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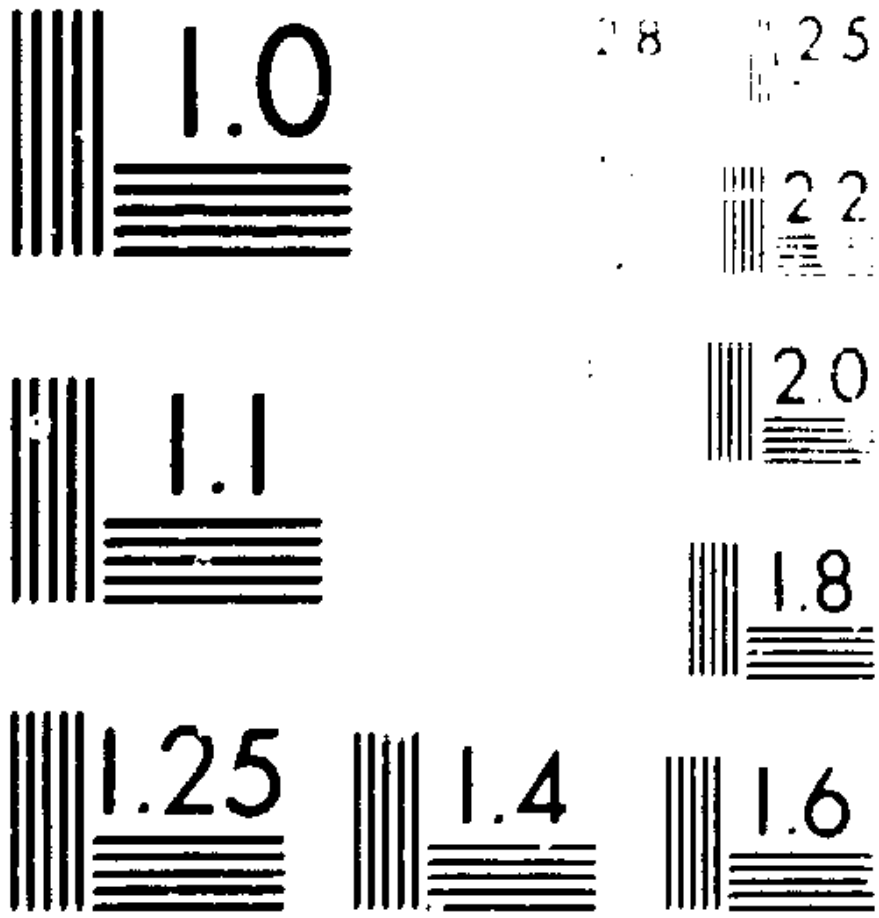
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APPLICATION OF GENETIC ENGINEERING FOR ENERGY AND FERTILIZER PRODUCTION FROM BIOMASS*

prepared by

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A(1). BACKGROUND AND JUSTIFICATION

In the light of the growing problems of fuel shortages and waste recycling it is necessary to establish efficient use and conversion of renewable resources (such as cellulosic materials and wastes) to liquid fuels. This will replace, at least in part, the need for the dwindling supplies of non-renewable materials such as petroleum oil.

In order to solve this important world-wide problem, input from genetic engineering and biotechnology is absolutely essential. Genetic engineering represents a multiple array of disciplines, which includes biochemistry, microbiology, genetics and recombinant DNA technology.

1. Cellulosic materials. Cellulose and hemicellulose are the most abundant renewable resources in nature. With lignin, they form about 95 per cent of the dry weight of plant wastes. World production of waste cellulose exceeds four billion tons per year (1). If cellulose materials can be efficiently converted into ethanol, a tremendous source for liquid fuel will be made available. Technically speaking, the process is relatively simple - the hydrolysis of cellulose to glucose and subsequent conversion to ethanol. The process was used for large scale production of ethanol during the First World War and the Second World War, and was further refined in the 1940s. Due to the low yield and corrosion by the catalytic acid needed to break down cellulosic materials, the process was not economically competitive with the petroleum-based processes. However, the recent energy crisis and the rapid exhaustion of the petroleum reserves, together with the development of microbial enzymatic hydrolysis of cellulose, has gradually reversed this situation. Through the implementation of genetic engineering and biotechnology, it is hopeful that the conversion of cellulosic material to ethanol will become economically competitive.

2. Cellulases. The major cellulase components were isolated from culture filtrates of many microorganisms such as Trichoderma reesei, the most thoroughly studied system. By using chromatographic procedures, the cellulases have been purified to homogeneity (2). These include:

(a) 1,4-β-glucano glucanohydrolase (also known as endoglucanase, CMCase - converts amorphous cellulose to free fibres.

(b) 1,4-β-glucan cellobiohydrolase (also known as exoglucomase, exocellulase, avicellase) - converts filter paper or avicel to cellobiose with terminal mode of attack. It is a decrystallizing enzyme. Cellobiose is a competitive inhibitor.

(c) β-glucosidase (also known as cellobiase) - converts cellobiose (also possible cellotriose and cellotetrose) to glucose. Glucose acts as a noncompetitive inhibitor Apparent high substrate inhibition at very high substrate concentration. It is the least stable of the three cellulases, and is present in the least amount in T. reesei.

3. Cellulolytic microorganisms. Many microorganisms contain cellulases. A few that have received recent attention are as follows (3):

(a) Bacteria
Cellvibro fulvus, cellvibro vulgaris - aerobe, mesophile;
Cellulomonas - aerobe, mesophile;
Pseudomonas fluorescens - aerobe, mesophile;
Clostridium thermocellulaseum - anaerobe, thermophile

(b) Actinomycete (aerobic)
Streptomyces QMB814
Thermoactinomycete; thermomonospora curvata;
Thermomonospora fusca

(c) Fungi (aerobic)
Aspergillus niger
Trametes sanguinea; Poria; Pestalotiopsis westerdijkii
Penicillium iriensis QM9624; Penicillium funiculosum;
Polyporus versicola; Polyporum tulipiferae;
Fusarium solani; sporotrichum pulverulentum QM9145
Trichoderma reesei (viride)
Trichoderma lignorium; Tricoderma koniugii
Thermoascus aurantiacus QM9383

Since the biomass is comprised of about 45 per cent cellulose, about 30 per cent hemicellulose and about 20 per cent lignin, the central theme and major objective of this work programme is the efficient conversion of cellulose and hemicellulose to ethanol. There are several experimental approaches to accomplish the above objectives. Only two of the most important ones will be discussed here:

(a) Using genetically manipulated microorganisms;

(b) Using recombinant DNA technology especially for molecular cloning and amplification of cellulase genes.

B(1). ACTIVITIES

For the effective conversion of cellulose and hemicellulose to ethanol, a number of fundamental as well as technical problems have to be solved. It is clear that several different problems will have to be addressed in parallel. Much of this work programme will involve basic research since a number of fundamental points in this complex system remain to be established.

1. Analysis of Cellulases from Cellulolytic Organisms. The three cellulases, the endoglucanase, the exoglucanase and the cellobiase, from a variety of known cellulolytic organisms (see section A(1)) will be analyzed for the amount excreted into the extracellular medium and in the cells for the β-glucosidase (e.g. units/ml), the specific activity of crude and purified enzymes (units/mg protein), the stability, the extent of product or substrate inhibition, etc. Other microorganisms should be screened for new sources of cellulases.

Once several suitable sources of cellulases are found, the amount or the properties of the enzymes can most likely be improved by applying genetic methods of mutation such as those used to select the favourable Trichoderma reesei mutants AM9414, MCG77 and MCG80 (4).

2. Cloning of the Cellulase Genes and Producing Favourable Mutants by in vitro mutagenesis. One suitable research approach that ICGEB can take is to clone the cellulase genes from cellulolytic organisms analysed in section one above. This can be accomplished by either making genomic libraries of these organisms or by making cDNAs from the mRNAs by applying the recombinant DNA techniques (5,6). Once the genes are cloned in a plasmid, placed next to a strong promoter and suitable ribosome binding sites, and introduced into E. coli or B. subtilis by transformation procedures (5,6), production of large amounts of these cellulases are expected.

The next step is to carry out in vitro mutagenesis on the cloned cellulase genes by base pair changes or by small deletions (7) with the hope of producing mutant genes which code for cellulases with increased resistance to product inhibition.

3. Cloning of fungal Cellulase Genes and Introducing them into Yeast. The fungus, Trichoderma reesei, produces and excretes large amounts of cellulases which can degrade cellulose to glucose. Yeast cells (such as Saccharomyces cerevisiae) that can efficiently convert glucose to ethanol cannot degrade cellulose. One suitable research approach that ICGEB may consider is to construct an improved strain of yeast which carries the cellulase genes and can excrete cellulases out of the cells to convert cellulose to glucose. This novel strain should be able to efficiently convert cellulose to ethanol. The construction of the strain involves two steps. The first step is to clone the genes for the cellulases of Trichoderma reesei (mutant MCG77 or MCG80) or of other cellulolytic fungi as described in the previous section. However, in this case the genes are joined to a yeast plasmid (such as YEp13 type plasmid shich has an Ars sequence and an easy selectable marker such as His3 or LEU2). The second step is to transfer the cellulase genes togecher with the yeast plasmids into yeast by transformation (8). The plasmid can be maintained in the yeast by growing the yeast in the absence of leucine. Another possibility is to allow the cellulase genes to be stably integrated into the yeast chromosome. Assuming that Trichoderma reesei signal peptides are recognized by the yeast (quite likely since both T. reesei and yeast are fungi) the cloned cellulase genes should include signal peptides for excretion of the cellulases into the medium.

If they are not recognized by the yeast, an alternative approach can be taken. The cloned gene coding for the T. reesei signal peptide will be removed by standard recombinant DNA techniques and only the structural genes of the cellulases will be included. These cellulase genes will be joined to a derivative of the YEp13 which also contains the promoter sequence and the signal peptide sequence of the yeast invertase gene. Since the invertase of yeast is excreted, the cellulase genes joined to its signal sequence gene should also be excreted. Yeast mutants which over-produce invertase have been isolated (9). If necessary, the invertase gene from this strain of yeast will be cloned.

The advantage of this approach is that common yeast cells such as S. cerevisiae (for which the transformation system has been established, and which can easily be grown in large scale fermenters) can then convert cellulosic materials directly to alcohol.

4. Cloning of Bacterial Cellulase Genes and Introducing them into Zymomonas mobilis. The bacterium Zymomonas mobilis can convert glucose to ethanol (10) with the following advantages:

(a) high specific ethanol yields;

(b) a tolerance to high glucose concentrations; and

(c) the ability to grow totally anaerobically so that the addition of oxygen in continuous fermentations is unnecessary.

Recently, Bland et al. (12) used an "attached film expanded bed" (AFEB) cell-immobilization reactor (13) and showed that the conversion of sugar to ethanol with the bacterium Z. mobilis can be increased to 105 gm/l-hr based on total fermenter volume (12). Although the AFEB fermenter is an efficient way to convert sugar to ethanol by Z. mobilis, this bacterium does not have the cellulase genes to convert cellulose to glucose.

Bacteria like Cellulomonas fimi contain cellulase genes and secrets cellulases that penetrate the wood matrix and attack the cellulose extracellularly to produce sugars. At least one cellulase from C. fimi has been cloned recently (11). The next step is to clone the gene coding for all three cellulases (endo-β 1,4-glucanase, exo-β 1,4-glucanase,

β-glucosidase) together with the signal peptide coding sequence, and join them to a bacterial plasmid such as pKC30 (14) next to the strong promoter sequence as described in section two above.

One project ICGEB can pursue is to transfer the cloned bacterial cellulase genes on a plasmid such as the pKC30 into Zymomonas mobilis by transformation. It may be necessary to first work out the transformation procedures for introducing plasmids into Z. mobilis by employing the principles and techniques used in introducing plasmids and foreign genes into E. coli and B. subtilis. If the plasmid and the cellulase genes are not stable in the bacterium, they can be forced to integrate into the bacterial chromosome by integrative recombination. This is accomplished by first cloning a gene Y from Z. mobilis in the same plasmid pKC30. Once the cellulase genes are also joined to this plasmid and transform the cells, the entire pKC30 derivative can be integrated into the chromosome of Z. mobilis at the site of gene Y by integrative recombination.

5. Cloning of xylose isomerase Gene and Introducing it into S. pombe.

High concentrations of hemicellulose-derived carbohydrates can be easily obtained by the use of dilute acids. The ideal organisms for the conversion of biomass-derived carbohydrates to products such as ethanol are the yeasts. However, most yeasts are not able to degrade pentoses. The recent successful demonstration of a quantitative production of ethanol (80 per cent of the theoretical yield) from D-xylose using yeasts and xylose isomerase would simplify the problems related to the pentose process (16).

One possible line of research is to clone the xylose isomerase gene and introduce it into a Schizosaccharomyces pombe, which is a more desirable yeast than baker's yeast (S. cerevisiae) in the conversion of xylulose to ethanol. This genetically manipulated yeast can then convert xylose to ethanol at high yield. This yeast strain should be much more efficient in ethanol production than the recently discovered strain of Pachysolen tannophilus strain NRRL 2460 (17).

In addition to the production of ethanol, the byproducts of hemicellulose degradation by microorganisms include methane, organic

acids, sugar alcohols, solvents and animal feed. Thus, other valuable materials can be produced.

In fact, this part of the work programme emphsizes the use of genetically manipulated yeasts or bacteria for the efficient conversion of cellulosic materials to ethanol. It is clear that the same yeasts or bacteria would also be useful in converting biomass into feed (such as single cell protein), and industrial chemicals as mentioned in the above paragraph.

The major component of the programme would be the research and development component, where research work would be conducted by at least four closely inter-related groups. The first to be led by a microbiologist, whose major function will be to analyze the cellulases from the known cellulolytic microorganisms and to look for new cellulolytic microorganisms. The second group, to be led by a biochemist, will function to purify the cellulases (selected by the microbiology group) and to study their properties. This group will also initiate work on the cloning of the cellulase genes. The third group, to be led by a recombinant DNA expert, will function to modify the cloned cellulase genes (by the biochemistry group), and also to transfer the cloned bacterial cellulase genes in Zymomonas mobilis cells. The fourth group, to be led by a expert in yeast, and will function to transfer the cloned Tricoderma reesei cellulase genes (or other fungal cellulase genes such as those from Penicillium funiculosum) into yeast cells, and to establish conditions for the optimal conversion of cellulose to glucose and to ethanol. Each of these four groups will include a postdoctoral fellow and/or a technician, and the group leader of these groups will be assisted by a junior scientist (Ph.D. level). Each group can train about two trainees from developing countries every two years.

Two meetings or workshops will be conducted by these four groups. International experts from each area of research and development will be invited. The topics of discussion will include studies on cellulolytic microorganisms and cellulases, the cloning and amplification of the cellulase genes, the transfer of cloned cellulase genes into fermentative yeast or bacteria, the improvements of the efficiency of conversion of cellulose (and hemicellulose) to sugar and then to alcohol.

The outputs of this programme will be produced in several steps. The first step will aim to identify the best cellulases (from one or more cellulolytic microorganisms), which give stable and high specific activity cellulases (the endoglucanase and the exoglucanase should be excreted into the growth medium). The next step is to clone several of these cellulases and join them to bacterial plasmids or yeast plasmids. The third step is to transfer the cloned fungal cellulases (such as those from T. reesei) into yeast cells and to maximize the direct conversion of cellulose to ethanol in this recombinant yeast. Parallel experiments will be carried out to transfer cloned bacterial cellulases (such as those from Cellulomonas fimi) into Zymomonas mobilis so that this recombinant bacteria can convert cellulose directly to ethanol. Substantial progress is expected; however, completion of research and development within the next five years would be unrealistic. However, even a partial sucess will be significant in ultimately solving the problem of efficiently and economically converting cellulosic materials to ethanol. Once basic R+D has been completed, the programme will be handed to the biotechnology group to adapt to large scale production of ethanol. Considerable technological improvements in the large scale recovery and drying of ethanol is also needed.

Advisory services will be provided to developing countries laboratories in these areas.

PRODUCTION OF FERTILIZER USING BIOLOGICAL SYSTEMS

A(2). BACKGROUND AND JUSTIFICATION

Biological nitrogen fixation is an essential process for all living organisms on the earth. The ability to reduce atmospheric nitrogen to ammonia is limited to a small number of bacteria, blue-green algae and actinomycetes. Some of these microorganisms can reduce atmospheric nitrogen in free-living form such as Klebsiella, Azotobacter, and blue-green algae. The economically important microorganisms, however, carry out the fixation process in close association with higher plants in the form of true symbiosis. During symbiosis the higher plant contributes the essential carbohydrates to the microorganism, and in turn the microorganisms supply the reduced nitrogen for the bacteria and higher plant protein synthesis. The nitrogen fixation, an anaerobic process, is carried out by the nitrogenase enzyme complex which is highly conserved in all nitrogen fixing microorganisms. The entire process in Klebsiella pneumoniae is encoded by a gene cluster containing 17 contiguous genes (three structural genes) which are transcribed unidirectionally in seven transcriptional units. N_2 fixation Nif operon requires the constant presence of the gene A protein which is functioning as an inducer. The gene L protein acts as a repressor of the Nif operon by regulating the production of gene A protein. Recent data of several laboratories demonstrate, not only in Klebsiella but also in Rhizobia and blue-green algae, that the Nif genes are organized in contiguous clusters. The regulation of Rhizobia nitrogen fixation process is presently not understood.

Symbiotic nitrogen fixation requires the interaction of microorganisms with higher plant cells. The proliferation of the plant root, induced by Rhizobia, results in nodule formation in leguminous plants. In contract to crown gall formation, the Rhizobia are inside of the plant cells and in a developmentally regulated process become bacteroids which carry out the fixation process.

Plant recognition, the infection process, and the host specificity seem to be carried as gene clusters in the bacterial genome. Recent findings in Rhizobium leguminosarum document the presence of nodulation genes and nitrogen fixation genes on a large plasmid. When this plasmid was transferred to Agrobacterium tumifaciens, ineffective non-fixing nodules were formed after infection of pea roots. Similarly, Rhizobium meliloti cells contain even larger plasmids called megaplasmids (larger than 500 KD in size). These megaplasmids also contain genes required for the nodulation process closely linked to the nitrogen-fixing gene cluster. Experiments to transfer the nitrogen-fixing capability from bacterial cells into a eukaryotic cell system by genetic engineering resulted in stable maintenance of the Nif gene cluster in yeast chromosome number 3. However, no translation products were observed in the transformed yeast cells, suggesting that the replacement of the bacterial Nif gene signals by yeast or plant signals is required to obtain gene expression. Further, more detailed studies will be required to understand the assembly of nitrogenase in a eukariotic cell.

B(2). ACTIVITIES

For the production of fertilizer using biological systems, a number of fundamental as well as technical problems have to be solved. Only two important projects are presented here. The research work would be conducted by a team of at least three closely inter-related groups, and in consultation with the plant genetic engineers as described in the programme of Improved Agricultural and Food Products.[1/] The first group, to be lead by a microbiologist, will work with nitrogen-fixing bacteria such as Rhizobium, Klebsella and Azotobacter, and to study the efficiency and host range for nitrogen fixation. The second group, to be led by a biochemist, will use biochemical methods to analyze the nitrogen fixation system, and use recombinant DNA technology to clone and to transfer genes between nitrogen-fixing strains of bacteria or between bacteria and plants. The third group, to be led by an expert in plants and plant cells, will study the mechanism and efficiency of nitrogen-fixation and photosynthetic systems in plants.

1/ "Improved Agricultural and Food Products through Genetic Engineering and Biotechnology", ID/WG.382/2/Add.5

As the systems becomes better understood, he will co-operate with the microbiology and biochemistry groups to use the genetically selected nitrogen-fixing bacteria or to transfer recombinant DNA modified bacteria into plants. He will check the rate of plant-growth, the protein content in seeds, etc., and try to maximize the yield.

Each of these three groups will include a postdoctoral fellow and a technician, and perhaps a junior scientist who will assist the group leader for this programme. Each group can train about two trainees from developing countries every two years. In other words, the three groups can train six trainees in two years, or 15 trainees in the first five years.

A joint annual meeting or workshop will be conducted by these three groups. Experts in this area of research from different countries will be invited to participate.

Advisory services from several major research groups in the world in this area of research will be needed. The advisory services will include inviting active researchers to come to the ICGEB for several days to several weeks. Perhaps three to six researchers will be invited each year. The services may also involve sending one or several researchers to certain major research centres around the world to work out very specific problems, related to soil conditions and competitive strains of nitrogen fixing bacteria in that region. Those strains may be brought back to the ICGEB for genetic modification.

The major output of this programme will be to improve the existing strains of Rhizobium to make them more competitive in their natural environment in different areas of the world. Since the soil conditions and the competing bacteria in the soils in different parts of the world will be different, tailor-made improved strains of Rhizobium will be made in each case. Although the principle of genetic modification is quite simple, this area of research is time-consuming and major breakthroughs within the first five years may not be possible. On the other hand, even minor improvements in nitrogen-fixation efficiency, which leads to a ten per cent increase in crop yield, will be economically significant.

Furthermore, any basic research at the ICGEB which leads to a better understanding of the nitrogen-fixing bacteria, and on the bacteria-plant interaction will be beneficial in improving crop productivity. Soybean and cowpeas are used as examples in this work programme. The principle behind this type of research and the technology gained can be applied to other systems.

The use of free-living microorganisms for improving crop productivity by studying nitrogen-fixing Pseudomonas and blue-green algae, is likely to yield positive results during the first five years.

1. Use of Genetic Engineering to Alter Thixobium and Other Mircroorgans to Fix More Nitrogen for Improved Crop Production

Alteration of Rhizobia for Improved Crop Productivity.

The major limiting factors in symbiotic nitrogen fixation are the lack of competitive strains of Rhizobium. It is well known that less than five per cent of the inoculated Rhizobia are found to be infective on growing crop plants. Secondly, Rhizobia are needed which are capable of nodulating and fixing nitrogen in the presence of fixed bacteria in the soil. Thirdly, the nodulation process and the nitrogen fixation process are sensitive to stress factors, e.g., drought, salt, and heat.

The major leguminous crop plants are soybean and cowpeas. Therefore, emphasis should be put on isolation of novel strains from soils and selection of competitive strains of Rhizobium japonicum and Rhizobium cowpea species. At the same time, these strains should be adopted to regional plant cultivars and to local soil conditions. Recombinant DNA technology, upon the availability of Rhizobium gene transfer systems, could be used to combine beneficial traits of natural Rhizobia. In addition to the generation of competitive strains, application of molecular biology to Rhizobia could lead to constitutive nodulator and constitutive fixer strains which are independent or which cannot be inhibited by soil nitrogen. Upon the availability of gene transfer systems and cloned genes in slow-growing Rhizobia nodulation genes, nitrogen fixation genes or the ability to overcome stress could be mobilized to create superior Rhizobium strains.

Efforts to understand response of the plant upon infection with microbes should also be approached by molecular biology. The ability of microorganisms to overcome natural plant defense mechanisms can lead to more effective Rhizobia infection and nodulation. Efforts also should be made to create nodules on other portions of the plant other than the root, for example, the stem or leaves.

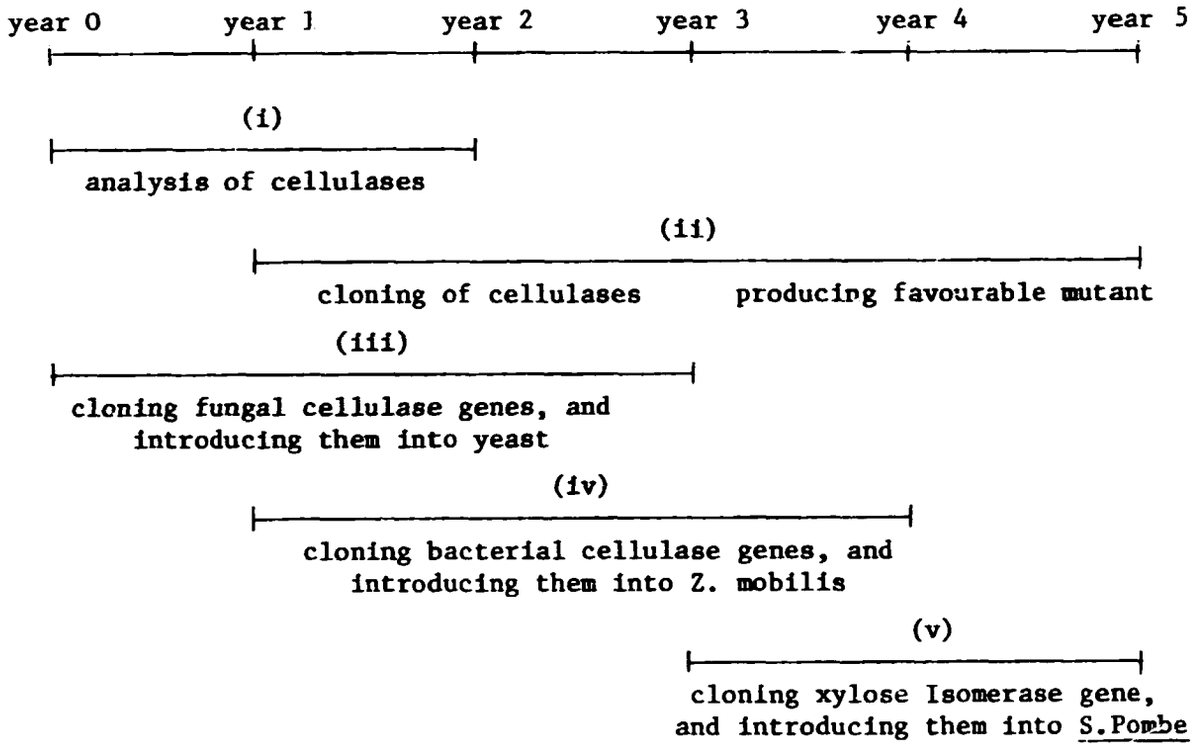
The supply of nitrogen in the form of nodules on stems or leaves, at the most needed time before pod filling, could be of tremendous economical importance in agriculture. Furthermore, the interaction between photosynthesis and nitrogen fixation should also be studied at the level of molecular biology. In summary, the development of superior strains, the more complete understanding of the infection process, and the response of the higher plant could lead to optimal plant-microbe association and, therefore, increased productivity in legumes. Recombinant DNA technology may allow us to transfer the nitrogen-fixing ability to important plants other than legumes. This research is strictly basic in nature and will take a long time to realize.

2. Use of Free-Living Microorganisms for Improving Crop Productivity.

Major nonleguminous crop plants are corn, rice, wheat, etc. The replacement of chemically reduced nitrogen by biologically fixed nitrogen in these crops is of immense economical importance; therefore, soil microorganisms, such as Azotobacter, nitrogen-fixing Pseudomonas and blue-green algae should receive more attention. The possible application of root or stem associated nitrogen-fixing Pseudomonas might become as important for fixation in cereal grains as Rhizobia are for increased legume production. In addition, genetic engineering on soil microorganisms in general could lead to better crop production by engineering microorganisms incapable of causing diseases or by altering microorganisms to become natural antagonists of pathogens and competitors. Further, attention should be directed to photosynthetic microorganisms which could be adapted as symbionts on leaves of higher plants; one such example is the water-fern (Azolla/blue-green algal interaction.

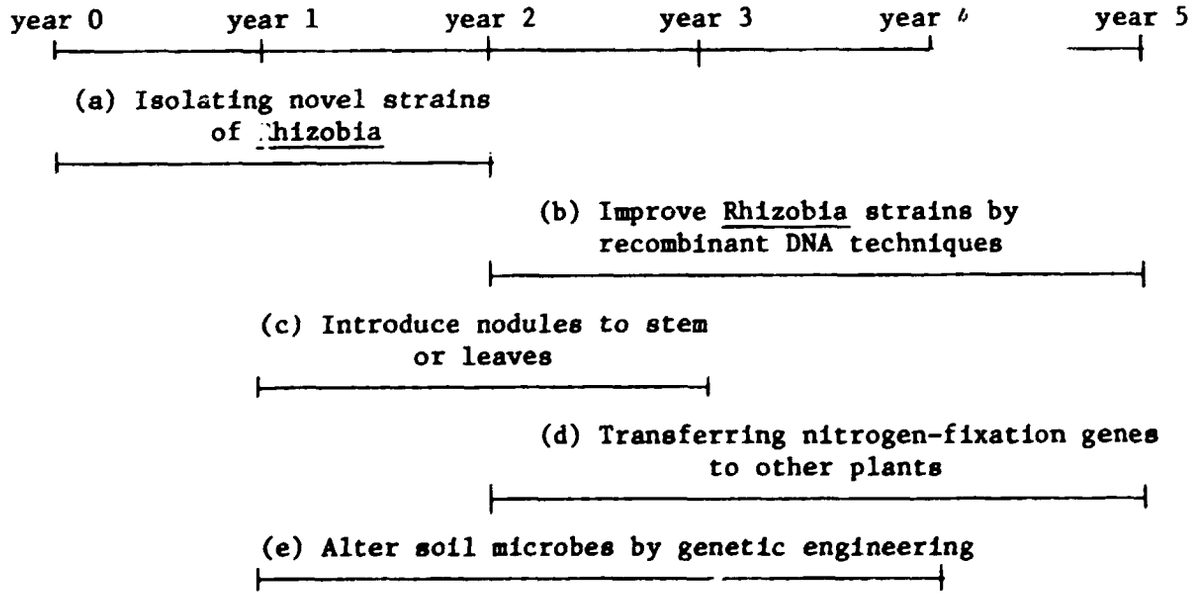
C(2). WORK PLAN

Time table for the work programme on fuel production from biomass:



Time table for the work programme fertilizers by biological system:

Some projects are expected to give positive results within the time frame indicated, others (such as (b) and (d)) may need much longer time for completion.



D(2). COLLABORATION WITH OTHER INSTITUTIONS

Contacts with several major research institutions will be needed. Those will include:

- Advanced research laboratories;
- relevant international organizations like the Food and Agriculture Organization of the United Nations (FAO);
- ongoing projects in this area, e.g. UNIDO programme on Conversion of Agricultural Wastes in the Philippines.

E(2). PREREQUISITES

The prerequisite necessary for ensuring the successful implementation of the programme is the appropriate academic qualifications of the trainees. The trainees must have an equivalent of a Ph.D. degree in biochemistry or microbiology. They must be familiar with the general laboratory techniques in biochemistry and microbiology. They must be highly motivated and eager to learn under close supervision from the scientific staff members or postdoctoral fellows at the ICGEB.

Initially, candidates for trainees may be recommended by a scientific board in a developing country. It is imperative that the final selection be made by the scientific staff at the ICGEB, because qualifications must be based on scientific merit.

F(2). FINANCIAL REQUIREMENTS

The following staff will be required when the programme will be in full operation:

- two senior scientists
- eight junior scientists
- seven postdoctoral fellows
- eight technicians

25 trainees are expected to be enrolled in this work programme.

Five-Year Budget

		(US$ thousands)
STAFF		
(first year 40 per cent, second year 60 per cent)		
Senior scientist	8 man year	600
Junior scientist	32 man year	1,440
Post-doctoral scientist	28 man year	672
Technicians	32 man year	544
Subtotal		3,256
Management of the Centre and Supporting Personnel		937
Total Staff		4,193
OPERATIONAL ACTIVITIES		
Visiting scientists	40 man months	320
Expert group meetings	4 man months	100
Advisory services	30 man months	300
Training	50 man year	1,125
Information material		30
Purchase of chemicals, etc.	104 man unit year	1,040
Associateship		150
Miscellaneous (travel, telephone, etc.)		138
Total Operational Activities		3,203
Total Work Programme		7,396

ANNEX I

EQUIPMENT REQUIREMENTS

The equipment requirement for this programme is as follows:

- 30 water baths and incubators
- 20 small rotary shakers
- 20 balances (one micro, one macro)
- 10 pH meter
- 20 desk top microcentrifuges
- 20 refrigerated microcentrifuges
- 10 floor model medium speed centrifuge (such as Sorvall RC-5)
- 10 spectrophotometer
- 30 refrigerators

The shared support facilities will include:

(a) Instrument rooms with:

- 1 scintillation counter
- 1 ice machine
- 2 large rotary shakers
- 1 recording spectrophotometer
- 10 electrophoresis sets
- 2 floor model medium speed centrifuges
- 2 high speed centrifuges (such as Beckman L8-70)

(b) A biological containment room (p2 level) with:

- 2 laminar flow hoods
- 2 large rotary shakers
- 2 medium speed centrifuges
- 1 high speed centrifuge
- 1 autoclave
- 2 microscopes
- 2 water baths
- 2 incubators

(c) A media preparation and sterilization room with:

- 1 autoclave
- 1 sterile hood
- 1 glass-distilled water still
- 2 balances

(d) A 4^{o} cold room with:

- 1 small centrifuge
- 2 water baths
- several columns for chromatography

(e) A -20^{o} cold room.

(f) A dark room with:

- 1 automatic x-ray film developer
- 1 enlarger
- 1 printer

(g) A dishwashing room with:

- 2 dishwashing machines
- 1 autoclave
- 2 ovens, etc.

Temperature and humidity controlled group chambers or greenhouses will be needed to grow plant cells and plants.

REFERENCE FOR THE LIQUID FUEL FROM BIOMASS

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