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COMMISSION ON FERMENTATION*

and

COMMISSION ON FOOD CONTAMINANTS†

GUIDELINES FOR TESTING OF SINGLE CELL PROTEIN DESTINED AS PROTEIN SOURCE FOR ANIMAL FEED—II

Prepared for publication by
J. C. HOOGERHEIDE and K. YAMADA
Commission on Fermentation

J. D. LITTLEHAILES and K. OHNO
Commission on Food Contaminants

GUIDELINES FOR TESTING OF SINGLE CELL PROTEIN DESTINED AS PROTEIN SOURCE FOR ANIMAL FEED (II)

Definition - By single Cell Protein (SCP) is meant isolated mass of non-viable, dried cells of micro-organisms (yeasts, bacteria, fungi or algae), generally produced by cultivating such micro-organisms on substrates such as alkanes, lower hydrocarbons (e.g. methane), lower alcohols (e.g. methanol or ethanol) or industrial and agricultural waste products (e.g. sulfite waste liquor) under standardized and well-controlled conditions in regard to organism, substrate and processing, and destined to be used as protein source in animal feed.

I. INTRODUCTION

On the premise that there will be a serious world shortage of proteins for both human and animal consumption in the forthcoming decades, a great deal of technological effort has been directed to the realization of a novel source of protein of microbial origin, which is referred to as single cell protein. The first generation technology of SCP, based on petroleum-derived hydrocarbon substrates including n-paraffin has already been firmly established from both engineering and product safety points of view.

IUPAC, in August 1974, published Technical Report No.12 entitled: "Proposed Guidelines for Testing of Single Cell Protein Destined as Major Protein Source for Animal Feed" in which "General Standards of Identity for SCP to be used for Animal Feed" are given and in addition "Specific Standards of Identity of SCP prepared from Hydrocarbons" are suggested. Although these specifications on the assessment of SCP in general and those specific for SCP based on hydrocarbon still stand, it is now considered that further information is required to specify the product. In addition specific consideration must be given to SCP based on methanol as substrate.

After a wide variety of carbon/energy sources have been explored for SCP production, methanol and methane have recently been of increasing interest as alternative feed stocks for SCP production. Methanol has advantages over liquid or gaseous alkanes in respect to several important points. Normal alkanes are hardly soluble in water, and the rate of microbial assimilation of these substrates is limited by their mass transfer rate into the culture fluid. Methanol, on the other hand, is completely miscible with water. In addition to this technical advantage, methanol is commercially available as a highly pure chemical of relatively low cost, which makes methanol highly promising as alternative feedstock for the second generation of industrially produced single cell protein.

It is the purpose of the present report to provide additional information on safety testing, to suggest specific standards for methanol based SCP and in addition provide a brief description of the new methanol based technology. This is in order to avoid some mistaken views that have previously arisen and may arise otherwise because of the unconventional nature of this feedstock and elaborated technology.

II. TOXICOLOGICAL AND NUTRITIONAL EVALUATION

1) General Requirements for Testing

"No novel source of protein should be admitted as animal feed ingredient unless it has been evaluated thoroughly with respect to safety and absence of toxicity. This evaluation must be conducted on samples derived from a fully stabilised process, yielding products of fully reproducible quality.

"In most cases such samples will be representative of pilot plant production. If on the basis of the outcome of the evaluation of such samples full plant production is undertaken, sufficient proof should be submitted that the plant production samples, in every essential respect, are identical to the pilot plant samples evaluated for safety, absence of toxicity and nutritional adequacy. To this end, chemical analysis and short term (i.e. acute) toxicity studies with laboratory animals should be carried out. Any change in organism or process should be publicly declared."

"Although in principle all yeasts, bacteria, fungi and algae may be considered for the production of SCP, adequate evidence will have to be given that the organisms are not pathogenic for man, animal or plant and that they produce no toxic product that cannot easily be removed to a level below an acceptable limit. The manufacturer of SCP should be required to declare all essential data regarding the strain, the manufacture and processing of the product, and in particular data necessary to establish efficient safety and quality control measures. As examples may be mentioned the type of micro-organisms used in the production, the raw materials used as major nutrients (e.g. gas oil, purified alkanes, methanol, methane, sulfite waste liquor,etc): whether unusual chemicals have been added (e.g. defoaming agents, substances to break emulsions); whether a solvent extraction process has been used; drying process used; the sanitary precautions taken during production and processing."

The above paragraphs largely taken from IUPAC Guidelines No. 12 are still pertinent. The above type of information is required for an understanding of any potential toxicity involved with either the process or the product. It is important for assessing potential hazard to the environment, the human population surrounding the factory, the factory workers, the animals fed the product, and finally the human population at the end of the food chain.

2) Toxicity Testing of the Product

In general the purpose of toxicological testing is to define the potential hazard to man. As direct testing is impracticable, evaluations are generally made on laboratory animals. The ability to carry out toxicological tests on the final target species, in this case farm livestock, is novel. As the understanding of the basic pathology, biochemistry, etc., of farm livestock is less well documented than that of the rodent, it is recommended that a toxicological evaluation should involve both rodent and target species. The extent of the testing procedure depends upon the chemical analysis of the product. Materials that have a chemical composition close to that of natural occurring raw materials are unlikely to produce abnormal effects. In contrast those products with an abnormal composition resulting from the biosynthesis of compounds not normally abundant in nature may need more careful evaluation. Thus an extensive chemical analysis of the product is required.

The following should be provided:

1. Proximate analysis: Quantification of moisture, lipids, protein, fibre, ash, gross energy.
2. Lipids: Identification and quantification of fatty acids, glycerols, phospholipids, non-saponifiable products.
3. Nitrogen Compounds: Identification and quantification of total nitrogen, amino acid nitrogen and profile, nucleic acid nitrogen, purine and pyrimidine bases, quantification of DNA and RNA.
4. Ash: Identification and quantification in terms of major minerals (Na, K, Mg, Ca, P, Cl) and trace elements in particular those of toxicological importance (Pb, Hg, As) and nutritional value (Mn, Zn, Cu, Se, Co, Mo).
5. Carbohydrates: Identification and quantification of principle carbohydrates.
6. Vitamins: Identity and quantity of both fat and water soluble vitamins.

Experience has shown that the potential for biological hazard is low for SCP's so far evaluated. Nevertheless, this hazard has to be continually evaluated by various biological studies.

Short term tests, such as 90 days rat studies, are valuable during the development stages of the process (see note a). As evaluation using longer term rodent studies should use material derived from the pilot plant. The objective of these tests is to evaluate potential carcinogenicity and they should therefore be of sufficient duration to induce tumour formation. The specific time length will depend upon the strain of the rat and the husbandry conditions under which the animals are kept. Individual laboratory experience will define this time period.

Experimental evidence should also be obtained from specific multigeneration and teratological experiments, again using the rodent. All conventional pathological, haematological, and biochemical parameters should be measured and evaluated. The tests should include bone marrow cytology to detect any host chromosome abberations. Reference should be made to Guidelines Numbers 6 and 15 issued by the Protein-Calorie Advisory Group of the United Nations System.

The main problem associated with these types of investigation is the inclusion level of the test material, and the subsequent effects that this has on the nutrient balance of the test diet. This in turn can induce changes in the parameters used for evaluation relative to the control. It is possible to induce a 'toxicological change' due to simple alterations of dietary minerals or energy. These changes could be wrongly ascribed to the SCP and hence extreme care must be exercised in the formulation of the test diets. To achieve a reasonably high level of the test SCP within the diet it is advisable to operate at two dietary protein levels (say 15 and 30%), adequate controls are needed using conventional protein sources of similar crude protein content to the test product. Considerable care must be exercised in equating the nutrient balance of the diet; simple changes in dietary levels of Ca, P, and Mg are known to induce nephrocalcinosis in rats, which if not recognised, could be ascribed to the use of the single cell protein as such. When evaluating the toxicological data it is necessary to bear in mind the changes that may be induced by such nutritional changes.

The toxicological evidence gained from the laboratory rodent species will form the principle basis for evaluation of hazard. Nevertheless it is still necessary to test the SCP in the diets of farm animals. Long term life-span studies equivalent to those carried out with the rodent are not necessary. The normal economic life span of a farm animal forms a sensible basis for the evaluation of risk in the actual target species.

These experiments with farm livestock should follow the same experimental concepts as those employed for the laboratory animal, this includes biochemistry, haematology, and terminal pathology. Mortality beyond that normally experienced in farm livestock must be carefully evaluated. The experimental designs used in these studies should simulate those employed for the laboratory animal, but due regard should be paid to the normal practices followed in animal production, thus experimental design must reflect conventional animal production systems used by the farming industry. The reproductive potential and the viability of the off-spring are important both scientifically and commercially. Multigeneration experiments made with both poultry and pigs need to have a sound statistical basis. Specific teratological investigations should also be made in at least one species apart from the rodent.

Care should be exercised in the interpretation of data. Within the intensive farming industry growth rate and productivity is sensitive to small changes in dietary nutrients. Changing dietary raw materials by the substitution of SCP will change the dietary nutrient profile. Poor productivity in the absence of any other toxicological symptoms should not be interpreted as a toxicological abnormality, but rather a change in nutrient balance. Such features are common place with naturally occurring raw materials.

Note a. Primary screening methods such as the *in vitro* Ames Test conducted on the soluble part of SCP and the more sophisticated *in vivo* Dominant Lethal Test may give some preliminary indication of possible cellular mutagenicity.

It should not be necessary to repeat the toxicological tests on every potential target species. If there are no abnormalities arising within the profile of studies undertaken on the rat, the fowl, and the pig, then work on other animals should be aimed at raw material acceptability and dietary formulation. This will indicate the potential limitations on the use of the product. Experiments of this nature are best regarded as applied animal nutrition and are of commercial significance only.

It is unlikely that single cell protein will provide all the supplementary protein within an animals diet: mixtures of proteins optimise animal productivity. Nevertheless the tissues resulting from the toxicological and nutritional experiments utilising large amounts of SCP should be evaluated for their potential effects upon the human food chain. Extensive tissue analysis of eggs, meat, fat and edible internal organs needsto be undertaken. The nature of the analysis will be dependent upon the basic analysis of the SCP product, they will include for instance fatty acids and trace elements, in particular the known toxic metals that may accumulate within the body system. Organoleptic evaluation on the meat is also desirable.

For the future it needs to be understood that the testing programme described above may not be necessary. At this stage in the development of our knowledge it is both prudent and necessary to accept that totally novel materials and products should be evaluated. In the future it is possible, given a satisfactory rate of industrial development, that these products will not be considered novel. The current testing programme as outlined above is long and costly. It is a testing programme that should cover any adverse eventuality as reflected by current knowledge within the subject. The hazards, if any, are to be derived from the substrate, or from cellular metabolites. The toxicity of the current substrates used for SCP production is well understood; further attention needs to be paid to the components that the cells manufacture themselves; with a potential understanding of this topic then the toxicological testing programme outlined above may become redundant. When this has been achieved SCP irrespective of source, can be regarded on an exactly similar basis to soya bean meal and fish meal.

3. Nutritional Testing of the Product

Conventional nutritional tests such as PER; NPU are of little practical value in animal nutrition although they may have some application during the early stages of product evaluation to monitor the effects of process changes at the pre-pilot plant stage. From a practical animal nutrition standpoint there is no substitute for the incorporation of the SCP with locally available raw materials and its evaluation under local practical animal husbandry conditions.

The toxicological evaluation of the SCP within the target species will have provided a considerable amount of evidence on the nutritional value and efficacy. However, the extent of this data will not be sufficient to demonstrate its nutritional potential as a novel material within the animal feedingstuff industry. These nutritional studies will require large animal populations housed under practical conditions which will define with statistical precision the correct mode of use for the product. This is considered to be a seller-customer relationship, and there is no need for the regulatory authorities to be unduly involved in the overall utilization of the SCP. If the livestock do not thrive in the toxicological tests it is unlikely that the product will be of any great value as a nutrient source.

To help the nutritionists in their role of dietary formulation the following information on nutrient value will be of assistance:

Available energy (defined as metabolizable energy, digestible energy, net energy, total digestible nutrients - depending upon the animal and usual mode of evaluation which varies from country to country); digestible crude protein; digestibility of amino acids, availability of amino acids.

Considerable attention needs to be taken over dietary nutrient balance. Abnormal profiles for minerals or amino acids within the SCP may give spurious results which need to be recognised at the time of dietary formulation and corrected.

III GENERAL AND SPECIFIC STANDARDS OF IDENTITY FOR SCP USED FOR ANIMAL FEED

1. General Standards of Identity for SCP

The final product must consist of the dead cells of the defined microbial species used for its production and contamination should be no more than that anticipated by good and standard food processing practices.

The product should conform to the following standards:

	Per gram
Viable bacteria count (total aerobic bacteria)	< 100 000
Viable moulds	< 100
Enterobacteriaceae	< 10
Salmonella	< 1 per 50 g
Staph. aureus	< 1
Clostridia total	< 1 000
Cl. perfringens	< 100
Lancefield Group D streptococci	< 10 000*
Moisture content (to be declared)	< 12%
Ash content	to be declared
Lead	< 5 ppm
Arsenic	< 2 ppm
Mercury	< 0.1 ppm
Total nitrogen	to be declared
Amino acid nitrogen and profile	to be declared
Nucleic acid nitrogen	to be declared
Ammonia and urea nitrogen	to be declared
Nitrate and nitrite nitrogen	to be declared
Available lysine as % of total lysine	to be declared

* to be aimed at 1000 per gram

For chemical and biological assay methods see IUPAC Technical Report No.12.

Recommended additional specifications:

Total lipids	to be declared
Fatty acid profile	to be declared
Glycerols, phospholipids	to be declared
non-saponifiables	to be declared
Fibre	to be declared
Proximate carbohydrate profile	to be declared
Vitamins	to be declared
Nutritional trace elements (Mn,Zn,Cu,Se,Co,Mo)	to be declared

2. Specific Standards of Identity for SCP

Benzo(a)pyrene (as indicator for presence of polycyclic aromatic hydrocarbons)	< 5 ppb
Acetylacetone reagent-positive substance (as formaldehyde)	< 20 ppm
a) SCP derived from alkanes: *	
Total hydrocarbons	< 0.5 %
Total aromatic hydrocarbons	< 0.05%
b) SCP derived from methanol:	
Residual methanol	< 20 ppm

* For analytical methods see IUPAC Technical Report No. 12

3. Determination of Methanol in Single Cell Protein

SCOPE

The method is suitable for the determination of methanol at the 8 ppm to 1000 ppm (w/w) level in single cell proteins.

METHOD

a) Summary

The method relies upon extraction of methanol from the cells by added water. The water is recovered by centrifugation and is then analysed for methanol content by the standard analytical procedure for aqueous methanol.

b) Apparatus

(i) Centrifuge tube
(ii) Centrifuge
(iii) Gas chromatograph, set up as in (d)

c) Procedure

About 5 g of sample is weighed accurately into the centrifuge tube. 10-20 ml of water is added to the tube by means of a pipette. The tube is allowed to stand for 2 hours, and then centrifuged at about 15,000 rpm for 20 minutes. 2µl of the supernatant is injected into the gas chromatograph.

d) Chromatographic Method for Determination of Aqueous Methanol

(i) Outline of Method

Gas chromatographic separations are used in the analysis. Quantitative results are achieved by the injection of reproducible volumes of sample, and the component peak heights obtained are converted to concentration by direct reference to peak heights from synthetic mixtures.

(ii) Apparatus and Materials

a)	Gas Chromatograph:	Any flame ionisation chromatograph capable of providing similar sensitivity to that in the specimen may be used.
b)	Column:	5 meters x ¼ OD s/s tubing.
c)	Column Packing:	20% UCON. HB 5100 on Chromosorb W. plus 4% NaOH.
d)	Diluent for Standards:	Water.
e)	Syringe:	5µl with 3" needle.
f)	Recorder:	1 mV 0.25 cms/min.

(iii) Preparation of Apparatus

a) Preparation of Packing

Weigh 50 grams of Chromosorb "W" and add 200mls of 4% NaOH to form a slurry. Remove all water by rotating in a heated rotary evaporator. Weigh 10 grams of UCON and dissolve in 125 mls of dichloromethane. Add this to the dried Chromosorb and remove solvent by placing in the rotary evaporator.

b) Preparation of Column

Bend a 5 meter length of ¼" OD tubing into convenient coils to suit the chromatograph oven. Close one end of the column with a glass wool plug, and connect this end via a catch pot to a vacuum pump. Connect a small funnel to the other end of the column and switch on the pump. Pour the prepared packing into the funnel vibrating continuously until the column is full. Leave the pump running for a further ten minutes then make up any fall in packing level. Shut down the pump and remove funnel and close the column end with glass wool. A note should be taken of the weight of packing used.

4. Determination of Formaldehyde in Single Cell Protein

SCOPE
This method is applicable for not less than 5 ppm of formaldehyde content of single cell proteins.

PRINCIPLE
The sample is suspended in a diluted phosphoric acid solution and the formaldehyde is steam-distilled. To the distillate is added a neutral solution of acetylacetone and ammonium salt, whereby a yellow colour develops due to the synthesis of diacetyldihydrolutidine. The colour is measured at the wavelength of 420 mμ.

REAGENTS
a) Acetylacetone Solution
(Analytical grade reagents, redistilled acetylacetone, which should be colourless)

150 g ammonium acetate
3 ml acetic acid
2 ml acetylacetone
distilled water
} to 1000 ml

b) Formaldehyde Stock Solution
Hexamethylenetetramine (311.2 g), analytical grade, is made up to 1000 ml in water. 5 ml of this solution is diluted with water to 100 ml to make the Stock Solution.

c) Formaldehyde Standard Solution.
20 ml of the Stock Solution is diluted with water to 100 ml (Standard Solution A: 1 ml = 4 μg HCHO). 5 ml of the Stock Solution is diluted with water to 100 ml (Standard Solution B: 1 ml = 1 μg HCHO).

APPARATUS
a) Steam Distillation Apparatus
b) Spectrophotometer
Any instrument capable of operating at the required sensitivity at the wavelength of 420 mμ.

PROCEDURE
a) Preparation of Sample Solution
About 10 g of the SCP is weighed accurately and added to 10 ml of water and 1 ml of 20% H_3PO_4. The solution is steam-distilled. Prior to the distillation 10 ml of water is added to the receiver, into which the leg of the cooler is to be dipped. Stop the distillation when nearly 200 ml of distillate is obtained. The distillate is made up to 200 ml with distilled water to make a sample solution.

b) Determination of Formaldehyde
The sample solution (5ml) is mixed with 5 ml of acetylacetone solution and heated for 10 minutes in a boiling water bath. At the same time, 5 ml of the Standard Solution, A or B, is treated in the same way. After cooling, optical absorption of each solution is measured as A and

CALCULATION

$$\text{Formaldehyde content (ppm)} = K \times \frac{A}{As} \times 200 \times \frac{100}{\text{sample weight (g)}}$$

K: content of HCHO (μg) in 1 ml of the Standard Solution

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1) Standard Methods of Analysis for Hygienic Chemists with Commentary. Pharmaceutical Society of Japan, p.223 (1973).
2) T. Nash, Biochem. J. 55, 416 (1953).

c) Preparation of Chromatograph

Install the column in the chromatograph leaving the detector end disconnected and the carrier gas flowing. Raise the oven temperature to 190°C and leave overnight. This conditioning period is necessary to allow all traces of solvent and possible impurities in the UCON to elute without contamination of the detector. After conditioning, connect the column to the detector and adjust the conditions to those specified.

(iv) Operating Conditions

Column temperature	:	108°C
Carrier gas	:	Nitrogen
Carrier gas pressure	:	8.5 lbs
Hydrogen pressure	:	17.5 lbs
Air pressure	:	29 lbs
2 µl sample injected	:	(3" needle)
Recorder	:	0 - 1 mV
Chart speed	:	0.25 cms/min

(v) Preparation of Calibration Standards

Calibration is carried out by the preparation of standard mixtures of pure methanol and distilled water covering the range anticipated in the sample. It is recommended that standards in the lower range i.e. 100 ppm to 1,000 ppm, are always freshly prepared.

Typical traces of aqueous standards are shown in Figure 1.

e) Calculation of Results

The concentration of methanol in the supernatant as found from the gas chromatogram is expressed in mg/l (ppm w/v).

$$\text{Concentration of methanol in single cell protein (ppm w/v)} = \text{Conc. methanol in supernatant} \times \frac{\text{ml water}}{\text{wt SCP}}$$

f) Limit of Detection

The limit of detection is fixed by

(i) The sensitivity of the chromatographic method and
(ii) the amount of water used in the extraction.

For the method described above it is 8 - 16 ppm (w/w).

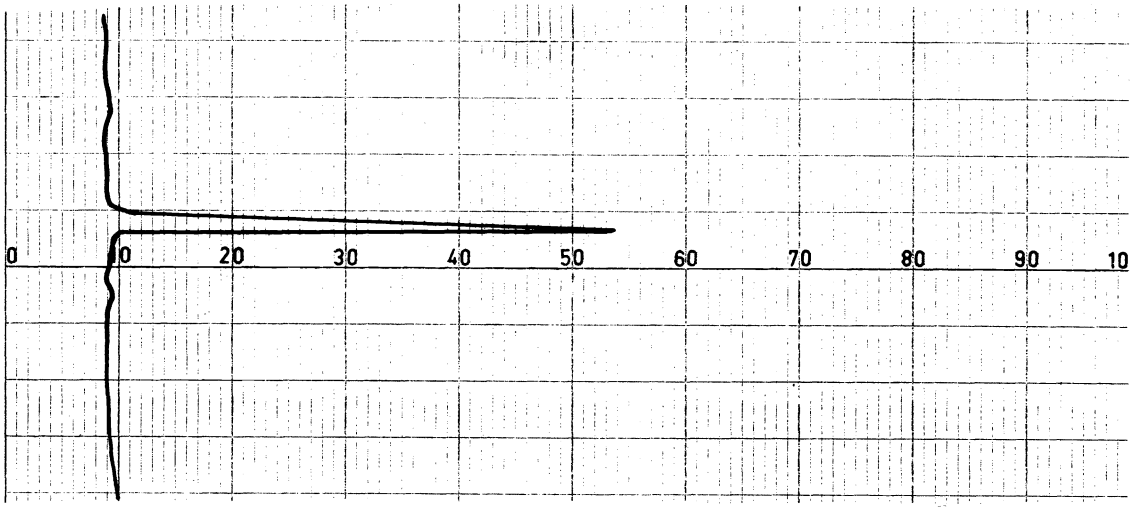

Fig 1.

No methanol could be found in manufactured SCP. A sample was therefore made up containing 780 ppm w/w methanol in solid.
This was extracted into 10 ml water.
Sensitivity setting was 1/2 max. 1 µl of water extract was injected.

APPENDIX 1

Methanol Production Process and Methanol Quality

Industrial methanol production has reached levels of 8-10 million tons per year. The three major process owners of methanol are Imperial Chemical Industries of Britain, Lurgi Apparate Technik of FDR and Mitsubishi Gas Chemical Company of Japan.

A brief description of the present technical process of methanol production might be in order since it might indicate possible (toxic) impurities present in crude or refined methanol that could be expected in methanol derived SCP.

The synthetic reactions of methanol syntheses are:

$$CO + 2H_2 = CH_3OH$$

$$CO_2 + 3H_2 = CH_3OH$$

As feedstock for production of the CO, CO_2 and H_2, light hydrocarbons are used, ranging from natural gas, LPG, naphtha to heavier hydrocarbons. Sulphur containing impurities are carefully removed since they are catalyst poisons. These gases are passed over a Zn-Cr or a Cu catalyst at a temperature of 200-400°C and at a pressure of 50-400 kg/cm².

After cooling this results in a crude methanol, containing water and small amounts of by-products.

Crude methanol is normally refined by distillation, yielding practically pure methanol. As a commercial industrial product it normally meets the standards represented by U.S. Federal Standards Specification Grade A or Grade AA (see TABLE 1).

TABLE 2 gives a typical analysis of crude and refined methanol.

On the basis of available information it must be concluded that commercial refined methanol contains only traces of impurities, mainly lower alcohols and their derivatives, all of relatively small molecular weight. Presence of such minor impurities in the substrate are not considered to give any adverse effect on the safety of SCP products produced from methanol.

Even if "crude" methanol were to be used for SCP production its composition does not indicate that an accumulation of impurities in the cell would occur as a consequence of substrate impurities, and hence this does not warrant establishing special limits for the use of crude methanol.

TABLE 1

U.S. Federal Standards

Characteristics	Grade A	Grade AA
	Requirement	Requirement
Acetone and aldehydes, per cent max.	0.003	0.003
Acetone, per cent max.		0.001
Ethanol, per cent max.		0.001
Acidity (as Acetic acid) per cent max.	0.003	0.003
Alkalinity, per cent max. as NH_3	0.003	0.003
Appearance	Clear and free from suspended matters or sediment.	
Carbonizable substances	No discolouration	No discolouration
Colour	Not darker than colour standard No.5 of ASTM platinum-cobalt scale	Not darker than colour standard No.5 of ASTM platinum-cobalt scale
Distillation range	Not more than 1°C and shall include 64.6° C ± .10°C at 760 mm	Not more than 1°C and shall include 64.6°C ± .10°C at 760 mm
Hydrocarbons	No cloudiness or opalescence	No cloudiness or opalescence
Specific gravity, max.	0.7928 at 20° /20°C	0.7928 at 20°/20 °C
Percent methanol by weight, min	99.85	99.85
Nonvolatile content	0.0010	0.0010
Odour	Characteristic, non-residual	Characteristic, non-residual
Permanganate test	No discharge of colour in 30 minutes	No discharge of colour in 30 minutes
Water, percent max.	0.15	0.10

TABLE 2

Typical Analysis of crude and refined methanol

Composition	Refined methanol	Crude methanol
Methanol (%)	99.96	84
Water (%)	0.012	16
Dimethyl ether (ppm)	n.d.	130
Methyl formate (ppm)	20	200
Acetone	trace	trace
Methyl acetate	trace	trace
Ethanol (ppm)	30	80
2-Propanol (ppm)	35	150
Methyl ethyl ketone	n.d.	n.d.
1-Propanol (ppm)	n.d.	30
2-Butanol (ppm)	15	150
1-Butanol (ppm)	n.d.	30

APPENDIX 2

Methanol-Utilizing Microorganisms

Since methanol has emerged as a novel feedstock for SCP production, a large number of microorganisms capable of growing on methanol has been isolated from nature. Both yeasts and bacteria have industrial potential. Difficulties may exist in their classification at species level using conventional taxonomic keys, as information on methanol-grown microorganisms is sparse due to the limited capability of organisms to grow on this simple alcohol.

Bacteria

Extensive taxonomic studies on methylotrophic bacteria have been undertaken (Refs 1-2). Methylotrophic bacteria are divided into two domains: obligate methylotrophs that can assimilate exclusively methane, methanol and other C_1-compounds, and facultative methylotrophs that can assimilate different carbon compounds besides C_1-compounds. Obligate methylotrophs include species of genera Pseudomonas, Methanomonas, Methylococcus and Methylomonas and, facultative methylotrophs involve species of genera Protaminobacter, Pseudomonas, Hyphomicrobium, Microcyclus, Achromobacter, Alcaligenes, Acinetobacter, Bacillus and some coryneforms. Strains so far identified as new species are based on differing physiological and biochemical properties relative to the established species. This situation requires new approaches of modern systematic bacteriology using chemo-taxonomical information and techniques in order to avoid confusion in strain identification.

Yeasts

Methanol assimilating yeasts include both those isolated from nature and those selected from among strains of stock cultures. No obligate methylotrophic yeasts are known. Sporogenic yeasts including Hansenula, Pichia and Saccharomyces as well as asporogenic yeasts such as Kloeckera, Torulopsis, Candida and Rhodotorula have been reported as methanol utilizers. A considerable number of new species has been identified but some of them are likely to be synonymous.

The fundamental principles of microbial taxonomy are based on morphology and this is given the heaviest weighting at the genera level. At the species, or strain level, differences in biochemical and physiological characters are more important and are used as classification criteria. Thus in recent years, new approaches in taxonomy involving subcellular structures, cell constituents, respiratory mechanism, specific DNA parameters, are being emphasized to clarify interrelationship of different microbial strains and species. Methylotrophs are a group of organisms that are difficult to deal with by the conventional methods of classification, and this in turn will necessitate new approaches and methods in taxonomy in order to eliminate undesirable confusion.

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1. R. Whittenbury, K.C. Phillips and J.F. Wilkinson, J. Gen. Microbiol. 61,205 (1970)

2. J. Jiybi and A. Ozaki, Microbial Growth on C_1-compounds, Osaka, 1975

APPENDIX 3

SCP Production Process Based on Methanol

Single cell protein production based on methanol is an area of applied microbiology where at present a great deal of activity is involved. Numerous scientific and technical publications as well as patent literature are proof of the great interest in this field.

Some of the pioneering work has reached the stage of commercial plant production; both yeasts and bacteria have shown to have industrial potential.

Table 3 summarizes the properties of some selected yeast and bacteria strains being considered for commercial development. A typical example of an SCP manufacturing process, applicable to both yeast and bacteria SCP production is schematically given in Fig. 2.

a) Feedstock Preparation and Sterilization
Nutrient medium for microbial growth containing methanol as carbon and energy source, ammonia, minerals and water (often recycled water from biomass separation process) is sterilized continuously in medium sterilizer, and fed continuously to a fermenter.

b) Aeration
Sterile compressed air is supplied continuously to the culture by means of a compressor after passing an air-filter and thus rendered free from contaminating organisms.

c) Biomass Propagation
Inoculum of the microbial culture used for SCP production is propagated batchwise until a certain cell density is obtained. Culture medium is then continuously fed to maintain a culture of microbial biomass under the desirable conditions. When the steady state of microbial culture is established, methanol is the limiting substrate in the continuous culture and the concentration of substrate methanol remains negligibly small in the microbial culture.

d) Biomass Separation
A microbial culture is continuously taken out of the culture vessel at the same rate of feeding. In the case of bacterial cultures the biomass aggregates and is sedimented prior to separation and concentration. Wet biomass is then subjected to a drying process. Culture medium after biomass removal is recycled to the fermenter after readjustment of mineral constituents and pH.

e) Drying and Pelleting
Wet biomass separated is usually spray-dried to a powdery SCP product. When necessary the powdery product is made into a pellet form of appropriate size to meet the requirements for proper feeding of farm animals.

Schematic Flow Diagram

of

Methanol-SCP Pilot Plant

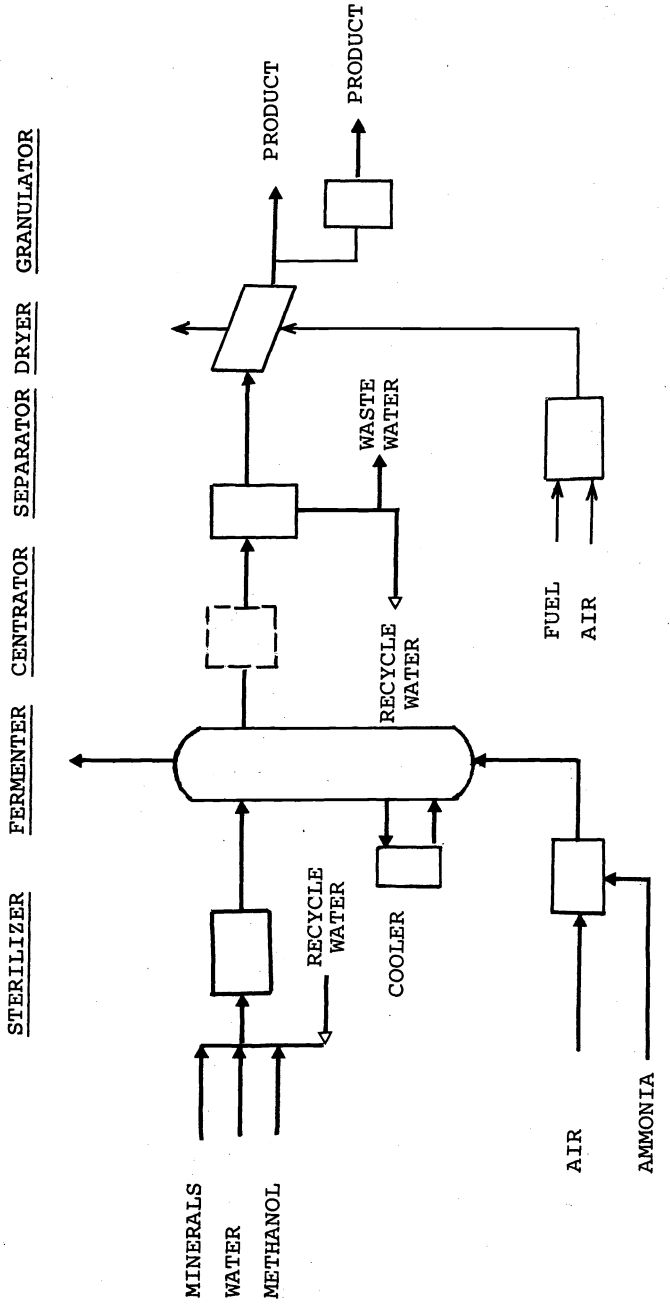

Fig. 2.

Table 3

Typical Microorganisms used for SCP production on Methanol and Analysis of the SCP

	Cultural Parameters			Typical Analysis		
YEASTS	Opt. Temp.	Opt. pH	Yield	Moisture	Crude Protein	Nucleic Acid
1) Pichia aganobii	27-30	3-5	40%	4.1%	57.5%	8.0%
2) Hansenula sp.	29	3-5	40	5.0	53.2	3.7
7) Candida sp.	28	3.5-4.5	31.5	8.0	45	
BACTERIA						
3) Methylophilus methylotrophus	36-42	6-7	50	5.0	78.9	15.9
4) Methylomonas methanolis	36-42	6-7	50	5.4	78	13.8
5) Aeromonas sp.	35-40	6-7	45-50	6.7	82.5	-
6) Methylomonas sp.	37	6.8	45	2-5	87	10
7) Methylomonas sp.	37	6.5-7.5	35	5	80	10

1) Mitsubishi Gas Chemical Co.
2) Unitica
3) ICI
4) Mitsubishi Gas Chemical Co.
5) Asahi Chemical Industries Co.
6) Hoechst
7) Institute of the Fermentation Industry, Poland.

APPENDIX 4

A. Natural occurrence of methanol

Like ethanol, methanol is a natural product that occurs abundantly in the plant kingdom, in fruits and vegetables belonging to our daily diet.

Methanol occurs naturally in (a) vegetables and fruits; (b) non-fermented fruit juices and (c) alcoholic products. It is produced from pectin containing substances in fruits as a result of the action of the enzyme pectin methylesterase and also possibly as a secondary product of alcoholic fermentation (1).

a) Occurrence in non-fermented fruit juices
The following Table of methanol concentrations in fruit juices is taken from a publication by Jacquin and Travernier (2) (TABLE 4).

Table 4

Fruit Juices		Country of Origin	Methanol Conc. ppm
Apple	(1)	France	25
	(2)	"	46
	(3)	"	64
Tomato	(1)	"	180
	(2)	"	218
	(3)	"	195
	(4)	"	204
Blackcurrant			680
Raisin/blackcurrant	(1)	France	145
	(2)	"	45
Gooseberry		"	65
Conc. sweetened blackcurrant		"	181
Orange		USA	31
Grapefruit		"	23
Bananas		"	17

Markh et al (3) found 0.7 to 12 ppm methanol in Russian grape juices treated with pectolytic enzymes and subsequently pasteurised. Similarly treated apple juices contained 7 to 15 ppm methanol.
b) Occurrence in fermented products
Brandy appears to contain the highest levels of methanol with concentrations quoted as high as 20,000 to 150,000 ppm; whiskies were found to contain 150 ppm (4). Zamorani (5) reported 100,000 ppm methanol in brandy. Nykanen (6) found 700-800 ppm in Russian brandy, 300-700 ppm in liqueurs, 100-200 ppm in whisky and 200-800 ppm in Finnish berry wine. Dyer (15) reported 180-670 ppm methanol in USA brandies, whereas foreign brandies contained 1700-3700 ppm methanol; whisky was found to contain 50-70 ppm. Rebellein (7) analysed 200 samples of German wines and found the following mean concentrations of methanol: white wines 50 ppm, red wines 14 ppm, rose wines 70 ppm and dessert wines 150 ppm. Radmine (8) found 138-340 ppm in 22 Yugoslavian wines.

Other levels observed in wines include:-

Table 5

Origin	Conc. ppm	Reference
Mendoza	7-150	9
Japan	180-240	10
France	220	10
Irpinia	170-500	11
Various	100-5000	12
Crimean	100-700	13
Various	50-325	14

Fermandez et al (16) found that in Spanish vinegar 400 ppm was rarely exceeded, a figure which he considered to be a safe level.

c) Other sources of methanol

Table 6

Source	Methanol Conc. ppm	Reference
Apricots (canned)	5-13	16
Bananas-ripe	1-6	17
-half ripe	18	17
Pineapples	1	18
Brussel sprouts (volatiles)	"very large" (but no conc. quoted)	19
Celery "	"	19
Parsnip "	"	19
Potato "	"	19,21
Onion "	"	19,20
Tobacco (Russian & Turkish)	0.2-0.5 mg/cigarette	22

A large dose of methanol is toxic for animals. The toxicity greatly varies depending on the species of animal. In man, when administered in a large amount, he often loses sight (5-10 g) or dies. The mechanism of the toxicity has not yet been clearly demonstrated.

Methanol has a coagulating action on albumin and results in irritation of mucous tissue particularly in the digestive system. The most serious effect, however, is its attack on the nervous system (particularly the optic nerves), the liver, kidneys and lungs (1).

The Handbook of Analytical Toxicology, (1969) (23) gives TLV = 260 mg/m^3 (air concentration to which it is believed that nearly all workers may be repeatedly exposed, day after day without adverse effect). The lethal dose, LD_{50}, for rates by ingestion of methanol was 10 g/kg body weight.

The physiological fate of the methanol liberated from pectin was studied by Summer (24) by means of suspensions of stomach, small intestine, large intestine, and appendix contents of guinea pigs. It was shown that methanol was released from pectin by pectinase in these organs. This is especially true in the appendix. The human and animal organism, therefore, when using pectin-containing fruit and fruit products must come into contact with small amounts of methanol, without any apparent ill-effect. Summer's studies were made to refute objections to the use of fruit juices treated with pectinase.

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B. Natural Occurrence of Formaldehyde

Formaldehyde and its aqueous solution, formalin, is a toxic substance for human and animal. Vapours are intensely irritating to mucous membranes. Topical application may produce an irritant dermititis. Ingestion may cause severe abdominal pain, haematemesis, haematuria, proteinuria, anuria, acidosis, vertigo, coma and death. LD_{50} orally in rats is 800 mg/kg. Formaldehyde acts as a powerful coagulant denaturing proteins of cell protoplasm, and thus destroys cell functions and causes death. For this reason, formaldehyde is widely used as an aseptic or a disinfectant.

In the Japanese food sanitation regulations, formaldehyde is among the chemicals listed as those prohibited for use in manufacture for preservation of foods destined to commercial sale. The State Standards on Foods and Food Additives, under the Food Sanitation Act state that formaldehyde detection should be conducted on the basis of a blue or indigo colour of Rimini's reaction and a purple colour of the modified Herner's reaction (Yanagizawa-Maruyama's reaction). In 1961, a processed product of cod meat obtained in the Tokyo Fisheries Market was found to be positive with the formaldehyde test. Soon after this it was demonstrated that formaldehyde is widely contained in cod and Alaska Pollock and the detection of formaldehyde in the previous instance was not the case of illegal addition of the chemical for the purpose of preservation. In the decree issued on February 12, 1962, the Health Ministry advised tentative measures including determination of formaldehyde using the chromotropic acid method with the cod samples that were found to be positive for formaldehyde using both Rimini's and modified Herner's reactions. The decree enforced disposal of cod with formaldehyde content of above a fixed level. Extensive studies undertaken by Amano, Yamada and Bito (1963) on the occurrence of formaldehyde in fish demonstrated that cod and Alaskan pollock contain considerable amounts of formaldehyde in their muscle and other tissues. Formaldehyde in cod muscle was isolated and chemically identified. The maximal level of formaldehyde determined in cod muscle was 15 mg/100g (150 ppm). (TABLE 7).

TABLE 7. Formaldehyde Content in the Muscle of Cod and Alaskan Pollock.

Sample No.	Species	Formaldehyde content (mg/100g)	Source and Status of Fish
TYM-1	Cod	4.2	Caught off West Kamchatka; frozen on board
TYT-5	"	7.4	Caught in Bering Sea; frozen on board
TYS-7	"	8.1	"
TYY-8	"	5.6	Caught off Olyutorskii Cape; frozen on board
HKR-17	"	6.6	Caught in Bering Sea; frozen on board
TYY-18	"	15	Caught off Olyutorskii Cape; frozen on board
W-19	"	2.5	Fishing ground was not certain; frozen
K-15	"	3.4	Caught off Kushiro, Hokkaido: iced
K-16	"	5.1	"
S-42	"	6.1	Purchased at Shiogama; iced
K-I-20	"	10	Fishing ground was not certain: salted and dried
Y-I-20	"	2.5	Caught off Olyutorskii Cape; salted and dried
N-55	Alaska Pollock	0.5	Caught at Essa Strait; treated with dry ice immediately after catching

In 1968, the Public Health Department of Osaka Prefecture reported that some commercial Chinese mushroom samples gave an odour of formaldehyde and were found to be positive in official formaldehyde tests. Three out of five fresh mushroom and five out of six dried mushroom samples were found to be positive. The National Institute of Hygienic Sciences, consequently, conducted extensive investigations on the occurrence of formaldehyde in various natural foods, particularly in some typical edible mushrooms. The investigation demonstrated that mushrooms particularly Chinese mushroom (Cortinellus edodos) contain small amounts of formaldehyde as natural constituent (TABLE 8).

TABLE 8. Formaldehyde Content in Vegetables

Vegetable	ppm
Tomato	0
Cucumber	2.3
Kikurage (Mushroom)	1.7
Hiratake (Mushroom)	34.5
Nameko (Mushroom)	7.5
Naratake (Mushroom)	13.2
Chinese Mushroom (fresh)	54.4
" " (dried, 9 samples)	100-406

Formaldehyde was also detected by acetylacetone method in the following vegetables: turnip, cucumber, chrysanthemum, radish, onion, egg plant, leek, parsley, lettuce, pimient and butterbur. The Japanese Food Sanitation Council advised that this low level of naturally occurring formaldehyde should not have any adverse effect on human health.

The Japanese Food Sanitation Act allows exceptional admission of chemical compounds (referred to as toxic or harmful) in foods, when they are specified by the Health Minister as not harmful to human health. On September 11, 1970, the Health Ministry issued a decree setting aside the provision that foods must contain no detectable formaldehyde. Since this decree there is no legal regulation, in Japan, of naturally occurring formaldehyde in foods. The use of formaldehyde as an additive is, however, strictly prohibited.

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APPENDIX 5

Typical Analysis of Pilot Plant Samples of SCP Produced from Methanol [1]

	Bacteria-SCP [2] Granular	Bacteria-SCP [2] Powder	Yeast-SCP [3] Granular
(Gross Proximate Composition)			
Moisture	10%	5%	4.5%
Protein	73	76	55.6
Fat	7.9	8.5	8.0
Ash	6.8	8.6	7.3
Fibre	not detectable		3.3
(Nitrogen Components)			
Total N	11.66%	12.16%	8.90%
Amino Acid N	8.86	9.24	7.20
Nucleic Acid N	2.26	2.35	1.32
Ammonia and Urea N	0.10	0.11	0.26
Ethanolamine N	0.14	0.15	0.015
Glucosamine N	0.18	0.19	0.006
Nitrate and Nitrite N			8 ppm
(Amino Acids)			
Alanine	5.15%	5.43%	3.20%
Arginine	3.35	3.53	2.79
Aspartic Acid	6.41	6.76	5.14
Glutamic Acid	7.31	7.71	6.88
Glycine	3.80	4.01	2.54
Histidine	1.49	1.57	1.18
Isoleucine	3.29	3.47	2.63
Leucine	5.08	5.36	4.01
Phenylalanine	2.61	2.76	2.40
Proline	2.24	2.37	2.12
Serine	2.54	2.68	2.71
Threonine	3.43	3.62	2.67
Tryptophan	0.75	0.79	0.66
Tyrosine	2.31	2.44	2.29
Valine	3.95	4.17	2.87
Cystine	0.45	0.48	0.82
Methionine	1.87	1.97	1.07
Lysine	4.70	4.96	3.66
Available Lysine	4.37	4.61	3.11
(Fats & Fatty Acids)			
Dodecanoic Acid	0.01%	0.01%	0.002%
Tetradecanoic Acid (14:0)	0.19	0.21	0.018
Tetradecanoic Acid (14:1)	0.005	0.005	0.001
Pentadecanoic Acid (15:0)	0.07	0.01	0.014
Hexadecanoic Acid (16:0)	3.16	3.39	1.03
Cis-9-Hexadecanoic Acid (16:1)	2.95	2.97	0.48
Heptadecanoic Acid (17:0)	0.01	0.01	0.060
Octadecanoic Acid (18:0)	0.26	0.27	0.084
Octadecanoic Acid (18:1)	0.79	1.02	1.43
Octadecadienoic Acid (18:2)	0.04	0.52	2.26
Octadecatrienoic Acid (18:3)			1.15
9,10-Methylene hexadecanoic Acid	0.25	0.25	0.000
2-Hydroxyhexadecanoic Acid	0.3	0.3	0.000
3-Hydroxyhexadecanoic Acid	0.3	0.3	0.000
Glycerols, phospholipids	7.5%	7.5%	4.53%
Non-saponifiables	1.0	1.0	1.67

(Vitamins)	Bacteria-SCP Granular and Powder	Yeast-SCP Granular
Thiamine (B_1)	3.6 mg/kg	137 mg/kg
Riboflavin (B_2)	33.4	219
Pyridoxine (B_6)	1.6	16
Cobalamine (B_{12})	0.00003	0.003
Folic Acid	12.1	0.32
Ca d-pantothenate	6.3	70
Nicotinic Acid	39.6	718
Biotin	2.5	0.51
Vitamin E	31.5	33
Inositol		8260
Choline	5.5	2600
Ergosterol		4000

(Nutritional Minerals, Dry Matter Basis)		
P	2.87%	1.80%
Mg	0.31	0.22
K	0.25	2.19
Na	1.14	0.093
Ca	0.03	0.033
Fe	0.016	0.034
Mn	21 ppm	30 ppm
Zn	26	317
Cu	21	4
Se	0.2	less than 0.05
Co	10	less than 0.1
Cl	500	2000

(Contaminants)		
Methanol	not detectable	not detectable
Formaldehyde		not detectable
Benzo(a)pyrene	less than 1 ppb	less than 1 ppb
Lead	0.05 ppm	0.74 ppm
Arsenic	0.01	less than 0.1 ppm
Mercury	0.01	0.04
Cadmium	0.1	less than 0.01

(1) Data are for guidance only; they should not be interpreted as specific production standards or recommendations.

(2) Data supplied by Imperial Chemical Industries Ltd., UK.

(3) Data supplied by Mitsubishi Gas Chemicals Inc, Japan.

IUPAC published the Technical Reports No. 12 entitled 'PROPOSED GUIDELINES FOR TESTING OF SINGLE CELL PROTEIN DESTINED AS MAJOR PROTEIN SOURCE FOR ANIMAL FEED' in August 1974. At the Joint Meeting of the Coordinating Committee on Food Chemistry and Commission on Fermentation in Madrid 1975, the need for a supplimentary volume of the Guideline, for the methanol based SCP's,was recognised and an ad hoc Working Group was organized. At the Joint Meeting of the Commissions in Warsaw 1977, the first draft was discussed and revised. The final version was approved by the Food Contaminants Commission in Budapest (August 1978) and the Fermentation Commission in Munich (September 1978).

Membership of the Working Group on Single Cell Protein responsible for the preparation of this report is as follows:

Dr J C Hoogerheide,	Commission on Fermentation
Dr K Yamada,	Commission on Fermentation
Dr J D Littlehailes,	Commission on Food Contaminants
Dr K Ohno,	Commission on Food Contaminants.

The Working Group acknowledges the cooperation extended by the following members within and outside of IUPAC:

Consultants: Dr. D A Stringer, Dr. M Kuraishi, Mr. I Nagai, Dr. A Kitai and Dr. S J Kubacki.

Japanese Drafting Committee: Dr. K Yamada (Chairman), Dr. K Kato (Secretary), Dr. K Ohno, Dr. J Takahashi, Dr. K Aibara, Dr. K Kameoka, Dr. A Kanbayashi, Dr. J Kato, Dr. K Komagata and Dr. Y Shirasu.