Pure & Appl. Chem., Vol. 59, No. 5, pp. 723–730, 1987.
Printed in Great Britain.

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

APPLIED CHEMISTRY DIVISION
COMMISSION ON BIOTECHNOLOGY*

RECOMMENDED METHODS FOR CHARACTERIZATION OF AGRICULTURAL RESIDUES AND FEED PRODUCTS DERIVED THROUGH BIOCONVERSION

Prepared for publication by
M. RINGPFEIL[1], B. NAGEL[1], TH. KREUTER[1], M. MOO-YOUNG[2] and C. ROLZ[3]

[1]Institute of Biotechnology, Academy of Sciences of the GDR, 7050 Leipzig, GDR (Parts 1 and 4)
[2]University of Waterloo, Institute of Biotechnology Research, Waterloo, Ontario, Canada N2L 3G1 (Part 1)
[3]ICAITI, P.O. Box 1552, Guatemala City, Guatemala, C.A. (Part 3)

*Membership of the Commission during the preparation of the report (1981–1985) was as follows:

Chairman: 1981–83 H. Dellweg (FRG); 1983–85 C. L. Cooney (USA); *Vice-Chairman*: 1981–83 C. L. Cooney (USA); 1983–85 M. Ringpfeil (GDR); *Secretary*: 1981–83 R. C. Righelato (UK); 1983–85 G. G. Stewart (Canada); *Titular and Associate Members*: H. T. Blachère (France; Titular 1981–83); V. K. Eroshin (USSR; Associate 1981–83); A. Fiechter (Switzerland; Associate 1981–83); T. K. Ghose (India; Titular 1981–85); P. P. Gray (Australia; Associate 1983–85); J. Holló (Hungary; Titular 1981–83); A. E. Humphrey (USA; Associate 1981–83); M. Linko (Finland; Associate 1983–85); R. C. Righelato (UK; Associate 1983–85); G. G. Stewart (Canada; Associate 1981–83); J. Takahashi (Japan; Titular 1981–83); J. E. Zajic (USA; Associate 1981–83); *National Representatives*: R. J. Ertola (Argentina; 1981–85); P. P. Gray (Australia; 1981–83); H. J. G. Wutzel (Austria; 1981–85); W. Borzani (Brazil; 1981–85); M. Moo-Young (Canada; 1983–85); B. Sikyta (Czechoslovakia; 1981–85); K. Von Meyenburg (Denmark; 1981–85); H. Dellweg (FRG; 1983–85); M. Linko (Finland; 1981–83); L. Penasse (France; 1983–85); M. Ringpfeil (GDR; 1981–83); J. Holló (Hungary; 1983–85); V. Jagannathan (India; 1983–85); L. Goldstein (Israel; 1983–85); F. Parisi (Italy; 1983–85); S. Fukui (Japan; 1981–85); B. G. Yeoch (Malaysia; 1983–85); O. Ilnicka-Olejiniczak (Poland; 1981–83); E. Galas (Poland; 1983–85); A. Fiechter (Switzerland; 1983–85); V. Johanides (Yugoslavia; 1981–85).

Recommended methods for characterization of agricultural residues and feed products derived through bioconversion

Abstract - The present set of guidelines have been put forth with the objective of providing a frame of reference which could be used to evaluate microbial biomass products grown on agro-industrial raw materials. They should be taken in a new perspective and should complement the previous guidelines prepared for SCP grown on synthetic raw materials. The guidelines make reference to important agro-industrial raw materials like animal waste, lignocellulosic crop residues and their mixtures. Bioconversion processes include submerged and solid substrate microbial growth.

Part 1:
Recommended methods for characterization of microbial biomasses produced from animal waste or mixtures of animal waste and defined organic compounds

The presented guideline deals with microbial biomasses produced from the liquid phase of animal waste or its mixtures with defined organic compounds, resp., and subsequently separated from the bulk liquid phase. Microbially enriched animal waste are no subject of this guideline. It relates to microbial biomasses for use as animal feed and not to human food.

1.1 RAW MATERIALS

1.1.1. Animal Waste. Animal waste must come from only one declared animal population. The conditions of husbandry should not be changed. The animal waste must not contain any other uncontrolled sewage such as municipal or sanitary. Auxiliary substances used in the animal production must be known. One has to give evidence that their use will not result in an impermissible accumulation of their residues or metabolites in the biomass produced.
Animal Waste from animals and stables which undergo special treatment has to be excluded.
Prefermentative treatment of animal waste, involving storage and separation of solids, should be carried out at constant conditions.

1.1.2. External Additional Carbon Compounds. Additional carbon sources must be well defined and of uniform quality. It must be ensured, that they do not contain any compounds, which would result in an accumulation of harmful substances in the feed. The additional carbon source must not be changed during the process. It should be added to the animal waste in an constant ratio.

1.1.3. Added Auxiliary Substances. Other added substances, such as mineral nutrients, antifoams, and coagulating agents, must have uniform quality. They must not contain any components, which would result in an accumulation of harmful substances in the feed. These substances must not be changed during the process. They should be added in constant ratios.

1.2 FERMENTATION

Continuous fermentation should be carried out keeping constant residence time, dissolved oxygen concentration, temperature, and pH-value.
Discontinuous fermentation should be carried out at reproducible conditions of dissolved oxygen concentration, temperature, and pH-value.
Initially chosen type and conditions of fermentation must be the same for producing the reference biomass as well as manufacturing the final product.

1.3 POST-FERMENTATIVE TREATMENT

The biomass must be separated from the bulk fermentation liquid and sterilized (devitalization of the microorganisms including spores, viruses, and permanent forms of higher-organized parasites, e. g. worm eggs), preserving the nutritive value, and not causing damages of the product.
Type and parameters of separation and sterilization must be the same for producing the reference biomass as well as manufacturing the final product.

1.4 CHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION; ANIMAL AND MEDICAL TESTS OF REFERENCE BIOMASS

1.4.1. Chemical Characterization.
Requirements according to the PAG/UNU Revised Guideline No. 15 (ref. 2)

- Total nitrogen, full amino acid profile, nucleic acids, and other non-amino components
- Total lipid, fatty acid profile, glycerols, phospholipids
- Carbohydrates
- Fibre content
- Ash, inorganic components
- Trace mineral components (beneficial: Co, Fe, Zn, Mn, Cu; harmful: As, Hg, Pb, Cd)
- Vitamins
- Gross and available energy values
- Nutritional value.

Additional Requirements

- Digestible raw protein
- Total amino acid content (pure protein)
- Hydroxy-butyric acid
- Benzo(a)pyrene
- Mycotoxins
- Bile pigments
- Substances acting as sexual hormones
- Polychlorinated bodies
- Residues or metabolites of auxiliary agents applied in animal production (disinfectants, antibiotics, hormones, insecticides).

1.4.2. Microbiological Characterization. The microbiological key flora should be kept under control during the fermentation.
After sterilization, the manufactured feed should meet the requirements of the PAG/UNU Revised Guideline No. 12, Table 1 (ref. 2).

TABLE 1. Limits for viable microorganisms in the sterilized material after permissible recontamination

Microorganism	Maximum Number per Gram
Viable bacteria	100,000
Viable yeasts and moulds	100
Enterobacteriaceae	10
Salmonella	1 per 50 gram
Staphylococcus aureus	1
Clostridia, total	1,000
Clostridium perfringens	100
Lancefield Streptococci Group D	10,000

1.4.3. Animal and Medical Test for Safety. The microbial biomass processed according to the settled technology and observing reproducible conditions must be tested according to the PAG/UNU Revised Guideline No. 6 and 15 (ref. 3, 1).
The choice of any analytical method adapted to the necessary determination is allowed. However, the analyst has to declare the chosen method and its origin in the literature.

Part 2 :
Recommended methods for manufacturing feed on the basis of agricultural crop residues

2.1 DEFINITION

Agricultural Crop Residues are those materials which remain as byproducts after the primary agricultural product has been harvested. These include such materials as cereal grain straws, of wheat, barley, rice, etc., cornstover (stalk and leaves), sugar-cane bagasse, etc.
The properties of these residues depend primarily on the plant species, growing conditions, and processing required to obtain the primary agricultural product.

2.2 REQUIREMENTS FOR USE OF AGRICULTURAL CROP RESIDUES AS RAW MATERIAL

It must be handled in such a way as to avoid contamination by other nearby materials (e. g. animal feaces, dirt).
It must not contain diseased plant material which has been found objectionable or plant material from a toxic species.
It must be stored in such a way as to avoid contamination and/or production of toxic metabolites as a result of microbial action (normally dry storage is adequate).
Any components used in crop production (e. g. pesticides) must be shown not to cause an excessive accumulation in the feed protein produced under the proposed fermentation and processing conditions.

2.3 REQUIREMENTS FOR SELECTION AND PROPAGATION OF MICROORGANISMS USED FOR FERMENTATION

The selected strain(s) must be non-pathogenic and satisfy non-toxicity regulations where they apply. Of particular importance is the case of heat stable toxins, where postfermentative treatment may have little effect on toxins present. These precautions are also of importance to the safety of personnel involved in production.

2.4 POST-FERMENTATIVE TREATMENT

Fermentation must be followed by treatment which ensures lack of viability of objectionable microorganisms present in the biomass produced. At the same time, or as the next step, toxic or non-nutritive substances may be removed if necessary or the nutritive value of the microbial protein may be enhanced by special treatment.
When these steps are completed, the product must meet the requirements of PAG/UNU Revised Guideline No. 15 (ref. 1).

2.5 PROCESS CONTROL

Control in fermentation step

- monitoring the flow of fermentation broth through the fermentor (for continuous processes)
- monitoring the composition of fermentation medium before fermentation
- monitoring the temperature, pH, pO_2 in the fermentation broth
- monitoring the composition of effluents (outlet gases, liquids) from the process
- monitoring of sterilization step, if applicable
- monitoring of downstream processing step(s) (e. g. postfermentative treatment, product drying, etc.).

2.6 CHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION; ANIMAL AND MEDICAL TESTS OF REFERENCE BIOMASS

cf. section 1.4.

Part 3: Recommended methods for characterization of upgraded agricultural residues through solid state bioconversion (IOBB* project)

3.1 THE PROCESS AND THE STRATEGY

3.1.1. Definition. Agricultural residues are an abundant biomass resource produced every crop year and in many tropical regions they are usually underutilized. They are commonly low in nitrogen and although they are rich in carbohydrates in most cases their availability is low. Agricultural residues can be upgraded by a controlled growth of microorganisms. One of the existing alternatives is the solid state growth of fungi.

3.1.2. Strategies. There are basically two operating strategies:

- Large conversion of the carbohydrate in the raw material to microbial biomass when a source of nitrogen and other nutrients are added. The resulting product is appropriate as a feed for monogastric animals.
- Controlled biodegradation and growth by providing very little extra nitrogen so that secondary metabolism is stimulated and toxic or inhibitory compounds originally present are metabolized. The final product usually is geared towards ruminant feeding.

3.1.3. Raw Materials. There are usually two classes of agricultural residues which can be upgraded with the proposed techniques; they are:

- Starchy products like wastes from potato, cassava and banana
- Lignocellulosic biomass like sawdust, stalks of crops, straw from cereals, bagasse from cane, lemon grass, citronella, pressed coffee pulp, coconut husks and similar
- Inulin rich plants like jerusalem artichoke, chicory, dandelion, dahlia and other members of the Compositae and Graminea
- Pectin rich byproducts.

3.1.4. Raw Materials Pretreatments. Upstream treatments can be included in the process in order to increase the substrate biodegradability or its ease of handling. They can be physical, chemical or a combination of both. In some instances pretreatments can also accomplish a partial removal of competing microflora.

3.1.5. The Microorganisms. The fungi to grow on a solid culture could be:

- Fungi imperfecti or filamentous
- Brown rot and white rot fungi in their filamentous form
- Fruiting fungi (fruiting can be allowed in the process to occur and in those instances a food product will also be produced. However no further comments will be directed to that end as the product should be treated like a mushroom.)
- Yeast with a high biomass yield, a low fermentation activity and an adequate production of hydrolytic enzymes.

3.1.6. The Process Reactor. The reactor geometry should have such characteristics suitable to handle solids and to satisfy the requirement to insure an homogeneous final product. The initial raw material moisture should be low enough in order to minimize bacterial contamination,but high enough to secure a healthy fungal growth. The raw material particle size should be appropriate to support growth but not so fine or large because of handling problems. It can be used as such, pasteurized or sterilized. These last two conditions could form part of the pretreatments. The inocula should be mixed thoroughly with the moist solids and it can be in the form of spores or a filamentous growth. During the growth cycle air should be supplied when needed but it

* International Organization for Biotechnology and Bioengineering

must be water saturated so that no solid drying takes place. Solids mixing might be continuous or intermittent depending on the system investigated. Gas composition could be monitored as temperature and pH of the solids.

3.1.7. Postfermentative Treatment. If the product will consist of the residual solids and the fungal filamentous biomass then it should be dried and reduced in size depending to which animal it will be fed. As an alternative for conservation, the total biomass could be ensiled and used as needed directly from the silo.
On the other hand, after growth the fungi could be induced to fructify. The fruiting bodies will be collected and the residual solid biomass could be processed as before.

3.2 CHEMICAL, BIOLOGICAL AND NUTRITIONAL EVALUATION

3.2.1. Chemical Requirements According to the PAG/UNU Revised Guideline No. 15 (ref. 1) cf. section 1.4.1.

3.2.2. Other Requirements.

- Organic acid profile (if ensiled)
- *In vitro* dry and organic matter digestibility
- Protein biological value
- Mycotoxins
- Growth inhibiting compounds (antibiotica)
- Residual pesticides.

3.2.3. Microbiological Characterization According to the PAG/UNU Revised Guideline No. 12 (ref. 2) cf. Table 1.

PART 4: ANNEX
Identification of the microbiological and chemical hazards of application of microbial biomasses from animal waste derived through bioconversion as animal feed

Microbial biomasses destined for food and feed are generally called SCP (Single Cell Protein).
SCP from pure initial materials, produced by fermentation with a well-known pure strain of microorganisms are approved protein feed components. In addition, agreeing with the statements of the PAG/UNU Revised Guideline No. 12 (ref. 2) and No. 15 (ref. 1), a microbial biomass produced by fermentation not involving the use of pure cultures as well as pure initial materials is also to designate as SCP.
The application of animal waste as raw material involves some problems not observed in processes hitherto applied.

4.1 CHEMICAL HAZARDS

The origin of animal waste gives rise to the presence of substances additionally introduced into the bulk feed or applied in the stables, resp.
The processing of animal waste gives rise to the presence of substances produced by microbiological action or applied in the processing steps, resp.
The artificially added substances can be defined, and subsequently the analytical determination is possible in the course of animal waste processing.
From the input materials, unknown substances can be introduced into the primary process of animal breeding as well as into the subsequent animal waste processing.
The situation is met also in the manufacturing of SCP produced on the basis of secondary products such as molasses, thin stillages as well as sulphite waste liquor. Moreover, in this processes microbial metabolites are formed too. In contrary to these processes, the recycling of the main chemical elements C, H, N and P of the animal waste components as newly synthesized biological material does not exclude the possibility of enrichment of accompanying substances remaining unchanged during the process. Therefore, to make sure that no harmful effects occur the following conditions should be maintained:

- Animal waste must come from only one declared animal population. The conditions of husbandry should not be changed.
- The reference biomass from animal waste to be tested for safety has to be produced under the same conditions as in the current production.

4.2 MICROBIOLOGICAL HAZARDS

The origin of animal waste gives rise to a higher concentration of living germs of microorganisms (10^5 - 10^7/ml), consisting of a variety of different families, genera, and species which cannot be defined comprehensively. It is possible that there are pathogenic species among them or species which generate pathogenic or toxic substances, resp. The presence of pathogenic microorganisms in a raw material is no reason to reject it is a basic material for manufacturing protein feed. This has been shown practically by processing animal bodies - i. e. carcasses of fallen or killed animals - into animal carcass meal for usage as animal feed, containing the same microorganisms as the animal waste. The more important condition for applying animal carcass meal in animal feeding is its sterility, effected commonly by heating the material at 403 K for 20 minutes to kill all viable species including spores, viruses and worm eggs. Consequently, the sterilization of the original animal waste should be taken into consideration. In contrary to animal carcass meal, animal waste will be processed by fermentation before its application in feeding. The biochemical conversion changes the organic compounds of the animal waste breaking them completely into small chemical units and synthesizing the new microbial biomass components.
To exclude the growth of pathogenic microorganisms, one has to adjust at least one of the fermentation parameters far off from the state of warm-blooded organisms, e. g. carrying out the fermentation under acidic conditions and/or elevated temperatures.
The whole range of factors affecting the composition of the microbial population during the fermentation gives rise to forming a biocenosis. If it is

kept constant, the microbial population will show a typical composition. This does not mean that there is only a limited small number of species in this culture, but that certain families, genera and species will dominate and form the major flora, and that other families, genera and species are scarcely appearing or generally absent.
The main factors describing the stability of the biocenosis are:

- Raw material used for fermentation (including the auxiliary substances)
- Temperature
- pH-value
- Dissolved oxygen concentration
- Residence time (if continuous fermentation is applied).

Only long-standing experience may justify a claim that the flora of a certain biocenosis is safe and without hazards for its use as feed.
As there exists no practical experience for using microbial biomasses from animal waste, a comprehensive testing programme is necessary which includes medical, toxicological and zoological testing of the biomass produced, and its result is only valid if a representative and reproducible product has been used.
Finally, in every case the presence of dangerous concentrations of harmful substances must be excluded by feeding tests. The feeding tests should be carried out corresponding to the PAG/UNU Revised Guidelines No. 6 (ref. 3) and No. 15 (ref. 1.).

REFERENCES

1. PAG/UNU Revised Guideline No. 15; UNU Food and Nutrition Bulletin 5, 67, (1983)

2. PAG/UNU Revised Guideline No. 12; UNU Food and Nutrition Bulletin 5, 64, (1983)

3. PAG/UNU Revised Guideline No. 6; UNU Food and Nutrition Bulletin 5, 60, (1983)