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ELABORATION OF A TECHNOLOGY FOR FEED ADDITIVE PRODUCTION

DP|MON|86|008|11-01

MONGOLIA

Technical report: Microbial synthesis *

Prepared for the Government of the Mongolian People's Republic
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of Zdenek Rehacek,
expert in microbial synthesis

Backstopping officer: B. Galat, Agro-based industries Branch

United Nations Industrial Development Organization

Vienna

* This document has not been edited.

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Explanatory note

During the course of the mission USD 1,00 was equal to Tughriks 3,00.

Acronym: MoAS - Mongolian Academy of Sciences
CAS - Czechoslovak Academy of Sciences

Abbreviation: R and D - Research and Development

Abstract

Elaboration of technology for feed additive production
DP/MON/86/008

The objectives of the mission /Sept 3 - Oct 3, 1989, in Ulan Bator Sept 7-30, 1989/ were fulfilled in close co-operation with UNIDO /Vienna/, the Institute of Biotechnology MoAS and the UNDP Office /both in Ulan Bator/

The Government Agency for the project is the MoAS, Institute of Biotechnology. Its section of microbiology is directly in charge of the project realization.

The project has started its activities in December 1988.

The UNDP contribution to the project is USD 358,000, the Government contribution is Tughriks 2,325,000.

The practical application of the project's results will be achieved through close co-operation of MoAS and the Ministry of Agriculture and Food Industry.

In the course of the mission experimental work carried out within the project was evaluated.

Detail plan was jointly elaborated of the forthcoming R and D of lyseine fermentation, yeast biomass production and ergosterol preparation.

The counterpart staff was made acquainted with the present and perspective R and D of lysine fermentation and yeast biomass production and was provided with the crucial procedures for enzyme assays and with the list of needed chemicals.

Urgent equipment and chemical requirements were specified.

Programme of scholarships and study tour visit was jointly

prepared.

Scientific discussions related to the fulfilling of the project goals were conducted with the counterpart personnel.

Guidelines for the production of lysine, biomass of yeasts, and ergosterol were jointly prepared.

Procurement and installment of fermentors become priority objectives for the scaling-up of the investigated fermentation processes.

Procurement of the high performance liquid chromatography /HPLC/ apparatus was found urgent.

Continuous co-operation with the Biocomplex for antibiotic and faccine production in Songino is utmost desirable.

Extension of the project and rational broading of its programme are recommended to increase the project's impact substantially.

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INTRODUCTION

Mongolia is predominantly an agricultural country. Agriculture, in particular cattle-breeding, is the most important part of Mongolian's economy. Modernization of the national agriculture becomes a crucial target. On of the major conditions both for raising the livestock output and increasing the production of cattle is the provision of enriched animal feed and feed additives.

At present, the lack of protein additives to animal feed is estimated at around 300,000 tons per year. The most economic way to produce feed additives, such as amino acids, single cell proteins, feed additives with antibiotic activity, and vitamins is the development of microbial industry.

The Mongolian Government has properly estimated the importance of biotechnology R and D for the country. Thus, research into biotechnology became one of the five leading directions in the national science policy which should be urgently developed. Special attention is paid to practical aspects of biotechnology.

The present project which has started in December 1988 aims at improving the quality and increasing the quantity of produced livestock products by developing a convenient technology for the microbial production of fodder additives. Within the project it is planned to develop microbial technology through the establishment of the appropriate laboratory facilities at the Institute of Biotechnology, MoAS, hiring of international consultants, training of national personnel, the application of modern process biotechnologies and the development of new products with biological activity.

It is expected that the biotechnological laboratory facilities will improve the feed additives quality, provide guidelines for industrial production of some feed amino acids in the country, and will also constitute a nucleus for the transfer of biotechnology to other feed processing plants and for personnel training in feed additives research, development and industrial production. However, in Mongolia there is a tremendous scope also for biotechnology in such fields as food processing and nutrition, health care and medicine, environment protection.

RECOMMENDATION

Mongolia, with its diversity of flora, possesses a large share of bioresources. Consequently, since genetic diversity is the primary feedstock for biotechnology, in this context Mongolia is favourably endowed. The challenge therefore is to seek for and identify new bioproducts and to device cost effective strategies for their productive exploitation.

Thus, it can be recognized that the extension of the project and broadening of its activities would substantially increase the project's impact. Biotechnologies of the following biosubstances appear utmost perspective:

a/ agricultural antibiotics,

b/ biologically active plant metabolites, e.g. heart glycosides of Adonis mongolica,

c/ immunomodulative peptides of fungal and animal origin.

Due to the outstanding importance of this proposal a soon assistance visit by the UNIDO representative to the Institute of Biotechnology MoAS is greatly recommended.

Potato pulp /PP/ is a major byproduct obtained from the starch industries. In Mohgolia, the total PP production is 100,000 tons per year. PP consists of cellulose, hemicellulose, starch and pectin. Some of these polysaccharides, namely pectin and starch, can be separated and recovered and others can be converted to valuable chemicals. PP problem has been deeply investigated at the Institute of Technical Chemistry of the Hannover University /GFR/. A training stage of a national scientist at that Institute would be useful.

Following difficulties encountered in the course of the mission deserve outstanding attention:

- Delay in the procurement of fermentors which are prerequisite for the scaling-up and the on-the-job training.
- Lack of a HPLC apparatus /High performance liquid chromatography/.
- Lack of chemicals for enzyme assays.
- Non-intensive research into physiology of production microorganisms.
- Scare co-operation with the Biocomplex for antibiotic and vaccine production in Songino.

There is a considerable activity in academic institutions regarding the use of computers for fermentation optimization and control. The national fermentation specialists should begin to learn about process control and sensors.

I. DEVELOPMENT AND IMMEDIATE OBJECTIVES

The project DP/MON/86/008 to which the mission of the microbial synthesis expert is related is to develop a new technology for the production of feed additives through the elaboration of microbial synthesis methods.

The immediate objectives of the expert's work are to carry out following duties in close co-operation with the Institute

of Biotechnology MoAS and the UNDP office in Ulan Bator;

- to draft the programme, conduct and carry out joint experiments and laboratory work in relation to microbial strains and the entire methodology for development of microbial synthesis technology,
- to assist in determining the equipment, chemicals, and nutrient media requirements and in preparing the corresponding specifications,
- to assist in the preparation of a training programme and in the identification of possible countries of training in the field of biochemistry, microbiology and fermentation technology,
- to conduct the on-the-job training programme for the counterpart personnel,
- to prepare guidlines and methodologies for the production of feed additives,
- to prepare a joint technical report setting out all the findings of the mission and recommendations to the Government on the follow-up actions which might be taken.

II. ACTIVITIES AND OUTPUTS

The infrastructure

The Government agency for the project realization is the Institute of Biotechnology MoAS, acting on behalf of the Mongolian Academy of Sciences and the Ministry of Foreign Relations and Supply.

The Institute ofBiotechnology MoAS is located in Ulan Bator and consists of following sections: microbiology, molecular genetics, cell engineering, biophysics. The Institute elaborates biotechnology R and D projects within MoAS. It employs around 50 professional, sub-professional, administrative and technical staff. Research and development activities are supported by MoAS. The section of microbiology /Annex I/ which is directly in charge of the project realization deals mainly with soil microbiology, microbial biochemistry and physiology, and microbial synthesis.

In addition to the Institute of Biotechnology MoAS, also the Institute of Chemistry MoAS participates in the project activities, particularly in respect of carrying out investigation of hydrolysis of raw materials for microbial technology.

On behalf of the Government of Mongolia, MoAS and the State Committee for External Economic Relations supervise the project activities and provide their guidance and support.

The activities relating to the project realization

In the framework of the project programme horizontal transfer of knowledge was improved by one study tour visit /Czechoslovakia/, three fellowships /Japan, GFR, CSSR/, and one participation in the International postgraduate training course UNESCO "On modern problems in biology and microbial technology" /CSSR/ /Annex II/.

The experimental work carried out within the project activities was accelerated by the procurement of scientific monographies /Annex III/, rare chemicals /Annex IV/, and sophisticated laboratory equipments /Annex V, VI/. However, the present submerged-fermentation facilities of the section of microbiology which is directly in charge of the project activities is unsufficient being represented by one rotary shaker machine /100 places for 500ml flasks/ and one fermentor ANCUM-2M /working volume 5 l, adjustment of temperature, aeration, stirring, pH/.

In the early stage of the project realization following topics have been investigated by the counterpart personnel: submerged fermentation of lysine, preparation of fodder yeast in fermentor, and ergosterol in yeasts.

The polyauxotrophic strain Brevibacterium sp.83 of the Culture Collection of the Institute of Biotechnology MoAS was used in the lysine fermentation experiments. The strain has requirement of methionine, threonine, cysteine and thiamine, respectively. The original wild strain was isolated from the soil sample by the Institute staff and was improved by mutation induced by UV-irradiation and by subsequent cultivation on nutrient media with increasing level of lysine. In this way the polyauxotrophic mutant sp.83 was prepared producing 12-20 g/l lysine when grown on a sucrose medium in flasks /500ml/ located on a shaker machine /200 rpm/. Through manipulation of cultivation conditions the yield of lysine was increased up to 35-40 g/l, i.e. by 100 %.

The final lysine fermentation medium consisted of following components /in 1 l dist. H_2O/: saccharose 100 g, soybeen hydrolysate 2 ml, methionine 200 mg, threonine 600 mg, thiamine 200 /ug, biotin 300 /ug, pH 7,0.

Using the 30 l-jar fermentor following cultivation conditions were found optimal: temperature 30 °C, aeration of 0,3 vvm /volume of air/volume of medium/minute/, agitation of 100 rpm.

Chicken treated with the dried feed lysine concentrate showed improvements of egg production /10-11 %/, egg weight /0,3-1,5 g/, and body weight /3,5 %/.

The attention of the yeast research group was focused on the production of yeast biomass by strains grown on media with wastes of the ethanol production. The local production of these wastes is 31,000 tons per year. From the set of tested strains of the genera Saccharomyces and Candida the strain Candida sp. B-3 was screened and used in further experiments. This strain is deposited in the Culture collection of the Institute of Biotechnology MoAS. For the cultivation of the strain at 27 °C in 500 ml flasks located on the rotary shaker machine /rpm 200/ following media /inoculation and fermentation/ were developed. The inoculation medium consisted of /g/l/: $/NH_4/_2SO_4$ 2,2, $MgSO_4$ 0,3, K_2HPO_4 0,6, NaCl 0,1, yeast hydrolysate 50 ml, pH 4-5. The fermentation medium contained : wastes of EtOH production 1 l, $/NH_4/_2SO_4$ 2,2, $MgSO_4$ 0,3, K_2HPO_4 0,6, NaCl 0,1, pH 4-5. The wastes of the EtOH production consisted of /%/: protein 14, starch 0,26, organic acids 2,1, phosphorus 0,03, nitrogen 0,1, ash 0,4.

The biomass suspension of the strain Candida sp. B-3 was tested on four months old piglets. The experiment lasted 56 days. The weight of the biomass treated piglets increased by 267 g per day while that of untreated animals increased by 188 g per day only.

Another attention of the section of microbiology was focused on the feed additive ergosterol. Within this topic a set of strains of the Saccharomyces and Zygosaccharomyces genera from the Culture collection of the Institute of Biotechnology MoAS was tested for intracellular ergosterol content. The best results were achieved with the strain Saccharomyces sp. 7. Its cells contained 0,88 % ergosterol when grown 48 h on a

rotary shaker machine /200 rpm/ in flasks /500 ml/ with medium containing following components /g/l dist H_2O/: glucose 100, $/NH_4/_2SO_4$ 4,3, K_2HPO_4 1,2, $MgSO_4$ 0,6, NaCl 0,1, $CaCl_2.2H_2O$ 0,1, yeast hydrolysate 5 ml, pH 6.

Comments by the expert

In order to improve the lysine fermentation R and D the expert on mission evaluated the experiments, results and procedures of the counterpart personnel and prepared an instructive paper /Annex VII/. Present state and perspectives of lysine R and D are overviewed in this paper stressing following topics: a/ physiology of the production strain, b/ continuous strain selection and improvement, c/regulation of lysine pathway and lysine production, d/ scaling-up. The crucial procedures for assays of enzymes of the lysine path are involved in the article as well as the list of the respective literature and chemicals needed for enzyme assays. The paper was jointly discussed with the counterpart personnel from the viewpoint of the forthcoming methodology, experiments and on-the-job training programme.

Yeast is the most popular organism used as a source of vitamins, as well as a protein additive. Annex VIII shows main components of yeast. The yeast cells can be easily separated from the fermentation liquid. They contain almost all essential amino acids, the quantities of which satisfy the physiological needs of growth and other metabolic activities. The bacteria cells have some advantages in comparison to other organisms, having faster division, higher protein content, and sulphur containing amino acids. However they also have drawbacks, e.g. they are liable to the attack of phaga, they contain increased quantities of nucleic acids, they are smaller in size if compared to yeast cells and require more energy for their separation.

The content of the nucleic acids in the bacteria and yeast cells amounts to 16 % and 6-12 %, respectively. Nucleic acids interfere and should be removed from the stockfeed when used in larger amount, Serious problem occurred during the utilization of single cell proteins in swine feeding because of uric acid deposited in the knees and as the consequence of that

animals could not get on their feed. Thus, increased quantities of nucleic acids in the single cell proteins should be reduced to those levels which would have no harmful effect.

In principle, there are three procedures for elimination of nucleic acids from the single cell proteins: chemical, enzymic, thermal. The removal of nucleic acids by chemical procedure involves the alkaline or acid treatment of the cells. Alkaline treatment is more efficient in eliminating nucleic acids from the biomass than the acid treatment. It must also be underlined that the alkaline treatment of the single cell proteins completely eliminates nucleic acids, but the protein is damaged in this process. The heating of protein and the alkaline environment lead to the formation of lysinoalanine and increased racemization of amino acids. Certain amount of lysinoalanine can be toxic. The acid treatment of the single cell protein isolate at increased temperatures completely destroys cysteine and tryptophan. Second procedure for the nucleic acid elimination is based upon the use of nucleases which break down nucleic acids. Endogenous nucleases can be induced inside the cell by heat, anions /acetate ions/, at the increased temperature /50 ^{o}C/, etc. The other procedure involves the use of exogenous nucleases.

Microbial cells have, as a consequence of their small size, high ratios of surface area to volume, and since the whole of the surface of a microbial cell is normally available for the uptake of nutrients and the excretion of waste products, growth rates of microorganisms are orders of magnitude higher than those of crop plants or domesticated animals. A young pig or chicken, for example, may double its weight in one month while a yeast cell will achieve the same amount of growth in about three hours. About one-half of a microbial cell grown under suitable conditions will consist of protein and the high rates of protein synthesis by microbial systems constitute their biggest single advantage in comparison with conventional agriculture.

Needs for technical co-operation/assistance

The major difficulties of the Institute of Biotechnology MoAS which deserve urgent solution are:

- lack of sophisticated fermentors,
- lack of HPLC apparatus
- lack of rare chemicals for advanced scientific research

- lack of up-to-date scientific literature
- lack of Xerox-machine
- shortage of experience in biotechnology R and D and of know how.

Needed chemicals and equipments are listed in Annex IX.

Training programme

In order to improve and strengthen both the transfer of scientific knowledge and the advanced scientific training in the field of microbial synthesis, technical microbiology, and fermentation technology one study tour visit /2 weeks/ of two national leading scientist, and a programme for two fellowships /3 months each/ were prepared to be realized in 1989 /Annex X/. The recent appropriate scientific monographies and papers were recommended to the counterpart personnel by the expert on mission /Annex XI/.

III. UTILIZATION OF RESULTS

National strategies are being developed which identify microbial technology as a substantial factor in the attainment of industrial and economic goals. Application of microbial technology will only arise as a result of systematic programme of R and D.

Within the Institute of Biotechnology MoAS biotechnology facilities are being improved. Some crucial laboratory equipments have already been procured and installed. The scientific staff has been trained abroad and on-the job, and high level expertise has been provided. The Institue is developing capabilities which will enable its effective assistance to the national biotechnology industry by providing an example, by a focal point for on-the-job training of biotechnology scientists and technicians, and by exploiting microbial activities for development of appropriate fermentation technologies for microbial products, especially the fodder additives for animal feed.

It has been recognized that the extension of the project and rational broadening of set of local bioresources to be investigated and applied would substantially increase the project's impact.

The Institute's linkage with the other food processing industries should be expanded so that the transfer of food techno-

logy will be achieved.

The procedure of the laboratory lysine fermentation elaborated by the counterpart personnel yields 40 g/l lysine in a 10 l fermentor. This fact challenges the scaling-up of the process. In addition, it supports the proposal for an establishment of a national pilot plant for amino acid production.

IV. CONCLUSIONS

The project reflects one of the most serious problems of the Mongolian technology R and D in solving the urgent need of the country. The special emphasis of the project is on setting up a microbial manufacture of fodder additives for cattle breeding. In order to accelerate research, development and application of the desirable fermentation processes it is particularly necessary to provide the Institute of Biotechnology MoAS, which is in charge of the project realization, with sophisticated laboratory equipments /especially fermentors/, rare chemicals, up-to-date scientific information, and advanced training of its junior scientists.

Training through research represents a permanent priority in each programme. Its activities essentially aims at providing young scientists with possibility to acquire specific knowledge and know-how in one or several of the complex disciplines which constitute modern biotechnology, i.e. from basic enzymology, microbial, plant and animal cell research /especially into physiology/, process engineering, or bioinformatics to risk assessment ard genetic engineering of industrial microorganisms. Training must necessarily involve transnational mobility for the selected junior scientists to receive the respective training in recognized foreign laboratories. Such activities are important because they contribute directly to improving the infrastructure for biotechnology training which, in turn, through the provision of tailor-made training activities at a low cost, leads to a rapid increase in the supply of skilled personnel.

International training in biotechnology does not only serve the specific interest of trainees and of host-laboratories, it also consolidates collaboration between the laboratories which share or exchange trainees and it slowly leads to the emergence

of policy makers, project leaders or department heads who, having started their scientific career, are fully aware of the potentialities, merits and difficulties of the international co-operation.

For the proposed extension of the project with broader activities additional financial support by the Government as well as additional external sources would be needed.

The creative and fruitful co-operation of all parties /Annex XII/ involved in the preparation of this Technical Report is greatly appreciated.

ANNEX I

Counterpart staff of the Institute of Biotechnology MoAS involved in the project realization

Name	Position held	Qualification
B. Dashnyam	Sci. secretary of the Institute	PhD
T. Puntsag	Chief of the Section of Microbiology	DSc
G. Urantsooj	Head of the Laboratory of microbial synthesis	PhD
L. Sandorj	Research scientist	Univ. Degree
B. Badrakh	Research scientist	Univ. Degree
D. Naranchimeg	Research scientist	Univ. Degree
Ch. Dulamsuren	Research scientist	Univ. Degree
T. Bold	Research Scientist	Univ. Degree
G. Dorj	Research scientist	Univ. Degree
D. Tserendulam	Research scientist	Univ. Degree

Semi-skilled emploees are not listed here.

ANNEX II

Realized study tour visit and fellowships of national scientists abroad

Name	Country	Institution visited	Date
T. Puncag G. Dorj	CSSR	- Agrocomplex Slusovice - Inst.Microbiology CAS - Kovo Prague - Mikrotechna Prague	19-29 July 89
		FELLOWSHIPS	
G. Urantsooj	Japan	- Univ. Tokyo - Amino acid factory Ajinamato Co, Kawasaki - Antibiotic factory Meiji	13 March - 1 August 89
B. Badrakh	FRD	- Inst.Techn.Chemistry, Hannover Univ. - Inst.Microbiology, Hannover Univ. - Inst.for Count Technique, Hannover Univ. - Biotechnol.Res.Society, Braunschweig - German Collection of Microorg. and Cell cultures, Braunschweig - Inst.for Fermentation and Biotechnology, West Berlin - Int.Food Exhibition, Frankfurt /Main/	28 Feb - 3 April 89
L. Sandorj	CSSR	- Inst.Antibiotics and Biotransformation, Roztoky near Prague - Inst.Microbiology CAS, Prague - Biotika, Slovenska Lupca - Mikrotechna Prague	3 April - 24 June 89
B. Mandah	CSSR	- International Postgraduate Training Course UNESCO "On modern peoblems in biology and microbial technology", Prague	14 Oct 88 - 30 Nov 89

ANNEX III

Scientific monographies procured by UNDP

C.H. Collins, P.M. Lyne /eds./: Microbial Methods. Butterworth Co, London 1985

G. Woodward /ed./: Immobilized Cells and Enzymes. A practical Approach. LRZ Press, Oxford 1985

L.C. Tauchstone, M.F. Dabbins: Practice of Thin Layer Chromatography. J.Wiley and Sons, New York 1983

ANNEX IV

Chemicals procured by UNDP

Chemical	Quantity
Vitamin B_{12}	1 g
Riboflavine	2 x 25 g
Rhodamine B spray reagent	2 x 100 ml
Ergocalciferol	5 g
Lytic agent from Arthrobacter luteus crude	2500 un
L-Amino acids /kit/	2 pieces
Orcinol ferrichloride spray reagent	2 x 100 ml
D-Arabinose cryst.	100 g
Thiamine HCl	25 g
Ninhydrin spray reagent	20 x 100 ml
Antimony pentachloride spray reagent	2 x 100 ml
Isatin spray reagent	2 x 100 ml
2-Mercaptoethanol	500 ml
Inulin from Dahlia tubera	100 g
Antimony trichloride spray reagent	2 x 100 ml
Silica gel Type G	500 g
Melibiose	100 g
D-Xylose	100 g
Pyridoxine HCl	25 g
Kit of 11 carbohydrates	1 piece
Gilsen Einwegspitzen gelb	1000 pieces
blau	1000 pieces
natur	1000 pieces
Gilson pipetmann P-20	1 piece
P-200	1 piece
P-1000	2 pieces
P-5000	2 pieces
Thomaplastmarkierungsstift	
Nr.61417	5 pieces
Nr.61418	5 pieces

Chemicals were supplied by Sigma,USA

ANNEX V

Laboratory equipments procured by UNDP

Equipment	Supplier	Model	Quantity
Dual-band UV-lamp	Cole-Parmer	K-9818-012	1
Lamp stand	Cole-Parmer	K-9818-50	4
Replacement filter	Cole-Parmer	K-9816-10	1
Repl. long-wave - watt tube	Cole-Parmer	K-9814-26	2
Repl. short wave 6-watt tube	Cole-Parmer	K-9813-27	2
Microscope Axioscop	Opton GFR		1
TLC Glass developing tank with lid	Sigma	T-9877	2
TLC tank lid	Sigma	T-7882	4
TLC Spotting guide and Rf reader	Sigma	S-2009	20
TLC Silica gel plates	Sigma		25

ANNEX VI

Equipments procured by the Government

Equipment	Model	Supplier	Quantity
Thermostat	TC-80M-2	USSR	3
Refrigerator	Minsk 16E	USSR	8
Technical balances	BAP-200	USSR	1
Compressor		USSR	1
pH-Meter		GDR	1
Centrifuge		USSR	2
Safe deposit big		MPR	6
small		MPR	5
Steam generator	Cs-318/40	HPR	1

ANNEX VII

Present state and perspectives of lysine fermentation R and D

Lysine was the first amino acid to be synthesized, if not the first biochemical synthesized. It is evidently necessary for protein function and so would be the first of the aliphatic amino acids to be depleted in the primordial soup. The presence of lysine in the genetic code must, therefore, be accounted for by special need for the amino group in proteins, for catalytic processes.

Lysine - a basic amino acid (positively charged) - is an essential amino acid which is very widely used to supplement cereal-based animal feed. L-Lysine is the form required in foodstuff. In the human food the necessary amount of lysine is 0,24 mmol per kg of the weight per day. The deficiency of lysine occurs especially in countries where the nourishment is restricted on grain. The content of lysine in grain is 2 to 3 times lower as in meat or eggs.

The bulk of the L-lysine production is by fermentation. Development of the fermentation production of lysine is associated with the isolation of auxotrophic mutants of bacteria capable of accumulation of significat quantities of lysine in particular. Commercially, the largest amonts are produced by Corynebacterium glutamicum, Brevibacterium flavum and Brevibacterium lactofermentum. L-Lysine is economically one of the most important amino acids.

Biosynthesis of lysine

Biosynthetically L-lysine is a member of the aspartate family (except in fungi). The aspartate family consists of aspartate, asparagine, methionine, threonine, lysine and isoleucine. Diaminopimelate, a lysine precursor, is needed for cell wall synthesis in bacteria but not for protein synthesis. It can therefore be considered another member of the aspartate family.

There are two pathways to lysine in living organisms: The pathway via diaminopimelic acid (DAP pathway) found in

bacteria, higher plants, diverse algae and lower fungi, and the pathway via α-aminoadipic acid (AAA pathway) found only in higher fungi and Euglena. The AAA pathway begins with acetyl CoA and α-ketoglutarate and proceeds via homoisocitric acid to α-aminoadipic acid, which is then reduced to L-lysine.

In the Brevibacterium strain synthesis of lysine proceeds via DAP pathway. This route begins with aspartic semialdehyde and pyruvate, which undergo an aldol condensation and lose water to yield a cyclic intermediate, 2,3-dihydropicolinic acid. At a later stage, meso-2,6-diaminopimelic acid is formed, which is converted to the meso form and then decarboxylated to yield L-lysine.

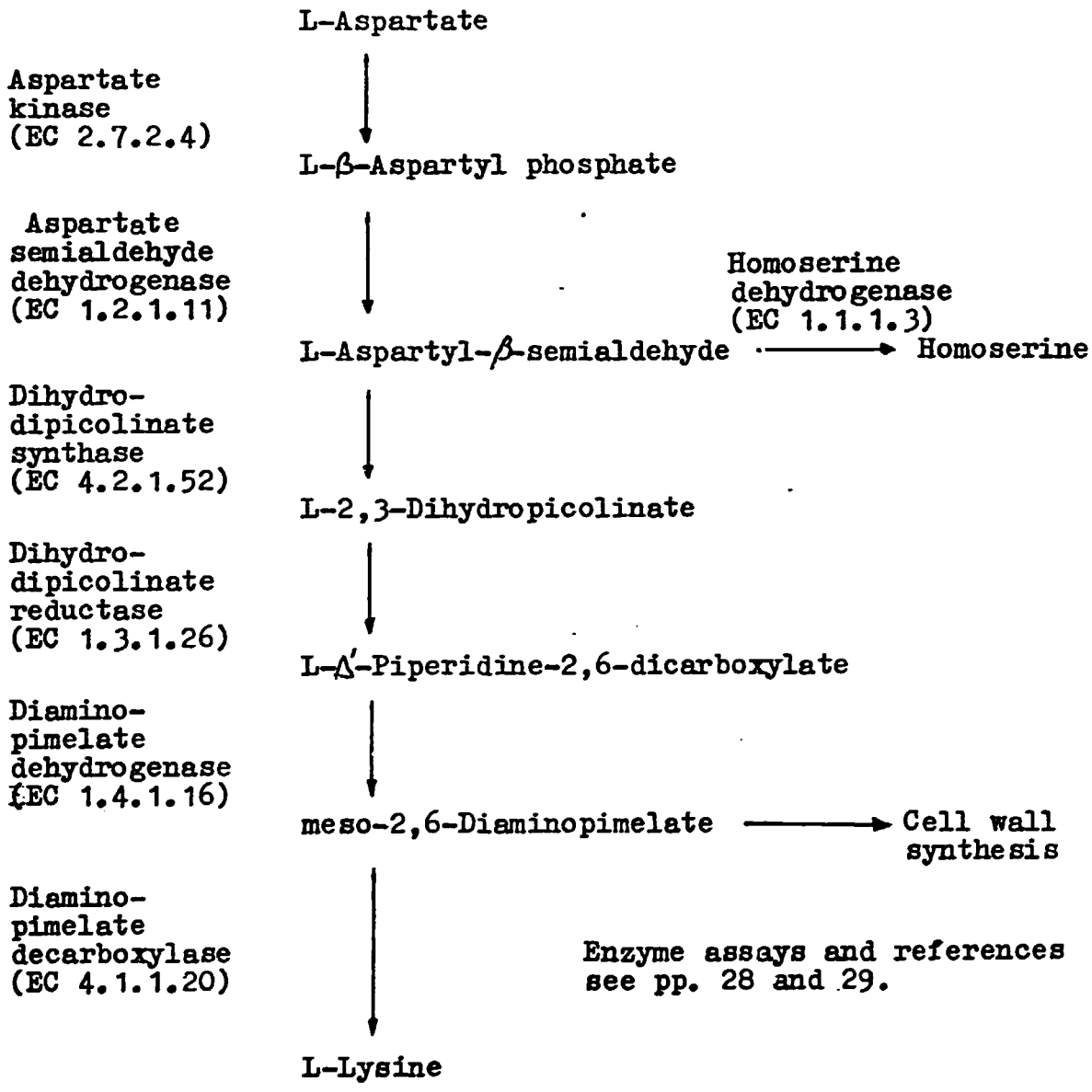
The biosynthesis of lysine occurred very early in the development of metabolism. It was probably the first amino acid to be synthesized. The original pathway was the DAP pathway.

Enzymes of lysine DAP-pathway

Lysine biosynthetic enzymes (Fig.1) are typically nuclear-encoded. They are synthesized in the cytosol. The entry of aspartate into lysine synthesis is controlled at the level of aspartate kinase activity (Fig.2). In contrast to E.coli. only one aspartate kinase is present in Brevibacterium. This enzyme is feedback-inhibited when both lysine and threonine are present in excess. If the enzyme is released from feedback inhibition, mutants excrete lysine in the range of 5-10 g/l. Other amino acids of the aspartate family are not excreted in these mutants, indicating the presence of further control elements at branching points. Thus, homoserine dehydrogenase is repressed by methionine and inhibited by threonine. Mutants with decreased homoserine dehydrogenase activity produce more lysine.

A farther control at this branching point is the synthesis of dihydropicolinate synthase. Upon leucin limitation a tenfold derepression of the enzyme specific activity occurs. Certain leucine-negative strains obtained by N-methyl-N'-nitro-nitrosoguanidine mutagenesis show increased lysine productivity. The blocking of homoserine synthesis at homoserine dehydrogenase results in the release of the concerted feedback inhibition by threonine and lysine on aspartokinase, and the aspartic semialdehyde produced proceeds to lysine through the lysine synthetic pathway on which no feedback inhibition is found.

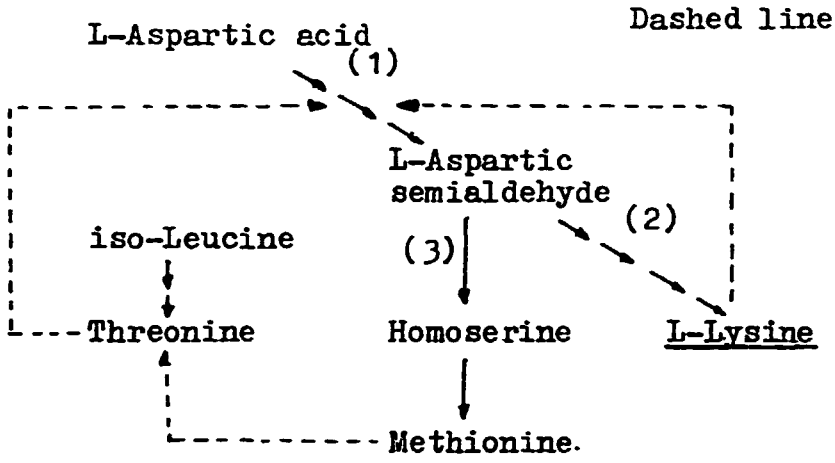
Fig. 1 Enzymes of lysine synthesis via diaminopimelic acid



Enzyme assays and references see pp. 28 and 29.

Limited concentration of biotin in the medium induces the formation of defective cell membrane with enhanced permeability to the amino acid, lowering its intracellular concentration and reducing the feedback control exerted over its biosynthesis. The promotion of lysine production by high concentrations of biotin is explained by the stimulation of pyruvate carboxylase by biotin which consequently leads to the increase of aspartate formation through the increase of oxaloacetate formation (Fig.3).

Fig. 2 Mechanism of L-lysine accumulation



(1) Aspartokinase
(2) Dihydropicolinate synthetase
(3) Homoserine dehydrogenase

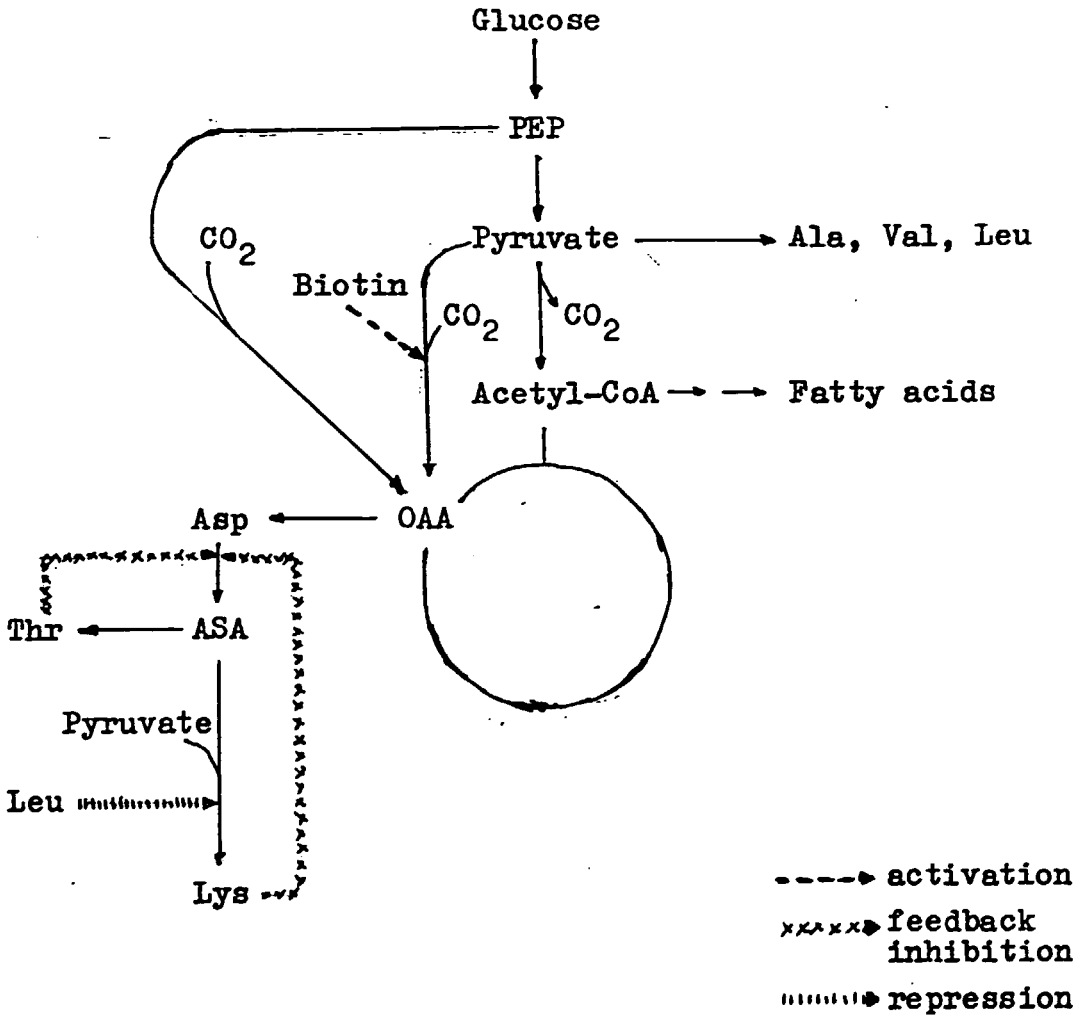
Fluoropyruvate inhibited pyruvate dehydrogenase and increased lysine production in the presence of high concentrations of biotin.

The metabolic relation between lysine, its precursors and related amino acids is shown in Fig. 2. The accumulation of lysine by production strains depends upon their having been selected for defects in key synthetic steps and also for defects in the feedback control mechanisms which normally control amino acid synthesis.

The development of a lysine fermentation process was initiated by the discovery that homoserine auxotrophs of the glutamate overproducer C.glutamicum will produce lysine if grown under the appropriate conditions. The importance of homoserine deficiency can be appreciated from the Fig. 2. In this species, the activity of aspartokinase, the enzyme which primarily governs lysine production, is controlled by multivalent feedback inhibition by lysine and threonine. Thus, homoserine auxotrophs (which lack homoserine dehydrogenase) will produce lysine because the threonine level can be kept low enough to avoid feedback inhibition of aspartokinase. Since both methionine and threonine are produced from homoserine(see Fig.2), both amino acids must be added to culture of the homoserine-deficient lysine producers. However, methionine helps to minimize inhibi-

tion of aspartokinase by threonine - presumably by competition for the allosteric binding site on the enzyme molecule.

Fig. 3 Pathway and regulation of lysine biosynthesis from glucose by Brevibacterium lactofermentum



Fermentation

The lysine fermentation is aerobic and is conducted in aerated stirred tank reactors (fermentors). The pH is maintained in the neutral range by addition ammonia, and temperature is controlled at 28 °C. A rather small inoculum is used, grown up in the minimum number of steps from an agar slope culture. A major problem of the process is back mutation to prototrophs which generally outgrow the auxotrophic lysine-producing strains. This is why a small inoculum is desirable and why continuous

processes have been unsuccessful. The addition of antibiotics, e.g. erythromycin, discourages the prototrophs. Alternatively, doubly auxotrophic lysine producers which are less prone to prototroph production may be used. The fermentation lasts about sixty hours and yields of 40-50 gL-lysine per litre are produced starting with sugar concentration of 100 g/l.

Sufficient supply of oxygen to satisfy the cell's oxygen demand is essential for the maximum production of lysine. The dissolved oxygen level must be controlled at greater than 0,01 atm, and the optimum redox potential of culture media should be above -170 mV. An extremely oxygen deficient condition leads to the production of lactic acid at the expanse of the lysine produced. The theoretical conversion yields calculated for the main pathways of lysine biosynthesis lead to the conclusion that the most efficient formation of lysine is via oxaloacetate formed by carboxylation of phosphoenolpyruvate (PEP). Therefore, the strain with high PEP carboxylase activity is desirable.

Investigation on dissolved oxygen and carbone dioxide in amino acid production shown that the conditions of oxygen supply and carbon dioxide removal are closely related to the fermentation results, influencing cell growth and the accumulation of amino acids. However, the physiological significance of carbon dioxide has not yet been sufficiently clarified.

Nature is basically very economically organized with several levels of control of metabolic fluxes in the cell. These controls avoid creation of high concentrations of one metabolite, while maintaining constant fluxes in the metabolic systems. If a metabolite could be efficiently removed one-line, there would be no inhibitory effects and thus the metabolic flux would not slow down.

Lysine recovery

Although the lysine hydrochloride may be directly crystallized from clarified broth after addition HCl and evaporation provided certain conditions are fulfilled (US Pat.3702341), the usual process consists of acidifying the clarified broth with HCl and adsorption of the lysine on a cation exchange

column in the ammonium form. The lysine is eluted from the column with aquous ammonia, reacidified with HCl and the L-lysine hydrochloride crystallized.

Breeding of lysine-overproducing mutants

Wild strains isolated from nature were not able to produce industrially significant amount of lysine. One reason for this is the regulation of cellular metabolism to avoid oversynthesis. With metabolic regulation, the permeality barrier is another mechanism which prevents microorganisms from allowing organic materials to escape into the environment, enabling cells to retain the intermediates and macromolecules necessary to maintain life.

In amino acid production from carbohydrates, the transport rate of glucose has never been reported to be a limiting factor. However, the transport rate of some substrates to cells has been found to be a limiting factor. A mutant with increased transport rate of a substrate or precursor into cells can be isolated by two methods. One is the selection of a rapidly growing large colony on a plate containing is compound which is the sole source of carbon. The other method is the isolation of a strain sensitive to a compound which is an analogue of the substrate compound and inhibitory to the growth of the microorganism.

L-Lysine has been produced by auxotrophic mutants. A combination of auxotrophy and genetic deregulation is generally useful for obtaining increased yields of primary metabolites. Multiple markers contribute to the yield of primary metabolite by stabilizing the productivity against reverse mutation during fermentation. A new industrial process has been developed for the production of certain amino acids using such a combination method. L-Lysine productivity of a homoserine auxotroph has been improved by endowing leucin auxotrophy and lysine-analogue (thialysine) resistance.

It is recommended to improve the Brevibacterium strain also by preparation of regulatory mutants selected as mutants resistant to S-(2-amino-ethyl)-L-cysteine (AEC), a lysine analogue, and mutants simultaneously auxotrophic and resistant

(e.g. mutants resistant to AEC and requirung leucine). Regulatory mutants of Brevibacterium producing lysine and selected for AEC-resistance in the presence of threonine have aspartate kinase desensitized to concerted feedback inhibition by lysine and threonine. Aspartaldehyd acid is utilized for the biosynthesis of lysine as homoserine dehydrogenase is inhibited in the presence of threonine.

Mutants having aspartokinase insensitive to the concerted inhibition were selected by resistance to S-(2-aminoethyl)-cysteine in the presence of threonine, and over-produced lysine but not threonine. When the mutants were derived from strains with low citrate synthase, the first enzyme of the tricarboxylic cycle, they produced more lysine with high frequency.

Mutants lacking homoserine dehydrogenase over-produced 35 g l^{-1} of lysine, which was almost equivalent to that by mutants having both feedback insensitive aspartokinase and low citrate synthase. Over-production of former auxotrphs was inhibited by excess threonine, in contrast to thet by the latter regulatory mutants.

The most common methods applied in the past were mutation and selection based on knowledge of molecular biology. However, recent developments of recombinant DNA technology and cell fusion have enlarged the scope of microbial production not only by increasing production capacity through increasing gene copies and promotion of gene expression but also by enabling combinations of different capacities between different species. Such methods even make possible the production of new metabolites which cannot be synthesized in the original organism.

Chemicals needed for enzyme assays (p.28)

Thialysine; leucine; N-methyl-N'-nitro-N-nitrosoguanidine; S-(2-aminoethyl)-L-cysteine; diethanolamine; NADP; NADPH; L-aspartate semialdehyde; Na_2HAsO_4; sodium pyruvate; o-aminobenzaldehyde; meso-diaminopimelic acid; pyridoxal phosphate; o-phtalaldehyde reagent (Pierce); TRIS; Tween 60.

Enzyme assays of the lysine DAP-pathway, and references

METHODS

Organisms and growth. The organisms used were *Corynebacterium glutamicum* ATCC 13032, the Leu⁻ strain ATCC 14310, *Brevibacterium lactofermentum* ATCC 13869, and two independently isolated mutants of *Corynebacterium glutamicum* ATCC 13032 with deregulated aspartate kinase (strains DG52-5 and MH20). These were obtained by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (250 μg ml⁻¹, 30 min) and selected on plates containing the lysine analogue *S*-(2-aminoethyl)-L-cysteine plus L-threonine (12 mM each). The medium for the plates was C.g. VIII, plus 2% (w/v) agar agar and 5% (w/v) urea, but with $CaCO_3$ omitted. C.g. VIII contained, per litre: 20 g $(NH_4)_2SO_4$, 0·5 g KH_2PO_4, 0·5 g K_2HPO_4, 0·25 g $MgSO_4.7H_2O$, 10 mg $MnSO_4.H_2O$, 10 mg $FeSO_4.7H_2O$, 1 mg $ZnSO_4.7H_2O$, 0·2 mg $CuSO_4$, 0·2 mg biotin. After sterilization, 4% (w/v) glucose and 2% (w/v) $CaCO_3$ were added.

For preparation of cell extracts and for measurement of amino acid production, a preculture was grown overnight at 30 °C in complex medium (C.g. III) consisting of, per litre: 2·5 g NaCl, 10 g yeast extract, 10 g peptone and 20 g glucose. Cells from this culture were washed once with 0·9% NaCl and transferred to medium C.g. VIII to give an initial OD_{600} of about 0·7 (corresponding to 2×10^8 cells ml⁻¹). All cultivations were done in 100 ml medium at 30 °C on a rotary shaker at 100 r.p.m.

Preparation of extracts and enzyme assays. The cells were harvested by centrifugation and, after washing with 50 mM-potassium phosphate buffer pH 7·5, were suspended in the same buffer. Sonic extracts were prepared by disrupting the cells with a microtip-equipped Branson Sonifier at maximal settings (5 min, 0 °C). The resulting homogenate was centrifuged for 30 min at 10000 *g*, and the supernatant used as cell-free extract.

Aspartate kinase (EC 2.7.2.4) was assayed in extracts of cells suspended in 50 mM-$(NH_4)_2SO_4$, 100 mM-Tris/HCl pH 7·5 according to the method of Black & Wright (1955). The assay mixture consisted of 100 mM-Tris/HCl pH 7·5, 13·3 mM-ATP, 24 mM-$MgCl_2$, 468 mM-$(NH_4)_2SO_4$, 613 mM-$(NH_3OH)Cl$, 93 mM-potassium L-aspartate, and extract. The assay was incubated at 30 °C for 30 min and the reaction stopped by the addition of 1 ml 10% (w/v) $FeCl_3.6H_2O$, 3·3% (w/v) trichloroacetic acid in 0·7 M-HCl to 1·5 ml assay mixture. After centrifugation, the absorbance at 546 nm was measured and compared with standards obtained with aspartyl hydroxamate.

Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) was assayed in extracts of cells suspended in buffer containing 33% (v/v) glycerol and was assayed according to Hegemann *et al.* (1970) in the reverse of the biosynthetic reaction. The assay mixture consisted of 60 mM-diethanolamine.HCl pH 9, 0·8 mM-NADP, 40 mM-Na_2HAsO_4, 0·12 M-NaCl, 1·5 mM-L-aspartate semialdehyde, and extract. The reaction was followed at 25 °C and 340 nm.

Dihydrodipicolinate synthase (EC 4.2.1.52) was assayed by the method of Yamakura *et al.* (1974). The assay mixture consisted of 0·2 M-Tris/HCl pH 8, 2 mM-L-aspartate semialdehyde, 4 mM-sodium pyruvate, and extract. After incubation for 10 min at 30 °C, the 500 μl assay mixture was mixed with 450 μl 1 M-HCl and 50 μl *o*-aminobenzaldehyde (20 mg per ml ethanol). After 50 min incubation in the dark, the samples were centrifuged and their absorbance at 540 nm determined ($\varepsilon = 1220$ M⁻¹ cm⁻¹).

For determination of dihydrodipicolinate reductase (EC 1.3.1.26) the *dapA* gene of *Escherichia coli*, coding for dihydrodipicolinate synthase (Richaud *et al.*, 1986), was transferred via plasmid pJC1 (see below) into the *dapB*-(reductase-) negative *E. coli* strain AT999 (Bukhari & Taylor, 1971). Extracts of this strain with a dihydrodipicolinate synthase specific activity of 3·3 μmol min⁻¹ (mg protein)⁻¹ were used to synthesize the reductase substrate in 900 μl of the following reaction mixture: 111 mM-Tris/HCl pH 7·5, 5 mM-aspartate semialdehyde, 1·1 mM-NADPH, 6 mM-sodium pyruvate, and 50 μl extract of AT999 (pJC1/*dapA*) (= 1 U dihydrodipicolinate synthase). This assay was incubated for 10 min at 30 °C and the reductase reaction started by the addition of extract previously incubated for 3 min at 70 °C (the heat treatment of the extract was necessary to inactivate the homoserine dehydrogenase and NADPH oxidase activities). The NADPH-dependent reduction was followed at 340 nm and 30 °C.

meso-Diaminopimelate dehydrogenase (EC 1.4.1.16) was assayed in the reverse direction at 30 °C in the following assay mixture: 200 mM-glycine/NaOH pH 10·5, 2 mM-NADP, 4 mM-*meso*-diaminopimelate.

Diaminopimelate decarboxylase (EC 4.1.1.20) was assayed in a system containing 50 mM-potassium phosphate pH 7·5, 0·1 mM-pyridoxal phosphate, 0·5 mM-EDTA, 25 mM-diaminopimelate, and extract. After incubation at 30 °C for various times (at least four points), the lysine formed was determined by amino acid analysis (see below).

Homoserine dehydrogenase (EC 1.1.1.3) was assayed at 25 °C in 66 mM-potassium phosphate pH 7·0, 0·2 mM-NADPH, 0·1 mM-aspartate semialdehyde. The activity was followed at 340 nm or determined by direct homoserine analysis (see below), giving comparable results.

All assays were checked for linearity with respect to time and protein concentration. They were optimized for the pH of the incubation buffer and for the concentrations of substrates and coenzymes. Determinations were done with at least three independently generated extracts. Specific activities are given in μmol min⁻¹ (mg protein)⁻¹.

DNA manipulation. The shuttle vector pJC1 and the plasmid pDA3 containing the *dapA* gene of *E. coli* (Richaud *et al.*, 1986) were isolated from *E. coli* DH5 and RDA8, respectively, by the alkaline lysis method (Birnboim & Doly, 1979). The construction scheme for the recombinant plasmid pJC1/*dapA* is shown in Fig. 2. The source of the DNA-modifying enzymes was Boehringer, whose recommendations for use were followed throughout. The ligation mix was used to transform *E. coli* DH5 (Hanahan, 1985) using the RbCl method, and Kan^r transformants were selected. Four of the transformants assayed had a specific dihydrodipicolinate synthase activity of 0·8–1·1 (host 0·04) and gave the restriction pattern of pJC1/*dapA*. This plasmid was isolated on a large scale by caesium chloride density gradient centrifugation (Maniatis *et al.*, 1982) and used to transform spheroplasts of *C. glutamicum* (Thierbach *et al.*, 1988).

Amino acid analysis. Amino acids were analysed as their *o*-phthalaldehyde derivatives by reversed-phase chromatography. Derivatives were formed via automatic pre-column derivatization using the *o*-phthalaldehyde reagent (Pierce) with an LC 1090 HPLC (Hewlett-Packard). The separation was achieved on a Lichrospher RP-18 column (Merck) using a gradient consisting of 0·1 M-sodium acetate pH 7·2 and methanol. Detection was via UV absorbance at 340 nm or fluorescence at 450 nm with excitation at 230 nm.

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ANNEX VIII

Main components of yeast

Component	Brewer's yeast /%/	Candida /%/
Dry mass	93,0	93,0
Ash	6,4	7,8
Crude fiber	3,0	2.0
Ether extract	1,1	2,5
Protein N x 6,25	44,6	48,3
Digestible	38,4	41,5
Major minerals		
Ca	0,13	0,57
Fe	0,01	0,01
Mg	0,23	0,13
P	1,43	1,68
K	1,72	1,88
Na	0,07	0,01
Trace elements /mg kg^{-1}/		
Co	0,2	-
Cu	33,0	13,4
Mn	5,7	38,7
Zn	38,7	99,2
Vitamins /mg kg^{-1}/		
Thiamine	91,7	6,2
Riboflawine	35,0	44,4
Nicotinic acid	447,5	500,3
B_6	43,3	29,5
Biotin	-	1,1
Pantothenic acid	109,8	82,9
Folic acid	9,7	23,3
Choline	3885,2	2910,6

ANNEX IX

Proposal of needed equipments and chemicals to be procured by UNDP .

EQUIPMENTS

- High performance liquid chromatography apparatus /HPLC/ for separation and identification of microbial metabolites and intermediates. Justification for the equipment procurement is enclosed. /Approx. USD 75,000/ (p.37).
- Microtip-equipped Sonifier for preparation of sonic extracts of bacterial cells for enzyme assays. /Approx. USD 2,000/
- Xerox machine for prepartion of copies of rare foreign scientific papers

CHEMICALS

Cat.Nr. /Kanto Chem.Co/	Chemical	Unit
01196	M-L-Amino acid Collection A /7999/	2 pieces
	M-L-Amino acid Collection B /8004/	2 pieces
	M-L-Amino acid Collection C /8001/	2 pieces
405402	Vitamin B_1 nitrate	50 g
14127	Ethylene Glycol Monomethylether /2-metoxyethanol/	2000 ml
56252	Ninhydrin	200 g
07824	Copper chloride	1000 g
40149	Thymol	200 g
40147	L-Threonine	100 g
40109	Thiamine HCl	50 g
25163	L-Methionine	100 g
25556	D-Melitose monohydrate	100 g
07160	D-Cellobiose	100 g
14019	meso-Erythritol	150 g
20030	myo-Inositol	100 g

ANNEX IX continued

97625	Vitamin A	1 g
40109	Vitamin B_1HCl	25 g
04630	Vitamin H /Biotin/	20 g
17557	Glucoso-J-lactone	500 g
14041	Ethylamine	500 ml
37005	Salicin	20 g
	Cycloheximide	25 g
	D(+)Trehalose	100 g

In total approx. USD 2,000

ANNEX X

Study tour visit and fellowships to be realized in 1989

Name	Country	Institution	Date
		STUDY TOUR VISIT	
T. Puncag and B. Arya	Sweden, Denmark, France		Oct 89 (2 weeks)
		FELLOWSHIPS	
Ts. Damdingaa	CSSR	-Inst.Microbiol.CAS Prague -Research Inst.of Antibiotics and Bictransformation, Roztoky near Prague	from Oct 89 (3 months)
D. Menchbat	CSSR	-Inst.Microbiol.CAS, Prague -Research Inst.of Antibiotics and Bictransformation, Roztoky near Prague	(3 months)

ANNEX XI

Recommended scientific monographies and papers

E.L. Vandamme /ed./: Biotechnology of vitamins, pigments and growth factors. Elsevier Applied Science, Barking 1989

A.H. Rose /ed./: Economic Microbiology. Vol. 2. Acad Press, London 1978

N.M. Fish, R.I. Fox, N.F. Thornhill /eds./: Computer applications in fermentation technology: Modelling and control of biotechnical processes. Elsevier Applied Sciences, Barking 1989

Z. Rehacek, P. Sajdl: Ergot alkaloids: Chemistry, biological effects, biotechnology. Elsevier, Amsterdam 1989

J. Bu'Lock, B. Kristiansen: Basic Biotechnology. Acad.Press, London 1987

K. Aida et al.: Biotechnology of amino acid production. Acad Press, London 1987

N.G. Alaeddinoglu, A.L. Demain, G.C. Lancini: Industrial aspects of biochemistry and genetics. Plenum Press, London 1985

W.M. Jaklitsch, M. Roehr, C. Kubicek (1987): Lysine biosynthesis in Penicillium chrysogenum - Regulation of general amino acid control and absence of lysine repression. Exp.Mycology 11, 141

J. Cremer et. al.(1988): Regulation of enzymes of lysine biosynthesis in Corynebacterium glutamicum. J.Gen. Microbiol.134, 3221

J.H. McClendon (1987): The relationship between the biosynthetic paths to the amino acids and their coding. In: Origin of Life 17, 401 (D.Reidel Publish.Co)

Papers related to enzyme assays of the lysine pathway see also in ANNEX VII.

ANNEX XII

List of persons met by the expert during preparation of the Technical Report in Ulan Bator (Sept 7-30, 1989)

Mrs. D. Elez	UNDP Assistant Resident Representative
Mrs. S. Solongo	UNDP Project Officer
Corr.member MoAS	Vicepresident of MoAS
D. Badga, DSc	Director of the Institute of Chemistry MoAS
Dr. J. Batsuur, DSc	Director of the Institute of Biotechnology MoAS
Dr. B. Dashnyam	Scientific Secretary of the Institute of Biotechnology MoAS Chief of the section of molecular genetics
Dr. T. Puntsag, DSc	Chief of the section of microbiology, Inst. Biotechnol. MoAS
Dr. G. Urantsooj	Head of the laboratory of microbial synthesis, Inst. Biotechnol. MoAS
Dr. Cieceg	Head of the laboratory of biochemistry and physiology of soil microorganisms, Inst. Biotechnol. MoAS
Dr. D. Badamochir	Head of the plant cell and tissue culture laboratory, Inst. Biotechnol. MoAS
L. Sandorj	Section of microbiology, Inst.Biotech.MoAS
B. Badrakh	dtto
D. Naranchimeg	dtto
Ch. Dulamsuren	dtto
T. Bold	dtto.
G. Dorj	dtto
D. Tserendulam	dtto
Dr. C. Dash	Scientific Secretary of the Institute of Chemistry MoAS
Dr. Tc. Zhanchiv, DSc	Director of the Institute of General and Experimental Biology MoAS

JUSTIFICATION FOR EQUIPMENT PROCUREMENT

In order to start reseach into physiology of formation of feed additives and other microbial products with biological effects, sophisticated equipment is necessary for separation and determination of mcg amounts of metabolic intermediates and final products. For this purpose the High performance liquid chromatography (HPLC) apparatus is generally used. At present, no equipment of this or similar type is available at the Institute of Biotechnology MoAs which is in charge of the project realization.

It is reasonable to expect that the HPLC apparatus will progressively foster and speed up both the elaboration of improved technologies for the feed additive production and research into rational regulation of formation of microbial metabolites.

Therefore, the procurement of the HPLC apparatus would substancially increase the project activities and enable very advanced scientific research to be carried out. This trend reflects the need for the development of biotechnology in Mongolia and application of its experimental results in practice.