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BASED ON BIOMASS

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ROMANIA

Technical report: Microbial Strains Screening, Selection and Evaluation*

Prepared for the Government of the Socialist Republic of Romania
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of Douglas E. Eveleigh
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I. ABSTRACT

Project DP/ROM/82/012/11-03/B/32.1.I

July 26 - August 9, 1986

Consultant on Microbial Strain Collection (DP/ROM/82/012/11-03/B/32.1.I).

The Institute for Energy Related Chemistry & Biochemistry (IECB) Bucharest is developing both enzymatic and fermentative approaches for the conversion of biomass (lignocellulose) into chemicals and feedstocks (ethyl alcohol*, industrial enzymes, single cell protein, biomass conversion to alcohol and L-methionine). Micro-organisms (bacteria, fungi, yeasts and actinomycetes) are the key "biological catalysts" in the development of these technologies. The major objective of the site visit was to assist IECB in methods of gaining highly productive microbial strains, their preservation and approaches to selection of further hyper productive genetic variants. During an 11 day tour, these objectives were addressed through a seminar series (seven lectures), plus discussions of the potential and operation of world microbial culture collections and analysis of the critical factors in laboratory screening approaches for selection of microbes that efficiently transform biomass.

The current IECB projects on biomass transformations were reviewed in light of this background and good progress noted. Recommendations were made of approaches for procuring further strains including via direct isolation from soil (inulinase), from industrial culture collections (α-amylase, protease) and from private collections (cellulase). Further strain improvement programs should incorporate classical microbial genetics approaches, while for large term development it is appropriate to consider developing some expertise in recombinant DNA technology.

*Key Words

Scientific communication appears dampened by interminable bureaucratic office manipulations resulting in slow information on transfer of knowledge of the world wide current status of Biotechnology and even in ordering equipment and supplies. In Biotechnology both dramatic and rapid advances occur relatively frequently. It is absolutely essential to know the current world wide status of research if Romani is to be competitive in Biotechnology. However, IECB has a restricted number of Biotechnology journals. Furthermore, little attention has been given to personal letter writing between researchers to exchange current research advances and ideas although this is the most widely used means of gaining current research status. Most difficulties with acquisition of strains can readily be solved by this latter approach. It is considered absolutely essential to improve communication such as via the use of the weekly journal Current Contents, personal letters to researchers with regard to availability of microbial strains and the current state of their research, purchase of a few key monthly journals and via computer using data bases and also via satellite/telephone communication to Moscow and New York on "Biomass Nets."

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INTRODUCTION

The Institute of Energy Related Chemistry and Biochemistry (IECB) is one of the twenty-two Institutes of the Central Institute of Chemistry. These Institutes address industrial goals of development of industrial technologies and range from the utilization of raw materials from minerals to biomass. The IECB established in 1981 emphasises the application of biological/biochemical solutions to energy related areas. A major part of their program focuses on the development of microbial fermentations and of the application of enzymes. It includes the utilization of lignocellulosic agricultural residues, novel ethanol fermentations, single cell protein and application of enzymes in dairy (catalase), leather/detergent (protease), cosmetic (esterification), food (D, L-methionine isomerization) and environment protection (phenol oxidase) industries (See Appendix V1). The group considers other products from phytomass; for instance trials with plant pigments (Medicago) have been initiated for use in margarine (Popescu et al., 1986). St. Bragagarea has specialized background interest in this field with a chapter in "Synthetic Dyes", ed. L. Floru, 1974.

The IECB projects are distinct from other Romanian "fermentation industries". For example, from general conversation Romania appears self sufficient for antibiotic production with major production facilities at Iasi which manufacture penicillin, cephalosporin, kanamycin, streptomycin, chloramphenicol, amphotericin and megamycin. Amino-acids such as mono-sodium glutamate, and vitamins are produced via fermentation and are sufficient for the Romanian market. Fermentative production of ethanol is at twelve major factories in Romania. Nucleic acids are routinely obtained from abroad (e.g. Chemapol, Czechoslovakia) but are produced locally by fermentation if the need arises. Fertilizers are produced for export.

The total staff of the Institute (about 60) are generally young and keen. Their training reflects the classic disciplines of chemistry, biochemistry,

engineering and biology. As the research projects focus on products for the chemical industry, the training in the three prior disciplines complements the research spheres. The biologists are well trained, but not all have major experience in the sub-discipline of microbiology.

The Institute for Energy Related Chemistry & Biochemistry (IECB) Bucharest is developing both enzymatic and fermentative approaches for the conversion of biomass (lignocellulose) into chemicals and feedstocks (ethyl alcohol, industrial enzymes, single cell protein, biomass conversion to alcohol and L-methionine). Micro-organisms (bacteria, fungi, yeasts and actinomycetes) are the key "biological catalysts" in the development of these technologies. The major objective of the site visit was to assist IECB in methods of gaining highly productive microbial strains, their preservation and approaches to selection of further hyper productive genetic variants.

These projects were reviewed in relation to microbial strains/biotechnology. A review of microbial strains is presented and then each research topic area addressed. The ethyl alcohol production and industrial enzyme projects should yield viable shorter term results. Upgrading of biomass and also the conversion of biomass are longer term projects that will give a firm basis for a 5-10 year program. The biochemical products (enzymes, mushrooms, etc) should be evaluated with regard to commercial reality.

RECOMMENDATIONS
(In Order of Priority)

Note that I acted as a UNIDO Consultant on Microbial Strain (Appendix V3). However, attention to the two primary recommendations would solve most of the problem areas, even though these recommendations do not specifically address microbial strains.

A. Library Needs:

Science progresses by challenge to accepted theory /method, quick change of direction following novel insights through experimental probing, all enhanced by broad exchange of ideas. In this clime, Biotechnology is a new and rapidly advancing field, its rate of progress is accelerating exponentially. In order for IECB to stay abreast of current advances and to be competitive, it is essential to have the means of communication at hand: via current Biotechnology articles, computer data bases and by personal communication. This is minimally addressed at present. The purchase of journals that address rapid publication of novel/significant results and of current reviews, and other means of communication should be explored. For example:

A1. Current Contents - a weekly journal of the latest research articles. This journal allows the researcher to maintain current awareness, and includes addresses of authors to facilitate writing requests for reprints. Note that the cost of postage for the requesting of such reprints is miniscule in relation to the cost of the full Journal, and thus a respectable amount of monies should also be set aside for the cost of such postage. (Personal Note: Following publication of a paper (cited in Current Contents), I receive numerous reprint requests from Czechoslovakia, Hungary and the U.S.S.R., but never a request from Romania).

A2. Current review journals are equally important: Bio/Technology (general articles); Enzyme and Microbial Technology, J. Fermentation Technology (Japanese focus).

A3. Computer instrumentation should be installed to permit assessment of current status of a research topic area via the generally available computer data bases. At the very least, annual computer searches of key words/titles for each project should be conducted at a major center, e.g. Moscow, Vienna or New York.

A4. Research problem solving is often most readily solved through discussion and can be via satellite/telephone "conversations". Computer communication via satellite should be established to the "Biomass Communication Networks." Dr. Murray Moo-Young was the leader of one such network, with participants including Canada, India, several African Countries, Guatemala, New Zealand, U.S.S.R., and the U.S.A. As Dr. Moo-Young will be a consultant in November, 1986, this topic should be discussed in detail with him.

A5. Distribution of the UNIDO reviews of topical research would further alleviate some library deficiencies.

B. Computers

B1. The advantages that are offered by the use of computers, plus the further programs that grow from them, and even including such mundane programs as graphics and chemical storage lists, make such instrumentation absolutely essential. For Romanian Biotechnology to be competitive, purchase of computers for the general laboratory is necessary.

B2. The Computerized Fermentor is a focus of the total IECB biomass research program.

(a) There shall be immediate phone calls to clarify the exact status of the purchase. Note: On my return, my phone call August 13,1986 to Robert Williams, UNIDO, Vienna now indicates that Chemap have promised delivery in November, 1986. An earlier delivery is recommended in that Dr. Moo-Young should visit IECB in November, and it would be best to have the machine operational by then. An assessment of the Chemap Company's demonstrated ability to service Olivetti computers is still appropriate to consider.

(b) Further monies be allocated to permit full purchase of the total fermentor, computerized control system and down stream processing apparatus.

(c) Consultations with Dr. M. Moo-Young (or alternate computer specialist) should be made.

C. Strains:

C1. Background: There is an immediate need for "world information" on culture collections. Thus it was strongly recommended that the culture catalogues of these institutions be purchased. Each catalogue contains an incredible assemblage of comparative data including citation to descriptive publications, keys to strains attributes (e.g. a listing of all cellulase or amylase producing cultures in a collection), preferred growth media and storage conditions. Note especially the German, British (NCIB) and Japanese catalogues.

C2. General training: It is felt that general expertise with strains is easiest developed through experience. However, should training abroad be envisaged, experience covering both culture storage and also microbial activities should be sought. i. e. though certain laboratories are excellent taxonomic centers they may have restricted programs in relation to Biomass investigations (e.g. the Centraalbureau voor Schimmelcultures, Baarn).

However, there are other centers that have good microbial collections plus biochemical research activities more in line with those of Biomass research at IECB. Three examples of yeast laboratories suffice: H. Schneider, National Research Council, Ottawa, Canada (pentose metabolism), B. Atkinson, Brewing Research Institute Mutley, Surrey, UK (a diverse yeast metabolic program), R. Bothast, U.S.D.A., Northern Regional Laboratory, Peoria, Illinois, U.S.A. (broad biomass program). Obviously there are many other research programs, for instance see Yeast Newsletter,(H. Phaff, Dept. Food Technology, University of California, Davis, CA, U.S.A.) - an informal but extremely informative "magazine" that covers the world's activities for yeast though reality demands a $5.00 per year subscription. Its excellent value.

C3. Genetics: The discipline of Microbiology is broad: the handling and nutrition of microbes, their biochemistry but also their genetics. The latter includes mutation, methods of gene transfer (transformation, transduction, conjugation, fungal sexual and parasexual cycles) and several aspects of recombinant DNA research. Time will permit the development of greater experience in these latter areas. I trust my lectures also gave a flavor of "total microbiology". However, IECB lacks an expert microbial geneticist. The running of a short intensive microbial genetics course by a visiting microbiologist would provide stepping stones in laboratory protocols. An alternate suggestion is for a staff biologist to attend a "bacterial genetics" course, for instance offered by EMBO (European Molecular Biology Organization) or at Cold Spring Harbor, (CSH) New York, USA. As further expertise is gained and entry into the recombinant DNA field becomes a reality, it is strongly recommended that a staff biologist be sent to a Molecular Cloning Course again either of EMBO or CSH. Note the EMBO and CSH courses are expensive ($400) and would require Western currency.

C4. Strains: There still remains the question of costs associated with gaining these cultures. Direct purchase is preferable with regard to receiving an authenticated strain but is restrictive with costs ranging from $20-65/culture. Note that cultures received from Eastern European Republics can be substantially cheaper that from the rest of the world. It is emphasized that these strains are often direct descendents of an original culture that has been deposited world wide e.g. at ATCC. CBS, IFO etc. Potential sources of free cultures are suggested.

D. Instrumentation.

D1. General: In order for UNIDO to gain a fuller understanding of the instrumentation facilities at IECB, it is still necessary for a consultant to conduct a survey. This consultant persumably M. Moo-Young, should request clearance to visit all the IECB laboratories and pilot plant laboratories. Additionally, it would be most beneficial to visit other associated Institutes to gain a full idea of the co-operation within the broad Biotechnology discipline. Thus permission should be requested for Dr. Moo-Young to visit other Departments that have a interests in "Biotechnology" at the University of Bucharest, at the Institutal de Cercetari Chimico-Farmacentice and also the Colectia National Institutal Cantacuzino which is developing a project involving recombinant DNA technology.

E. The cellulose/ligno cellulose conversion projects

This is a longer term project. Focus should be on utilization of available hypercellulolytic mutant strains with regard to both enzyme production and effectiveness of the cellulase in substrate conversion. Maximal saccharification should be evaluated using practical pulp and paper industry substrates. More effective strains can be developed, e.g. with higher specific activity or

catabolite repression resistant strains which grow an soluble substrates. An outline for such approaches is outline in Appendix V4.As noted in Recommendation G, my preference would ultimately be for the use of cellulase in a combined saccharification/fermentation approach (SSF - simultaneous saccharification fermentation). Of the IECB projects, this one is the most complex and needs monitoring in relation to advances by other world biomass centers. However, it will provide a firm basis for the microbial/fermentation program.

F. Enzymology:

Development of industrial enzymes falls into both short (5 years) and longer term developments. The selection of which specific enzyme processes to develop is best addressed by (a) those familiar with Romanian industrial needs besides (b) plus consideration of novel applications. A major program, the enzymatic oxidation of alcohols to aldehydes is very appropriate to the general research thrust of IECB. The immediate needs are for gaining adequate comparison to other microbial sources. One Hansenula strain is awaited (for six months), and further strains have been requested during this site visit. Though the enzymatic oxidation of alcohols to aldehydes is of general interest and has been addressed worldwide, it is still necessary to evaluate the potential commercial application of enzymatically derived aldehydes; for instance in relation to current industrial usage and also in relation to competitive chemical processes that address making the same feedstocks.

Further novel approaches such as the use of continuous culture to aid in gaining total enzyme productivity, await delivery of supplementary apparatus. The delivery delay is a cause of concern. New channels of communication need to be developed with UNDP Vienna/vendor in order to simply gain a response! The reasons for the lack of reply for 6 months to the Bucharest telex communications puts a demoralizing aura on research there. No doubt there is a

rationale reason for the delay but IECB needs at least the courtesy of a reply to indicate the status quo.

In the long run it is essential to develop expertise in recombinant DNA technology. Perhaps one novel longer range approach could be the production of restriction endo-nucleases (REN's), a basis for Romanian recombinant DNA studies.

G. Ethanol production

Major studies should remain focussed on development of the immobilized yeast cell reactor with soluble substrates.

With specialized substrates such as inulin (from Jerusalem artichoke) or starch (from sweet sorghum) there should be co-ordinated programs involving microbiologists (strain development), biochemists (characterization of enzyme preparations) and fermentation engineers (combined simultaneous-saccharification-fermentation-SSF). The latter methodology appears the better, when insoluble substrates are employed.

H. Single Cell Protein from Biomass

Upgrading of lignocellulose by growth of yeast on a residual 3% 'sugar' is being developed. I question the economics of this up-grading process as little increase in total protein value would occur. If protein is the focus, I suggest upgrading via culture of ligno-cellulosic microbes. Phanerochaete chrysoporium (Sporotrichum pulverolentum) immediately springs to mind (Smith et al., 1986). Further details should be sought from K. Gregory, Dept. Microbiology, University of Guelph, Canada. This organism is also being used to upgrade cellulosics in Cuba based an Swedish technology (via K. Eriksson, Swedish Forest Products Laboratory). Dr. M. Moo-Young will also wish to discuss the benefits of his SCP-upgrading process based on the fungus Chaetomium.

I also believe an evaluation is appropriate of demonstrated application of such materials in relation to the "market" - chickens and pigs or with ruminants; what percentage of such upgraded material can be employed as a supplement and what is the total projected market in relation to these figures?

II. MICROBIAL STRAINS

The major focus of my consultant activities revolved around microbial strains (Appendix V2). The overall impression was that the microbial strains used by IECB comprised a practical and useful assemblage. However, assembling them had been a tortuous journey. Some strains had been gleaned from 'friendly' culture collections, e.g. Micrococcus lysodeikticus NCTC 2665 (Sandulescu, et al., 1986) received via the Colectia Nationala Institutal Cantacuzino, Bucharest. Other microbes had been selectively isolated from soils including a variety of cellulolytic fungi (Zamfir et al., 1986) and also an alcohol-oxidase producing Candida sp. IECB -P14m (Chirvase et al., 1986). However, it was also evident that the group at IECB had gone through the "typical growing pains" of a new institution setting up a culture collection. Hopefully our discussions should have resolved such questions as the best approaches to gaining free cultures via exchange or friendly co-operation; practical storage methods, etc. These points are discussed below.

Many of the IECB programs are based on microbial fermentations and enzymes and it is necessary to:

(A) have a comparative selection of micro-organisms at hand for research projects.

(B) the background data available for requesting/receiving strains from other culture collections

(C) methodologies for selective isolation of particular microbes.

(D) screening approaches to obtain hyper-production of microbial metabolites

(E) facilities for storage of microbial cultures

IIA. An "active working" microbial collection.

Though a collection may have numerous strains, only a few are kept active (e.g. cultures an slants). It is obviously impossible to keep a large collection active in this manner (the ARS collection has 77,000 strains - see Kurtzman; Appendix V3). Such large collections may appear impressive but there no need to develop an excessive collection. Probably the only current ICEB requirement is to gain further standard comparative strains from other researchers/collections in order that a realistic evolution of the working strains can be made. For instance methanol oxidase of Candida sp. IECB - P14m should be compared with Hansenula, Pichia, Torulopsis and other Candida spp.

IIB. Receipt of strains from other collections/individuals.

A lecture covering the general status of recognized culture collections was given. The major collections included the American Type Culture Collection (ATCC), the National West German Collection and the Japanese Fermentation Institute, Tskuba and also the Osaka University Collections for bacteria and fungi (plus viruses, cell lines and monoclonal cultures); the British National Industrial Collection of Bacteria for bacteria; specifically for fungi the Centraalbureau voor Schimmelcultures (CBS), Holland and the Commonwealth Mycological Collection, Kew, UK (See Appendix V3). It was strongly recommended that the culture catalogues of these institutions be purchased. Each catalogue contains an incredible assemblage of comparative data including citation to descriptive publications, keys to strains attributes (e.g. a listing of all cellulase or amylase producing cultures in a collection), preferred growth

media and storage conditions. Note especially the German, British (NCIB) and Japanese catalogues. I was able to donate certain catalogues to IECB (Appendix V3).

There still remains the question of costs associated with gaining these cultures. Direct purchase is preferable with regard to receiving an authenticated strain but is restrictive with costs ranging from $20-65/culture. Note that cultures received from Eastern European Republics can be substantially cheaper that from the rest of the world. It is emphasized that these strains are often direct descendents of an original culture that has been deposited world wide e.g. at ATCC, CBS, IFO etc. The problems in ordering apart from cost, could be working through bureaucratic documentation, etc. I assume the UNDP could help in this vein, and indeed promised help in tracking down a Hansenula yeast strain ordered six months back.

There are at least three further options for gaining original strains. First is via the ARS, USDA, Peoria Collection (Appendix V3) which permits one individual a selection of twelve free cultures twice per year. As a culture catalog is not published, it is desirable to write the Curator with regard to the specifics of what strains are available. The more specific the request, the easier the reply. R. Latta, Curator of Microbial Collections, Division of Biology, National Research Council, Sussex Drive, Ottawa, Ont, Canada is perhaps another free source of cultures. The second option is to write to a fellow worker who is using the required strain. Though some requests may fall on deaf ears, other positive replies may often include current unpublished results. I would opine that most cultures in university circles in the USA are obtained through letter writing plus offers of reciprocity. The personal contact approach should also be emphasized at national/international meetings, besides obtaining cultures by technical staff visiting or studying at laboratories abroad.

The Rutgers hypercellulolytic Trichoderma reesei strains RUT-C30 and RUT-P37 will be given to IECR.

A third approach for enzyme producing strains is to order the enzyme and then attempt to culture the strain from the enzyme. Patent position must be considered in the latter approach.

IIC/D. Methodologies for Screening Hyper-enzyme Producing Strains.

A few general comments. First the principal method for employment of the enzyme should be considered especially with cellulose substrates, i.e. does one require a secreted or cell-bound enzymes. Utilization parameters with regard to pH including a range of 1-11, optimal temperature for activity and stability (e.g. α-amylase at 102-103°C!) salt concentration should present no major problems. More novel situations such as activity in organic solvents present intriguing hurdles in screening protocols (e.g. see Luisi and Laane, 1986). For intracellular enzymes, facile methods of release are critical. I personally favor the use of temperature sensitive lytic strains that following production, can be lysed simply by raising the temperature a few degrees. In the longer term recombinant DNA (rDNA) approaches will facilitate specific enzyme production. In this instance temperature sensitive strains can be caused to switch an enzyme synthesis via a change of temperature following the initial culture of cells to high density (Caulcott and Rhodes, 1986) (e.g. E. coli at 70 g dry wt/l - see J. Fiesko, Soc. Industrial Microbiol., Abstracts, 1985, Boston, MA). Note that in the rDNA approaches the enzymes may be precipitated within the cell and sometimes are difficult to solubilize. Secretion vectors can be used to overcome such problems (Inoue and Halegova, 1980).

Specific screening procedures were discussed at length including focus on simple observation, approaches to circumvent regulatory systems, the need to develop screens for enzymes of high specific activity, and with cellulase

production the utilization of soluble substrates to potentially gain enhanced growth rate and greater productivity/unit time. An illustrative outline for cellulase screening is attached (Appendix V4).

IIE. Storage of cultures

Routine storage includes use of refrigeration, storage in dry soil, over silica gel or in lyophile. The latter should be readily adaptable to the Edwards lyophilyzer-see Kurtzman (Appendix V3). Though storage in liquid nitrogen is developing into a method of choice, it is expensive and periodic failures occur, e.g. lack of delivery of liquid nitrogen. Such accidents introduce a note of caution with regard to storage suggesting that a variety of methods should be used in case of individual failure of one method. Cultures of major interest should also be deposited at international culture centers that specialize in storage and maintenance.

In our most general long term laboratory storage method, we mix a culture with 10-20% glycerol and then lightly coat glass beads with the suspension. The microorganism/beads are then frozen -20 to -80°C. For recovery, the tube is removed from the freezer and allowed to warm until a few beads can be removed. The residual cells/beads are rapidly returned to the freezer, thus preventing alternate freeze/thaw of the stored culture. Storage is critical. A lost culture can mean years of work to regain an equivalent strain.

III. BIOMASS FERMENTATION PROJECTS OF IECB

The activities of IECB outlined in Appendix V1 can be sub-divided into several broad areas:

IIIA. Ethanol Production Application of Immobilized Yeast

Ethanol is considered a prime production target for the Romanian chemical industry. A focal point of the IECB program is directed to optimizing methods of ethanol production by standard fermentation techniques, investigation at new fermentation methodologies (immobilized yeast) and by expanding the substrate base to include alternate materials crops (Jerusalem artichoke, sweet sorghum, fodder beet, and waste cellulosics). The substrates (Jerusalem artichoke, sweet sorghum and fodder beets) are discussed further under the enzymology section.

The IECB group are well known experts in ethanol fermentation, and indeed the ethanol pilot plant group are currently writing chapters in a new Romanian text (The Yeast, ed. I. Anghel - "Enzymes from yeasts" and "Ethyl Alcohol from Yeast"). Their expertise is further demonstrated in the text "Bioengineering of Microbial Enzyme Preparations", G. Zarnea, Gh. Mencinicopschi and St. Bragarea. Editur Technica, Bucaresti, 419 pp. 1980).

The ethanol fermentation studies are conducted at the pilot plant near Titan, Bucharest. The focus is on scaling up the use of immobilized yeasts. (trapped in Kappa - carragheenan). A four meter partitioned column is under investigation. I did not discuss the project in detail. The strain employed, Saccharomyces sp. P 196 was isolated locally.

There was brief discussion of the fermentation of glucose syrups (30-40%) by osmotolerant strains. IECB have considered this as one attractive route to gain high ethanol concentrations by (15-20%) starting with 30-40% sugar solutions. Though this approach has several attractive features, it also has I believe crucial drawbacks. The most critical is how to store "high glucose"

syrups, for osmophilic bacteria, yeasts and fungi could also prove to be hardy contaminants. Furthermore initial rates of conversion at high sugar concentrations would be slow, while final rates would also be slow due to the high ethanol concentrations. Perhaps IECB can develop an optimal process. In which case, comparison of osmophilic strains is appropriate. I suggest writing to the following two centers to gain comparative data:

Mrs. B. Kirsop (Chairman)
National Collection of Yeast Cultures
Food Research Institute
Colney Lane
NORWICH NR4 7UA, UK

Osmophilic Yeast Collection
Tate and Lyle Ltd., Research Centre
Westerham Road
Keston, Kent
UNITED KINGDOM

In contrast to the use of osmophilic yeast, I envisage an optimal process in which glucose is consumed directly as it is produced from cellulose, i.e. a continuous saccharification/fermentation process. The glucose is not subject to degradation by microbial contaminants and as it is directly fermented, and thus it does not inhibit the action of beta-glucosidase through end-product inhibition. Furthermore cellobiose utilizing yeasts (e.g. Brettanomyces) are now being evaluated in dual culture with Saccharomyces, the removal of cellobiose preventing its end-product inhibitory effects on the cellulase components endo-glucanase and cellobiohydrolase (see SERI reports Grohman et al., Bioconversion Processes Contractors Reports, July, 1986, SERI pub.-donated). Simultaneous fermentation saccharification plants with starch as a substrate, have recently been put into operation in the USA (see Raphael Katzen Associates International, Inc., 1050 Delta Avenue, Cincinnati, OH 45208).

IIIB. Applied Enzymology

Applied Enzymology is a blossoming field. As Romania imports enzymes from Western sources, it is pertinent to consider enzyme production within the country. One role of IBCB is to implement such a program by first performing characterization and application of enzymes and then feasability pilot scale operations.

The initial program has concentrated an catalase, an enzyme of commercial application in removal of H_2O_2 (and "pasteurization") of food and of certain use in the plastic/rubber industry. Micrococcus luteus has been shown to be an excellent source of catalase (and is acceptable to the food industry). Optimal growth has been achieved via growth an a "yeast extract" medium. The latter is inexpensive (1leu/kilo) in that it is based on the lysis of "waste brewery yeast cells" via lysis in 5% sodium choride. M. lysodeikticus is tolerant of high salt concentrations. Extraction of catalase has been optimized using egg white lysozyme. Egg whites are again inexpensive. The program has been effective in developing low cost approaches to enzymology.

A second project is the Enzymatic Oxidation of Alcohols (Methanol Transformation). Methane is a generally available resource but requires processing to yield a more utilizable chemical feedstock. Microorganisms can aid in such transformations, such approaches being more efficiently conducted utilizing liquid methanol rather than the relatively insoluble methane gas. The conversion of methane to methanol is chemically facile. Two research veins have been considered. The first was direct microbial conversion of methanol to single cell protein (SCP). This process is currently considered to be uneconomic, a view also reached by Imperial Chemical Industries U.K. and Hoechst W. Germany; both companies have major interests in this area. The SCP project has been terminated. However, the C_1 metabolic studies have been continued with regard to methanol oxidation. Enzymatic oxidation of methanol yields

formaldehyde, which can then be reacted with phenol under alkaline conditions to yield the insulating resin "Bakelite", while acid conditions yield a lacquer ("Novolac"). The primary oxidations of alcohols occur enzymatically via two independent routes: by NAD dependent alcohol dehydrogenases or by alcohol oxidases. The latter employ oxygen as a substrate, do not require cofactors and are easier to regulate hence the IECR project has focussed on alcohol oxidases for the enzymatic conversion of a variety of alcohols; for example the yeast (Hansenula) methyl alcohol oxidase. (Note: in this instance, IECB still await the Hansenula strain ordered about six months ago).

However studies to date using a Candida sp. methyl alcohol oxidase show maximal synthesis to occur early in the exponential phase of growth. Direct assays are time consuming and are inappropriate for analysis in relation to the brief peak period of enzyme sythesis. Indirect growth assays have been shown to give good correlation. However, as the project develoops to include means of gaining enhanced enzyme yields through semi-batch and continuous culture, more rapid assays for alcohol oxidase will be considered. It is now proposed to develop a firmer base to the project by comparison with other microbial alcohol oxidases (a variety of strains have been requested from the U.S. Department of Agriculture Culture Collection, Peoria, Ill., U.S.A. as a result of my visit). The project objectives also consider alcohol oxidases that act towards a broad range of alcohols thus permitting production of a variety of higher aldehydes that can used as feedstocks in a several chemical process. Fermentations for the production of alcohol oxidases require high oxygen transfer rates and thus an air-lift tower fermentor has been employed. Advances employing continuous culture apparently await the delivery of peristaltic pumps, these being readily adaptable to operate perhaps two simplified small tower fermentor units - this is discussed further under "Purchase of a Fermentor" VIB1.

The intent of the program is to develop further with commercially expensive enzymes. The selection of which specific enzyme processes to develop is best addressed by (a) those familiar with Romanian industrial needs (b) plus consideration of novel applications. This will need careful evaluation of cost effectiveness, availability of production strains or needs to develop a microbial screening program, and also the levels to which scale-up in fermentors is possible. Potentially useful strains are available from world culture collections (noted in the section on "Microbial Strains").

In the long term it is essential to develop expertise in recombinant DNA technology. One novel longer range approach could be the production of restriction endo-nucleases (REN's), a basis for recombinant DNA studies. UNIDO has supported this general research area with the establishment of International Centers for Genetic Engineering and Biotechnology (ICGEB) in Italy and India. The preparation of REN's could be a first step by IECB into this important area, though it should be considered cautiously in relation to the development of recombinant DNA technology in Eastern Europe: market areas, plus rapid means of delivery (in dry ice).

In brief, enzyme project is making good progress. I would suggest that a market analysis of the application of higher aldelydes should be made, and also on economic assessment of competitive chemical process that could achieve the same goals.

IIIC. Single Cell Protein (SCP) for Fodder

The current focus is to up-grade the protein content of agricultural ligno cellulosic wastes. [Note: a prior SCP project based on methanol as a substrate has been stopped based an economic costs (see Section IIIB)]. The project now focusses on waste agricultural ligno-cellulosics. One area is on corn residues. Xylan is first removed by dilute acid extraction (H_2SO_4) yielding xylose in the

product stream which can then be converted to furfural (Milen et al., 1986; Zorzon et al., 1986). Patents applications covering this process are pending. The residual cellulo-lignin material is now more digestible to ruminants. However IECB consider two further alternate processing stages. In the first, upgrading of a high value product, the mushroom Pleurotus ostreatus is being considered. This project is in co-operation with another institute (Institute for Animal Biology and Nutrition). This appears as a most useful project with a valuable product though, I was unsure whether exporting these specialized mushrooms was an achievable final goal (as a canned product?) or was it for a specialized internal culinary market. If neither, perhaps the common Agaricus campestris should be considered for more general consumption. However, the project was not directly part of the IECB discussions. The alternate processing of the extracted ligno-cellulose was by SCP-upgrading via culture of yeast on the residual (approx. 3%) sugar. I question the economics of this up-grading process as little increase in total protein value would occur. If protein is the focus, I suggest upgrading via culture of ligno-cellulosic microbes. Phanerochaete chrysoporium (Sporotrichum pulverolentum) immediately springs to mind (Smith et al., 1986). Further details should be sought from K. Gregory, Dept. Microbiology, University of Guelph, Canada. This organism is also being used to upgrade cellulosics in Cuba based an Swedish technology (via K. Eriksson, Swedish Forest Products Laboratory). Dr. M. Moo-Young will also wish to discuss the benefits of his SCP-upgrading process based on the fungus Chaetomium.

I also believe an evaluation is appropriate of demonstrated application of such materials in relation to the "market" - chickens and pigs or with ruminants; what percentage of such upgraded material can be employed as a supplement and what is the total projected market in relation to these figures? This project could well develop into a major use of waste ligno-cellulosics and

has several aspects which are unique to Romanian agriculture. However, what is the best microbe: fungus or bacterium or a mixed culture? What are the best fermentation approaches - low scale or high scale technology? For example, low scale fermentation with such acidophiles as Scytalidium, which is capable of growth at pH 1 would probably result in no need for sterilization of the substrate.

IIID. Conversion of Cellulosics to Ethanol

Major world schemes have recently been presented for the conversion of biomass into energy sparing chemicals. All processes for the large scale production of chemical feedstocks, are heavily dependent on the cost of the substrate which may reflect 50% of the final product price. It is thus essential to utilize all components of biomass, respectively cellulose, hemicellulose and lignin. The research costs to consider such a three pronged approach are substantial.

The IECB approach recognizes the cost factor and thus proposes a three stage program. These are:

(1) Utilization of waste materials from the pulp and paper industry.

(2) Utilization of cellulosic wastes from the chemical industry

(3) Utilization of ligo-cellulosic residues from the tanning industry

This sequential approach facilitates gradual developmnet of methodologies and allows initial focus an cellulosic substrates and in the longer term considers utilization of lignins. Hemicellulose utilization is addressed in a further aspect of the IECB program with production furfurals (See Section III).

IIID1. Utilization of waste materials from the Pulp and Paper Industry

The Pulp and Paper industry produces considerable amounts of fine particulate cellulose which forms a pollution problem. This material has been collected and used as material for the production of "particulate board" or is burnt for production of energy. An alternate approach is to convert the

cellulose to glucose and hence via fermentation to a chemical feedstock (ethanol, acetone-butanol). The current IECB project addresses this latter approach. The first step is to develop hypercellulase producing microorganisms. Over twenty cellulolytic isolates have been obtained from soils and the four best further characterized. This is useful headway but the strains todate are not as effective as strains already available world-wide. I will supply hyper-cellulolytic mutants Trichoderma reesei (RUT-C30 and RUT-P37) which are state-of-the-art mutants. It is now necessary to develop fermentation conditions to gain enhanced cellulase production, and to evaluate the effectiveness of the cellulase with the pulp/paper industry substrate. Move effective strains can be developed, e.g. with higher specific activity or catabolite repression resistant strains which grow an soluble substrates. An outline for such approaches is outline in Appendix V4.

IIID2,3. Cellulosics from Chemical Industry/Ligno cellulosics from the Tanning Industry.

These two projects will develop after IIID1. The substrate materials include wastes from the production of carboxymethyl-, hydroxyethyl- and methyl-celluloses while the latter are residual ligo cellulose from the tanning industry. Each substrate has unique characteristics which deserve special attention in due course. For instance, carboxymethyl celluloses are characteristically produced with 70% degree of substitution or conversely with 30% free glucose. De-esterification will be necessary to finally gain free glucose. With ligno-cellulosic wastes, pretreatment schemes for disrupting the lignin-polymeric complex will be necessary.

However, the initial study of cellulase in part (i) will facilitate gradual development of methodologies to address these aspects.

IV. INFRASTRUCTURE

IVA. Library Resources

The library resources were minimally adequate. Inspite of this limitation the comparative analysis of world literature cited in the IECB papers was good [illegible] citation to a variety of high quality resources - journals [Biochim. Biophys. Acta, Analyt. Biochem., Enzyme Engineering, Science], patents and foreign governmental reports. However, it was also obvious that there was a considerable time lag in gaining this literature, and that journals sometimes had volumes missing. These deficiencies are serious in the fast moving biotechnology world and are disappointing especially when there are several outlets readily available that specifically circumvent such problem areas. These include the weekly listing of new scientific papers [Current Contents], and science update journals such as Bio/Technology, Enzyme and Microbial Technology, which select and discuss the more important advances. See Recommendation A.

IVB. Instrumentation

The laboratories and pilot plant appeared to be equipped with standard apparatus (a) at the pilot plant there was a general analytical laboratory. Specialized equipment noted included a CO_2/O_2/pH monitor (Hungarian). For microbiology there were two laminar air flow hoods and also good microscopes available. At the Bucharest IECB laboratories, I was shown two laboratories and an office, instrumentation room (excellent spectrophotometers). After continued requests, I was permitted to see the general enzymology laboratory. There seemed to be some prior barrier in communication in order to gain such permission but Dr. Giurca was most helpful in facilitating this request. I did not see any fermentors or the other laboratories, and was not granted permission to visit other institutes.

IVB1. Overview of the Purchasing Status of a Computer Controlled Fermentor

Many of the IECB Biomass projects focus around fermentation engineering. Thus, IECB approached UNIDO for a Chemap-computer controlled, state-of-the-art, fermentor. Though approved in 1982, the delivery of the fermentor is set for November 1986. This delay was result of problems associated with international purchasing procedures. Several lessons have been learned. First is of communication. It is well known that direct communication via telephone is often more effective than letters or even telegrams. If not to maintain momentum it appears that UNIDO and IECB need an ombudsman (of senior status) to intercede and activate such delayed purchase agreements should the momentum be lost. It requires a senior official to be nominated with such authority. Secondly, as alternate components are to be purchased, one must be sure of the compatability of these materials with the major equipment. This requires a legal notice of warranty and of guarantee. There is also the question of maintenance and repairs. The original Chemap fermentor is to be serviced through the original company which is skilled with both its fermentor and its computers. However, one now has the hurdle of either Chemap servicing auxillary computers with which they have much less practical experience, or alternatively dealing with two companies; Chemap for the fermentor and the Italian Olivetti Company for the computers. Whichever approach is followed it is essential to have a warranty and guarantee to ensure successful operation. There is also the question of cost of repair parts for a West European built computer. There will definitely be a need for repairs, spareparts etc. with time. I do not know how much "Western" currency has been put aside for these needs.

In terms of practicality, I emphasize that during the interim between the original purchase order and later delivery, that the total purchase price has risen and now the UNDP allocated funds are insufficient to purchase the total

equipment i.e the fermentor plus down-stream processing apparatus (Dynamill and Alfa-Laval centrifuge). A major recommendation is that UNIDO reconsider the budget in order that both the fermentor and the down stream-processing equipment may be purchased in order to yield an effective total operational fermentation unit.

A further matter is that smaller accessory apparatus such as peristaltic pumps could have been delivered and this would have permitted initiation of continuous culture studies. In terms of value for money, these pumps are a bargain. IECB should ensure their purchase from either Eastern or Western European sources immediately.

From the above there is:

(a) Concern regarding the operational/maintenance/repair status of a "modified" instrument.

(b) there is a budgeting problem regarding the purchase of both fermentor and down-stream purchasing equipment due to the delay of purchase and concomitant price increase via inflation. The UNIDO budget should be reconsidered.

(c) there is an immediate need for small associated instrumentation (e.g. 3 or 4 peristaltic pumps). Perhaps these can be bought from Eastern European sources.

(d) Dr. Giurca has emphasized the delicate and current status of the purchase negotations. He obviously does not want further complications with regard to purchase/delivery. However, as Professor M. Moo-Young a consultant with considerable fermentor expertise is to visit IECB soon, his evalutation should be taken into account.

IVB2. Computer Facilities

Computers are now in general world use with regard to administrative applications, accessing data bases, word processing, instrumentation control, scientific computations and specific programs such as "chemical activity structure relationships". At IECB there is a central facility available for major computational studies and there will be developmental studies on computer controlled fermentation following acquisition of the "UNIDO Fermentor". However, I find the usefulness of computers to be minimally addressed at IECB as evidenced by the absence of such computer facilities in offices and laboratories.

IVB2a. The outlook for catching up with the necessary biotechnological computer expertise appears bleak. In general, researchers at IECB appeared frustrated with the lack of availability of small computers to be used for routine office/laboratory tasks, but they preferred not to discuss this topic area. This is a demoralizing research aura. One wonders how the operation, repair and further development of the computer controlled fermentations will develop in this sterile climate. In comparison UNIDO projects in Lesser Developed Countries have computers at hand for the general researcher, e.g. Biomass Program; Lourena, Brazil.

IVC. Teaching/Outreach/Long Range Planning

(i) Dr. Guirca is associated with the L'Institute Nationale de Chimie, (L'ecole polytechnique. La facultie de technologie de chemique. Section de biochimie) and gives a current course entitled "Biotechnology". The course is designed for senior students. In the final year, students may pursue biotechnology projects, with work and practical experience at IECB. Three IECB researchers were trained at L'Institute Nationale de Chimie.

(ii) The local universities have active research programs. Some project co-ordination is possible through students performing research study at IECR in the final year of their baccalaureate degree. The input into such project is dependent on the student's other educational committments. On the average two students/year participate in the IECB program. Each student subunits a full project report with distribution to both University IECB.

(iii) Studies in recombinant DNA research necessitate an available infrastructure such as the availability of restriction endo-nucleases and of short lived radio-isotopes. These aspects can be addressed through inter-Institute co-operation. Thus each "biological" institute could become the production of a set number of restriction endo-nucleases. Direct co-operation with those with rDNA experience is strongly recommended e.g. at the Instututal Cantacuzino.

APPENDIX VI. RESEARCH PROJECT AREAS AT THE INSTITUTE FOR ENERGY RELATED CHEMISTRY AND BIOCHEMISTRY (UNIDO PROJECT 84/460/ROM/012)

G. MUSCA, DIRECTOR

Head of Unit - Dr. Radu Giurca

Research Topic Areas (Modified via D.E.E.*)

IIIA. Fermentative Production of Ethanol Immobilized Yeast (St. Bragagerea)

IIIB. Industrial Enzymes/Fermentation (S. Niculescu, C. Sandulescu, A. Chirvase)

IIIC. Furfural and Single Cell Protein from Biomass (I. Milea)

IIID. Enzymatic Hydrolysis of Lignocellulose (R. Giurca, M. Zamfir)

* I have slightly modified the original IECB outline - specifically one project original 'Single Cell Protein (SCP)' from Biomass Processing for Fodder. This project included conversion of methanol to S.C.P., a project currently discontinued. However, the major substrate methane (but used as methanol) and the developmental fermentations are still addressed, the research focus now including fermentative production of alcohol oxidases and their use to convert alcohols to aldehydic chemical feedstocks IIIB. The prior S.C.P. aspects are well covered in Project IIIC.

APPENDIX V2. PROFESSIONAL DISCUSSIONS AT THE ICECHIM - ICEB

Tuesday 29.07.86 Arrival and settling in at the Dorobanti Hotel

(Met by Drs. Stelian Niculescu and Alexandru Slavov)

Wednesday 30.07.86 Welcome address and Concepts of Bioenergy/Biotechnology with General Director Maria Ionescu

Tour of Exhibitions of Science Program, Discoveries and Patents with Dr. Radu Giurca, Head of the Laboratory of Biotechnology and Dr. Stelian Niculescu, Head of Biochemical Laboratory.

Lecture and Discussion Program:

The general format included lectures in the morning followed by small group laboratory discussions in the afternoon.

Lectures included:

Wed. 30.07.86

I. MICROBIOLOGY AND BIOENERGY: AN OVERVIEW

(3 pm Visit to PNUD Strada Aurel Vlaicu 16 79362 Bucharest - Financial Aspects)

Thurs. 31.07.86

II. CELLULASE - MOTHER NATURE IS COMPLEX

Laboratory

Friday 1.08.86

III. SCREENING TECHNIQUES FOR HIGH YIELDING CELLULASE STRAINS

Monday 4.08.86 Meeting at PNUD 3 pm. Noel Eichhorn

IV. RECOMBINANT DNA APPROACHES TO CELLULASE: ADVANCES AND PROSPECTS

Tuesday 5.08.86

V. ETHANOL PRODUCTION IN THE U.S.A.

ZYMOMONAS - a Potentially Useful Ethanologen

APPENDIX V2. (CONTINUED)

Wednesday 6.08.86

VI. MICROBIAL STRAINS
BIOMASS - PROBLEMS/PERSPECTIVES

Friday 8.08.86

VII. BIOTECHNOLOGY FOR FUN + PROFIT?

(a review)
Meeting at PNDP 3 pm
Noel Eichhorn - review.

Saturday 9.08.86

Depart from Bucharest 10:30 am

APPENDIX V3. MICROBIAL STRAINS

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Appendix V4. CONCEPTUAL OUTLINE FOR

SELECTION OF HYPER-CELLULOLYTIC STRAINS

An outline is presented to gain strains capable of growing on soluble substrates. These substrates will facilitate fermentor operation (in comparison to the use of insoluble cellulose) and will result in faster growth rates and hence greater cellulase productivity. The preferable sequence would be first gain good lactose (milk whey) inducible strains and then use these to gain "glucose-constitutive" strains.

A. High yielding
B. Rapidly growing
C. Adequate β-glucosidase - cell bound
D. Constitutive production when grown on soluble substrates.
E. Stable strains

General approaches were noted in the Rutgers papers.

1. Mutation

Nitrosoguanidine is the most effective mutagen in our hands. UV light is also effective. Other mutagens can be tried but have not yielded as productive results. However, a particular type of mutant may become available only through use of further mutagens.

2. Strains

Initiate the program with hyperproductive strains e.g. T. reesei RUT-C30 or RUT-P37.

3. Restriction of colony size

Use oxgall or deoxycholate - NOT phosphon D. No "restrictors" are required with the use of 2-deoxyglucose.

4. Detection of activity

Total cellulase - use acid-swollen cellulose to give a translucent agar layer. Incubate pregrown cultures overnight at 50°C. Observe the degree of clearing.

Endo-glucanase - use carboxy-methyl cellulose (CMC) overlay in combination with Congo red.

β-Glucosidase: Methyl umbelliferyl β-D-glucose (MUG) is the most sensitive and easy to use. It could even be too sensitive for selection of high yielding strains. Note - if cell-bound enzyme is required, one should not select on the basis of activity (fluorescence) around (outside of) the colony, but for greater intensity actually at the colony surface.

If MUG is too sensitive, p-nitro-phenyl-ß-D-glucoside could be used. Similarly, salicin or esculin could be used as alternate substrates (see prior papers).

Cellobiohydrolase: No good assay but try methyl umbelliferyl-ß-D-cellobiose (MUCB) [Sigma Chemicals, Chicago or Koch-Light, Colnbrook UK. supply this chemical].

On the one hand, one should focus on detection of total activity. However, as acid-swollen cellulose is difficult to prepare and there is also evidence of co-ordinate regulation of the cellulase components, detection simply of endo-glucanase or ß-glucosidase mutants could yield mutants with increased total activity.

5. Constitutive Strains

Mutate as described and plate on rich medium (malt extract or potato dextrose agar. After the colonies have grown up, overlay with "detection agar". For MU-derivatives one can observe under UV light after 10 min - 30 min. Congo red c.f. endo-glucanase detection is perhaps 30 min after the addition of the CMC overlay. For total cellulase activity, the overlay must be incubated overnight at 50°C and then clearing observed. In this instance, induction of cellulase could occur. This can be prevented by incorporation of an inhibitor of protein synthesis into the overlay medium (actidione). If this kills the colonies, then prior replica plating must be used. However, one can assume that incubation at 50°C will inhibit protein synthesis and induction, and thus only constitutive mutants will be selected for. Again if 50°C is too stringent lower the temperature (40-45°C) or in advance of heating at 50°C.

6. Best mutant selection:

To date this has been through the use of 2-deoxyglucose as an antimetabolite and also as a catabolite repressor. Mutate and plate with 2-deoxyglucose (2DG) plus an alternate substrate (cellobiose, CMC, acid-swollen, cellulose or Avicel). Our first mutants were with 0.2% 2-DG + 2% cellobiose. Following selection of the first mutant series, these proportions are changed to have a greater proportion of 2DG. A series of mutants can be developed by this technique alone.

Note: In this method, mutants can be only just alive. Some simply grow and then die lysing. Tender loving-care! Perhaps initially subculture in sloppy agar to give osmotic support.

7. Selection of best mutant from several hundred mutants

Culture in small wells, using the agar covered by a polycarbonate sheet with 5-10cm holes. Use either the relative direct clearing of cellulose agar or an overlay method. 50 mutants at a time can be evaluated by using a pyrex cooking dish (25cm x 50cm) as the culture dish. Select the optimal mutant.

Notice also the sequential comparative method of Labudova et al., FEMS Microbiology Letters 20: 211-215 (1983).

Critical use of these two approaches can also lead to selection of stable strains.

8. All mutant activities must be confirmed in liquid media. Activities in agar need not correlate with that in liquid media! Practical media should be used (cheese whey, molasses, xylan) note the whey lactose could enhance yeilds by induction. Selection of faster growing strains can be by use of liquid media (continous culture) (Bungay et al.) but care should be made of not concomittantly selecting fast growing, but low yielding strains.

NOTES

* Evaluation of cellulase production should include studies of <u>total</u> saccharification of crystalline cellulose, and not just initial enzyme reation rates.

* Remember mutational anaylses are random. <u>Expect to evaluate several hundred mutants!</u>

* Store mutants carefully - (deep freeze - lyophilizer). Once lost, you have to begin the total process again.

* All types of mutant should be considered. Altered morphology may indicate enhanced enzyme production (see Labudova et al., cited above).

* Following isolation of good constitutive mutants, one can only select further mutants based on relative activity [i.e. increased size of clearing zones].

Perhaps at this stage industry should take over.

<u>PROTOPLAST FUSION</u>

This technique (see attached papers) could be useful in combining the total cellulolytic attributes of differing strains (see Toyama references). Success in other programs has been limited, e.g. in antibiotic screening programs. However, one attribute is to revitalize "hyper-mutated" strains by crossing to gain hybrid vigor. Extra-chromosomal inheritance with fungi and yeasts (e.g. mitochondria) could play a vital role here.

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