ₂ SO ₄ (conc.)	1 ml/l

The inoculum described above was added to a fermenter equipped with stirring and aeration means and having therein two liters of the medium made with tap water described below:

FM-12 Medium

Component	Amount
H ₃ PO ₄ (85%)	2.0 ml
KCl	1.0 g
MgSO ₄ · 7H ₂ O	1.5 g
CaCl ₂ · 2H ₂ O	0.2 g
NaCl	0.1 g
Trace Mineral Solution*	10.0 ml
Distilled Water	To make 1 liter

*This trace mineral solution was formulated as given in the recipe shown below:

Trace Mineral Solution

Component	Amount
CuSO ₄ · 5H ₂ O	0.06 g
KI	0.08 g
FeCl ₃ · 6H ₂ O	4.80 g
MnSO ₄ · H ₂ O	0.30 g
Na ₂ MoO ₄ · 2H ₂ O	0.20 g
ZnSO ₄ · 7H ₂ O	2.00 g
H ₃ BO ₃	0.02 g
H ₂ SO ₄ (conc.)	3 ml
Distilled water	To make 1 liter

The methanol content was initially 1.5% (v/v), pH was about 6.7, and temperature was about 55°C. Stirring rate of the contents was at about 1000 rpm, and air was introduced at about 2 liters per minute. Ammonium hydroxide was added continuously to keep the pH in the range of about 6.7—6.9 and also to provide a source of nitrogen.

After good growth of the inoculum was observed, a continuous addition of the nutrient medium having 5% by volume methanol was started and a corresponding volume of fermenter effluent was withdrawn. The feed rate for the nutrient mixture varied from about 150 to 800 ml/hour during the course of the run of about 500 hours. The average retention time for cells in the fermenter varied from about 2.4 to 5 hours. The fermenter effluent was sampled from time to time so as to recover some of the cells, which were dried, weighed, and subjected to protein analysis. The effluent samples were processed to recover only cells, i.e., solubles were not analyzed. Cell concentration values ranged from about 12 g/l (dry cells) early in the run (25 hours), to 18 g/l at 190 hours and about 24 g/l near the end of the run. Methanol conversion to recoverable cells was 44% of that charged. The protein content for a sample of cells taken during the course of this run has been presented hereinabove in the discussion describing product recovery.

The results above show that the novel *Bacillus* species NRRL B-8066 of this invention could be readily cultured continuously at 55°C. with methanol as the source of energy and carbon. Furthermore, the cells of this species have been shown to be high in protein content.

EXAMPLE III

Another continuous fermenter run was carried out using the culture NRRL B-8066 of Example II. In this run the fermenter was inoculated with 500 ml of an aqueous dispersion of the culture which had been grown for 26 hours on IM2 medium plus 1.5% by volume methanol:

IM2 Medium

Component	Amount
KH ₂ PO ₄	2.0 g/l
K ₂ HPO ₄	3.0 g/l
MgSO ₄ · 7H ₂ O	0.4 g/l
CaCl ₂ · 2H ₂ O	0.04 g/l
NaCl	0.1 g/l
(NH ₄) ₂ SO ₄	2.0 g/l
*Trace mineral solution	0.5 ml/l

*The trace mineral solution was composed of the following materials for one liter aqueous solution:

Trace Mineral Solution

Component	Amount
CuSO ₄ · 5H ₂ O	0.06 g
KI	0.08 g
FeCl ₃ · 6H ₂ O	4.80 g
MnSO ₄ · H ₂ O	0.30 g
Na ₂ MoO ₄ · 2H ₂ O	0.20 g
ZnSO ₄ · 7H ₂ O	2.00 g
H ₃ BO ₃	0.02 g
H ₂ SO ₄ (conc.)	3 ml

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The inoculum described above was added to a fermenter of the same type as described in Example II. The fermenter contained two liters of the same medium, FM-12, described in the fermentation run of Example II except that only 5 ml per liter of the above trace mineral solution was added to the medium.

The methanol content was initially limited to about 1.5% v/v, pH was about 7.1, and the temperature was about 53°C. During the first two hours, stirring and air volumes were increased slowly up to 800 rpm and 2 liters per minute, respectively. Ammonium hydroxide was added as described in Example II to keep the pH in the range of about 7.0—7.2, and as well to provide a source of nitrogen.

After good growth of the inoculum was established during the first 24 hours, the continuous addition of the nutrient medium having 7.5% by volume methanol and 0.05 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ was started and a corresponding volume of fermenter effluent was withdrawn. The feed rate for the nutrient medium mixture was about 200 to 400 ml/hour during the course of the run of about 94 hours. Average retention time in the fermenter varied from about 3 to 5 hours. The fermenter effluent was sampled from time to time to recover and dry the cells which were weighed. The effluent samples were processed to recover only the cells, i.e., solubles were not analyzed. Cell concentrations after 24 hours ranged from 17 to 26 g/l (dry cells). Methanol conversion to recoverable cells was about 43% of that charged.

The above results demonstrate again a good yield of microbial cells from the novel *Bacillus* species can be obtained by using methanol as the sole source of energy and carbon at a high fermentation temperature.

EXAMPLE IV

A continuous fermentation run was conducted with Culture 47 NRRL B-8065.

A fermenter was used of the same type as previously described. The fermenter contained two liters of the FM-12 medium as described in Example III, but in this instance made up with deionized water, was charged with 500 ml of an aqueous dispersion of Culture 47 NRRL B-8065 grown for 11 hours on the same inoculum IM2 medium as described in Example III with 1.5% by volume methanol.

The methanol content was initially 1.5% (v/v), pH initially was about 6.45, and the temperature was maintained at about 54°C. As in Examples II and III, ammonium hydroxide was added to keep the pH at about 6.3—6.6 and as well to provide a source of nitrogen. Stirring and air volume rate were gradually increased to 400 rpm and 0.5 liter per minute respectively over a period of about 7 hours. After about 11 hours the continuous addition of nutrient medium, FM-12 made with tap water, having 2.5% by volume methanol and additionally 1 g/l KH_2PO_4 , 1 g/l K_2HPO_4 , and a small amount of antifoam agent was started. After 28 hours the feed was modified by providing additional $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ to double its concentration in the feed. This adjustment appeared to stimulate to some extent oxygen uptake by the cells. These unique species appear to have a relatively high Mn^{++} requirement for optimum growth.

During the course of the 101.5 hour run, additional amounts of the other trace minerals were added but none seemed to have a stimulatory effect on cell growth rate. In addition to air, the fermenter also was charged after 28 hours with oxygen at an increasing rate of 0.12 up to 0.4 liters per minute. The stirring rate was correspondingly increased from 400 to 800 rpm during this period. The effluent was sampled from time to time to recover the cells, dry, and weigh them.

The feed rate of the media ranged from about 300 ml/hr at 28 hours to about 850 ml/hr at 71 hours to 1070 ml/hr at 95 hours. The residence time for the cells ranged from about 1.8 to 2.5 hours during the last 30 hours. Cell concentrations ranged from about 7 to 10 g/liter (dry cells), and methanol conversion to recoverable cells was about 50% of that charged.

The above results demonstrate that Culture No. 47 NRRL B-8065 also provides a good yield of microbial cells when grown on methanol as the sole source of carbon and energy at relatively high fermentation temperatures.

EXAMPLE V

The crude protein content of the cells of the novel *Bacillus* species is in the range of about 70 to 85% by weight. The crude protein value is obtained by multiplying the weight percent N (Kjeldahl analysis) of the dried cells by the factor 6.25. Subjection of these dried cells to a hydrolysis step followed by analysis of the amino acids by gas chromatography shows the protein content to be in the range of about 55 to 70% by weight. An amino acid distribution for one sample of bacterial cells prepared according to this invention using Culture No. 72 NRRL B-8066 is shown below:

	Essential Amino Acid	g/100 g dried cells	
	leucine	5.52	
	isoleucine	4.68	
5	lysine	5.36	
	methionine	1.38	5
	cystine	0.05	
	threonine	2.93	
	phenylalanine	2.72	
10	tyrosine	2.00	
	tryptophan	0.79	10
	valine	5.25	
	Nonessential Amino Acid	g/100 g dried cells	
	alanine	5.83	
	arginine	2.93	
15	aspartic acid	6.38	15
	glycine	3.76	
	glutamic acid	9.95	
	histidine	1.18	
	proline	2.37	
20	serine	1.96	20
	Total	65.04	
	Total Essential Amino Acids	30.68	

It can be noted that the content of sulfur-containing amino acids, e.g., cystine and methionine, is relatively low. In fact, some samples of cells from the above *Bacillus* species have shown no cystine per 100 g of dried cells. This situation is not unusual in single cell protein SCP processes and can be adjusted by simply adding suitable amounts of synthetic cystine or methionine to the feed ration employing such SCP.

WHAT WE CLAIM IS:—

1. A process for the production of single cell protein, which comprises aerobically culturing a microorganism of the genus *Bacillus* in an aqueous nutrient medium containing a water-soluble oxygenated hydrocarbon providing a source of assimilable carbon, a source of assimilable nitrogen, and such other inorganic and organic growth factors as may be necessary for the growth of the microorganism, and recovering the single cell protein from the culture liquor, wherein the microorganism used is a *Bacillus* species deposited as NRRL B8065 or NRRL B8066.
2. A process according to claim 1, wherein the microorganism is cultured at a temperature in the range 45° to 65°C.
3. A process according to claim 2, wherein said temperature is in the range 50° to 60°C.
4. A process according to any of claims 1 to 3, wherein said oxygenated hydrocarbon comprises an alcohol, ketone, ester, ether, acid or aldehyde of up to 10 carbon atoms, or a mixture of two or more thereof.
5. A process according to claim 4, wherein the oxygenated hydrocarbon comprises an aliphatic monohydric hydrocarbyl alcohol.
6. A process according to claim 5, wherein said alcohol contains from 1 to 4 carbon atoms per molecule.
7. A process according to claim 4, wherein the oxygenated hydrocarbon comprises one or more of the following: propanol, butanol, pentanol, hexanol, 1,7-heptanediol, 2-heptanol, 2-methyl-4-pentanol, pentanoic acid, 2-methylbutanoic acid, 2-pentanol, 2-methyl-4-butanol, 2-methyl-3-butanol, 2-butanol, 2-methyl-1-propanol, 2-methyl-2-propanol, 2-propanol, formic acid, acetic acid, propanoic acid, formaldehyde, acetaldehyde, propanol, butanal, 2-methylpropanal, butanoic acid, 2-methyl-propanoic acid, pentanoic acid, glutaric acid, hexanoic acid, 2-methylpentanoic acid, heptanedioic acid, heptanoic acid, 4-heptanone, 2-heptanone, octanoic acid, 2-ethylhexanoic acid, glycerine, ethylene glycol, propylene glycol, 2-propanone, 2-butanone, diethyl ether, methyl ethyl ether, dimethyl ether, di-n-propyl ether or n-propyl isopropyl ether.
8. A process according to claim 4, wherein said oxygenated hydrocarbon comprises methanol or ethanol.
9. A process according to any one of the preceding claims, wherein the concentration of the oxygenated hydrocarbon in the culture medium is maintained in the range 0.0 to 5 vol. per cent.

(21)

Essential Amino Acid

g/100 g dried cells

leucine	5.52
isoleucine	4.68
lysine	5.36
methionine	1.38
cystine	0.05
threonine	2.93
phenylalanine	2.72
tyrosine	2.00
tryptophan	0.79
valine	5.25

Nonessential Amino Acid

g/100 g dried cells

alanine	5.83
arginine	2.93
aspartic acid	6.38
glycine	3.76
glutamic acid	9.95
histidine	1.18
proline	2.37
serine	1.96
Total	65.04
Total Essential Amino Acids	30.68

It can be noted that the content of sulfur-containing amino acids, e.g., cystine and methionine, is relatively low. In fact, some samples of cells from the above *Bacillus* species have shown no cystine per 100 g of dried cells. This situation is not unusual in single cell protein SCP processes and can be adjusted by simply adding suitable amounts of synthetic cystine or methionine to the feed ration employing such SCP.

WHAT WE CLAIM IS:—

1. A process for the production of single cell protein, which comprises aerobically culturing a microorganism of the genus *Bacillus* in an aqueous nutrient medium containing a water-soluble oxygenated hydrocarbon providing a source of assimilable carbon, a source of assimilable nitrogen, and such other inorganic and organic growth factors as may be necessary for the growth of the microorganism, and recovering the single cell protein from the culture liquor, wherein the microorganism used is a *Bacillus* species deposited as NRRL B8065 or NRRL B8066.

2. A process according to claim 1, wherein the microorganism is cultured at a temperature in the range 45° to 65°C.

3. A process according to claim 2, wherein said temperature is in the range 50° to 60°C.

4. A process according to any of claims 1 to 3, wherein said oxygenated hydrocarbon comprises an alcohol, ketone, ester, ether, acid or aldehyde of up to 10 carbon atoms, or a mixture of two or more thereof.

5. A process according to claim 4, wherein the oxygenated hydrocarbon comprises an aliphatic monohydric hydrocarbyl alcohol.

6. A process according to claim 5, wherein said alcohol contains from 1 to 4 carbon atoms per molecule.

7. A process according to claim 4, wherein the oxygenated hydrocarbon comprises one or more of the following: propanol, butanol, pentanol, hexanol, 1,7-heptanediol, 2-heptanol, 2-methyl-4-pentanol, pentanoic acid, 2-methylbutanoic acid, 2-pentanol, 2-methyl-4-butanol, 2-methyl-3-butanol, 2-butanol, 2-methyl-1-propanol, 2-methyl-2-propanol, 2-propanol, formic acid, acetic acid, propanoic acid, formaldehyde, acetaldehyde, propanol, butanal, 2-methylpropanal, butanoic acid, 2-methyl-propanoic acid, pentanoic acid, glutaric acid, hexanoic acid, 2-methylpentanoic acid, heptanedioic acid, heptanoic acid, 4-heptanone, 2-heptanone, octanoic acid, 2-ethylhexanoic acid, glycerine, ethylene glycol, propylene glycol, 2-propanone, 2-butanone, diethyl ether, methyl ethyl ether, dimethyl ether, di-n-propyl ether or n-propyl isopropyl ether.

8. A process according to claim 4, wherein said oxygenated hydrocarbon comprises methanol or ethanol.

9. A process according to any one of the preceding claims, wherein the concentration of the oxygenated hydrocarbon in the culture medium is maintained in the range 0.0 to 5 vol. per cent.



10. A process according to any one of the preceding claims, wherein air or oxygen is fed to the culture medium at a rate of from 0.02 to 2.1 mol. of oxygen per minute per vol. of liquid in said culture medium and wherein the culture medium is maintained at a pressure in the range 0.1 to 100 atmospheres and a pH in the range 5 to 9.

11. A process according to any one of the preceding claims, wherein the oxygenated hydrocarbon contains one or more aldehydes and is treated with ammonia or an ammonium compound in a ratio of 0.01 to 10 molar equivalents per mole of aldehyde prior to employment in the culture medium.

12. A process according to any one of the preceding claims, wherein the culture medium is treated with a manganous compound during culturing.

13. A process according to any one of the preceding claims, wherein the culture is conducted in a substantially foam-filled fermentation vessel.

14. A process according to any one of the preceding claims, wherein the pH of the culture medium is in the range 6—7.5.

15. A process according to claim 14, wherein the microorganism is NRRL B-8065, the oxygenated hydrocarbon is methanol, and the pH is in the range 6.2—6.8.

16. A process according to claim 14, wherein the microorganism is NRRL B-8066, the oxygenated hydrocarbon is methanol and the pH is in the range 6.2 to 6.5.

17. A process according to any one of the preceding claims, wherein ammonium hydroxide is employed as the nitrogen source.

18. A process according to claim 1 substantially as hereinbefore described in any one of the preceding Examples II to IV.

19. Single cell protein, when prepared by a process according to any one of the preceding claims.

For the Applicants:—
D. YOUNG & CO.,
Chartered Patent Agents,
9 & 10 Staple Inn,
London, WC1V 7RD.

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