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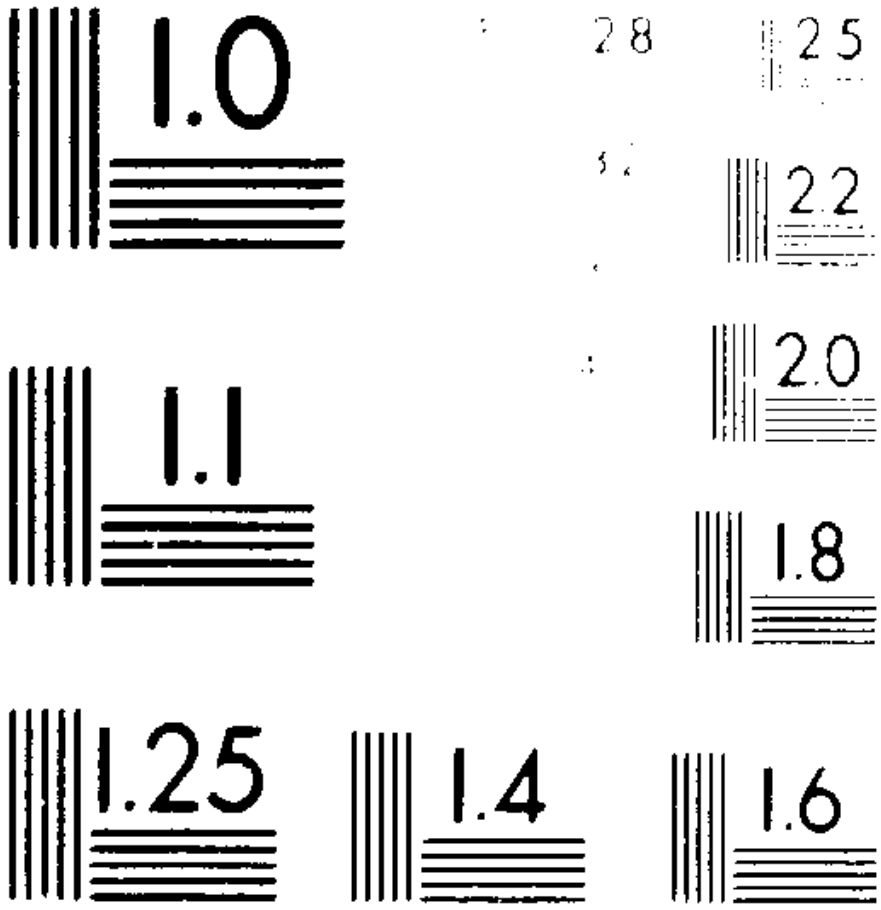

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L. McKay
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Second Draft

# PROPOSAL FOR INTERNATIONAL COOPERATION ON LACTIC BACTERIA FERMENTATION

## I. Background and justification

Cassava is a major root crop in the tropics and it serves as the major source of calories for people in many parts of the world. While cassava constitutes a major portion of the diet for either man or domestic animals, the diet must be supplemented with protein-rich foods or feed supplements since cassava contains a very low percentage of protein nitrogen. A potential protein-rich supplement for cassava-based diets is microbial protein produced on cassava itself as a substrate. Amylolytic fungi have been grown on cassava and studies are being conducted on using thermophilic fungi for single cell protein (SCP) production using cassava as the substrate. Many other indigenous fermented foods also contain starch as the primary carbohydrate source, i.e. corn- or rice-based fermentation.

The present proposal will require the concerted efforts of several laboratories both in developing and industrialized countries, setting up the basis for an international cooperation in the field of lactic bacteria.

## II. Objectives

From the Symposium in Mexico on the Importance of Lactic Acid Fermentation in the Food Industry, which was sponsored by UNIDO, a paper was presented on the ecology of lactic fermentations of starchy food. It was reported that amylase producing thermophilic lactic acid bacteria were isolated. This type of organism, as stated by the authors, could be

from E Kurz

used for developing new industrial processes based on traditional fermentations. Furthermore, such an organism would have the same advantages and may even be better than thermophilic fungi for SCP production from cassava of other starchy raw materials. For example, the amylolytic lactic acid bacteria could directly utilize starch, it would grow faster than fungi, there would be less chance of contamination due to its acid production and growth at high temperature, and unlike fungi would not have the potential of producing mycotoxins.

The objectives of this proposal are to determine the feasibility of developing the amylase producing lactic acid bacteria into an industrially useful starter culture for use in starch-based indigenous fermented foods. The specific objectives would be:

1. To determine the occurrence of amylase producing lactic acid bacteria in starch-based indigenous fermented foods and the role played by these strains. Examine physiological and biochemical properties of these bacteria.

2. To examine the protein-excreting potential of these strains as this property may be of value in this, as well as other biotechnology applications.

3. To apply recombinant DNA technology to amylase producing lactic acid bacteria.

4. To evaluate the use of high amylase producing lactic acid bacteria as starters in starch-related indigenous fermented foods and to evaluate their use as producers of SCP on starchy substrates.

III. Activities

In conjunction with physiological and biological properties of these amylolytic bacteria the following activities could be undertaken:

(a) To examine classification of the organism;

(b) Measurement of amylase activity in different strains;

(c) Environmental factores contradicting amylase production;

(d) Enzymatic and genetic regulation of amylase production;

(e) Isolation of regulatory mutants producing high levels of amylase.

The understanding of protein-excretion of this bacteria could open the door to develop a more efficient amylolative bacteria and the utilization of this type of bacteria as a new vehicle for the production of gentically engineered bio-products. In this connection the following activities could be undertaken:

(a) Variation of membrane permeability as a function of nutritional requirements (i.e. biotin deficiency);

(b) Physiological characteristics of the cell membrane

(c) Mechanism of protein excretion by lactic acid bacteria;

(d) Isolation of mutants capable of excreting proteins at high-level.

For the application of recombinant DNA technology to the amylase producing strains of lactic acid bacteria, the following steps are necessary:

1. Develop methods for lysing the cells.

2. Isolate and characterize the plasmids:
   (a) determine sizes;
   (b) determine functional properties, i.e. is a plasmid coding for the ability of the strain to produce amylase? Secrete proteins?
   (c) if amylase producing plasmid exists, can a high copy number mutant be isolated which overproduces amylase?

3. Vector preparation. Can any of the plasmids found in these strains be used to construct a cloning vector applicable to these organisms?

4. Protoplast formation and regeneration. In order to perform recombinant DNA technology in the organism, a transformation system will be needed. This would necessitate a study on formation of protoplasts and their regeneration.

5. Transformation and cloning. Techniques to transform the propoplasts with plasmid DNA would be developed.

6. Formation of clone banks and gene isolation. Experiments would also be conducted to isolate the gene responsible for amylase production whether it would be chromosomal or plasmid linked. This gene could then be cloned into a high copy number plasmid for maximum expression of the enzyme. The genetic regulation of amylase production would also be examined and the gene could possibly be linked to an efficient promotor.

The above-mentioned studies are designed to acquire the fundamental genetic knowledge necessary to apply biotechnology to this potentially useful organism. The study should also lead to a strain which produces high levels of amylase which could then be evaluated under fermentation conditions.

In the process of evaluation of the amylase superproduced strain as suitable starter culture for indigenous fermented food or SCP, the following activities should be undertaken:

(a) Identification of the process parameters;

(b) Quantification of the influence of those parameters in the final product;

(c) Nutritional evaluation of the final product;

(d) Set the criteria for scaling-up.

IV. Inputs

The undertaking of the foregoing, will require a syncronized effort among experts in different institutions having previous experience and know-how in the field.

It is envisaged that the inputs required to fulfil each of the four objectives will be similar in terms of scientific equipment, supplies, technical staff, etc. The following is a summary of the estimates presented in tabular form. As indicated, the funds could be provided in kind or in cash by the participant laboratories and the same international funding organization.

ESTIMATIONS FOR EACH OBJECTIVE

| | Concept | Quantity | Cost US $ | Source of funding |
|---|---|---|---|---|
| Lab. space | 50 $m^2$ | | 2,000 | Host Lab. |
| Scient. equipm. | Centrifuges E.F. apparatus (preparative, ultra) spectrophotometer cameras, incubators, separation columns | | 4,000 | Host Lab. |
| Supplies | Reagents, Restriction Enzymes, Radio-labelled chemicals ... | | 30,000 | Internat. |
| Technical staff | Professor | 2 m/y | 8,000 | Host Lab. |
| | post-doc. (from (developing country) | 3 m/y | 88,000 | Internat. |
| | Technician | 2 m/y | 4,000 | Host Lab. |

In view of the nature of the project, a good coordinating mechanism should be articulated, and good communication lines be established among

participating institutions. It is envisaged that four working meetings will be organized (at the beginning of the project and at the end of each year). The participants at the meeting should be the senior and junior scientists involved in the project. To reduce cost involved, the meetings should be held at the facilities of the most centrally located institution. In the assumption that four laboratories will participate, 6 scientists will be involved.

## V. Budget

| | | |
|---|---|---|
| Research activities | US $136,000 x 4 lab. | US $544,000 |
| Coordination | 6 scientists x 4 meetings x US $2,000 | US $ 48,000 |
| Consultant | 2 m/m | US $ 10,000 |
| Staff travel | | US $ 10,000 |
| Miscellaneous | | US $ 20,000 |
| | TOTAL | US $642,000 |

## VI. Project work plan

The activities foreseen in the present proposal are expected to be initiated concurrently with the exception of those involved in the evaluation of the superproduction strain. It is planned that a three-year period should be adequate to have an analysis of the

accomplished results and serve as a basis for future recommendations.

The activities geared to evaluate the use of the superproducer as starter cultures for indiganous fermented food or SCP should start two years after the whole project has been initiated. At this stage it is not relevant to provide a more detailed time-table.

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# CONCLUSIONS AND RECOMMENDATIONS FROM THE SYMPOSIUM ON THE IMPORTANCE OF LACTIC ACID FERMENTATION IN THE FOOD INDUSTRY

Recent advances in the field of molecular biology are presently creating a revolution in agriculture and well do the same in the food industry. The food industry is, no doubt, the oldest user of biotechnology. For example, for centuries humans have used milk from various sources to make fermented milk drinks and a large variety of cheeses. Vegetables are also fermented all over the world resulting in products such as sauerkraut, kimchi, pickles, gari, pozol, etc. Fermented meat, fish, and cereal products are also common in the diet of many cultures. Indigenous fermented foods, which have essentially remained unstudied, are also used by many populations in the third world countries to supplement their diet. Some beneficial characteristics of these many types of fermented foods include:

1. Growth of spoilage and/or pathogenic microorganisms is prevented and thus the shelf-life is extended;

2. In many cases the digestibility of the end-product is increased over the digestibility of the raw material;

3. The nutritional value of the fermented food may be increased; and

4. The fermentation may reduce the processing time before the food is consumed.

The applications of the new technologies of genetic engineering and biotechnology could vastly improve the characteristics of fermented foods

and consequently could result in the development of local industries where new and traditional technologies could be merged without affecting the eating habits of the native population. In this regard, a workshop on Technology Transfer, Management and Development and Implications on Newly-emerging Advanced Technologies, held in Spain, November 1983, emphasis was placed on the impact that biotechnology could have on traditional techniques for the production of fermented food. At the same time, the need for assessing the state of the art in fermented foods was stressed. The importance of lactic acid fermentation for developing countries was also discussed in a meeting held in Argentina in March 1984 on the establishment of a biotechnology network in Latin America. At this meeting it was agreed by the representatives of Argentina, Brazil, Costa Rica, Cuba, Chile, Mexico and Venezuela that lactic acid fermentation should receive priority consideration in the selection of topics for collaborative research and development. Further impetus in this area was provided by the current Symposium on Lactic Acid Fermentation sponsored by UNIDO in Mexico City during November 1984. This Symposium dealt with traditional fermented foods in third world countries and the state of the art with respect to applying genetic engineering to the lactic acid bacteria involved in the fermentation processes. As will be seen later in this section, the technique for applying recombinant DNA technology to dairy fermentation organisms is rapidly being developed, but this type of information is lacking in lactic acid bacteria found in other traditional or indigenous fermented foods.

The following recommendations and/or conclusions are drawn from the Symposium on Lactic Acid Fermentation (no priority has been given to the listings):

A. A listing of all organizations involved in research and development of indigenous fermented foods in developing countries should be made available. Technical and scientific exchange programmes among groups having similar interests and objectives should be encouraged. A well-documented and coordinated survey of indigenous fermented foods is essential. Such listings exist in the book by Steinkraus on Indigenous

Fermented Foods and if possible this listing needs to be kept up to date as new information is gathered. As stated by Steinkraus in his 1982 UNIDO report (UNIDO/IS.336):

> "Those involved in research on indigenous fermented foods recognize that we have only investigated the surface of a gold-mine of knowledge available on other indigenous fermented foods used daily in many relatively remote parts of the world. To complete our scientific knowledge requires that be bring all these fermentations to light, determine the essential microorganisms involved, study the biochemical changes that occur in the proteins, lipids, vitamins, and other components in the substrates, determine the flavours and textures produced and how they can be controlled, and finally give the world a broader view of how microorganisms can be grown on edible substrates and contribute more to the total proteins and nutrients available for men in the future."

Having isolated, identified, and studied the essential lactic acid bacteria in indigenous fermented foods and studied their products, growth conditions, and enzymology, it is then possible to optimize the organism through genetic engineering techniques. This would result in the production of high quality fermented foods in regions where it is needed.

To accomplish the above-mentioned objective of improving upon these traditional fermentation processes the following questions must be asked:

1. What raw materials are involved in the fermentation process?

2. What are the processing methods?

3. What are the types and functions of the microorganisms involved in the fermentation process?

4. What is the role of the fermented food in the diet and nutrition of the consuming population?

5. What are the potential mechanisms for improving the fermentation and/or process?

5. Work should also proceed on development of improved processing methods for those fermented foods being or having potential of being consumed by the largest number of people. Small scale industries should be developed in those countries where the food will be consumed. An important prerequisite for meaningful small- or even large- scale industrial production of these selected fermented foods is the development of an acceptable pure starter culture. Meaningful projects associated with the development of acceptable starter cultures and processing must be implemented on a collaborative basis between the developing country and centres of excellence. This should encourage effective and efficient characterization of results obtained where they are originally needed.

The rationate for the above-mentioned approach is that in many third world countries fermented foods are consumed in large quantities. For example, in Thailand over 50 types of fermented products are consumed involving vegetables, fruit, fish, meat, cereals, or milk as the raw material. About 200 fermentation type industries aid in producing many of these products. Similar industries could be established in other developing countries. Some of the technical problems that must be resolved are:

1. control of the lactic acid fermentation has not been adequately studied;

2. no inoculum of lactic acid bacteria is used;

3. tools and other implements are very simple;

4. process of production is labour-intensive;

5. sanitation conditions are usually inadequate; and

6. no technique of genetics or genetic engineering have been used to improve upon the fermenting microorganisms.

To summarize, the application of emerging concepts for the improvement of traditional fermented foods would be made easier by knowing:

1. Mechanism and function of lactic fermentations in traditional food systems;

2. Basic studies on the mechanisms and function of lactic fermentations will also provide a common ground for the cooperative work between different regions. These indigenous fermented foods within an area should be studied and developed into industries to serve as a food source for the native population.

C. There also appears to be a need for the development of a Center in Latin America for the production of commercial dairy cultures. Lactic acid bacteria starters are currently being imported and these imports may be cut. Therefore it will be necessary to establish a Center in Latin America to mass produce commercial cultures. The Center of Reference for Lactic Acid Bacteria located in Tucuman, Argentian could be one such Center. It has many years of experience working with this group of organisms and already has expertise in the area of taxonomy and dairy cultures. Other possible locations would be the Instituto de Technologia de Alimentos in Brazil, where such studies are being initiated and laboratories in Mexico. They would need to develop, possibly in collaboration with other laboratories, the expertise for mass production, preserving and the shipping of active cultures. This expertise could then be used to develop cultures for inocula to be used for indigenous fermented foods. Latin American countries also appear to need dairy cultures that function under their manufacturing conditions. New strains native to Latin America need to be isolated and made available to Latin American cheese-makers. Also the group at Tucuman has the opportunity of developing a starter culture for their native Argentina cheese. The latter could then be developed into a small business for the local farmers.

The specific questions which need to be addressed in this area include:

1. Can suitable strains of lactic acid bacteria be isolated from raw milk or green plant materials that will function in Latin American cheeses? These strains would need to be characterized with respect to stability, acid-producing ability, heat tolerance, salt tolerance, proteolytic activity, phage sensitivity, and ability to produce good flavoured cheese.

2. Can phage resistant strains be developed?

3. A critical question that must be answered is how will the cultures be mass produced, preserved, and distributed? On the tropical areas will the cultures be made available as liquid, frozen, lyophilized, spray dried or other? How will stability and activity be maintained during distribution? Can "dry" cultures, such as used in bread making, be developed to circumvent the distribution problem of liquied or frozen cultures?

D. Dairy fermentation processes and the lactic acid bacteria involved were discussed extensively. These studies reached from the isolation and characterization of strains involved in the production of a native Argentina cheese, to biochemistry and genetics of phage infection, and to strategies for genetic engineering of dairy streptococci and for the lactobacilli. There is no doubt that this group of bacteria will probably be the first lactic acid bacteria where genetically improved strains will come about. This is mainly due to the fact that a critical mass of investigators exist in the field which provides the impetus for major achievements. Fundamental advances have been made in the genetics and recombinant DNA technology with the dairy lactic acid bacteria. These studies should open new opportunities for both strain improvement and process development for a wide variety of lactic acid fermentation processes. The genetics and plasmid biology of lactic acid bacteria

isolated from indigenous fermented foods could be facilitated by cooperation with those laboratories having expertise in these areas, such as at Minnesota, MIT, North Carolina State, and many European laboratories.

E. A question that needs to be answered is whether the lactic acid bacteria which predominate in many of the traditional indigenous fermented foods have unique biochemical and genetic properties? It appears that unique lactic acid bacteria are being isolated and identified in various parts of the world. For example strains of lactic acid bacteria which excrete proteins and produce amylases have recently been reported. These starch hydrolyzing strains could be essential in improving upon those fermentations in which starch is the primary carbohydrate source (solid state fermentation processes). Studies on the genetics, plasmid biology, and mode of protein secretion by these strains is urgently needed and the results of such studies could lead to the genetic engineering of such strains for use in many indigenous fermentation processes. Collaboration is needed between those laboratories in Mexico working with these unique strains of lactic acid bacteria and those laboratories having expertise in plasmid biology and genetics of lactic acid bacteria.

Specific questions in this area include:

1. Can lactic acid bacteria with unusual ability to produce organic acids, essential amino acids, such as lysine or methionine, vitamins, such as thiamine, riboflavin, or B-12, or antagonistic compounds against spoilage and pathogenic organisms be isolated and then used in producing indigenous fermented foods?

2. Can lactic acid bacteria producing enzymes such as proteinases, lipases, pectinases, or others be found and then explored?

3. Can the amylase producing strains of lactic acid bacteria be used, to improve upon indigenous fermented foods based

on starch as the carbohydrate source? Can these strains be used to produce SCP from starchy foods? Can the protein content of fermented foods such as gari be increased by using amylase producing lactic acid bacteria? Can genetic engineering principles be applied to these amylase producing strains to improve the organism? Such questions as regulation of amylase production, plasmid biology, and mechanisms of protein excretion would need to be answered.

F. As we have seen, lactic acid bacteria alone or in conjunction with other microorganisms are utilized for the manufacture of many different fermented foods. One unique association that needs to be studied and exploited is that observed in pozol, a fermented maize product consumed in Mexico. The fermentation not only involves lactic acid bacteria but also a nitrogen fixing organism. The nitrogen content of the final product is greater than the original starting material. Basic studies need to be conducted on this microbial association.

Questions that need to be addressed in this area include:

1. Has the nitrogen fixing microorganism been fully characterized and what is the mechanism of nitrogen fixation?

2. What is the role of the lactic acid bacteria in the ability of this organism to fix nitrogen?

3. Can the responsible microorganisms be isolated in pure culture and then combined to form a nitrogen-fixing starter culture that could be used to increase the nitrogen content of those indigenous fermented foods naturally low in nitrogen?

4. Could the genes responsible for nitrogen-fixation be transferred to a lactic acid bacteria species to develop a new culture that could be used in food fermentation processes?

5. Could the laboratories in Mexico, which have the expertise on this nitrogen-fixing strain, work with other interested laboratories in order to rapidly develop the system?

This is a very exciting system and UNIDO should do whatever possible in order to foster research in this area.

6. The preservation of grass, i.e. silage, by the use of lactic acid bacteria would have a global impact if it could be consistently accomplished. A suitable starter needs to be developed that will function in tropical regions. Such a culture would aid in preserving animal feed (silage) during that time of year when green grass is not available. Collaboration needs to be conducted among the various laboratories working in this area.

H. Lactic acid fermentations are also being extended to non-traditional raw materials for production of animal or human food. These efforts should continue as they may lead to ways of converting a waste material into an edible food source. Examples under study include production of fish silage with sugar cane wastes to produce animal food, the lactic acid fermetnation of banana puree as a means of preserving bananas for human consumption, and the recycling of agricultural waste by lactic fermentations.

Biotechnology in the lactic acid bacteria will occur. While it may occur first in those strains used in dairy fermentation processes, the true impact may come when this technology is applied to those strains found in indigenous fermented foods. To accomplish this goal we must understand the fundamental biology (metabolism, genetics, plasmids) of

the strains involved. This could be accomplished by joint research ventures between the developing country where the properties of the indigenous fermented food could be studied and the organisms isolated and a laboratory which has the expertise in the genetics and plasmid biology. Between the two efforts, strains which improve upon the fermentation process could be developed. For international cooperation however, there has to be a funding source for investigative studies to proceed.