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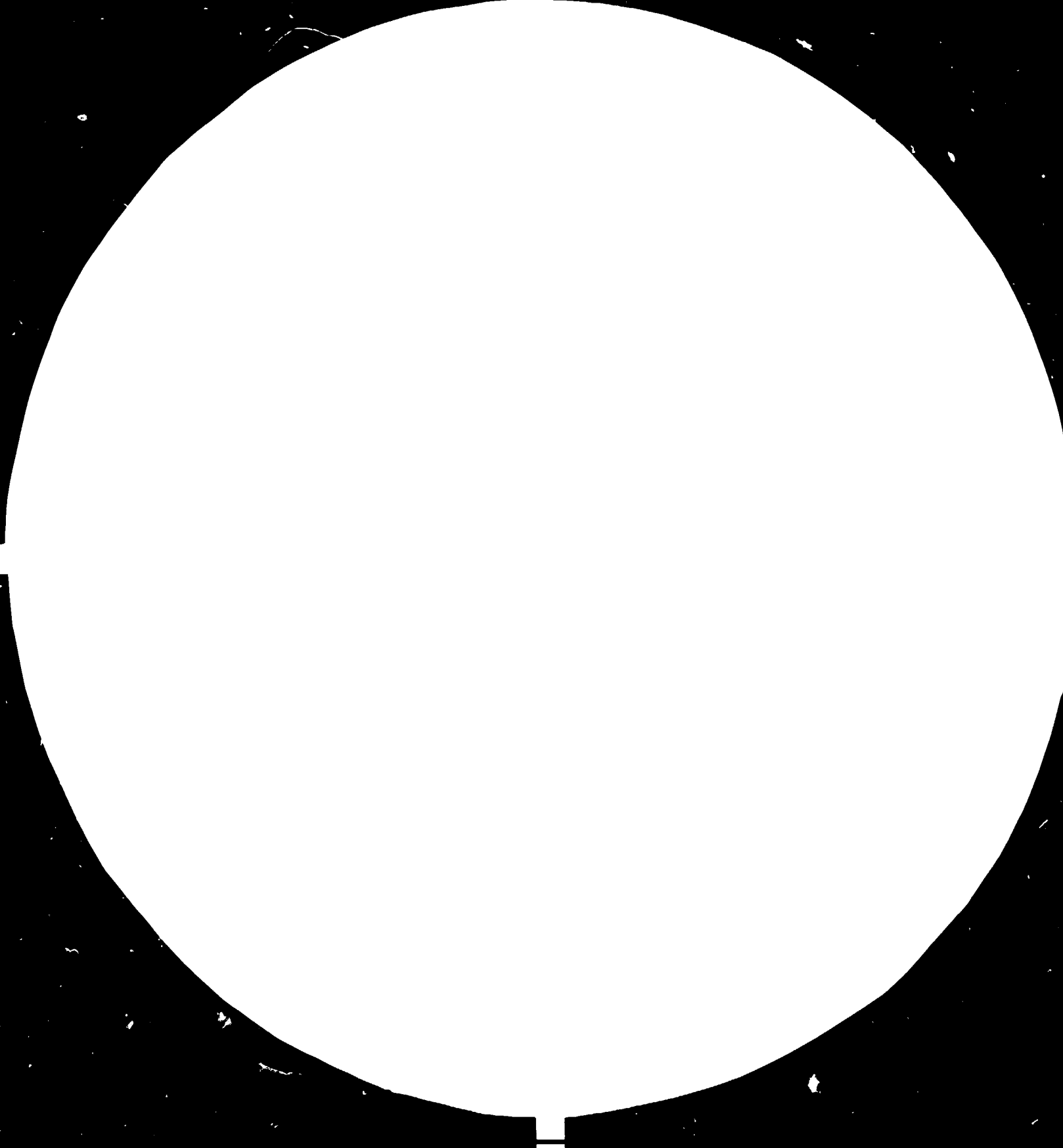

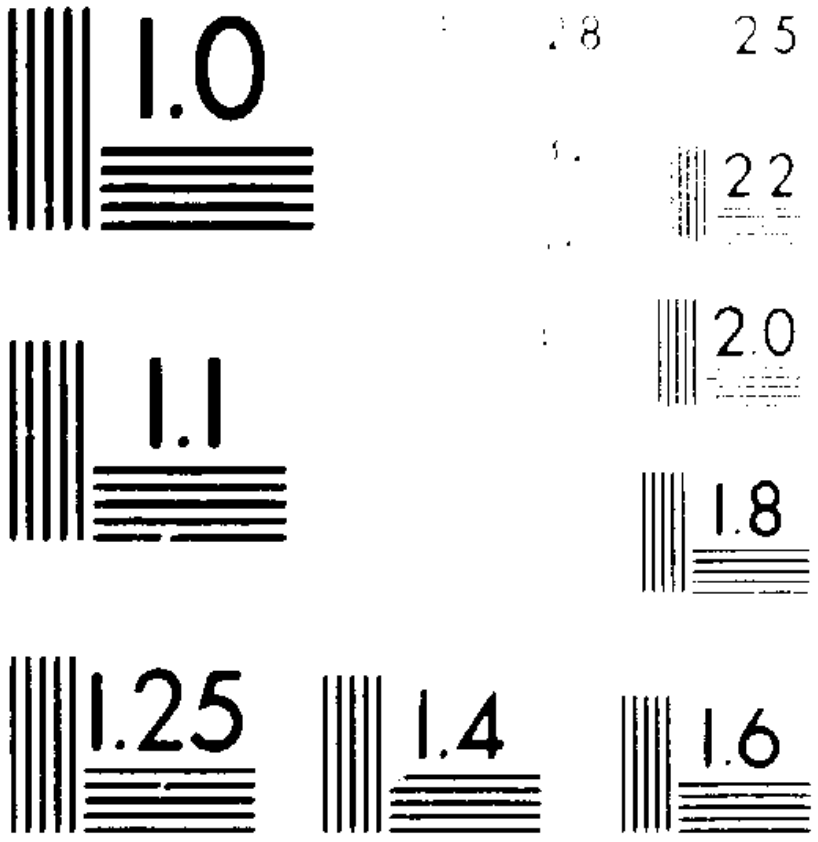

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BIOSCIENCE AND ENGINEERING.

DP/IND/80/003

INDIA

Technical Report*

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

Based on the work of Yu-Yen Linko,

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Cellulolytic Enzymes and Ethanol Production by Immobilized Cells

United Nations Industrial Development Organization
Vienna

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ABSTRACT

The project Bioscience and Engineering DP/IND/80/003 has been reviewed on the subjects concerning cellulolytic enzymes and ethanol production with immobilized yeast. Comments and recommendations for further research and development are reported here.

One of the immediate objectives, development of an ethanol process based upon immobilized microbial cell reactor has progressed well according to schedule, and biggest ethanol producer in the country has shown great interest in the technology and is willing to set up pilot plant or prototype plant in the factory in cooperation with NCL. This is very encouraging since most alcohol producers in the world have "wait and see" attitude to wait for the first ethanol plant to be set up. In the area of cellulolytic enzymes, NCl scientists have isolated and improved the strain Penicillium funiculosum which has the highest activity so far reported in one of the enzymes β-glucosidase. With the skill of scientists, further improvement is possible, and make bioconversion of cellulose a step nearer to reality.

Because of the UNIDO project, Laboratory is now well equipped for carrying out biotechnology research, except for difficulties in obtaining spareparts and repairing. Overall impression is that the project is progressing satisfactorily with good results.

INTRODUCTION

Project background

The objective of the UNDP/UNIDO project DP/IND/80/003 Bioscience and Engineering is to strengthen the expertise and research facilities at the National Chemical Laboratory (NCL) for the development of biotechnologies for exploitation of ligno-cellulosic resources and of technologies for controlled release pesticide formulation. The five year project is divided into four areas:

(1) Development of a fermentation process for the production of microbial biomass products from cellulosic materials.

(2) Development of an enzymatic process for hydrolysis of cellulose to glucose.

(3) Development of a process for glucose conversion to etnanol based on immobilized microbial whole cells.

(4) Development of processes for the production of controlled release pesticides by microencapsulation and monolithic and matrix binding.

Purpose of the mission

The purpose of this mission was to review the R&D work carried out at NCL in the field of cellulolytic enzymes and ethanol production by immobilized cells, give advice and guidance on general methodology and specific techniques in order for the R&D programme to achieve the goals set out in the project document and within the time frame indicated in the project document, and prepare a report on finding and recommendation for future work programme.

PROCEDURES

Two technical reports on project were supplied by UNIDO after signing of Special Service Agreement. This facilitated the review of relevant NCL publications before arriving in India. On arrival at NCL, the meeting with Director, Dr. L.K. Doraiswamy was arranged immediately and again on the last day of the mission. After the project description by Dr. C. SivaRaman and Dr. M.C. Srinivasan, visits of individual scientists in Biochemistry, Chemical Engineering including Biochemical Engineering, and Process Development divisions were arranged to obtain overall impression of the expertise and facilities at NCL for biotechnology research. After that most of the time were used for interviewing individual scientists of the groups involved in the project and discussing the problems being encountered in their work.

I was asked to give two seminars at NCL Auditorium, which were attended by scientists from NCL, MACS and the University of Poona. The topics and dates were:

- Immobilized Biocatalyst Technology Research at Helsinki University of Technology. 10.2.1984.
- Current status of immobilized microbial cell technology for ethanol production. 13.2.1984.

A seminar was also held at Hidustan Antibiotics Ltd., Pimpri, Poona on 14.2.1984.

GENERAL COMMENTS

NCL under the guidance of the Director, Dr. L.K. Doraiswamy is a dynamic research institute with great number of highly qualified scientists and many remarkable achievements. The excellence of the Laboratory was evident as it has been

able to attract distinguished Indian scientists to return from abroad. Its organization is ideally suited for Biotechnology Research which is multidisciplinary in nature. Biochemistry division includes among others an enzyme chemistry group, a microbiology group and a genetic engineering group. After bench development the process can be continued to be developed by Biochemical Engineering group in Chemical Engineering division and Process Development Division for optimization, modelling, economic evaluation and process design. The close interactions of different groups in various disciplines will greatly facilitate the completion of the project. It is my feeling that communication among the different groups in the project should be closer at each phase of research and development so that each expertise can be utilized to maximum advantage. For instance, for alcohol production with immobilized yeast, the process is similar to heterogenous catalysis. Expert in catalysis group and polymer group could give some ideas as to types and properties of supports and the reactor engineering group on catalyst support suitability in reactor performance to prevent encounter of unexpected problems during pilot tests.

NCL is today well equipped for biotechnology research, which is one of the objectives of the project. However, once an instrument is out of order, the repairwork and replacement of parts are difficult to obtain, even though there is a $ 5000 annual allocation for urgent purchasing of replacement parts and chemicals. This fund could not be utilized at all because field purchase facilities have not been made available to the National Project Director, resulting in wasteful delays in the project.

Training of scientists abroad has been progressing well. This year, four scientists will be trained at the laboratories of prof. L.T. Fan (Kansas State University), prof. Rogers (The University of New South Wales), prof. Moo-Young (University of Waterloo) and Dr. Jeffries (Forest Products Research Lab.). UNDP trainee programme is valuable because it makes possible for choosing an institute of excellence for training since no financial burden is involved in accepting a trainee. Similarly, funding of consultations accelerate greatly the progress of research. There was a constant flow of scientists supported by various organizations visiting NCL during my stay in India, due to the 7th Biotechnology symposium held in Delhi, and it was stimulating and useful to have interactions with professors Holland, Rogers, Bungay, and Moo-Young, and Dr. Jeffries and Dr. Gray and others. Both NCL and I had the privilege of such a beneficial opportunity.

REVIEW OF THE PRESENT STATE OF STUDIES

Cellulolytic enzymes

Two promising fungi *Penicillium funiculosum* and *Sclerotium rolfsii* were isolated during extensive screening of several hundred cultures from various sources.

The shake flask study showed that both fungi produced in addition to extracellular endo- and exo-β-D glucanases, high extracellular β-glucosidase. In comparison to most studied *Trichoderma reesei*, *P. funiculosum* and *S. rolfsii* produced ten times higher β-glucosidase activity extracellularly. This is very important for cellulose hydrolysis because it prevents the accumulation of inhibitory cellobiose. However, exo- and endo-β-D-glucanase activities were lower than with the best *T. reesei* cultures. In comparison with *T. reesei* QM9414 cellulases, same degree of saccharification was reached

employing same unit of exo-β-D-glucanase activity, even though endo-β-D-glucanase activity of QM9414 is 2-3 times higher. In summary, although the extracellular β-glucosidase activities achieved with the new strains were significantly higher than reported elsewhere, the exo-β-D-glucanase and end-β-D-glucanase activities were below such strains as QM9414 or Rutgers C. 30.

With a wild strain of P. funiculosum grown in instrumented fermentor employing high concentration of cellulose in the medium, exo-, endo-β-D-glucanases and β-glucosidase activities obtained were 3-3.5, 18-30 and 9-13 units/ml, respectively, which is quite high in extracellular β-glucosidase activity and reasonably good in exo-β-D-glucanase activity.

Further strain improvement by mutation, and optimization of culture condition resulted in a hypersecretive mutant Cu-1 with extracellular β-glucosidase activity of 30-36 IU/ml, which is among the highest reported for fungi.

Similarly, a S. rolfsii mutant has been isolated and the enzymes purified and characterized.

In addition, the performance of P. funiculosum cellulase on alkali-treated cellulose powder and bagasse for simultaneous saccharification and fermentation with free and immobilized yeast cells have been carried out.

Further two strains of Neurospora crassa have also been used for direct fermentation of alkali-treated cellulose powder and bagasse to ethanol, utilizing both cellulose and hemicellulose.

P. funiculosum cellulases have been immobilized on a soluble polymer, poly(vinyl alcohol) using carbodiimide, resulting in 90 % activity yield. The bound enzyme catalyzed the hydrolysis of alkali-treated bagasse with a greater efficiency than free cellulase. The immobilized enzyme could be used for 3 times. Similarly, β-glucosidase from P. funiculosum was immobilized on polyamide by glutaraldehyde and reused for 6 times without loss of activity. Also total mycelial biomass of P. Funiculosum (48 h growth) was succesfully used for saccharification of alkali-treated bagasse and 56 % saccharification was obtained in 48 h. The rate of hydrolysis was equivalent to that of 14 day-old culture filtrates.

Ethanol production by immobilized yeast

Ethanol- and substrate-tolerant yeasts were isolated and a method developed for production of yeast in a 100 l fermenter. In order to increase ethanol tolerance, addition of whole hen egg yolk to the fermentation medium have been tried in batch and continuous process, while promising results were obtained in a batch operation but not in a continuous operation.

Yeast cells were immobilized in open pore gelatin matrix developed at NCL, and used in a column reactor for continuous conversion of cane molasses and sweet sorghum juice with reported good productivity. Operational properties of column fermentors with gelatin immobilized yeast have been studied in detail. However, during the long term stability study employing a 10 l ethanol/day column, some operational problems were encountered. Agar

immobilized yeast cells were also tested, but difficulties in preparing agar bead with existing apparatus and poor operational stability was observed. Operational performance declined drastically after 8 days, and the activity was not restored by washing the column.

In order to decrease polution problems in ethanol production, studies on recycling spent wash for dilution of concentrated molasses feed stock, and on biomethanation of distillery stillage have been initiated.

COMMENTS

In India petrochemicals such as acetic acid, acetic anhydride, styrene, synthetic rubber, polyethylene, butylacetate, and PVC are also produced from fermentation alcohol. Molasses is the most important source for ethanol by fermentation, with installed capacity of 900 million liters of alcohol and an out-put of 600 million liters. Approximately 60-65 % ethanol produced is used for the chemical industry.

Alcohol is an important renewable feedstock, which in India, is usually fermented in open vessels at 141 distilleries with the existing efficiency of 210-220 l alcohol/tonne molasses. In open vessel, a significant quantity of ethanol is lost with CO_2. Technological improvements are urgently needed to increase ethanol yield to about 250 l ethanol/ tonne molasses. The immobilized yeast system for producing ethanol could be the most promising and reachable technology for improvement. During my discussion session with Dr. Srinivasan at NCL, Dr. H.C. Bhandari, project manager of D.C.M. Sugar Division came to visit Dr. Srinivasan, saying that he has heard about NCL's research on ethanol production with immobilized yeast. He was very interested

in the technology and prospects of improving Daurala distillery's ethanol production which today has a capacity of 45 000 kl/year (production 50 000 l/day), the largest in India. Daurala distillery was established in 1943, the first distillery to be installed in India with totally in-company design, and since then new technologies have been introduced in the plant such as energy conserving distillation columns with the scientific application of waste heat recovery systems, modern technology for the recovery and recycling of yeast, etc to increase overall efficiency. Therefore it is natural that the company is interested in new immobilized yeast technology. Dr. Bhandari attended my seminar held at NCl, and invited me to visit the Daurala plant. During the 7th International Biotechnology Symposium in New Delhi the following week, I had the opportunity to discuss the subject further with him, and a meeting was held with Dr. N.G. Karanth, NCL., Dr. Bhandari, D.C.M., prof. P. Linko, myself, and Dr. N. Vaidyanathan, general manager, D.C.M. Sugar Division. Visit to Daurala Sugar Works (100 km from Delhi) was made with Dr. Karanth, prof. P. Linko, myself and Dr. Bhandari during the symposium week.

I am convinced that cooperation between NCL and D.C.M. sugars could be fruitful and I shall be most happy to assist in anyway I can. Dr. Bhandari expressed his willingness to set up a pilot plant or anysize of prototype plant at the Daurala distillery and to provide the personnel and other assistance for duration of such experimental period. At this stage of research at NCL, when the 100 l ethanol/day pilot plant is in the planning, the interest shown by the biggest and most modern distillery in India to come forward in asking for cooperation and willingness to try out NCL developed technology is most valuable and encouraging from the UNDP project point of view.

RECOMMENDATIONS

In biotechnology of cellulose utilization, several problems have still to be solved such as more efficient economic pretreatment of lignocellulosic raw materials for increasing reactivity, higher enzyme activities and productivity of cellulose complex production, total utilization of the lignocellulose components through pentose fermentation, by-product processing, etc.

P. funiculosum cellulases have an advantage in the high extracellular β-glucosidase activity. Mutations and improvements of strain should be continued.

In this project, several substrates have been used such as rice straw, bagasse, etc. Since cellulose conversion processes are species dependent, it is important at this stage to identify realistically one potential feasible source of cellulose. Although 60 million tonnes of rice straw is produced annually, much is used for building houses. How much can really be collected economically for bioconversion? Same applies to bagasse, of which 52 million tonnes are produced annually, but how much would be in practice available for bioconversion? Much is today burned for energy production in sugar factories.

Enzyme production can be accelerated in continuous culture or fed batch culture as compared to conventional batch process. Therefore such work should be carried out either by P. funiculosum or in comparison with employing immobilized growing cells in continuous or repeated batch processes. An immobilized cell process could be feasible because P. funiculosum cellulases are all extracellular.

May be this is an interesting research topic for a trainee abroad. According to our experience in producing an α-amylase and glucoamylase mixture, immobilized Aspergilus niger spores are first grown in a growth media for cell mass, then transferred to enzyme producing media containing solid indurer to obtain very high enzyme activities in shorter time as compared with conventional fermentation. This principle may also be applied to cellulose production. The pH, nutrient composition and concentration, etc. will also affect the activity obtained.

John and E. Sturge Ltd. produces cellulases by Penicillium funiculosum. It would be very interesting to compare their activities with NCL's.

Several cellulose pretreatment methods have been used during the course of the project. It is important to critically review the results and pick up the most efficient and realistic method of pretreatment with economic and engineering aspects of scale up in mind. A promising pretreatment could be a steam explosion type treatment. Water soluble xylans and xylose could be separately or simultaneously utilized. Xylose isomerase from Chainia sp. isolated at NCL can convert xylose to xylulose, which yeast can further convert to ethanol. This enzyme is attractive because it is extracellular and no Co^{++} is necessary for isomerization. If enzyme can be modified to have a lower pH optimum, it may be an important spin-off result for fructose syrup production.

Instead of immobilizing β-glucosidase with soluble support, and to recover immobilized enzyme from reaction mixture by ultrafiltration for reuse, a more attractive method would

appear to be using an ultrafiltration reactor employing suitable membrance cut off for retaining cellulases and to pass glucose through. But even so, productivity may still be too low and concentration polarization too high.

I recommend work on immobilization of P. funiculosum mycelial biomass with glutaraldehyde and a plastisizer to extrude as pellets for reuse. It may prolong the bio-catalyst life-time and thus be more economical. In our experience with an immobilized cellulase by cross-linking method, the hydrolysis was more efficient than that with free cellulase. NCL results with polyamide immobilized cellulase are in agreement with our results.

Alternative stable immobilized yeast systems are urgently needed, due to the operational problems encountered with the gelatin immobilized yeast in a 10 l ethanol/day column fermentor, which were not observed in the laboratory scale operation. I was told that each consultant suggested a different method of immobilization, such as flocculation of yeast, immobilization on wood chips, etc. I suggested Ca-alginate beads or porous alumina beads (obtained from the NCL catalysis group). According to our several years of experience on ethanol production by immobilized yeast, alginate bead immobilized yeast gave best results in long term operational stability, productivity and ease of preparation. Experiments were initiated during my stay at NCL using alginate immobilized yeast column and porous alumina adsorbed yeast column in addition to flocculant yeast column already in operation. Ca-alginate beads are very sensitive to the presence of phosphate ions which dissolve the beads. Therefore the molasses should be first analyzed for Ca^{++} and phosphate content. If too much

phosphate is present in molasses, beads will swell and disintegrate. There was no problem in operational stability with the Cuban molasseswe have used. Another reason in favor of recommending alginate beads is that Kyowa Hakko Kogyo Co, Ltd. Japan, has just concluded a MITI supported ethanol project employing Ca-alginate immobilized yeast for ethanol production in pilot columns with a total bed volume of 4000 l, and demonstration plant of 20 000 l column has been set up since April, 1983. This is the largest scale so far tested using an immobilized system in ethanol production and it is claimed to have 6 months continuous operational stability, which we have also proved in our laboratory scale experiments.

For maintaining yeast cell viability and growing during operation, small aeration ($\frac{1}{10}$ column volume per minute) to column is essential and addition of unsaturated fatty acid and/or a sterol to feed stream is of advantage and thus should be tested for the NCL yeast strain.

For 100 l ethanol/day pilot scale, 2 separate columns in series are recommended in order to obtain high conversion yield. Residence time in the column should not be the shortest possible since inactivation is faster at shorter residence times. Usually a compromise of 3-5 h residence time is suitable.

It is recommended that the tolerance of the NCL yeast strain to low pH ~ 4 is studied. In order to prevent contamination, the pH of the fermentation should be as low as possible, at least 4.

The flocculation of yeast might be attractive if it works in large scale since no support is needed, and it could be

more economical. I would like to suggest to construct two more 10 l/day columns and try out Ca-alginate yeast bead, alumina adsorbed yeast and flocculated yeast, respectively to save time , since testing one system takes long time.

To develop an economic, simple and efficient molasses pretreatment is a difficult task. I recommend that NCL contacts DCM Sugar to find out if the new DCM process could be compared with NCL technology. It is important to minimize precipitation in the biocatalyst reactor.

An integrated ethanol production system should be designed. Anaerobic digestion of distillation stillage has the advantages of effluent treatment, producing fertilizer and methane fermentation for process fuel. However the high salt content in stillage may cause problems. A combination of heat pump for energy cost saving and anaerobic fermentation of stillage should be considered in developing a complete integrated ethanol production system.

After successful pilot tests with the NCL 100 l ethanol/ day columns for about 3 months, 1000 l/day columns could be built in D.C.M. factory for further testing in cooperation with NCL.

CONCLUSION

NCL has highly qualified research groups for carrying out the project. Scientific quality and quantity of publications from the last few years are impressive, and methods used are innovative. No doubt the results obtained during the project's duration will increase the knowledge in biotechnological utilization of cellulose.

As for the ethanol production with immobilized cells, possibility of success is visualized as targeted and it could have an impact on improving the alcohol fermentation industry and entire alcohol-based chemical industry in India. The objective of the ethanol project is to obtain information for designing a commercial plant producing 45 000 l/d, which equals the production figure of Daurala plant. Therefore constructing a 1000 l ethanol/d system at Daurala near the end of the project will be very useful.

The UNDP project certainly makes NCL one of the best equipped laboratories to carry out biotechnology research.

International contacts of scientists are good, and cooperation with foreign institutes can be further built up through the trainee programme.

In all, the project is in good hands of the Director Dr. Doraiswamy, Dr. SivaRaman, Dr. Srinivasan, Dr.(Mrs.) SivaRaman, Dr. Karanth and Dr. Venkitakvishnan.

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