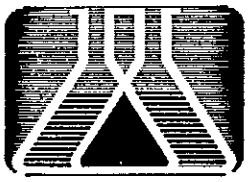

JICA - BIOTECH

joint study project

1986

Annual Technical Report

The National Institutes of Biotechnology and Applied Microbiology (BIOTECH) is a research and development unit of the University of the Philippines at Los Baños. It is located on a 77.5 hectare Agriculture and Life Sciences Complex.

BIOTECH is mandated to develop technology for microbial-based industries.

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Introduction

The potential use of locally produced alcohol fuels to reduce the burden of foreign exchange payments for petroleum products can be harnessed under a national alcohol program through research in biotechnology.

The Philippines contains some essential biotechnological ingredients that could be utilized in the development of an alcohol fermentation technology. It has an abundant supply of cheap agricultural materials for the production of alcohol. It has also a vast diversity of microorganisms which can be engineered to include or enhance desirable characteristics for increased performance and productivity.

The JICA-BIOTECH Joint Study Project on Biofuels aims to promote the goals of the national alcohol program through applied research in biotechnology. Three areas were identified including a component of waste treatment studies for the alcohol fermentation industry.

These studies are categorized as follows:

Study 1: Isolation and genetic improvement of yeast for alcohol production

Study 2: Large-scale production of amylolytic enzymes capable of saccharifying raw starch

Study 3: Microbial decolorization of fermentation and distillery wastes including biogas production

- Substudy 3A: Biogas production from distillery slops
- Substudy 3B: Microbial decolorization of distillery slops

Study 1: **Isolation and Genetic Improvement of Yeast for Alcohol Production**

NESTOR R. APUYA
TAKASHI YAMAKAWA and TORU KODAMA (The University of Tokyo)

Local distilleries presently use inferior yeast strains which have low alcohol production and sensitivity to elevated temperature (greater than 40°C) and low pH. This results in very low fermentation efficiencies and low volumetric productivities of these distilleries. The use of improved yeast strains is expected to substantially reduce the cost of ethanol production by Philippine distilleries.

The objectives of the study are: a) to isolate yeast strains with desirable characteristics, especially high alcohol productivity, heat tolerance, and acid tolerance, and b) to study genetic improvement of yeast in order to obtain strains of heat tolerance and high alcohol productivity.

Study 2: Large- scale Production of Amylolytic Enzymes Capable of Saccharifying Raw Starch

BERNADETTE C. MENDOZA and MA. AUSSIELITA L. LIT
YOSHIKI TANI (The University of Kyoto)

The Philippines has several raw materials for ethanol production which may be classified as saccharine, starchy, and cellulosic. At present, large-scale production of ethyl alcohol is based on saccharine sources, largely sugarcane-based. The utilization of starchy materials as alternative sources of ethanol is constrained by such factors as lack of local industrial experience and technology. In addition, the conventional process employs a costly and cumbersome procedure for starchy materials.

The joint study project on the large-scale production of raw starch digestive-amylase eliminates the need for such uneconomical step by the isolation of fungal strains able to digest raw or uncooked starch. Specifically, the main objectives are: 1) to isolate and screen local fungal strains which are capable of saccharifying raw starch, 2) to decide optimum culture conditions and 3) to produce the enzyme on a large-scale basis.

Study 3: Microbial Decolorization of Alcohol Fermentation and Distillery Wastes Including Biogas Production

Molasses alcohol slop, the residue from molasses alcohol distillery is one of the most difficult wastes to dispose of. It is generated at quantities approximately 10 times the volume of alcohol produced, and has exceptionally high BOD. It is acidic, has deep dark brown color and has a near boiling point temperature at discharge. The high pollution load of this waste on the water environment necessitates the development of a treatment system for the alcohol fermentation industry.

The JICA-BIOTECH Joint Study Project on Biofuels has added the "environmental" component through Study 3. Research focus is on the use of biological treatment methods to remove the prganic matter content (as BOD or COD) and color. The general objective of the study is to develop practical techniques for the treatment of alcohol fermentation wastes through microbial decolorization and thermophilic anaerobic digestion.

The specific objectives of each of the 2 sub-studies are listed below:

Substudy 3A: **Biogas Production From Distillery Slops**

ELIZABETH BUGANTE
SUSUMU OI (The Osaka City University)

a) to find ways to maximize gas production and increase the methane content from slop waste using selected methanogenic sludge.
b) to isolate and characterize promising strains of acidogenic and methanogenic microorganisms from sludge samples.

Substudy 3B: **Microbial Decolorization of Distillery Slops**

MA. LUZ F. PAJE
KIYOMOTO UEDA (Resident JICA Expert)

a) to isolate agar bacteria from soil sample
b) to screen the isolates for decolorization in conjunction with biogas production.

JICA-BIOTECH JOINT STUDY PROJECT ON NITROGEN FIXATION

The Philippines at present is in the process of extensive economic recovery due to huge foreign debts and shortage of dollar reserves. A very important step towards a fruitful recovery is the improvement of agriculture and forestry. However, since the total land area devoted to agriculture and forestry is not getting any larger, our scientists are confronted with one serious task — increase production by the use of fertilizers and pesticides. It is clear enough that our country cannot afford imported chemical fertilizers. It was projected that by 1988, the demand for urea will increase by 49%. This means that we shall need ₱1.92 to ₱3.72 B to meet the demand of our farmers. Only 30% of this demand could be produced locally while the rest has to be imported. The Philippine government acquired a loan amounting to $260 M in 1984, primarily for fertilizer importation. The present conditions leave no option but to look for alternative fertilizers.

The country is blessed with a rich supply of useful soil micro-organisms like the rhizobia. The association of rhizobia and legume results into a symbiotic nitrogen-fixation which could be utilized as an alternative to expensive chemical fertilizers. The use of rhizobia as microbial inoculant holds great promise. The production of microbial inoculants however, is possible without the necessary basic researches dealing on biological factors involving nitrogen-fixation. The JICA-BIOTECH joint study on nitrogen-fixation was conceived in this

direction. Five major study areas were identified with a supplementary study on genetic engineering as applied to nitrogen-fixation:

Study 1: Isolation, classification and screening of bacterial strains for high nitrogen-fixation
Study 2: Studies on conditions for mass production of rhizobial inoculants
Study 3: Assay techniques for nitrogen-fixation measurements
Study 4: Development of process for preserving N-fixing bacteria

Study 1: Isolation, Classification and Screening of Bacterial Strains for High Nitrogen-Fixation

ROSARIO G. MONSALUD and GLORIA D. REYES
SHIRO HIGASHI (The University of Kagoshima)
YOSUKE MINO (Obihiro University of Agriculture and Veterinary Medicine)

Nitrogen fixation research covers the various biological systems which could be beneficial to plants. One particular system of great importance is the rhizobium legume symbiosis.

The ultimate goal of research in this area is to discover and develop effective rhizobia which could be used as inoculants to a wide variety of crops or which are tolerant to a wider range of conditions. This would need first the identification and classification of rhizobial isolates, and screening them under greenhouse and field conditions.

The first study under the JICA-BIOTECH Joint Project on Nitrogen Fixation deals with the isolation and characterization of rhizobial strains from different locations in the country and screening them for nitrogen fixing ability.

Study 2: Studies on Conditions for Mass Production of Rhizobial Inoculants

IRENEO J. MANGUIAT, ANGELA DE LA CRUZ, and VIRGINIA PADILLA
YOSUKE MINO and TOSHIKAZU TAKAHASHI
(Agricultural Research Institute of Tokachi Nokyoren)

Mass production of rhizobial inoculants is hampered by the high cost of chemicals used for making the traditional medium, i.e., yeast extract mannitol broth (YEMB). It is therefore necessary that a substitute medium which is both locally available and inexpensive be used in mass-inoculant production. The most likely substitute for YEMB which could satisfy these criteria is coconut water. Consid-

ering the tremendous volume of coconut water disposed by our coconut processing industries which is over 1.2 M tons in 1981, the use of coconut water as a growth medium for rhizobia will not only lower production costs but may also help solve our pollution problems. In 1985, researchers at BIOTECH reported the successful use of coconut water as a medium for growing rhizobia. However, different strains of rhizobia have different nutritional requirements. It is very important therefore to know if our promising strains could also grow on coconut water as well as or better than they do on the traditional medium (YEMB) (Conducted in 1985).

However, it is also necessary to continue searching for alternative growth media using locally available substrates.

It is likewise important to conduct studies on the optimization of culture conditions for the growth of promising rhizobial strains. The most important physico-chemical factors which should be studied are temperature, pH and dissolved oxygen concentration.

The 1986 study aims to determine the optimum pH, temperature and oxygen requirements necessary for the growth of promising rhizobial strains from agro-forest legumes and to determine the possibility of using modified YEMB as growth medium for these isolates.

Study 3: Development of Assay Techniques for Nitrogen Fixation Measurements

JUANITA C. MAMARIL and PABLITO SANDOVAL
TOMIO YOSHIDA and TOSHIKAZU TAKAHASHI
(The University of Tsukuba)

The rhizobium-legume symbiosis fixes about 35 million tons of nitrogen annually. Despite these huge figures, however, the benefits which may be derived from the association have only been partially explored.

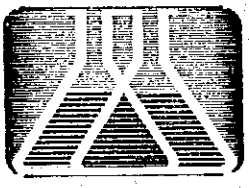
High on the priority list of legumes with great potential is *Leucaena* sp. or ipil-ipil because of its possible role in the improvement of soil fertility, pasture development, reforestation and generation of electric power.

A successful rhizobium-legume symbiosis requires the presence of an effective strain of rhizobia i.e., bacteria capable of fixing nitrogen in the root nodule of the host plant, and a suitable soil environment that will favor the growth of both the bacteria and the host plant. Many types of soil do not contain the power nodule bacteria to bring about nitrogen fixation and the successful growth of legumes. This problem may be solved by inoculating the host plant with the proper strain of rhizobia. This study was thus conducted to determine the nitrogen fixing ability of certain rhizobial strains and to test their effectiveness on ipil-ipil under field conditions.

Study 4: Development of Processes for Preserving Nitrogen-Fixing Bacteria

FE TORRES
TOSHIKAZU TAKAHASHI and SHIRO HIGASHI

One indispensable aspect in studying nitrogen-fixing microorganisms particularly rhizobia is the maintenance and preservation of pure cultures. Two of the recommended methods by which this can be done are by freeze drying or by storing cultures on porcelain available. On the other hand, the latter method is faster and allows for easy access to the stored culture. Contamination and mutations resulting from maintaining the cultures in agar slants can also be overcome by this technique. Furthermore, the method offers the advantage of extending the survival of rhizobia over long periods even at room temperature. One drawback however is that these beads are not locally available. The suitability of volcanic cinder and perlite as alternative carriers for rhizobia was therefore investigated. (Conducted in 1985).



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BIOFUELS

STUDY 2: LARGE-SCALE PRODUCTION OF AMYLOLYTIC ENZYMES CAPABLE OF SACCHARIFYING RAW STARCH

SUB-STUDY 2A: LARGE-SCALE PRODUCTION OF RAW STARCH DIGESTIVE GLUCOAMYLASE: SCREENING, SELECTION AND OPTIMIZATION OF CULTURAL CONDITIONS FOR GLUCOAMYLASE PRODUCTION

ABSTRACT

Six local fungal isolates were tested for glucoamylase production in comparison with *Rhizopus* sp. 46, *Rhizopus* sp. 62 and *Aspergillus* sp. N2 in liquid and solid culture media using different types of substrate. Isolate 432, which belongs to the genus *Aspergillus* was selected as the best strain for glucoamylase production in liquid culture medium. The cultural conditions for maximum enzyme production in terms of the physiological and nutritional requirements were studied. Maximum enzyme production was attained when a mixture of 0.5% rice bran and 1.0% raw cassava starch was used as substrate and supplemented with 0.1 to 0.5% peptone, 0.2% malt extract, 0.5% $MgSO_4.7H_2O$ and 0.1% KH_2PO_4, pH 4.0. Cultivation was carried out at 30°C under reciprocal shaking with 110 rpm for 72 to 96 h. Using the optimum conditions, glucoamylase production in a 2.0-L fermentor was conducted at different agitation speeds. Higher enzyme production was obtained at 300 rpm.

INTRODUCTION

Starch and starch-containing materials are of key importance in the food and chemical industries being an excellent source of glucose for the fermentation process. Cassava (*Manihot esculenta* Cranzt), one of the major agro-industrial crops produced worldwide, contains 25 to 32% starch. Due to the high starch content, cassava has attracted much attention as a starting raw material for the food and fermentation industries.

The utilization of starchy materials as energy source conventionally employs the cooking of raw starch before enzymatic hydrolysis. The cooking step renders the process of ethanol production from raw starch to be more uneconomical and cumbersome. Recently, the elimination of such cooking step has been developed and made possible through the use of glucoamylase preparation from bacteria and fungi. These microbial enzyme preparations have been widely used in the industrial conversion of starch into useful products such as acetone, butanol and ethanol, which in turn can be used as direct energy sources or as industrial chemicals.

This research work was conducted from October 31, 1986 to January 15, 1987. Partial results of the entire research work during the training is hereby presented.

The amylase enzyme which catalyzes the complete conversion of starch into D-glucose units is glucoamylase, which is also known by other names such as amyloglucosidase, fungal amylase, gamma amylase and glucase. Unlike the alpha-amylase (1,4-alpha-D-glucan glucanohydrolase), which randomly cleaves and fragments the starch molecule at the alpha-D 1,4 linkages, or the beta-amylase (1,4-alpha-D glucan maltohydrolase), which catalyzes the hydrolysis of the maltose units from the non-reducing end of the substrate, glucoamylase is able to attack the branched alpha-D-1,6, as well as the alpha-1,4 and alpha-1,3 linkages to a complete degradation of starch to glucose (6). Glucoamylase can, therefore, act on a wide variety of carbohydrate substances including starch, amylopectin, amylose, amylodextrin, dextrins and glycogen. The broad specificity of this enzyme may, however, be partly due to contamination by other amylases (12, 22).

Glucoamylase is mostly derived from microorganisms, usually from the fungal species of *Aspergillus* and *Rhizopus* (23). Among the *aspergilli* group, strong activity has been detected in *A. niger* (21), *A. phoenicis* (9), *A. awamori* (4, 17), and *A. oryzae* (7, 10, 22). Glucoamylase in the *Rhizopus* species has also been documented (17). Other potential sources of this fungal amylase are *Penicillium oxalicum* (20), *Mucor rouxianus* (19), *Schizophyllum commune* (14), *Corticium rolfsii* (6), *Lentinus edodes* (1) and the yeasts such as *Saccharomyces diastaticus* (2) and *Endomycopsis* sp. (3) . In bacteria, only selected strains of *Bacillus subtilis* are employed for commercial production since this microorganism produces high levels of enzyme activity.

The method used in glucoamylase production includes the most widely used submerged culture process (14, 18) and the mold bran process using solid culture (5,21). At present, research studies have been undertaken in different countries to improve the production as well as to increase the activity of the enzyme for maximum efficiency.

The present study was undertaken to isolate a local fungal strain capable of saccharifying raw or uncooked starch efficiently. The purification and characterization of the glucoamylase and the production in large scale were also studied. The study was carried out at the Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Japan under the guidance and supervision of Professor Yoshiki Tani.

MATERIALS AND METHODS

Microorganisms. Six hundred and fifty-three isolates were screened for their glucoamylase activity. One hundred and thirty-five isolates were evaluated for the secondary screening and six fungal isolates

were selected for the tertiary screening. The enzyme activity of these six isolates were compared with that of *Rhizopus* sp. 46, *Rhizopus* sp. 62 and *Aspergillus* sp. N2 in the tertiary screening. The best strain selected belongs to the genus *Aspergillus.* The strain was designated as *Aspergillus* sp. 432 and was used throughout the experiments.

Preliminary screening. A pure culture of the fungal isolate was aseptically inoculated on the agar plate containing 2% raw cassava starch using the three point inoculation technique. The inoculated starch agar plate was incubated for 4 to 7 days at 35°C. After incubation, glucoamylase activity was checked according to the development of a clear zone (halo) at the peripheral region of each colony. The presence of such clear zone on the agar medium was taken to mean the secretion of the glucoamylase enzyme active on raw cassava starch.

Secondary screening. The selected fungal strain was allowed to grow and sporulate on a slant of malt agar (0.3% malt extract and 1.5% agar) for 5 days at ambient room temperature.Spores were harvested and inoculated at 5% (v/v) in 100 ml of liquid medium consisting of 10% raw cassava starch (commercial grade, oven sterilized at 120°C for 2 h), 0.5% $(NH_4)_2SO_4$, 0.1%, KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 0.3% yeast extract in distilled water in a 500-ml shaking flask. The pH of the medium was adjusted to 3.5. Reciprocal agitation at 110 rpm at 35°C was done for 5 days. The cultured broth was filtered and the filtrate was used as the crude enzyme preparation.

Tertiary Screening: Glucoamylase production in liquid and solid culture. Two kinds of liquid culture media were used in the experiment. The first culture medium consisted of 1% rice bran in 100 ml of the glucoamylase basal medium, pH 3.5, whereas the second liquid culture medium consisted of 5% raw cassava starch (separately sterilized) in 100 ml of glucoamylase basal medium, pH 3.5. Using 5% inoculum, each culture medium was aseptically inoculated with the fungal spores and then cultivated at 35°C with reciprocal shaking at 110 rpm.

The solid culture consisted of a mixture of 0.9 g rice bran and 0.3 g rice hull in a 30-ml Erlenmeyer flask. After autoclaving the mixture for 20 min, 1 ml sterilized glucoamylase basal medium and 0.1 ml spore suspension were added to the medium. The cultivation was carried out at 35°C for one to five days.

Preparation of the culture filtrate. The liquid broth was filtered through cotton and gauze, and the filtrate was centrifuged at 8000 x G at 5°C for 15 min. To the solid culture, 10 ml of 0.01 M potassium phosphate buffer, pH 7.0 was added and then the mixture was incubated in a rotary shaker for 30 min at room temperature. After incu-

bation, 5 ml of 0.01 M potassium phosphate buffer, pH 7.0 was added and the extract was filtered through cotton and gauze. The culture filtrate was centrifuged at 8000 x G at 5°C for 15 min. The culture filtrate obtained from both liquid and solid cultures were dialyzed against 0.01 M potassium phosphate buffer, pH 7.0, twice for 2 h. The culture filtrate obtained was used as the crude enzyme preparation.

Assay of crude glucoamylase activity. The activity of the crude preparation on both cooked and uncooked starches was determined. The reaction mixture for the assay was composed of either cooked cassava starch (1 mg/ml) or raw cassava starch (100 mg/ml), citrate phosphate buffer (50 μmoles/ml, pH 3.5), and the crude enzyme (1 ml) to make a total volume of 3 ml. Cooked cassava starch was prepared by boiling raw cassava starch at the concentration of 1 mg/ml in water bath for 15 min. The citrate phosphate buffer was prepared from 0.1 M citric acid and 0.2 M Na_2HPO_4 with the final pH of 3.5. The reaction mixture tubes were shaken at 35°C for 60 min in water bath and then 0.12 N NaOH was added to stop the reaction. Reducing sugar was determined according to the method of Nelson and Somogyi (10, 15) with glucose as the standard. One unit of enzyme activity was defined as the amount of enzyme which produces one μmole of reducing sugars (expressed as glucose) per h.

Determination of reducing sugar concentration. A 0.5 ml aliquot (0-60 μg glucose) from the reaction mixture was made to react with 0.5 ml copper solution and immersed in boiling water for 10 min. and then cooled in ice water. To this was added 0.5 ml arsenomolybdate solution and 5.0 ml distilled water. Absorbance was read at 500 nm using a Shimadzu double beam spectrophotometer UV-140-02 (for initial screening studies) and a Hitachi 220-A spectrophotometer. Blanks and standards for the standard dilution curve were likewise prepared. Reducing sugar concentration of the samples, expressed as μg glucose per ml was calculated from the curve.

Optimization of cultural conditions for glucoamylase production. The production of the glucoamylase enzyme of *Aspergillus* sp. 432 was studied under various cultural conditions such as carbon sources and concentrations, temperature, pH, addition of growth factor and aeration with respect to the volume of the basal medium. The basal medium used in these experiments was the same as that previously mentioned in the secondary screening supplemented with 0.5% rice bran and 1.0% raw cassava starch as carbon source. The experiment on the effect of agitation was carried out using the 2.0-L jar fermentor. All conditions were carried out using the above mentioned procedures except otherwise indicated.

Production of glucoamylase in jar fermentor culture. The optimum conditions for glucoamylase production were used in this exper-

iment. The inoculum for the jar fermentor culture was prepared as previously described. Fifty milliliters of the fungal spore suspension was inoculated in 1-L of the medium in a 2-L jar fermentor KMJ (Mitsuwa Rikagaku Kogyo Co., Ltd., Osaka). The cultivation was carried out at 35°C for 96 h with 200, 300 and 400 rpm agitation, and 1 vvm aeration.

RESULTS and DISCUSSION

Isolation, Screening and Selection

A total of 653 fungal cultures were isolated and acquired from different sources. One hundred and thirty-five isolates showing positive enzyme activity on raw cassava starch agar plates were screened for enzyme production using raw starch liquid culture medium. Six isolates exhibiting the highest enzyme activity were selected and further examined for enzyme production in comparison with those of *Rhizopus* sp. 62, *Rhizopus* sp. 46 and *Aspergillus* sp. N2 in both solid and liquid culture media (Table 1). Based on the overall performance of these isolates in enzyme production on different culture media, isolate 432 was superior among the isolates tested and was therefore selected as the best isolate for glucoamylase production.

Glucoamylase Production in Liquid and Solid Culture Media

The preliminary and secondary screening proved to support that the fungal isolates selected have the ability to hydrolyze raw cassava starch in liquid culture medium. Since glucoamylase production also makes use of the solid culture process, parallel experiments on glucoamylase production by these isolates were studied using both processes. Results showed that 10- to 100- fold increase in enzyme production was attained when the liquid culture process was used (Table 2). However, between raw cassava starch-liquid medium and rice bran-liquid culture medium, the latter was found to be a better substrate for enzyme production in terms of activity per gram substrate.

In the solid culture, the maximum enzyme activity was obtained after 96 h of cultivation (Table 3). Isolates 432 and 1519 gave the highest enzyme activity of 39.0 U/ml and 38.9 U/ml, respectively. In the liquid culture, where two different types of fermentation media were used, maximum enzyme activity was exhibited after 48- and 144- h of cultivation. As shown in Table 4, isolates 240 and 432 gave the highest enzyme activity of 25.0 U/ml after 144- h cultivation and 19.4 U/ml after 48- h cultivation in the rice bran-liquid culture medium. When raw cassava starch-liquid culture medium was used, the maximum enzyme activity was attained after 48- and 72- h cultivation by isolates 896 and 334. Glucoamylase activities of isolates 896 and 334 were 13.9 U/ml and 11.2 U/ml, respectively (Table 5).

Table 1. List of selected fungal isolates with the highest glucoamylase activities from the secondary screening.

Strain	Activity (units x 10^{-2} per ml culture filtrate)
896	14.0
1040	8.8
1519	8.6
334	7.2
432	7.2
240	7.1
1000	6.4
926	5.9
471	5.9
921	5.8
547	5.7
936	5.7
920	5.5

Table 2. Glucoamylase activities of crude enzyme preparation of selected fungal isolates in liquid and solid cultures using different kinds of substrates after 72- h cultivation.

Strain	Activity (units per g of solid medium) SRHRB[a]	LRB[b]	LRCS[c]
896	0.2	19.4	3.1
1040	0.2	25.0	9.4
240	0.5	26.9	17.8
432	0.3	9.4	8.7
334	0.2	7.3	18.8
1519	0.3	2.4	7.6
62	0.3	6.8	6.5
46	0.5	21.7	4.3
N2	0.2	1.7	2.9

[a] SRHRB – Solid culture, rice hull and rice bran
[b] LRB – Liquid culture, rice bran
[c] LRCS – Liquid culture, raw cassava starch

Table 3. Glucoamylase activities of crude enzyme preparations of selected fungal isolates at different hours of cultivation using rice hull-rice bran-solid culture medium.

STRAIN	Activity (units/ g of solid medium) Cultivation time (h)				
	24	48	72	96	120
896	6.4	10.4	11.3	8.6	10.4
1040	4.2	6.4	11.0	18.7	6.7
240	4.0	9.5	32.4	34.2	17.2
432	11.5	21.0	23.1	39.0	27.9
334	5.8	16.9	16.5	12.9	12.7
1519	6.8	2.7	25.2	38.9	12.9
62	23.1	21.5	24.3	34.8	25.4
46	12.7	10.6	37.8	25.8	20.0
N2	5.1	21.2	18.6	29.5	10.6

Table 4. Glucoamylase activities of crude enzyme preparations of selected fungal isolates at different hours of cultivation using rice bran-liquid culture medium.

STRAIN	Activity (units/ ml culture filtrate) Cultivation time (h)			
	24	48	72	144
896	3.1	4.6	11.6	13.1
1040	2.9	4.3	15.0	2.4
240	2.7	5.9	16.1	25.0
432	11.2	19.4	5.6	6.0
334	4.8	8.5	4.4	3.6
1519	2.1	3.1	1.4	1.0
62	4.0	2.4	4.0	2.9
46	3.9	10.7	13.0	2.4
N2	3.4	2.5	1.0	0.7

Table 5. Glucoamylase activities of crude enzyme preparations of selected fungal isolates at different hours of cultivation using raw cassava starch (5% w/v) culture medium.

STRAIN	Activity (units/ ml culture filtrate) Cultivation time (h)				
	24	48	72	96	120
896	0.1	13.9	1.9	1.2	2.0
1040	0.1	12.9	5.6	6.4	2.1
240	1.4	0.5	10.7	5.4	1.8
432	0.2	0.8	5.2	4.7	2.9
334	0.1	0.4	11.2	8.6	1.8
1519	0.1	0.5	4.6	3.1	2.0
62	0.8	3.2	3.9	1.0	2.0
46	0.3	1.0	2.6	0.6	0.3
N2	1.4	6.3	1.7	0.8	0.5

Optimization of Cultural Conditions for Enzyme Production

Effect of carbon source and concentration. Maximum glucoamylase production was achieved after 48- and 72- h cultivation using either raw cassava starch, rice bran or combined raw cassava starch-rice bran mixture. As shown in Table 6, both rice bran and raw cassava starch can be used as suitable carbon sources for the production of the enzyme. However, when rice bran and raw cassava starch were used in combination, a relatively higher enzyme production was achieved. When using the mixture of 0.5% rice bran and 1.0% raw cassava starch, 2- to 3- fold increase in enzyme production was observed. The increase in enzyme production may be due to the presence of the essential nutrients necessary for growth and enzyme biosynthesis.

Effect of nitrogen source and concentration. Ammonium sulfate was used as the source of nitrogen during the screening of the glucoamylase-producing fungi. Since variation occurs in the utilization and uptake of nitrogen in different fungal species, it is worthwhile to evaluate the ability of the fungal strain to utilize other nitrogenous compounds. Based on the results shown in Fig. 1, peptone was found to be the best nitrogen source followed by ammonium nitrate, sodium nitrate and urea in a decreasing order. Maximum enzyme production was attained when peptone was supplied at 0.1% to 0.5% concentration. However, when the concentration of the other nitro-

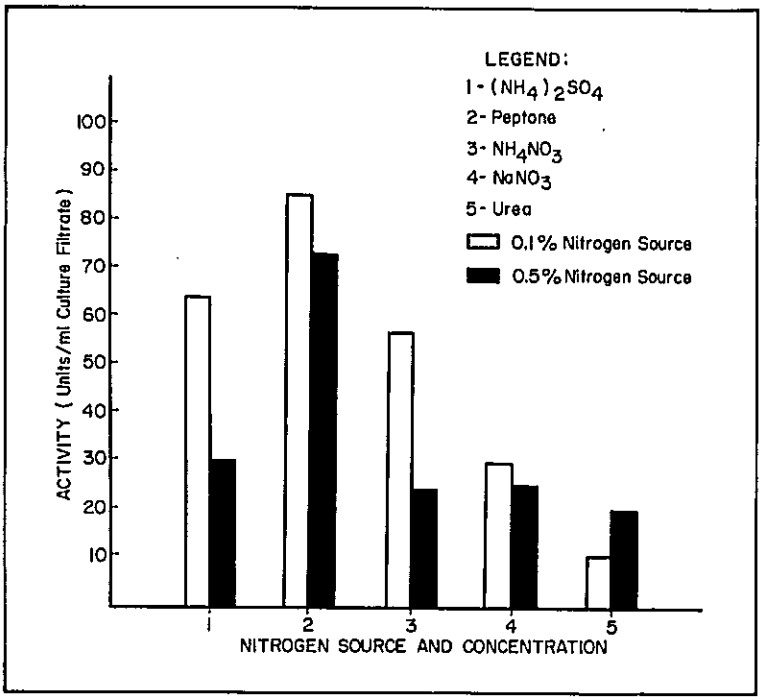

Figure 1. Effect of nitrogen source and concentration.

genous supplements was increased from 0.1% to 0.5%, low enzyme production resulted except in the case of urea. The strain showed its ability to produce high amount of enzyme using either organic or inorganic source of nitrogen.

Effect of initial pH. As shown in Fig. 2, pH 4.0 appeared to be the optimum pH for enzyme production. Above or below this pH level, the decrease in enzyme production was observed. Increasing the pH near neutrality caused a drastic decrease in enzyme production.

Effect of incubation temperature. The maximum glucoamylase production was attained at 30°C (Fig. 3). Above or below the optimum temperature, the activity of the enzyme decreased. Enzyme production was intensely stimulated at this temperature since a relatively higher activity of the enzyme was observed at 30°C among the temperatures tested.

Effect of the addition of growth factor. The effect of growth factor on enzyme production was tested using yeast extract, beef extract and malt extract at 0.2% concentration. The data in Table 7 showed that malt extract was preferred as the additional nutrient for supplementing growth and enzyme biosynthesis. The addition of malt extract to the fermentation medium enhanced enzyme production to a greater degree as compared with yeast extract and beef extract. The

Table 6. Glucoamylase activity of **Aspergillus** *sp. 432 grown on different substrate concentrations of raw cassava starch and rice bran using liquid culture medium at different h of cultivation at 35°C.*

Substrate Concentration (gram per 100 ml of the basal medium)	Activity (units per ml of culture filtrate) Cultivation time (h)		
	24	48	72
0.0 Starch: 0.5 Rice Bran	0.2	10.6	2.5
0.0 Starch: 1.0 Rice Bran	0.4	2.8	7.4
0.0 Starch: 2.0 Rice Bran	1.0	8.0	13.0
0.0 Starch: 3.0 Rice Bran	1.6	1.2	24.7
0.2 Starch: 0.0 Rice Bran	0.1	1.7	2.8
0.2 Starch: 1.0 Rice Bran	0.3	9.3	15.1
0.2 Starch: 2.0 Rice Bran	1.3	2.1	10.3
0.2 Starch: 3.0 Rice Bran	2.1	3.2	6.2
0.5 Starch: 0.0 Rice Bran	0.5	5.5	4.8
0.5 Starch: 1.0 Rice Bran	0.2	2.0	25.9
1.0 Starch: 0.0 Rice Bran	0.1	4.0	7.5
1.0 Starch: 0.5 Rice Bran	0.3	3.2	45.5
1.0 Starch: 1.0 Rice Bran	0.2	1.4	16.8
3.0 Starch: 0.0 Rice Bran	0.1	1.7	14.9
3.0 Starch: 0.5 Rice Bran	0.3	1.5	6.4
3.0 Starch: 1.0 Rice Bran	0.3	2.0	17.2

Table 7. Glucoamylase activity of **Aspergillus** *sp. 432 grown on raw cassava starch-rice bran liquid culture medium containing 0.2% growth factor supplement after 72- h cultivation at 35°C.*

Growth factor	Activity (units per ml culture filtrate)
Yeast extract	33.0
Beef extract	47.4
Malt extract	55.8

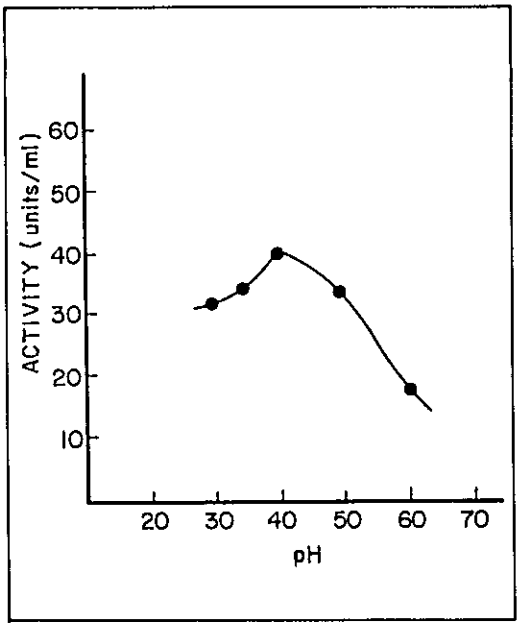

Figure 2. Effect of pH.

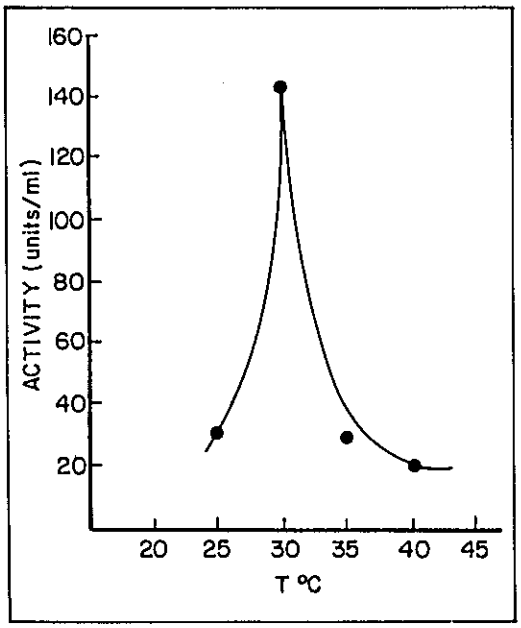

Figure 3. Effect of temperature.

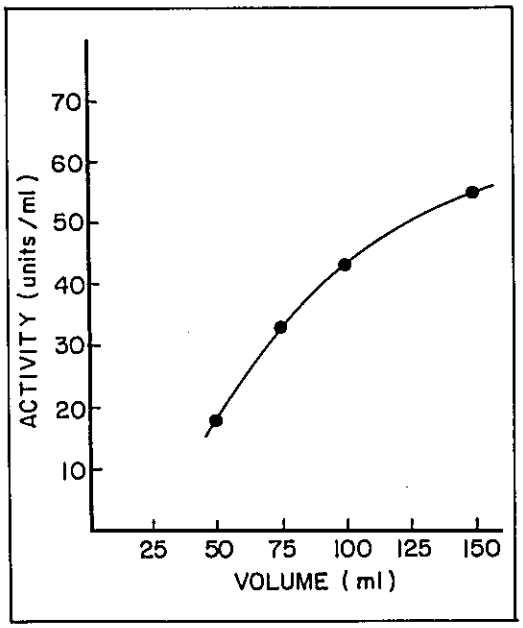

Figure 4. Effect of aeration.

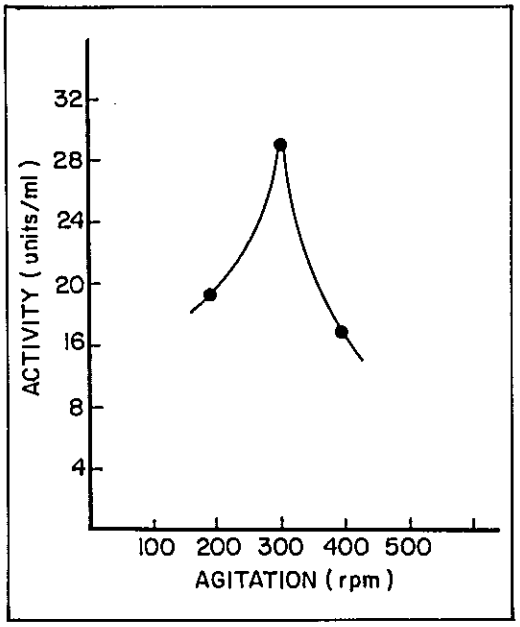

Figure 5. Effect of agitation.

addition of growth factor was necessary to increase the production of the enzyme.

Effect of aeration in different volumes of the medium. The change in aeration during fermentation brought a big difference in the production of the enzyme. In the initial phase where smaller volume of medium was used and the oxygen supply was higher, enzyme production was observed to be relatively low. When the volume of the fermentation medium was increased and the oxygen supply decreased, the production of the enzyme by the strain was not affected in anyway. Results in Fig. 4 showed that the decrease in oxygen supply was followed by an increase in enzyme production to some extent. This would mean that oxygen supply may affect the enzyme production of a strain but it would be very minimal. Oxygen supply is not a critical factor for growth and enzyme biosynthesis during enzyme production.

Optimum conditions for glucoamylase production. The optimum cultural conditions necessary for the maximum production of the enzyme is summarized in Table 8. For maximum enzyme production, a mixture of 0.5% rice bran and 1.0% raw cassava starch is necessary. The nitrogen source is supplied by peptone from 0.1% con-

Table 8. Optimal conditions for enzyme production of Aspergillus *sp. 432.*

Cultural Conditions		Requirement
A.	Nutritional	
	Substrate	Raw Starch Rice Bran
	Substrate concentration	1.0% (w/v) Raw starch 0.5% (w/v) Rice bran
	Nitrogen source	Peptone
	Nitrogen concentration	0.1% – 0.5% (w/v)
	Growth factor	Malt extract
	Growth factor concentration	0.2% (w/v)
	Minerals	KH_2PO_4 $MgSO_4.7H_2O$
	Mineral concentration	0.1% (w/v) KH_2PO_4 0.05% (w/v) $MgSO_4.7H_2O$
B.	Physiological	
	Initial pH (medium)	4.0
	Temperature (°C)	30
	Aeration (ml/500 ml flask)	100

centration with the addition of 0.2% malt extract, 0.05% $MgSO_4.7H_2O$ and 0.1% KH_2PO_4 in distilled water contained in 500-ml shaking flask. The initial pH of the fermentation medium required is 4.0 and the cultivation should be carried out at 30°C with 100 ml of the medium under reciprocal shaking at 110 rpm.

Production of Glucoamylase in Flask Culture

Glucoamylase production was evaluated using four types of culture media (Table 9). GAMP-1 and GAMP-2 were the newly formulated culture media for glucoamylase production based on the optimization studies described above. GAMP-3 and GAMP-4 were modified culture media for glucoamylase production based on Tani *et al.* (18). As shown in Fig. 6, a relatively higher enzyme activity was attained after the 72- h cultivation using GAMP-2. A drastic decrease in enzyme production was observed using the other types of culture medium. It could also be noted that although GAMP-1 and GAMP-2 had the same basal media composition, a lower enzyme activity was obtained with GAMP-1. This may be due to the absence of starch in the medium. Previous experiments showed that the presence of both rice bran and raw cassava starch in the culture medium stimulated glucoamylase production of *Aspergillus* sp. 432.

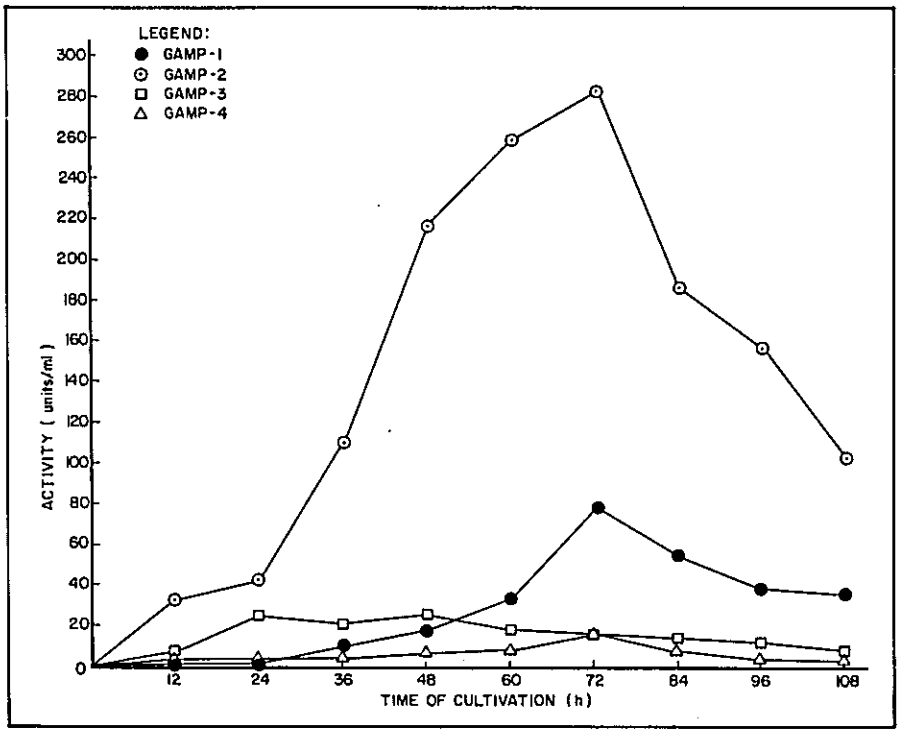

Figure 6. Effect of medium composition.

Table 9. Media composition for glucoamylase production in flask culture of **Aspergillus** *sp. 432.*

	Composition	Requirement (% w/v)
GAMP-1	Peptone	0.5
	Malt extract	0.2
	KH_2PO_4	0.1
	$MgSO_4.7H_2O$	0.05
	Raw cassava starch	1.0
	Rice bran	0.5
GAMP-2	The same basal medium composition as GAMP-1, however, only 0.5% rice bran was used as carbon source.	
GAMP-3	$(NH_4)_2SO_4$	0.5
	Yeast extract	0.3
	KH_2PO_4	0.1
	$MgSO_4.7H_2O$	0.05
	Raw cassava starch	1.0
	Rice bran	0.5
GAMP-4	The same basal medium composition as GAMP-3, however, only 0.5% rice bran was used as carbon source.	

Note: The pH of GAMP-1 and GAMP-2 was 4.0 whereas the pH of GAMP-3 and GAMP-4 was 3.5. The pH of the medium was adjusted prior to the sterilization of the basal medium.

Production of Glucoamylase in Jar Fermentor Culture

Glucoamylase production was tested in the 2-L jar fermentor using the optimum conditions for enzyme production with 200, 300 and 400 rpm agitation. The highest enzyme activity was obtained during the 72- h cultivation. A two fold increase in enzyme production was observed when 300 rpm agitation was used (Table 10). At 200 and 400 rpm agitation, maximum enzyme activity was attained during the 96- h of cultivation. The best results using the jar fermentor were obtained using the above mentioned optimum conditions with 300 rpm agitation and 1 vvm aeration for 72- h cultivation at 30°C.

Table 10. Glucoamylase activity of **Aspergillus** *sp. 432 at different agitation speeds using the 2.0-L KMJ fermentor at 30°C at different h of cultivation.*

AGITATION (r p m)	Activity (units per ml culture filtrate) Cultivation time (h)			
	24	48	72	96
200	12.55	13.13	19.47	24.16
300	10.49	13.95	28.89	17.47
400	11.94	13.83	16.60	22.11

SUMMARY and CONCLUSION

The production of glucoamylase by a local fungal isolate of *Aspergillus* sp. 432 was carried out using either liquid and solid culture processes. The best culture medium for production was developed in the liquid culture consisting of 0.5% rice bran, 1.0% raw cassava starch, 0.1% to 0.5% peptone, 0.2% malt extract, 0.05% $MaSO_4.7H_2O$ and 0.1% KH_2PO_4, pH 4.0. The culture filtrate derived from *Aspergillus* sp. 432 showed its ability to digest raw cassava starch as well as raw cassava waste to some extent. Glucoamylase production was best attained when the cultivation was carried out at 30°C for 72 to 96 h under reciprocal shaking at 110 rpm for the flask culture. A large scale production of glucoamylase was performed with a jar fermentor using the optimal cultural conditions established. The productivity in the jar fermentor varied with the change of agitation speed.

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STUDY 2: LARGE-SCALE PRODUCTION OF AMYLOLYTIC ENZYMES CAPABLE OF SACCHARIFYING RAW STARCH

FUNDAMENTAL STUDIES ON THE PRODUCTION OF THE ENZYME BY SOLID CULTURE USING LOCALLY AVAILABLE MATERIALS

ABSTRACT

Corn medium supported sporulation of BJRS 1429 better than cassava, sweet potato and glutinous rice, yielding 1.8 x 10^8, 5.2 x 10^8, 1.0 x 10^9 and 6.4 x 10^8 spores/gram substrate at 3,6,9 and 15 days, respectively after inoculation. Wheat bran was found to be a very good medium for "koji" enzyme production but rice bran, a local material, could be used as substitute. Evaluation of different combinations of rice bran and cassava starch as "koji" medium components showed that raw starch active-glucoamylase activity was optimum in the *15 g rice bran: 0 cassava starch* formulation yielding 0.54 IU/ml and 6.8 IU/g of "koji" substrate.

INTRODUCTION

The use of solid culture or the mold bran process for the production of fungal glucoamylase preparations has been well-documented (1, 2, 3, 4, 5, 7, 8, 9, 10). Despite more sophisticated advances and high technology packages in the field of microbial enzyme production, conventional solid culture, or more popularly known as "koji" process, still remains a more economically attractive process because it is not as capital-intensive as the submerged culture process, requiring only the minimum of production inputs. Other advantages include easier, simpler and lesser management during production and recovery of a more concentrated product. Very recent studies comparing the efficiency of both liquid and solid processes for use in the production of raw starch-active glucoamylase under optimized conditions indicate that the latter, is, in fact, three times more efficient than the former (8).

This paper describes efforts to find cheap and locally available substrates for mold sporulation and for "koji" enzyme production. Traditional media to induce and enhance spore formation include very expensive and imported commercially prepared culture media. In "koji" making, the main component is usually wheat bran, non-tropical crops, and thus, subject to supply limitations.

MATERIALS AND METHODS

I. Selection of locally available and inexpensive medium for sporulation by the mold

A. Organism

A local isolate, BJRS (JICA-BIOTECH Raw Starch Code) 1429, was used. It was found to produce black spores when grown on potato dextrose agar (PDA) and on malt agar. This organism was one of promising producers of glucoamylase active on raw starch selected in earlier studies (see report of A. Lit).

B. Raw Materials

In the preliminary experiment, the following starchy substrates were used, namely raw tubers of cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*), grains of corn (*Zea mays*) and rice (*Oryza sativa*) of the indica, joponica and glutinous types. On the basis of spore formation, the substrates were narrowed down to cassava and sweet potato tubers, corn and rice of the glutinous type.

C. Preparation of sporulation substrates

The sweet potato and cassava tubers were washed, peeled, cut into small cubes less than 0.5 cm^2 and soaked in water overnight. The rice and corn grains were soaked in water for two consecutive days. All substrate materials were drained of water and five grams of each substrate per test tube was used. Sterilization was done at 15 pounds per square inch for 30 minutes.

D. Preparation of mold inoculum

A loopful of the stock culture was aseptically inoculated onto a slant of PDA and was allowed to produce vegetative mycelia and spores for six days at ambient room temperature. Ten milliliters (ml) distilled water was poured into each of the sporulated cultures and a spore suspension was made by scrapping the spores from the agar surface. All spore suspensions collected were mixed thoroughly. One ml was inoculated into each sample. The amount of spores per ml of the suspension was determined using a hemacytometer according to the following equation:

$$\text{spores/ml} = \frac{\text{Average spore count X dilution factor}}{2 \text{ X } 10^{-5} \text{ (ml)}}$$

where, 2×10^{-5} = volume (ml) delivered to the area

II. Evaluation of rice bran as a substitute for wheat bran in "koji" enzyme production

A. Preparation of the solid culture medium

The medium consisted of 16 grams of bran (rice bran or wheat bran), and in some treatment, 2 grams of raw, commercial grade cassava starch and tap water to make a final moisture content of 50%. The medium was then sterilized at 15 lbs. per $in.^2$ for 20 minutes. To each sample was inoculated one ml of spore suspension prepared as previously described. Incubation was done for 7 days at ambient room temperature (Figure 1).

B. Extraction of the crude glucoamylase enzyme

Four gram samples were collected from each treatment. Fifty ml of 1%NaCl solution and 0.5 ml toluene were added. Extraction was done overnight at room temperature. Each sample (Figure 2) was filtered through to a 50-ml volumetric flask using a Whatman #1 qualitative filter paper. This consisted the crude enzyme or the culture filtrate.

C. Assay of enzyme activity on raw cassava starch

The activity of the crude enzyme extract on raw cassava starch and in the culture filtrate was assayed following the procedure described elsewhere (JICA-BIOTECH Annual Report for 1985). Absorbance was read at 500 nm using a SPECTROPLUS-D spectrophotometer. One international unit (IU) of enzyme activity was defined as the amount of enzyme which produces one micromole of reducing sugars (expressed as glucose) per minute.

III. Determination of optimum rice bran: cassava starch ratio

A. Rice bran and cassava starch combinations

The following combinations were evaluated in terms of effects on glucoamylase activity on raw starch substrate:

0 Rice bran	:	2, 4, 6, 8, 10, 15, 20 g cassava starch
5 g Rice bran	:	0, 2, 4, 6, 8, 10, 15, 20 g cassava starch
10 g Rice bran	:	0, 2, 4, 6, 8, 10, 15, 20 g cassava starch
15 g Rice bran	:	0, 2, 4, 6, 8, 10, 15, 20 g cassava starch
20 g Rice bran	:	0, 2, 4, 6, 8, 10, 15, 20 g cassava starch

B. Determination of enzyme activity (IU) per gram of solid substrate

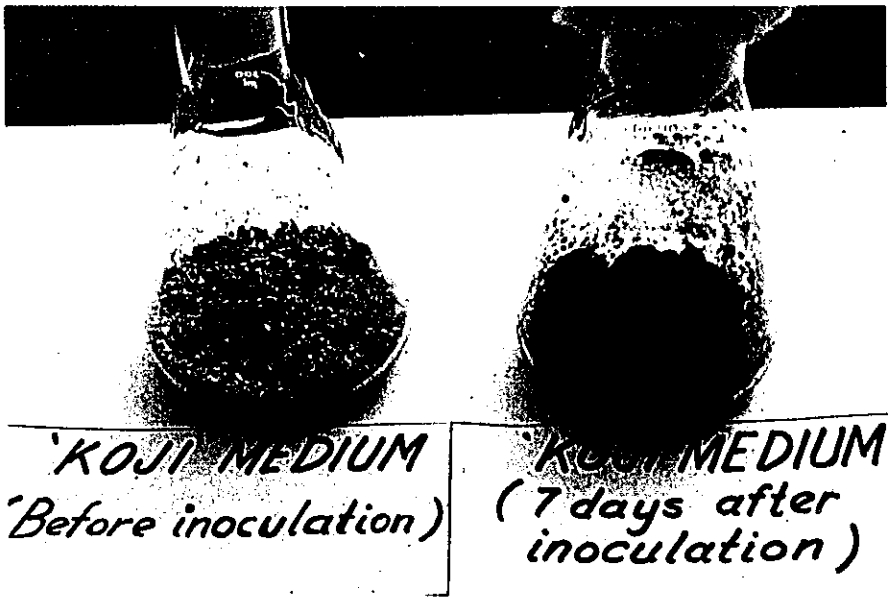

Figure 1: The "koji" after 7 days of incubation at 30°C.

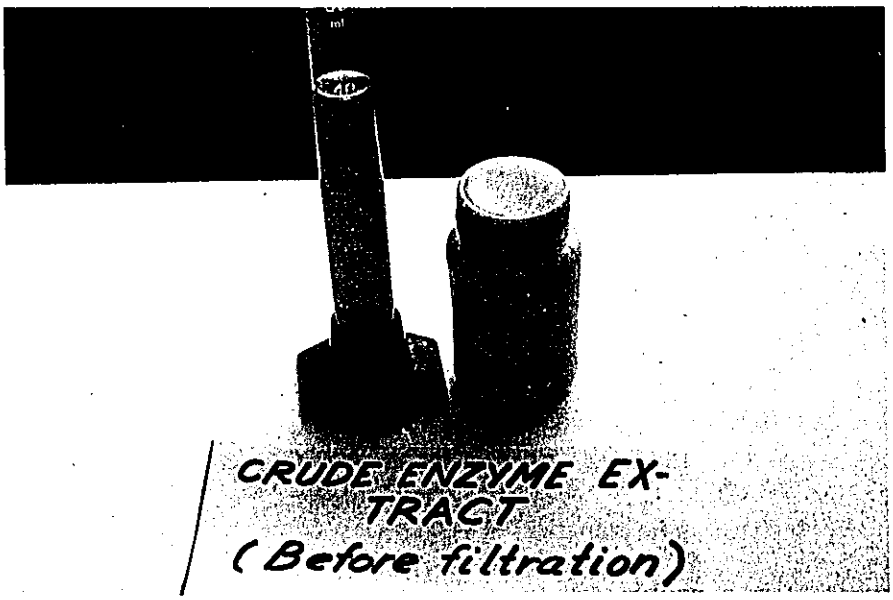

Figure 2: The crude enzyme sample after extraction using 1% NaCl solution and 0.5 ml toluene but prior to filtration.

The activity of the glucoamylase enzyme per gram of solid substrate was determined according to the equation:

$$IU/g = \frac{IU/ml}{\%\ \text{initial total solids (in "koji")}}$$

RESULTS AND DISCUSSION

I. Selection of locally available and inexpensive medium for sporulation by the mold

In the preliminary experiment, a visual comparison between the starchy substrates was done in terms of supporting sporulation. It was observed that sweet potato, cassava, corn and glutinous rice produced more spores than the other raw materials used.

Using these substrates, the time course of spore formation was checked for 15 days. The spore count at each sampling period was determined using the hemacytometer. Data obtained showed that maximum sporulation by the organism, BJRS 1429, was obtained from the corn substrate after 9 days since inoculation (Figure 3). The spore concentration obtained at this time period was 1.0×10^9 spores per ml of suspension. Results obtained from using corn as the sporulation substrate showed that it was consistently better compared with the other three raw materials.

II. Evaluation of rice bran as a substitute for wheat bran in "koji" enzyme production

A. Bran medium

Two kinds of bran were used, namely, wheat bran and rice bran. The wheat bran, obtained from the Ajinomoto Chemical Company, was used for comparison. The rice bran was purchased from the local market.

The starch substrate used was cassava starch produced commercially. It was either sterilized (at 120°C for two hours in the oven) or unsterilized.

Tap water was added to a final moisture content of 50%. The medium was sterilized at 15 lbs. per $in.^2$ for 20 minutes.

B. Moisture determination

The moisture content of each component of the bran medium, as determined by drying to a constant weight at 100°C, ranged from 8.8% (wheat bran) to 17.5% (cassava starch) (Table 1). Wheat bran was found to contain 8.8% moisture.

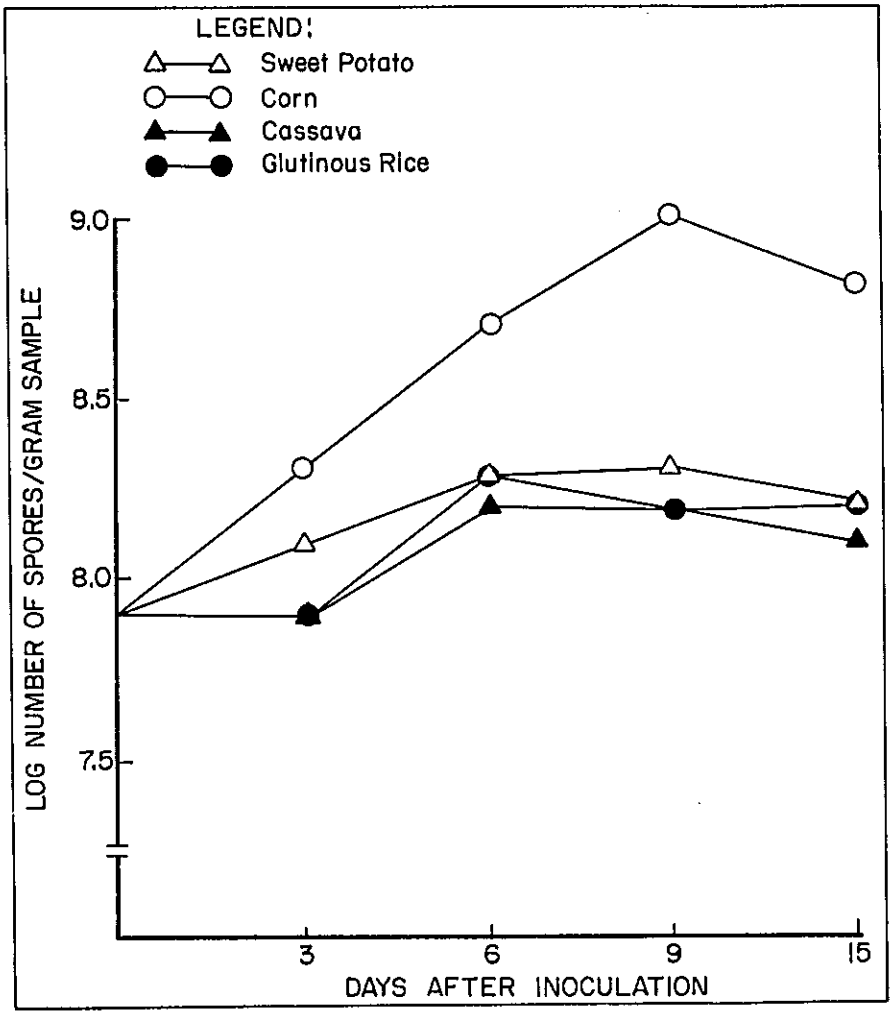

Figure 3. Spore production profile at BJRS 1429 at various time periods after inoculation using sweet potato, corn, cassava and glutinous rice as substrate.

Table 1. Moisture content determination of the bran components.

Material	Total weight of solids (g)	Moisture Content (%)
Rice bran	16	10.3 ± 0.3
Wheat bran	16	8.8 ± 0.7
Cassava starch	2	17.5 ± 2.5

C. Comparison of reducing sugar formation and volumetric glucoamylase activities in media containing rice bran or wheat bran as substrates for "koji" enzyme production

The amount of sugar formed from raw cassava starch during reaction with the crude glucoamylase extracts and the volumetric enzyme activities were determined (Table 2). The former, expressed as micrograms of glucose per ml of reaction mixture, was obtained by taking the difference between the sugar concentration values for the reacted and unreacted culture filtrate samples. The volumetric activity of the enzyme, expressed as IU/ml, was obtained by calculating the micromoles of glucose produced per ml per minute of reaction time.

Wheat bran was undoubtedly a better medium for "koji" enzyme production as compared with rice bran (Table 2). Higher reducing sugar values of 1.88, 1.53 and 1.87 x 10^3 micrograms glucose per ml were obtained in most of the treatments containing wheat bran instead of rice bran. However, in treatments with rice bran, reducing sugar values of 1.10, 0.97 and 0.91 x 10^3 micrograms glucose/ml were obtained, indicating that rice bran can be used to support fungal enzyme production although to a lesser extent than wheat bran. Expectedly, similar trends were observed for the volumetric enzyme activities.

Saccharification values were found not to differ much between sterilized and unsterilized starch treatments in formulations containing cassava starch. In WB + CS + TW formulation, reducing sugar values obtained for the sterilized and unsterilized starch treatments were 1.88 x 10^3 and 1.87 x 10^3 micrograms glucose/ml, respectively. In the RB + CS + TW formulation, values of 0.91 x 10^3 and 0.97 x 10^3 micrograms glucose per ml were obtained under sterilized and unsterilized starch conditions, respectively. The same phenomenon was not found true for the formulations without cassava starch. The enzyme activities exhibited similar trends.

On the basis of experimental findings that rice bran can also be used as substrate for raw starch digestive-glucoamylase production, optimization experiments were conducted to determine the best conditions using rice bran as the major substrate.

Table 2. Relative volumetric activities of crude enzyme filtrate obtained after growing BJRS 1429 on wheat bran and rice bran media at 30°C for 7 days.

Bran Composition*	Condition of sterilization of raw cassava starch	Reducing sugar concentration (X 10^3 µg/ml)	Volumetric glucoamylase activity	
			micromole gluc/hr/ml	IU/ml
WB + TW	No starch	1.53	8.52	0.14
RB + TW	No starch	1.10	6.08	0.10
WB + CS +TW	Sterilized**	1.88	10.43	0.17
RB + CS + TW	Sterilized	0.91	5.03	0.08
WB + CS + TW	Unsterilized	1.87	10.41	0.17
RB + CS + TW	Unsterilized	0.97	5.40	0.09

* Legend: WB = Wheat bran
RB = Rice bran
CS = Cassava starch
TW = Tap water

** Sterilized in the oven at 120°C for two hours.

III. Determination of optimum rice bran: cassava starch ratio

The different combinations of rice bran (0, 5, 10, 15 and 20 g) and cassava starch (0, 2, 4, 6, 8, 10, 15 and 20 g) were evaluated in terms of volumetric enzyme activity (IU/ml) (Figure 4) and units of activity per gram of "koji" substrate (IU/g) (Figure 5).

The highest volumetric activity of 0.54 IU/mi was obtained from the crude extract produced from the formulation containing **15 g rice bran and no cassava starch** added (Figure 4). Other treatments consisting of **20 g rice bran: 4 g cassava starch, 20 g rice bran: 8 g cassava starch** and **15 g rice bran: 2 g cassava starch** also gave high yields of 0.47, 0.43 and 0.40 IU/ml, respectively. These values are definitely higher than data obtained from unoptimized conditions (Table 2) even with wheat bran as substrate.

Consequently, the same formulations which gave higher volumetric activities also gave higher enzyme activites per gram of "koji" substrate (Figure 5). The **15 g rice bran: O cassava starch** treatment gave the highest activity of 6.8 IU/ g, followed by 5.9, 5.4 and 5.0 IU/g obtained from **20 g rice bran: 4 g cassava starch, 20 g rice bran: 8 g cassava starch,** and **15 g rice bran: 4 g cassava starch** combinations respectively. Data obtained form the Central Analytical Services Labora-

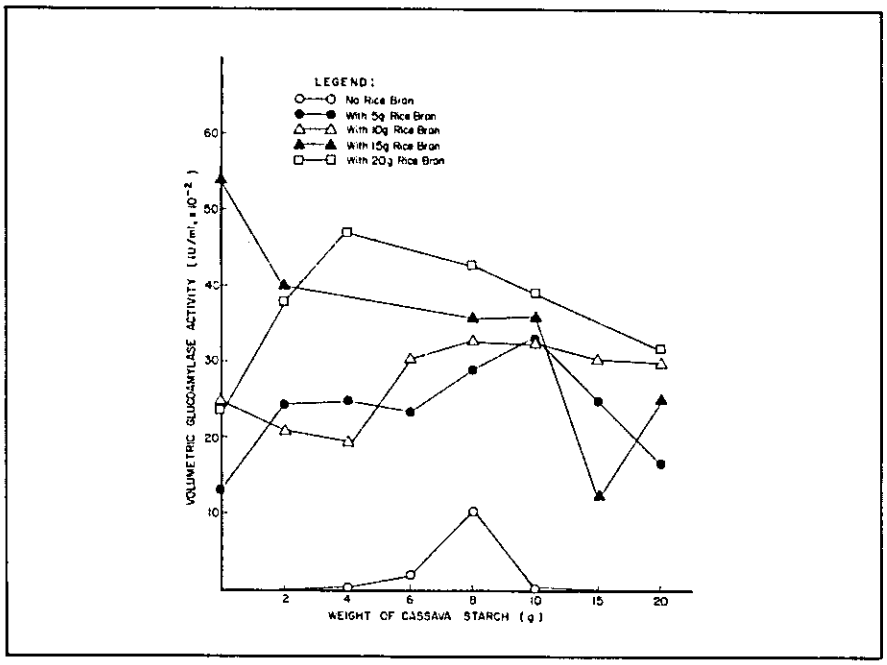

Figure 4. Effect of varying rice bran: cassava starch ratio on the volumetric activity (IU/ml) of crude glucoamylase in raw starch produced by BJRS 1429 in solid culture at 30°C for 7 days.

tory (CASL) of BIOTECH showed that rice bran has a high carbon content of almost 39.85% (Table 3), possibly accounting for the non-requirement of an additional source of carbon, that is cassava starch, for enzyme production. It was also found to contain 5.76% hydrogen, 0.71% nitrogen and a C/N ration of 56/1.

The lowest enzyme activities were observed for treatments containing only cassava starch and no rice bran. Increases in enzyme activities were noted following addition of rice bran to the cassava starch. Relatively, higher enzyme activities were obtained at higher concentrations of rice bran in the medium. On the basis of these findings, the best combination recommended for raw starch active-glucoamylase production by BJRS 1429 is **15 g rice bran: 0 cassava starch.**

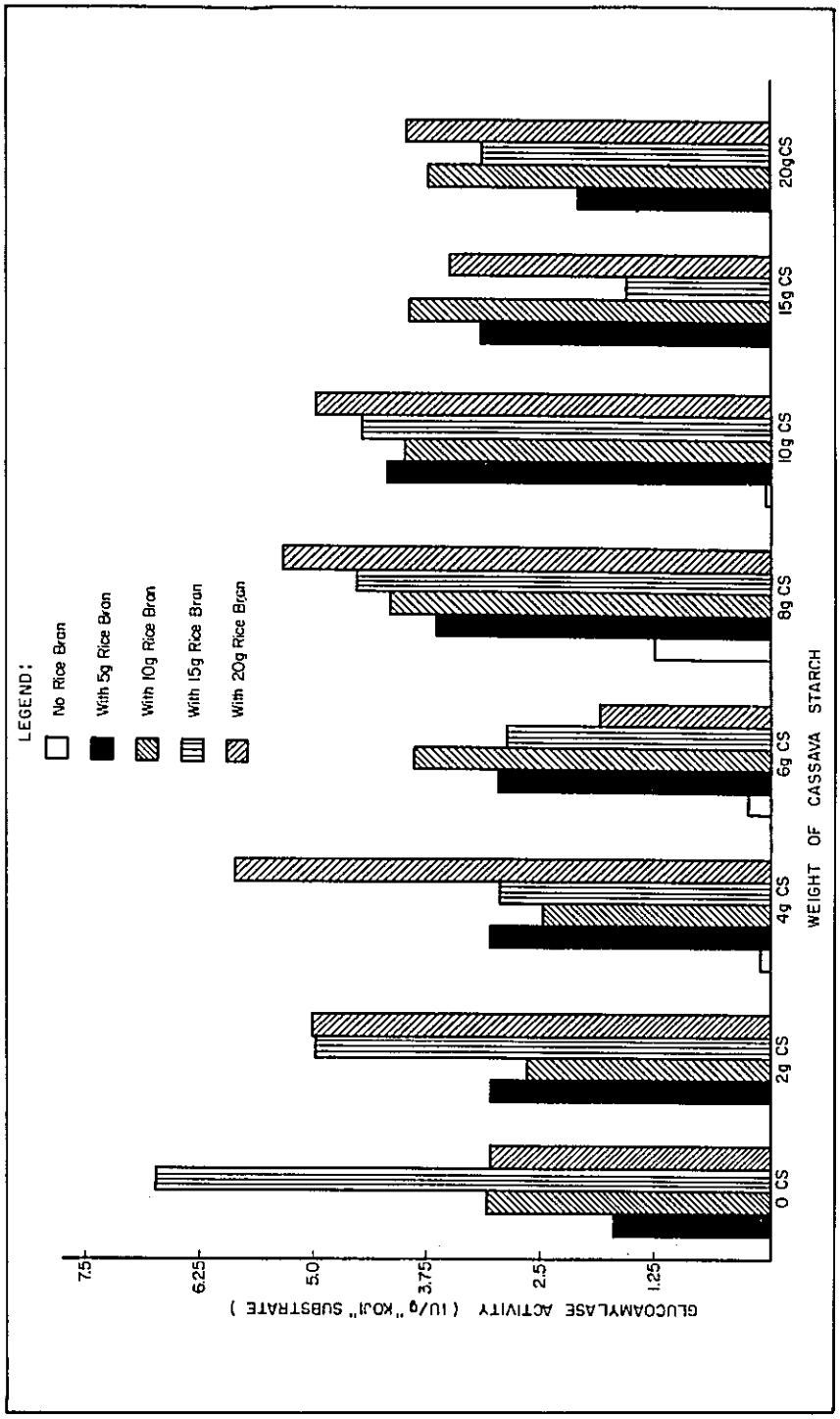

Figure 5. Glucoamylase activity (IU/g of "koji" substrate of BJRS 1429 evaluated in varying combinations of rice bran and cassava starch incubated at 30°C for 7 days using the solid culture process.

Table 3. Carbon, hydrogen and nitrogen content of rice bran (Partial CASL data, 1986).

ANALYSIS	DATA (%)
Carbon (C)	39.85 ± 0.14
Hydrogen (H)	5.76 ± 0.02
Nitrogen (N)	0.71 ± 0.01
C/N Ratio: 56/1	

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STUDY 3: MICROBIAL DECOLORIZATION OF ALCOHOL FERMENTATION AND DISTILLERY WASTES INCLUDING BIOGAS PRODUCTION

SUB-STUDY 3A: BIOGAS PRODUCTION FROM DISTILLERY SLOPS

SINGLE AND TWO-PHASE FERMENTATION OF SLOP WASTE UNDER MESOPHILIC AND THERMOPHILIC CONDITIONS

ABSTRACT

Performance of single and two-phase methane fermentation of slop waste under mesophilic and thermophilic conditions were tested to determine the most efficient process. The efficiencies of these processes were measured in terms of biogas produced, reduction in COD value and utilization of volatile fatty acids (VFA) formed at different COD loadings.

The result of the studies showed that two-phase thermophilic fermentation was the most efficient process with 20.5 liters/day of biogas (60% methane) produced, 19% COD/day removed and effective utilization of VFA formed at a COD loading rate of 0.012 kg/L.

INTRODUCTION

Industrial waste water particularly slop waste contains high COD and BOD values and therefore needs prior treatment before being discarded to rivers and streams. Since waste water leaving the factories have a high temperature (70-80 degrees centigrade), thermophilic methane fermentation seems to be a viable pretreatment.

Anaerobic methane fermentation is composed of two distinct steps: acid formation by a heterogeneous group of facultative anaerobic. microorganisms and methane generation by a group of obligate anaerobic methanogens. The rate of acid formation was reported to be 10 times faster (Ghose et al., 1981) than methane generation, thus methane generation is the rate-limiting step. Separation of these two steps, therefore, insures maximum production of their respective products.

Fermentation at thermophilic conditions also improved the efficiency of methane fermentation as worked out by Sriprasertsak et al., (1985) in addition to the benefits derived from the separation of the two steps involved (Oi et al., 1985).

The study aimed to compare the efficiency of methane gas production, VFA accumulation and utilization and COD reduction in slop waste using single and two-phase fermentation processes under mesophilic and thermophilic conditions.

MATERIALS AND METHODS

Substrate

Slop waste was produced by alcoholic fermentation of molasses on a laboratory scale or obtained from a local distillery plant in Nasugbu, Batangas. It was autoclaved and stored at freezing temperature to prevent possible secondary microbial attack during storage. Before feeding, thawed slop waste was boiled once to expel any dissolved oxygen and pH adjusted to 7.0 with 3N NaOH.

Inoculum

The inoculum for the thermophilic fermentor was collected near the La Carlota distillery plant in Negros Island. It was maintained in a water bath at 55 degrees centigrade and acclimatized by gradual addition of slop waste. The mesophilic inoculum came from hog manure, conditioned to slop waste for more than two years.

Fermentor

A 5-L Marubishi fermentor equipped with a stirrer, pH and thermostat control was used in the single-stage fermentation. Rubber tubings were connected to two sampling ports for feeding and withdrawal of fluids. Gas was collected in 10-L capacity rubber balloons attached to a sampling port. A 3.8-L carboy bottle was connected to the 5-L fermentor to complete the set-up in the two-phase fermentation system. The 3.8-L bottle served as the acidogenic phase while the 5-L fermentor served as the methanogenic phase. A mini-pump was used to transfer effluent from the first to the second fermentor (Figure 1.).

Procedure

The 5-L fermentor was seeded with 2 liters of inoculum and 1 liter of slop waste to make a working volume of 3 liters (υ) and allowed to ferment until steady-state condition. Addition of known volume of slop waste was initiated and analyses of fermentation parameters was done at set time intervals.

Effluent withdrawn from the fermentor was collected and placed in a 3.8-L bottle. Two liters of effluent were mixed with 1 liter slop waste to start the acidogenic phase. At steady-state condition, known volume of slop waste (υ) was added, allowed to ferment then pumped to the 5-L fermentor. Mesophilic fermentation was done at room temperature (28-30 degrees centigrade) while thermophilic fermentation was done at 55 degrees centigrade.

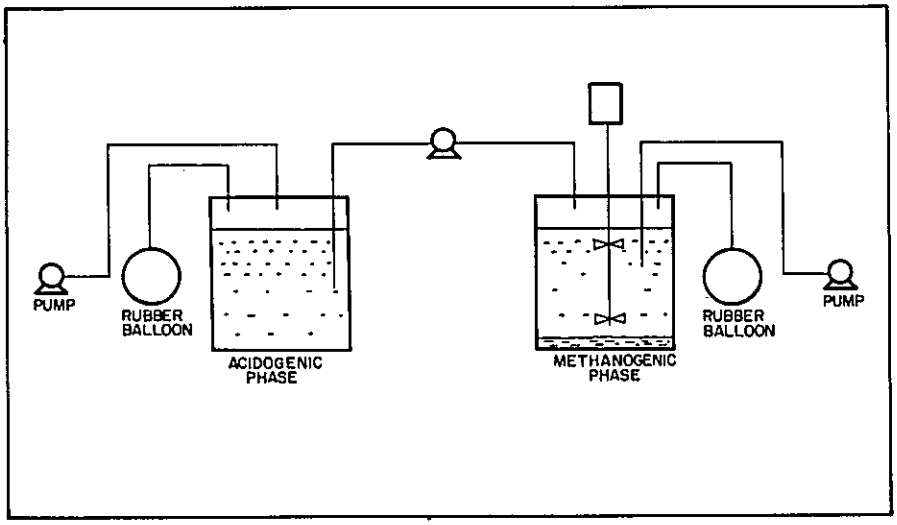

Figure 1. Schematic diagram of a two-stage anaerobic fermentation system.

Chemical Analysis

The fluids sampled during fermentation were analyzed for pH and COD (Standard Methods, 1981). Volatile fatty acids were analyzed using a gas chromatograph (Shimadzu GC-7A and Chromatopac C-R3A; column 2.1m x 3.1mm, PEG 6000 column, carrier gas: nitrogen, 50 ml/min.; 160 degrees centigrade, flame ionization detector). The methane composition was analyzed by the same gas chromatograph (column 3.2m x 3.2mm, activated carbon; carrier gas: helium, 50 ml/min; 70 degrees centigrade; thermal conductivity detector).

RESULTS AND DISCUSSION

Mesophilic Fermentation

Single-Phase Fermentation. Single-phase fermentation of slop waste produced a maximum of 2.5 liters of gas per day (41% methane) at 0.0055 kg/L-day COD loading for a hydraulic retention time (HRT) of 5 days (Fig. 2). The rate of gas production and COD reduction generally decreased with increase in COD loading. The percent methane composition increased to 53% at 0.0065 kg/L-day COD load then decreased. The % COD removal at this loading rate was 5/day. The accumulation of VFA (maximum of 3.86 mg/L-day) at increased COD loading may be attributed to the accelerated activity of acidogens and gradual elimination of methanogens resulting in

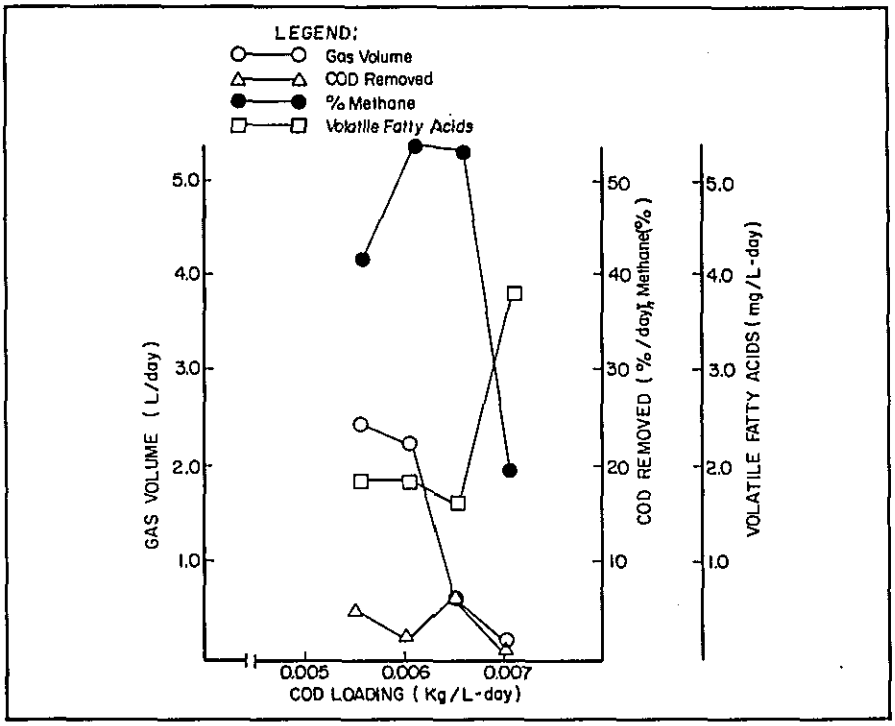

Figure 2. Single stage fermentation of slop waste (3.0 L reaction mixture, 30°C).

the overall reduction of gas produced. The limit of COD loading for balance VFA formation and gas generation appeared to be 0.0055 kg/L-day. Any further increase in the COD loading seemed detrimental to the process desired.

Two-Phase Fermentation. In the two-phase fermentation system, the formation of VFA was concentrated in the first fermentor. Except for the COD load of 0.0118 kg/L-day where there was a surge in gas production and abrupt decrease in % COD removed and VFA accumulated, acidified slops (2.71-8.65 mg/L-day) resulted in the production of small amounts of gas (less than a liter) with minimum methane concentration (2-10%) (Fig. 3). The abnormal trend obtained at the above loading rate may be due to "loading shock" which altered microbial activity. After acclimation to high loading, chemical changes which occurred followed the usual trend typical of an anaerobic fermentor. The highest COD reduction (28%) was obtained at 0.007 kg/L-day COD loading.

In the second fermentor, gas produced was slightly lower than that obtained in the single-phase fermentation system at the same COD loading. A maximum of 1.48 L/day of gas was produced at

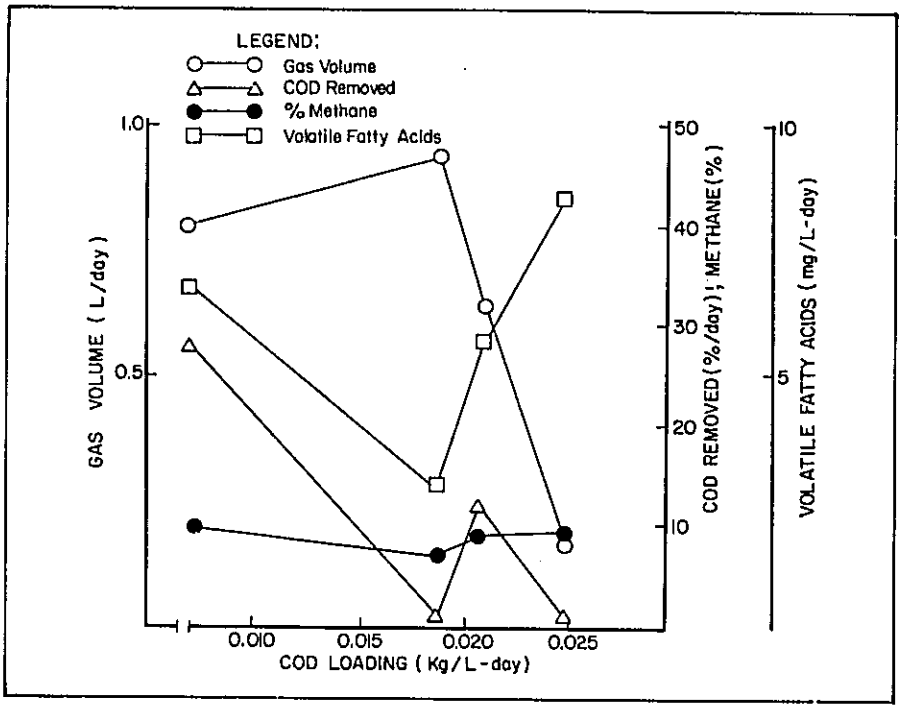

Figure 3. Acidogenic phase in two-phase methane fermentation of slop waste (3.0 L reaction mixture, 30°C, pH 5.6).

0.0065 kg/L-day COD loading but 11% COD removal for a hydraulic retention time of 7 days (Fig. 4). The decrease in VFA concentration in the second fermentor effected an increase in gas production. Any increase in VFA after transfer to the second fermentor was not high enough to affect the whole methanogenic process.

Thermophilic Fermentation

Single-Phase Fermentation. Since slop waste coming from factories have a relatively high temperature (80-90 degrees centigrade), thermophilic methane fermentation of slop waste was tried with the same range of COD loading as those at mesophilic condition.

Single-phase fermentation of slop waste showed a dramatic increase in gas production and % COD removal over that performed at mesophilic condition. At a COD loading rate of 0.008 kg/L-day, total gas produced was 4.23 L/day, (45% methane), an almost two-fold increase over mesophilic fermentation. The % COD removal increased with a corresponding increase in COD loading (Fig. 5). However, the 17% COD removed with the approximate loading rate as in mesophilic condition was a great improvement. The VFA level was maintained in the range 4-6 mg/L at the loading rate tried indicating efficient conversion of acids to gas.

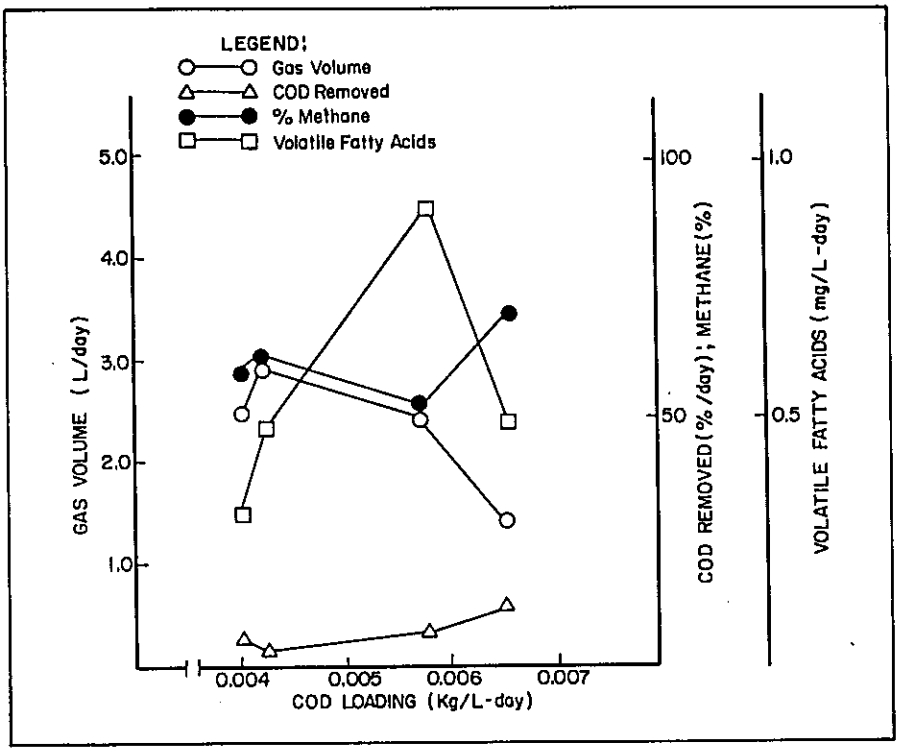

Figure 4. Methanogenic phase in two-phase fermentation of slop waste (3.0 L reaction mixture, 30°C).

Two-Phase Fermentation. In the acidogenic phase, the amount of gas evolved was generally higher than that obtained at mesophilic condition. The highest volume of gas produced (24L/day-20% methane) was obtained at 0.057 kg/L-day COD loading. The VFA formed was in the range 11-13 mg/L-day except for the lowest COD load (0.018 kg/L-day) which accumulated 2 mg/L-day and % COD removal, 11-14% (Fig. 6).

In the methanogenic phase, the highest amount of gas produced was 20.5 L/day (60% methane) obtained at COD loading of 0.012 kg/L-day (Fig. 7). The volume of gas produced with comparable COD loading rate, 0.0065 kg/L-day in mesophilic fermentation was 8 times greater. The highest reduction in COD was 19%/day at 0.0065 kg/L-day loading rate. The % COD reduction was relatively close to the reduction effected in the thermophilic single-phase fermentor but slightly higher than the reduction obtained under mesophilic conditions. The slightly high VFA formed in the single-stage and methanogenic phase may mean incomplete methanogenesis. Additional gas may still be produced should fermentation be allowed to proceed further.

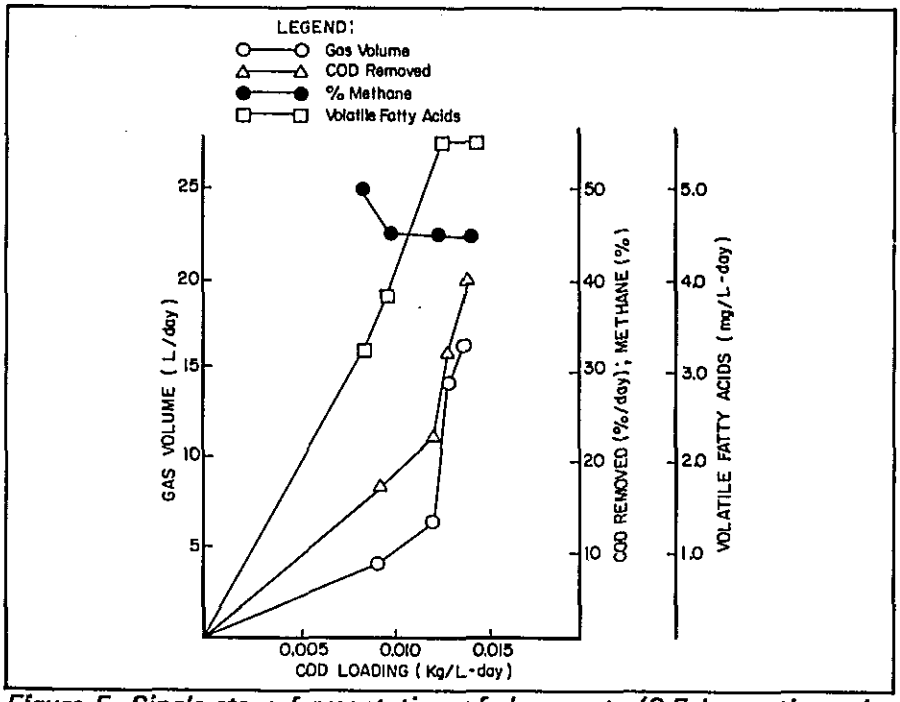

Figure 5. Single stage fermentation of slop waste (3.5 L reaction mixture at 55°C).

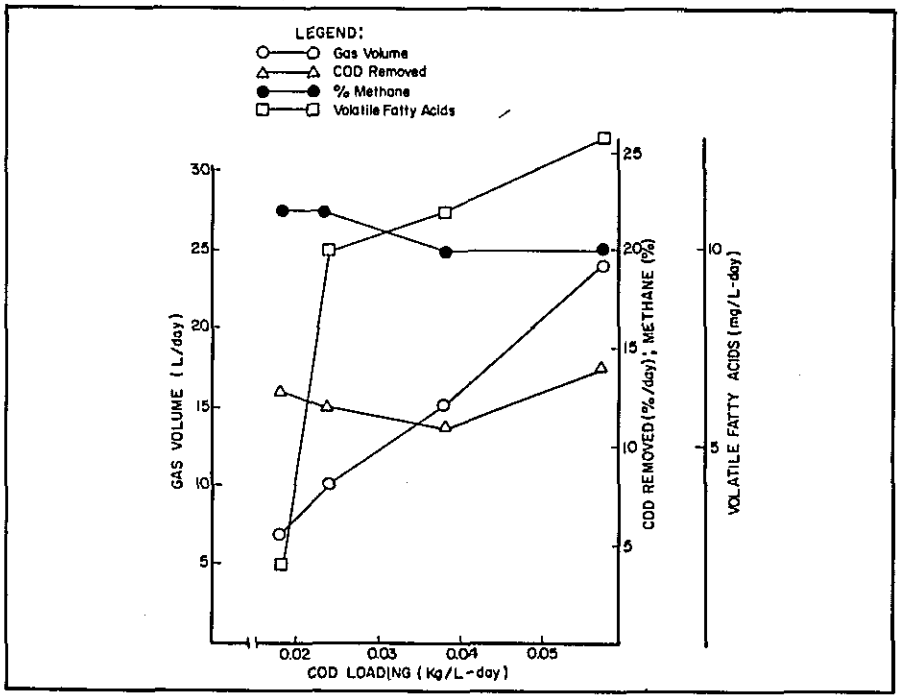

Figure 6. Acidogenic phase in two-phase methane fermentation of slop waste (2.0 L reaction mixture, 55°C pH 6.4).

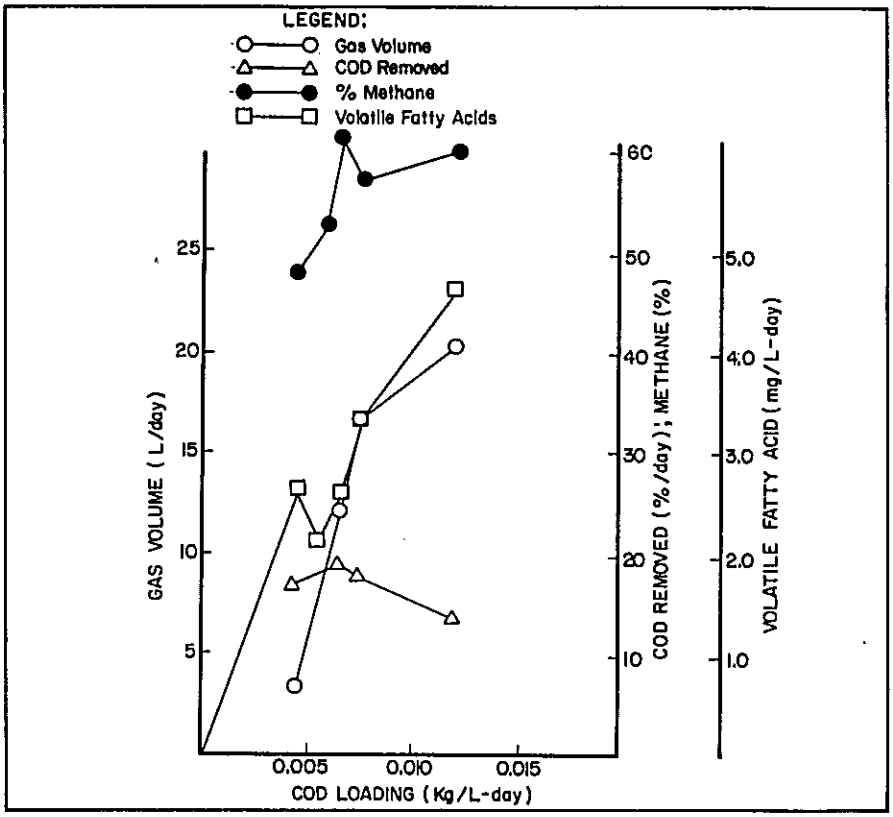

Figure 7. Methanogenic phase in two-phase fermentation of slop waste (3.5 L reaction mixture, 55°C).

Summarizing therefore the performances of the two fermentation systems under two different conditions (Tables 1 and 2), it may be concluded that thermophilic two-phase fermentation was the most efficient based on the volume of gas produced, % methane composition and VFA utilization. The % reduction in COD value may be further improved in future studies with the proper manipulation of existing parameters, development of new fermentor designs and hopefully the selection of high methane yielding methanogens.

Table 1. Chemical changes in a single and two-phase fermentation system under mesophilic condition.

	Vol. of slops, ml (v)		Equiv. COD Ld. (kg/L day)	Gas evolved, rate (L/day)	(%CH4)	COD redn. rate (%/day)	HRT = V/v (days)	VFA Acc (mg/L day)
Single-Phase	200		0.0065	1.85	(53)	7.0	15.0	1.63
	400		0.0060	2.30	(53)	2.0	7.5	1.91
	600		0.0055	2.50	(41)	5.0	5.0	1.93
	800		0.0060	1.29	(36)	4.2	3.75	2.36
	1000		0.0070	0.18	(20)	1.7	3.0	3.86
Two-Phase Acid.	200		0.0070	0.800	(10)	28.0	15.00	7.70
	400		0.0210	0.575	(9)	12.5	7.50	5.75
	600		0.0250	0.165	(9)	1.0	5.00	8.65
	800		0.0190	0.940	(7)	0.7	3.75	2.71
	1000		0.0118	0.274	(2)	0.4	3.00	2.93
Meth.	205**	(14)***	0.0450	0.715	(9)	22.0	14.6	0.26
	429	(57)	0.0065	1.480	(70)	11.0	7.0	0.48
	600	(128)	0.0057	2.500	(50)	7.7	5.0	0.92
	800	(228)	0.0040	2.59	(58)	5.0	3.7	0.30
	1000	(333)	0.0041	2.98	(60)	2.8	3.0	0.47

* v = vol. of slops fed/day; V = working volume (3000 ml)

** Vol. transferred from acid. phase

*** Vol. corresponding to original slop waste

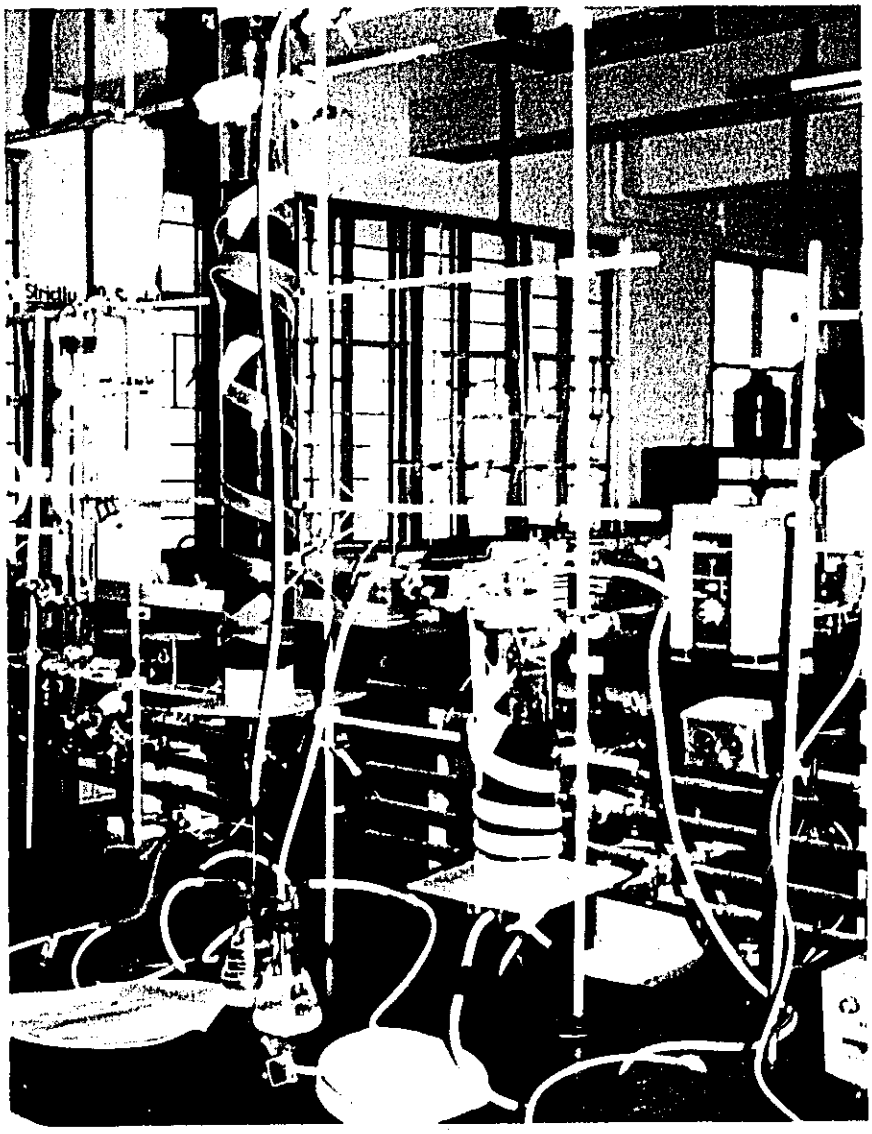

Figure 8: Set-up of two-phase fermentation of slop wastes under thermophilic condition.

Table 2. Chemical changes in a single and two-phase fermentation system under thermophilic condition.

	Vol. of slops, ml (v)	Equiv. COD Ld. (kg/day)	Gas evolved, rate (L/day)	(% CH4)	COD redn. rate (%/day)	HRT = $\frac{V}{v}$ (days)	VFA Acc. $\left(\frac{mg/L}{day}\right)$
Single-Phase	60	0.0090	4.23	(45)	17.0	50.0	3.87
	120	0.0120	6.68	(45)	23.0	25.0	5.54
	200	0.0130	16.40	(45)	40.0	15.0	5.54
	270	0.0080	9.00	(50)	30.0	11.1	3.21
	400	0.0125	14.5	(33)	32.5	7.5	3.19
Two-Phase	200	0.0180	6.75	(22)	13.0	15	2.00
Acid	300	0.0240	10.00	(22)	12.0	10	10.00
	500	0.0380	15.00	(20)	11.0	6	11.00
	1000	0.0570	24.00	(20)	14.0	3	13.00
Methanogenic	403 (54)	0.0055	2.45	(53)	14.0	7.4	2.15
	480 (76)	0.0045	3.25	(48)	16.5	6.3	2.66
	710 (168)	0.0065	12.25	(61)	19.0	4.2	2.78
	949 (300)	0.0075	16.70	(57)	17.5	3.2	3.55
	1342 (600)	0.0120	20.50	(60)	13.5	2.2	4.68

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STUDY 3: MICROBIAL DECOLORIZATION OF ALCOHOL FERMENTATION AND DISTILLERY WASTES INCLUDING BIOGAS PRODUCTION

SUB-STUDY 3B: MICROBIAL DECOLORIZATION OF DISTILLERY SLOPS AND BIOGAS EFFLUENT

ABSTRACT

Anaerobic decolorization of biogas effluent and slops was investigated using agar bacteria cells (isolate no. 6) immobilized in 2% sodium alginate solution at 35°C. The highest decolorization obtained was 72.04% in effluent at pH 7 after 48 hrs using 1.92 g cells/100 ml sodium alginate solution. Decreasing rates of decolorization were observed both for effluent and slops at pH 7 and 4 within 25 days using the same immobilized cells. Enzyme activity, however remained from 25 to 40% of the original.

Semi-continuous decolorization process was compared with the batch process for a period of 20 days. The highest decolorization yield was 47.38% in the semi-continuous process and 53.49% in the batch process using 2.13 g cells/100 ml sodium alginate solution after 2 days. Decreasing rates of decolorization were also observed both in the batch and semi-continuous processes. Enzyme activity remained up to 20% in both processes.

The relationship between agar liquefaction ability and decolorization activity was studied. The isolate which lost its agar liquefaction ability was able to decolorize effluent.

INTRODUCTION

Molasses wastewater from the alcohol fermentation industry is usually treated by biological processes such as methane fermentation and activated sludge treatment. The wastewater obtained has a dark brown pigment called melanoidin.

Several studies have been done on the decolorization of molasses wastewater by fungi and bacteria. In 1973, Hongo and co-workers (1) isolated some strain of Basidiomycetes which are efficient decolorizers of distillery waste pigments. Enzymatic decolorization of melanoidin by *Coriolus* sp no. 20 was reported by Watanabe et al. (2) in 1982. This organism was able to reduce 77% of the darkness of the melanoidin solution. In 1984, Aoshima et al. (3) selected a strain of Basidiomycetes *Coriolus versicolor* Ps4a possessing a high decolorization activity of approximately 80% under culture condition. Ito and Ueda (4) reported an 80% decrease in the original color intensity in 8 hrs using the same strain in a continuous decolorization (OD = 3.5 at 475 nm) of molasses fermentation residue in a bioreactor.

In 1981, Atthasampunna and Ohmomo (5) were able to isolate a strain of yeast which is capable of decolorizing molasses pigment by 50% after 2 days of incubation at 40-45 °C. This yeast requires glucose and yeast extract for growth and decolorization activity. Sirianuntapiboon (6) isolated *Aspergillus* sp. no. 2-6-1 which has a melanoidin decolorization activity at 60% at 45 °C within 3 days.

Ueda and Tozawa (7) in 1984 reported a 60% decrease in original color intensity of molasses pigment and 80% in synthetic pigment using immobilized agar bacteria cells under anaerobic conditions.

The objectives of the study were to: 1) optimize growth conditions of the agar bacteria for decolorization experiments; 2) establish immobilization procedure for agar bacteria cells; 3) determine decolorization activity of immobilized agar bacteria cells in slops and biogas effluent under anaerobic conditions and; 4) design batch and semi-continuous processes using biogas effluent in bioreactors.

MATERIALS AND METHODS

A. Cell preparation

Fifty fermentation flasks were each coated with 60 ml of the agar medium (Appendix A) with 2 g agar. Forty ml of the same medium were added to a 48-hr slant culture of the local isolate and then added in each of the fermentation flasks as inoculum. The fermentation flasks were shaken on a reciprocal shaker at the conditions of 120 rpm, 7 cm stroke at 35°C for 2 to 3 days.

The cells were separated from the medium after shaking by centrifugation at 415 x G for 30 min to remove the residual agar. The supernatant was again centrifuged at 10,387 x G for 15 minutes.

The cells collected were suspended in 100 ml distilled water. Two ml were dried at 103°C overnight to determine the dry weight of the cell.

The cell suspension was centrifuged again to collect them as a paste for immobilization.

B. Immobilization procedure for agar bacteria

Sodium alginate solution was prepared by adding 2 g of sodium alginate to 60 ml distilled water and steamed until sodium alginate was completely dissolved. The solution was allowed to cool to remove the air bubbles.

Distilled water was added to the cell paste to make up a volume of 40 ml. The cell suspension was thoroughly mixed and transferred to the cooled sodium alginate solution.

The mixture of cell and sodium alginate solution was added dropwise with the use of a pipette or a microtube peristaltic pump to an ice-cold 1% calcium chloride ($CaCl_2$) solution with constant stirring. The beads formed were allowed to settle in $CaCl_2$ solution overnight at refrigeration temperature. The volume of the beads was determined by water displacement.

C. Method of Decolorization

For the initial screening for the decolorization activity of the immobilized cells, 15 ml of the beads were added to 30 ml of the effluent or slops with 1% glucose and 0.1% CaCl (absorbance was adjusted to 3.5 to 4.0 at 475 nm with distilled water) at two pH values 4 and 7. Monitoring of the pH was done daily for 2 to 3 days. Likewise, 5-ml samples were taken daily for absorbance readings.

The stability of the immobilized cells was determined by replacing the slops and effluent every 2 days but using the same beads. This was done for more than 500 hrs.

To determine the decolorization activity, the samples and control (untreated and kept in the refrigerator during reaction time) were centrifuged at 10,079 x G for 15 min. One ml was added to 9 ml of either buffer solution of pH 4 or 7 depending on the original pH of the sample. This was done to bring back the pH of the resulting solution to its original value so as to eliminate the effect of pH on the color of the solution. Absorbance readings were taken using a spectrophotometer (Spectroplus D) at 475 nm. Percent decolorization was computed by dividing the difference of the final and intial absorbance with the initial absorbance.

RESULTS AND DISCUSSION

1. Decolorization of slops and effluent by immobilized cells

a. Determination of shaking time for cell preparation

Two shaking times for the cell preparation of isolate no. 6 were tested to determine optimum shaking period for high cell yields. Cell yields of isolate no. 6 are shown in Table 1.

Results showed that a reproducible cell yield cannot be obtained. Besides, the cell yields obtained were very low from a 5-li medium. This could be attributed to the following: 1) inefficient separation of the residual agar from the cells; 2) shaking time may not be enough to maximize cell growth; 3) composition of the medium used and; 4) rate of oxygen supplied for growth may not be optimal.

It was observed that after more than 72 hrs of shaking, isolate no. 6 produced a slime-like product which made the separation of the cell from the residual agar more difficult.

b. Batch decolorization process using isolate no. 6

Although the organism was aerobic, the decolorization process proceeded anaerobically with the use of the immobilized cell system.

Table 1. Cell yields of isolate no. 6 from a 5-1i medium after 48 and 72 hours shaking at 120 rpm.

Shaking time (hr)	Trial No.	Cell yield (g, DCW)
48	1	1.92
	2	2.45
	3	1.55
72	1	1.33
	2	1.47

DCW — dry cell weight

Resting cells could also be used but new cells have to be prepared for every treatment since the cells can be washed out during replacement of slops or effluent. With the cells entrapped in an immobilized state, there was no washout during the replacement of the treatment solutions.

Using 1.92g cells/100 ml sodium alginate solution, the highest decolorization activity obtained was 72.04% in effluent at pH 7 after 48 hrs. Effluent at pH 4 was decolorized by 54.40% while slops were decolorized by 49.99% and 39.70%, respectively at pH 7 and 4 after 48 hrs. These results are presented in Figures 1 and 2.

The figures represent a decreasing trend in the decolorization activity both for effluent and slops for more than 500 hrs at two pH values. There was a drastic decrease (72.04 to 38%) in the decolorization rate for effluent at pH 7. Slops at pH 7 and 4 gave a relatively stable decolorization yield at 25 to 30% after more than 500 hrs.

When 2.45 g cells/ 100 ml sodium alginate solution was employed, the highest decolorization activity was 71.18% using effluent at pH 7 after 48 hrs. Effluent at pH 4 gave 65.83% decolorization while slops at pH 7 and 4 had 56.76% and 47.18% decolorization respectively, after 48 hrs. These results are presented in Figures 3 and 4.

As illustrated in Figures 3 and 4, replacement of the treatment solutions was done every 3 days to check if further decolorization occurred after 2 days. The result however showed that there was a decrease in the decolorization activity after 48 hrs. Compared to trial 1, higher decolorization activities were obtained from the effluent at pH 4 and slops at pH 7 and 4. This could be due to a higher cell concentration of the immobilized cells used.

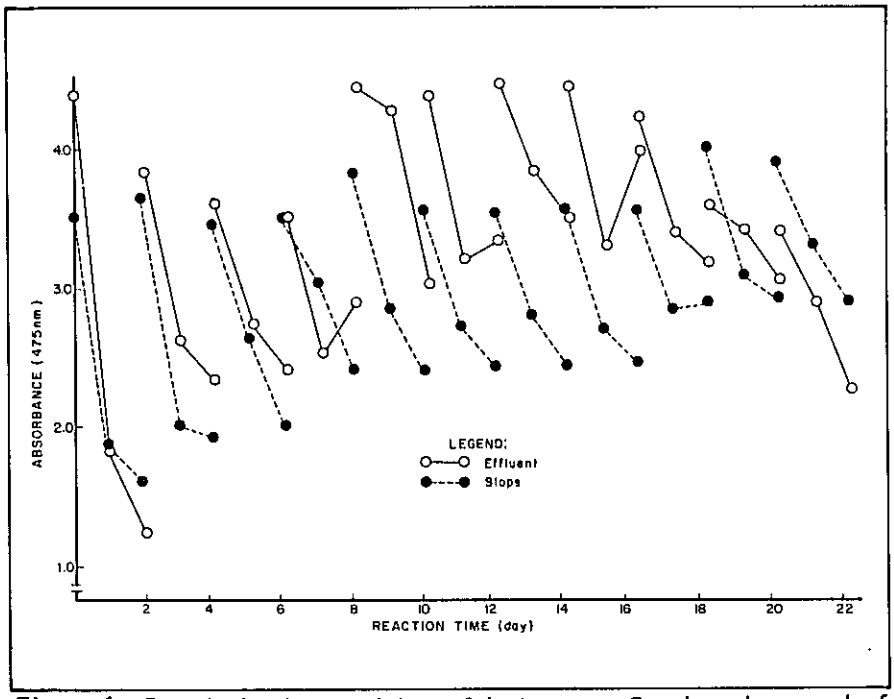

Figure 1. Decolorization activity of isolate no. 6 using slops and effluent at pH. 7.0, trial 1.

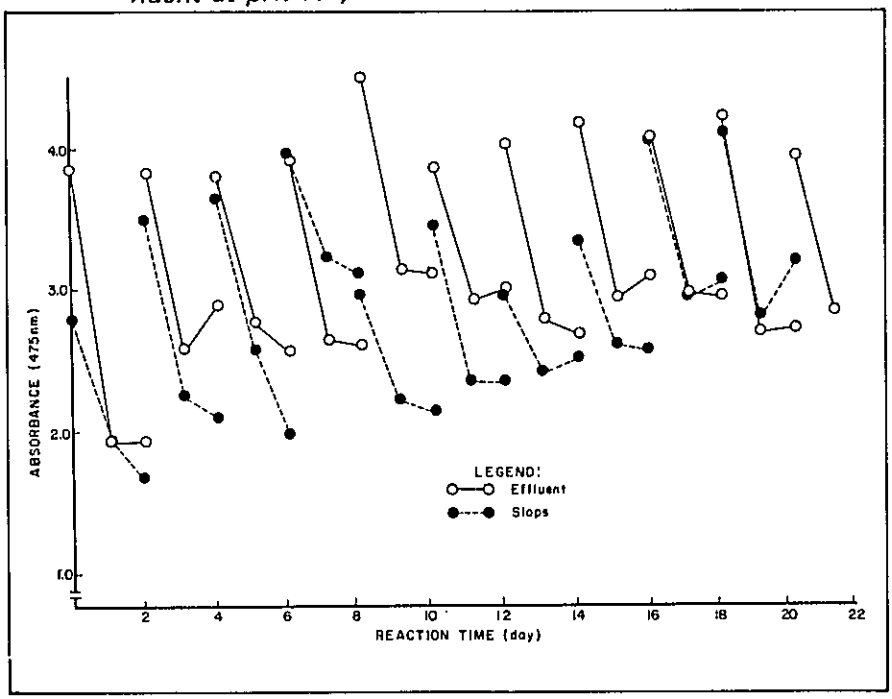

Figure 2. Decolorization activity of isolate no. 6 using slops and effluent at pH. 4.0, trial 1.

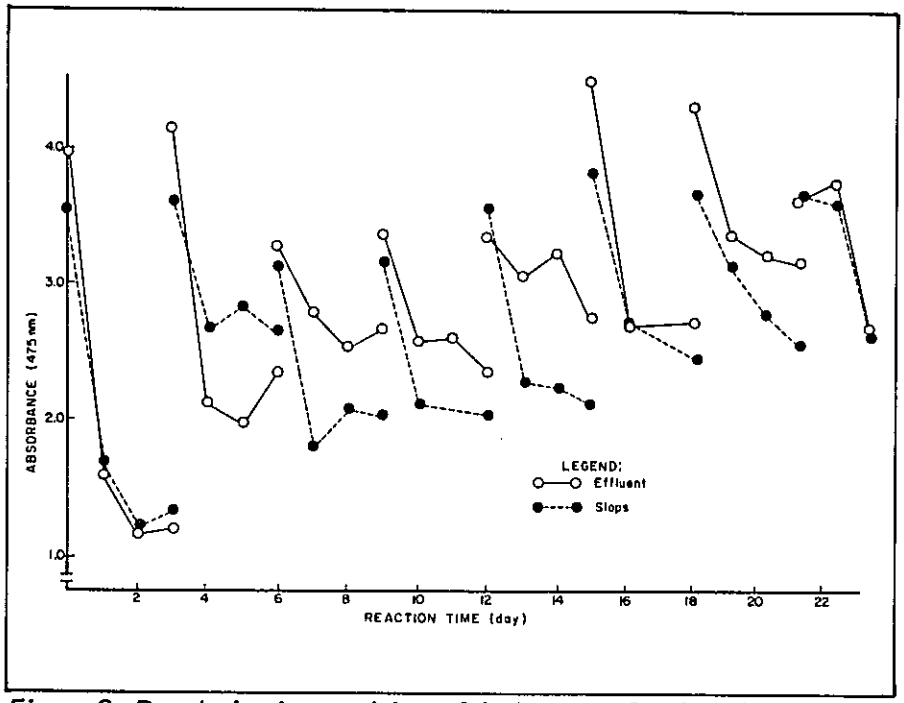

Figure 3. Decolorization activity of isolate no. 6 using slops and effluent at pH. 7, trial 2.

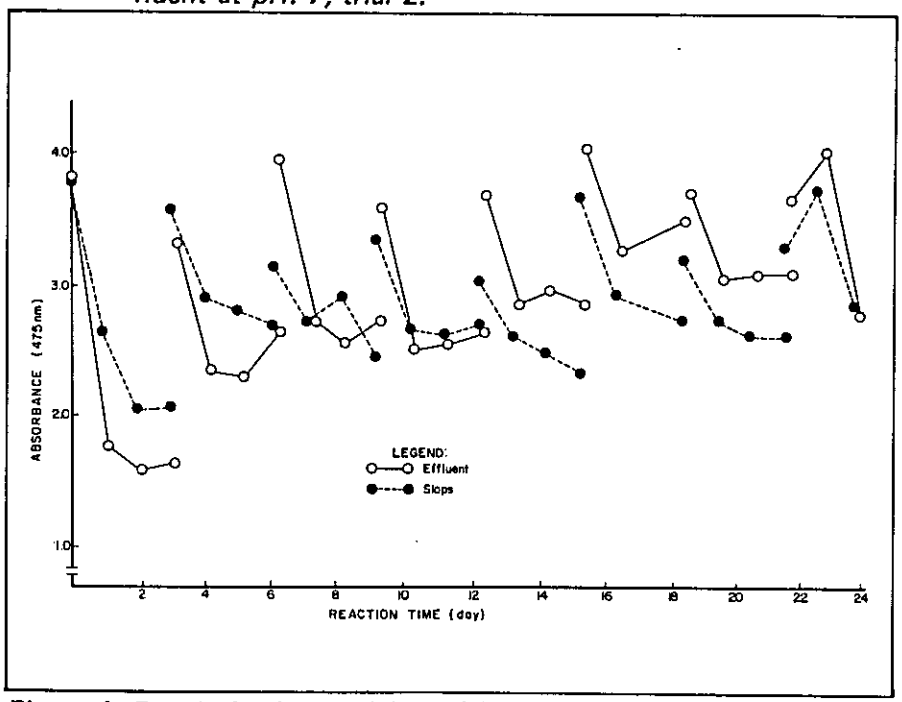

Figure 4. Decolorization activity of isolate no. 6 using slops and effluent at pH 4.0, trial 2.

Results of Trial 2 likewise showed that there was a decreasing trend in the decolorization of slops and effluent after more than 500 hrs. For effluent at pH 7, the decrease in the decolorization activity was not as abrupt as in Trial 1. For effluent at pH 4, decolorization values of 35 to 40% were retained after 500 hrs while for slops, 25 to 35% and 15 to 20% activities were retained at pH 7 and 4 respectively.

Both trials indicated a decrease in the decolorization activity of the immobilized cells after more than 500 hrs. This could be attributed to the leaking of the enzyme from the beads as a result of the breaking up of the beads. Although the beads were rigid, handling like washing and straining during replacement of the treatment solution could cause damage to the beads. This could be prevented by a semi-continuous process of decolorization wherein no physical handling of the beads would occur during replacement of the treatment solution. If the gel strength of the beads is increased, enzyme activity would be affected. However, further experiments should be done to prove this claim.

Another factor which has to be considered is the presence of a protease enzyme which could destroy the enzyme responsible for decolorization. The protease could be produced by the organism itself or by contaminating microorganisms. The presence of inhibitors in the treatment solutions could also affect the decolorization activity of the immobilized cells.

Both trials indicated that effluent at pH 7 was the best treatment solution for decolorization. The resulting pH for all the treatments after 2 to 3 days was in the range of 3.5 to 4.0 regardless of whether the starting pH was 4 or 7.

The beads formed by immobilization were rigid enough to carry out decolorization activity for more than 500 hrs. However, some of the beads also opened up during the process. One tenth % $CaCl_2$ was added to the treatment solutions to ensure the stability of the beads, but this would also add up to the production cost. Hence a more stable immobilizing agent has to be considered.

c. Effect of slops of the decolorization activity of the immobilized cells

The immobilized cells were immersed in pure slops with 0.1% $CaCl_2$ solution at pH 7 to determine if the substrate (slops) would enhance the decolorization activity of the cells. A control was made by immersing the same amount of immobilized cells in 0.1% $CaCl_2$ solution only. Decolorization activities for both treatments were determined.

The results of the activity of the immobilized cells immersed in slops prior to decolorization experiments are shown in Figure 5.

Lower decolorization yields were obtained from the immobilized cells immersed in slops. Immobilized cells immersed in water decolorized the effluent by 40.09% as against 4.25% when immersed in slops. When slops was used as the treatment solution, 44.58% decolorization was obtained in immobilized cells immersed in water against 10.17% in cells immersed in slops. This could be due to a protease enzyme present in the immobilized cells that was enhanced by the substrate (slops) having nitrogen content. The protease enzyme could have destroyed the enzyme responsible for decolorization, hence the low decolorization activity. Water with $CaCl_2$ does not have any nitrogen content, hence the protease enzyme could not be induced resulting in higher decolorization rates.

d. Effect of toluene on the decolorization activity of the immobilized cells

The immobilized cells were immersed in distilled water with 2 drops of toluene to prevent the growth of contaminants and to accelerate autolysis of the agar bacteria. The same amount of immobilized cells were also immersed in distilled water without toluene to serve as control. Both treatments had 0.1% $CaCl_2$ added. Two incubation periods were used, 4 and 7 days, at 35°C. Since toluene is volatile, a few drops were added every day for the set-up with toluene during the incubation period. The cells from each treatment were tested for their decolorization activity.

Results in Table 2 show that there was not much difference in the decolorization rates of the beads immersed in distilled water with toluene from that with only distilled water. After 4 days in toluene, effluent and slops were decolorized by 33.49% and 17.28% respectively. When the immobilized cells were immersed in distilled water for 4 days, the decolorizations rates were 26.08% and 23.23% for effluent and slops respectively. After 7 days in toluene, effluent gave 20.83% and slops 26.48% decolorization rates in immobilized cells with toluene.

Toluene causes lysis of the cells hence its effect on the immobilized cells was determined. If toluene can cause lysis, then the enzyme would be released from the cells and be made more available for reaction than the enzyme still intact within the cells. Since not much difference in the decolorization rates were observed, it can be said that even if toluene caused lysis of the cells, the enzyme released from the cells were still in the immobilized state, hence decolorization activity proceeded. Likewise, even if the cells were not lysed, the enzyme still reacted thus decolorization also proceeded.

Table 2. Decolorization rates of immobilized cell immersed in toluene or distilled water.

Substrate	Treatment of the Beads					
	Days in T	Days w/o T	Reaction Time (Days)	Abs, Co	Abs, Sple	% Decol.
Effluent	4		1	4.18	3.12	35.36
			2		2.78	33.49
	7		1	3.65	2.88	21.10
			2		2.89	20.83
		4	1	4.18	3.55	15.07
			2		3.09	26.08
		7	1	3.65	2.96	18.90
			2		2.74	24.93
Slops	4		1	3.53	3.35	5.09
			2		2.92	17.28
	7		1	3.70	2.80	24.32
			2		2.72	26.49
		4	1	3.53	2.89	18.13
			2		2.71	23.23
		7	1	3.70	2.45	20.27
			2		3.36	9.20

e. Determination of the best working volume

Working volumes of 1:2, 1:3 and 1:4 (beads: solution) were used. Decolorization activity was monitored for 2 days.

The best working volume was 1:2 beads to solution ratio as shown in Figure 6. This could be attributed to the effect of cell concentration to the final volume of substrate. At a fixed cell concentration, increasing volumes of substrate will give a decreasing rate of decolorization. This trend however could be observed before optimum cell concentration is achieved.

g. Semi-continuous decolorization process using isolate no. 6

The decolorization process was scaled up from 30 ml volume of substrate to 500 ml with a beads: solution ratio of 1:3. Two 700-ml column reactors were fabricated (Figure 7). The semi-continuous process was achieved by daily feedings of 500 ml volumes in the reactors for a period of 20 days using the same immobilized cells.

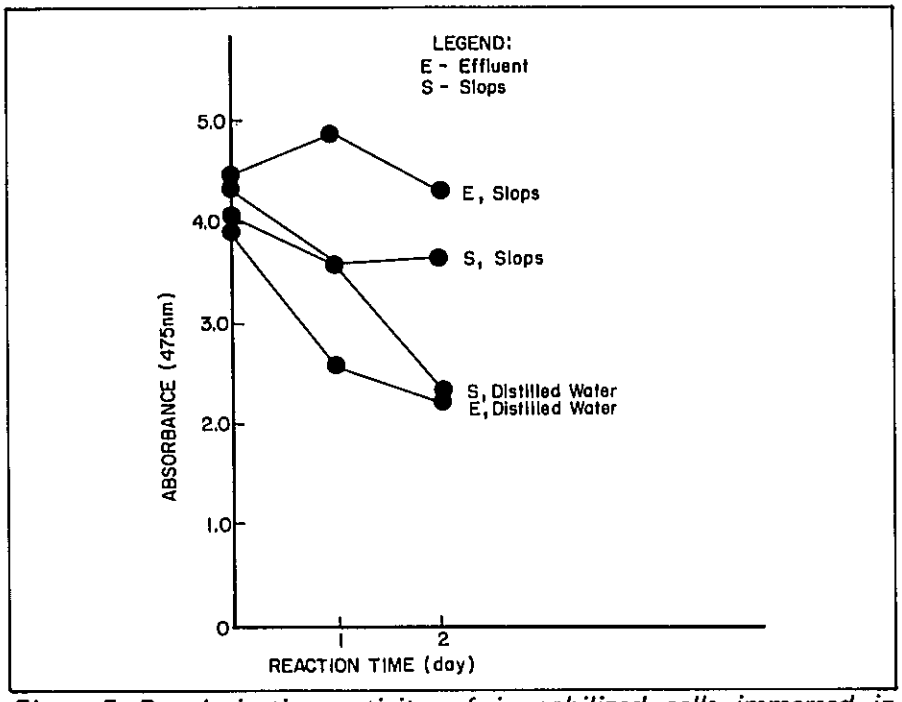

Figure 5. Decolorization activity of immobilized cells immersed in slops and distilled water.

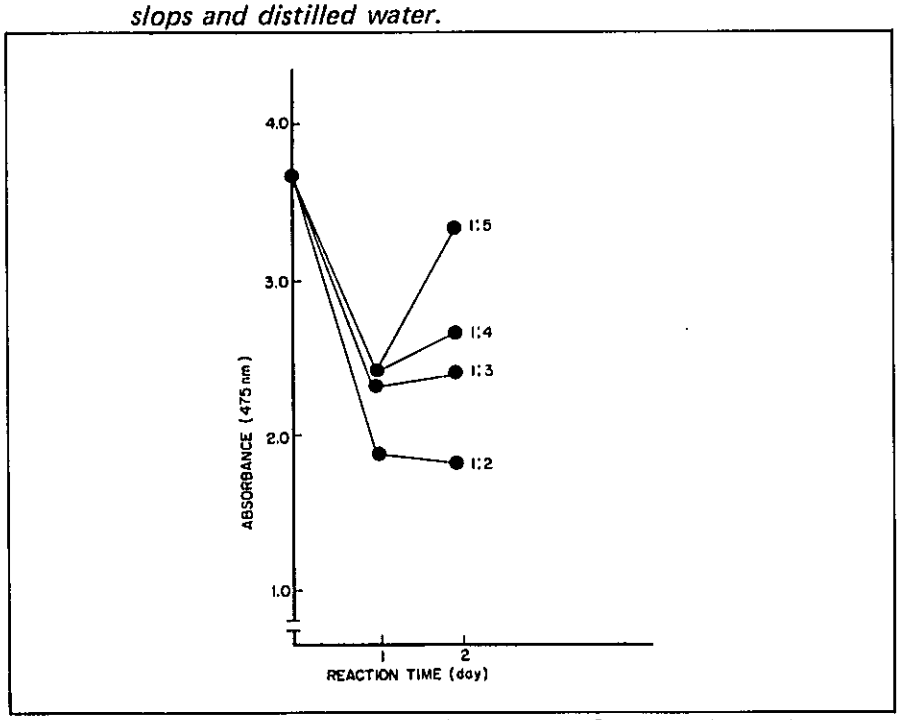

Figure 6. Decolorization rates of isolate no. 6 at varying volumes of treatment solution.

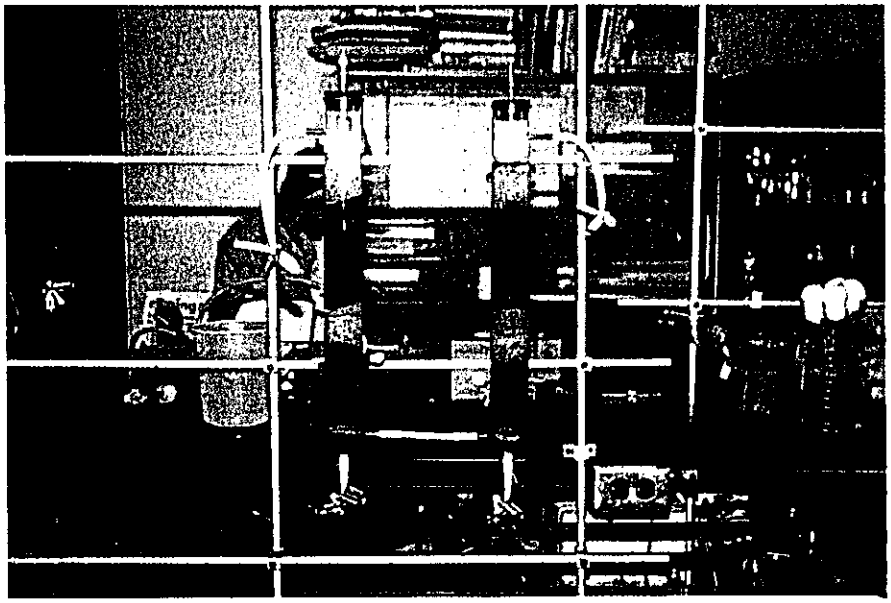

Figure 7. Semi-continuous decolorization set-up.

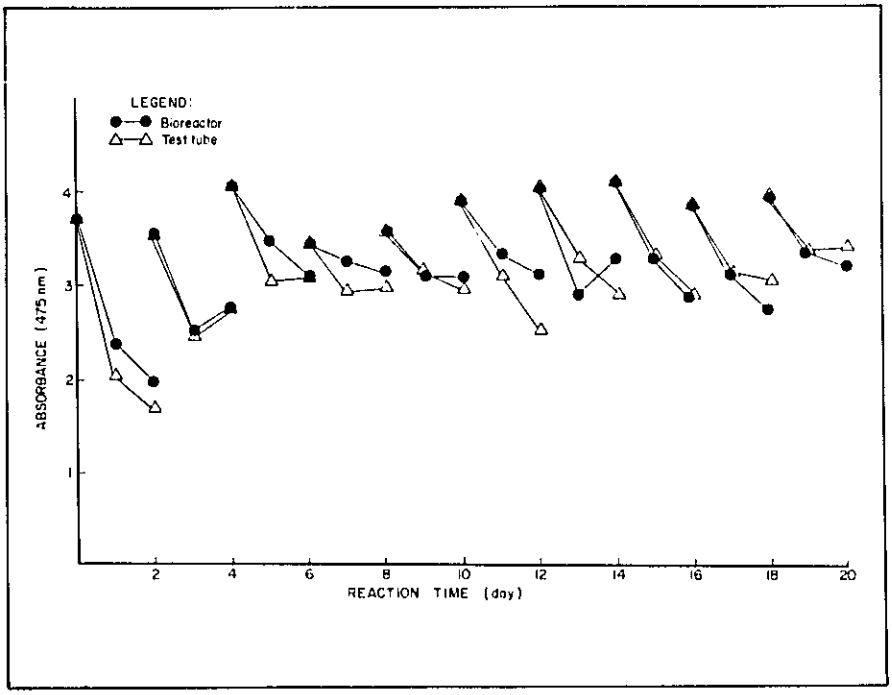

Figure 8. Semi-continuous and batch decolorization processes using isolate no. 6.

This operating period (20 days) corresponding to 10 cycles, with a retention time of 2 days per cycle. A cycle consisted of 1 day retention time in reactor 1 and another day in reactor 2. Daily pH readings and 5 ml samples were taken for absorbance readings. Simultaneous with the column reactor experiment, batch process using test tubes was also done using the same beads: solution ratio.

Using 2.38 g cells/100 ml sodium alginate solution, the highest decolorization yield obtained in the column reactors was 47.58% after 2 days (Figure 8). The succeeding cycles show a decreasing trend in the decolorization activity with about 20% activity retained. In the batch process, the highest decolorization yield was 53.49% after 2 days. Decreasing trend in the decolorization activity was also observed with 20-25% activity retained (Figure 9).

Compared with first and second batch processes, the decolorization yields were much lower. This could be due to the difference in the composition of the substrate. The slops in the first and second batch processes came from Nasugbu, Batangas while the semi-continuous process used slops from Tarlac. In the first and second batch processes, about 20% substrate was used to obtain an absorbance reading of 3.5 to 4.0, whereas in the semi-continuous process, as much as 50% substrate was used to get the same initial absorbance reading. The substrate used in the semi-continuous process was more concentrated.

Another problem which arose during the semi-continuous process was the presence of contaminants which could have affected the decolorization activity of the immobilized cells.

2. Relationship between agar liquefaction and decolorization activity

The isolate which can grow on glucose agar and broth and has lost its liquefaction ability was tested for its decolorization activity. The isolates which can grow on glucose agar are shown in Table 3. After the first transfer from glucose medium, liquefaction was still observed in all the isolates. After the second and third transfers, liquefaction was no longer observed. These results indicate that the ability to liquefy may be lost after repeated transfers to glucose medium. The isolates when grown on agar medium used agar as the main carbon source. When the isolates were transferred to glucose medium, the isolates utilized glucose as the carbon source, hence no liquefaction was observed.

All the isolates grew in glucose broth as presented in Table 4. However, some of the isolates did not grow on agar medium after the first transfer from glucose broth. Isolate no. 7 was able to liquefy after the first transfer from glucose broth. In the following transfers,

Table 3. Growth of the isolates on glucose (GM) and agar (AM) media.

Isolate No.	GM1	AM1	GM2	AM2	GM3	AM3
8_1	+ (−L)	+ (+L)	+ (−L)	+ (−L)	+ (−L)	+ (−L)
8_2	+ (−L)	+ (+L)	+ (−L)	+ (−L)	+ (−L)	+ (−L)
6	+ (−L)	+ (+L)	+ (−L)	+ (−L)	+ (−L)	+ (−L)
7	+ (−L)	+ (+L)	+ (−L)	+ (−L)	+ (−L)	+ (−L)
13	+ (−L)	+ (+L)	+ (−L)	+ (−L)	+ (−L)	+ (−L)

\+ : positive growth
− : negative growth
+L : with liquefaction
−L : without liquefaction

Table 4. Growth of the isolates on glucose broth (GB) and agar medium (AM).

Isolate No.	GB1	AM1	GB2	AM2	GB3	AM31	AM32
8_1	+ (−L)	− (−L)	+ (−L)	− (−L)	+ (−L)	− (−L)	− (−L)
8_2	+ (−L)	− (−L)	+ (−L)	− (−L)	+ (−L)	− (−L)	− (−L)
6	+ (−L)	+ (−L)	+ (−L)	+ (−L)	+ (−L)	± (−L)	− (−L)
7	+ (−L)	+ (+L)	+ (−L)	+ (−L)	+ (−L)	± (−L)	− (−L)
13	+ (−L)	+ (−L)	+ (−L)	+ (−L)	+ (−L)	± (−L)	− (−L)

\+ : positive growth
− : negative growth
± : poor growth
+L : with liquefaction
−L : without liquefaction

isolate nos. 8_1 and 8_2 did not grow on agar medium from the broth. Inability of the organism to grow on agar medium from a broth medium is due to the absence of the agar in the broth medium. Since no other carbon source was present in the agar medium except agar, the organism utilizing glucose as the carbon source could not grow on agar medium.

Isolate nos. 6 which lost its liquefaction ability was tested for its decolorization activity. It gave a decolorization yield of 40.84% after 2 days in effluent at pH 7 using 0.3375 g cells/50 ml sodium alginate solution. After 3 cycles, enzyme activity remained at 20% (Figure 9).

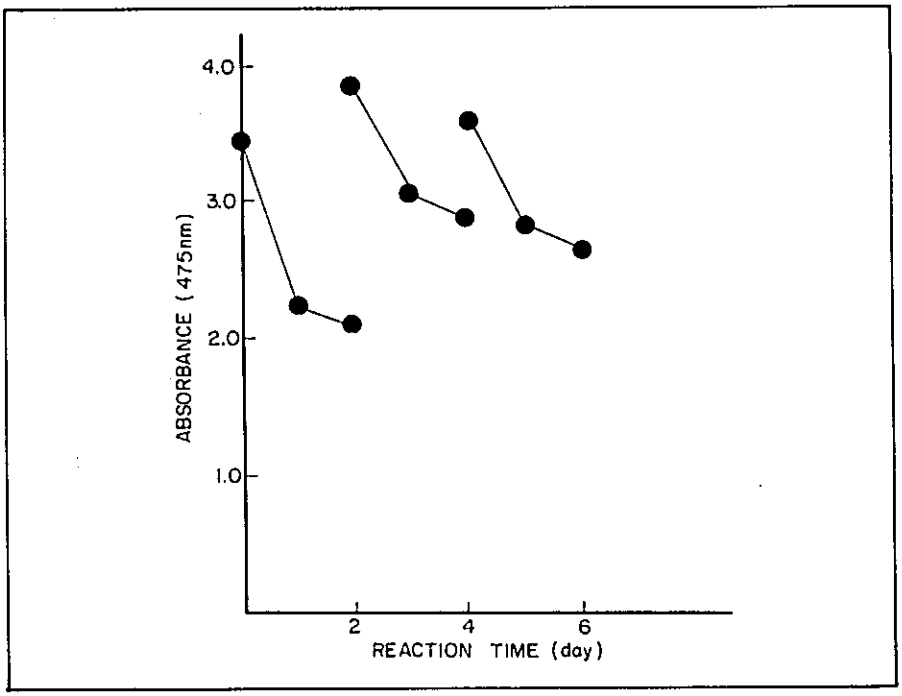

Figure 9. Decolorization activity of isolate no. 6, which has lost its agar lique faction ability, in effluent.

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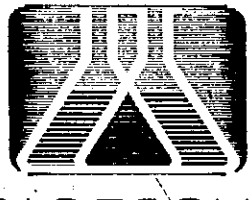
APPENDIX A

Composition of medium

$NaNO_3$	– 2 g
K_2HPO_4	– 1 g
$MgSO_4$	– 0.5 g
distilled water	1 L
pH	8.5



JICA ~ BIOTECH
joint study project on

NITROGEN FIXATION

STUDY 1: ISOLATION, CLASSIFICATION AND SCREENING OF BACTERIAL STRAINS FOR HIGH NITROGEN FIXATION

CHARACTERIZATION OF NITROGEN-FIXING RHIZOBIAL ISOLATES FROM ROOT NODULES OF *LEUCAENA LEUCOCEPHALA* AND *VIGNA RADIATA*

ABSTRACT

There were variations in the growth and cultural characteristics of the 38 rhizobial isolates from ipil-ipil and mungbean. Although they exhibited characteristic properties in yeast extract mannitol agar (YEMA), they differed in colony diameter and growth rate. Thirty isolates were fast-growers and only eight were slow-growers. Ninety percent of the isolates including all ipil-ipil rhizobial isolates exhibited acidic reaction in yeast extract mannitol medium; 55% exhibited marked growth in peptone glucose agar and almost 50% caused a slight pH change in litmus milk.

The ability of the isolates to cause nodule formation on homologous host proved that they are indeed rhizobia. Among the mungbean rhizobial isolates, only BJVr-6 and BJVr-4 were effective in fixing nitrogen under screenhouse conditions. Others were either ineffective or were not able to compete for nodule sites with the native rhizobia. The screening experiment further indicated no significant differences among five control treatments.

Screening among ipil-ipil rhizobial isolates, however, indicated that all were as effective as the control strain CB 81 in fixing nitrogen under screenhouse conditions. Likewise, nitrogenase activity was comparable except isolates BJL1-1, BJL1-7 and BJL1-10, which had significantly lower nitrogenase activity than CB 81.

Among the 26 isolates characterized biochemically, BJLI-14, BJLI-30, BJVr-7 and BJVr-12 exhibited a wide tolerance to NaCl, pH and antibiotics. These isolates could grow well even at 4-5% NaCl and at both pH extremes. They were also resistant to streptomycin and penicillin G. In addition, four other mungbean rhizobial isolates were resistant to tetracycline. The NaCl tolerance of the rest of the isolates, however, ranged from 0-1% only while pH tolerance was either one-sided or a narrow one. All isolates were catalase positive and tyrosinase negative. Finally, hypertrophy initiating ability test indicated that all the isolates do not belong to *Agrobacterium*.

INTRODUCTION

The diversity of the root nodule bacteria makes adequate characterization indispensable in any attempt to classify and identify rhizobial strains of high nitrogen fixation potentials. A combination of morphological, cultural, physiological and serological tests are usually employed in addition to infective properties. It is important, however, that when assessing these characteristics a strain must be effective in fixing nitrogen.

MATERIALS AND METHODS

The growth and cultural characteristics of rhizobial isolates were determined on four media, i.e., YEMA + Congo red, YEMA + brom-

thymol blue, peptone glucose agar + bromcresol purple and litmus milk (Somasegaran, et al., 1985). Observations were done after three to five days and 14 days for litmus milk at 28°C.

For infectivity test, pre-germinated seeds were transplanted into growth pouches containing Jensen's seedling medium. After five days they were inoculated with broth cultures of the isolates. Harvesting was done after six and nine weeks for mungbean and ipil-ipil, respectively. The number and weight of nodules formed were determined and analyzed statistically for significance.

To test the effectivity of the isolates in nitrogen fixation, screenhouse experiment in three successive batches was conducted using Annam clay soil ameliorated with 1.65 g lime and 2.0 solophos/2 kg soil. Mungbean seedlings were inoculated with broth culture inoculum right after planting and were harvested six weeks thereafter while ipil-ipil seedlings were inoculated five days after planting and were harvested 11 weeks thereafter. Nodulation, nitrogenase activity of the nodules, dry matter yield and nitrogen fixation by plants were determined and analyzed statistically for significance. The acetylene reduction assay (ARA) was used to determine the nitrogenase activity (Somasegaran, et al., 1985) and the Kjeldahl method was used to determine the amount of nitrogen fixed by the plants (AOAC, 1980)

For biochemical characterization, selected isolates were studied using physiological/biochemical tests. These tests were as follows: hypertrophy initiating ability, NaCl tolerance, pH tolerance, antibiotic resistance, tyrosinase, and catalase. Standard methods for these tests defined in the Bergey's Manual of Systematic Bacteriology (1984) were followed.

RESULTS AND DISCUSSION

The growth and cultural characteristics of 26 ipil-ipil and 12 mungbean rhizobial isolates were determined using four media usually employed for presumptive identification of rhizobia (Table 1). In yeast extract mannitol agar (YEMA) containing bromthymol blue, 34 isolates exhibited acidic reaction and only four (all mungbean isolates) exhibited alkaline reaction (Figure 1). In peptone glucose agar, 21 isolates had marked growth, four had very little growth and 13 had no growth at all. In litmus milk, however, almost half of the isolates caused a slight change in pH while the rest did not. Some isolates also formed clear serum zone on litmus milk. Four ipil-ipil rhizobial isolates (BJLI-8, BJLI-9, BJLI-12 and BJLI-15) exhibited strange reactions like curdling of litmus milk and acidic reaction in peptone glucose agar and were therefore considered non-rhizobial isolates.

All isolates exhibited characteristic properties of the root nodule bacteria in YEMA containing Congo red, i.e., whitish, circular and

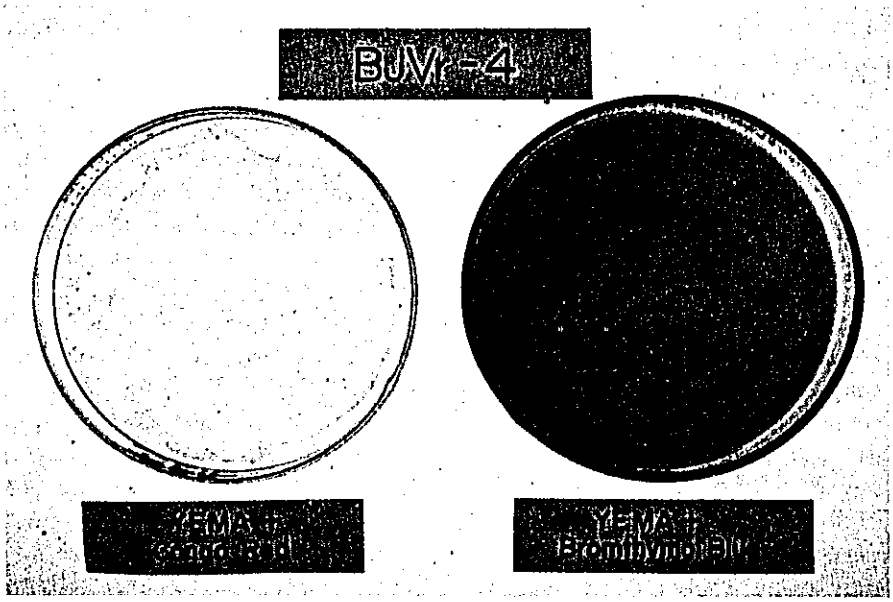


Figure 1. Alkaline production of BJVR-4 in yeast extract mannitol agar after 7 days incubation at 28°C.

mucoid colonies but differed in colony diameter and growth rate (Table 1). Colonies with diameter of 2-4 mm within two or three days at 28°C are fast growers (Jordan, 1984) while those which grow after 5-7 days are slow-growers (Somasegaran et al., 1985). Of the 26 ipil-ipil rhizobial isolates, 22 were fast-growers and four slow-growers while among the mungbean rhizobial isolates, eight were fast-growers and four slow-growers.

To test the identity of the isolates at the generic level their nodulating ability was studied. Further screening for effectivity to fix atmospheric nitrogen in symbiosis with a homologous host was also undertaken. The isolates were indeed rhizobia since they caused nodule formation on homologous hosts. Among the mungbean rhizobial isolates only BJVr-4 was comparable with control strain R-302 in terms of the number of nodules produced. However, seven produced high nodule dry weights which were comparable with that of control strain R-302 under growth chamber conditions (Table 2). These isolates were BJVr-1, BJVr-2, BJVr-3, BJVr-4, BJVr-7, BJVr-8 and

Table 1. Cultural characteristics of ipil-ipil and mungbean rhizobial isolates in four media after 3-5 days incubation at 28° C.

Isolate[1/]	YEMA + Btb		Peptone glucose agar + Bcp			Litmus Milk: (14 days)		YEMA + Congo red[3/]			
	Acid Reaction	Alkaline Reaction	Marked growth no pH change	Very little growth, no pH change	No growth No pH change	Slight pH change	No pH Change	Colony 3 Days	dia (mm) 5 Days	Growth rate Fast (F)	Slow (S)
BJL1- 1	X				X		X	2.0-3.0			F
2	X		X				X	2.0-4.0			F
3	X				X	X		1.0-2.0			F
4	X				X		X[2/]	1.0-2.5			F
5	X				X		X	1.0-2.0			F
6	X				X		X	1.5-2.0			F
7	X				X		X	1.0-2.5			F
10	X			X			X		1.0 - 2.0		S
11	X		X				X[2/]	2.0-3.0			F
13	X		X			X[2/]		1.0-2.0			F
14	X		X			X[2/]		2.0-3.0			F
16	X		X			X[2/]		1.0-2.0			F
17	X				X	X[2/]		1.0-1.5			F
18	X				X		X[2/]	1.0-1.5			F
19	X				X		X[2/]	1.0-1.5			F
20	X				X		X	1.0-1.5			F
21	X				X	X[2/]		1.0-2.0			F
22	X			X			X		1.0 - 2.0		S
23	X			X			X		1.5 - 2.0		S
24	X		X				X		1.5		S
25	X				X		X	1.0-1.5			F
26	X		X				X[2/]	1.0-2.0			F
27	X		X				X[2/]	1.0-2.0			F
28	X				X		X	1.0-2.0			F
29	X			X			X[2/]	1.0-2.0			F
30	X		X			X		1.0-3.0			F

Table I. Continued . . . . .

Isolate[1]	YEMA + Btb		Peptone glucose agor + Bcp			Litmus Milk (14 days)		YEMA + Congo red[3]		
	Acid Reaction	Alkaline Reaction	Marked growth, no pH change	Very little growth, no pH change	No growth No pH change	Slight pH change	No pH change	Colony 3 Days	dia (mm) 5 Days	Growth rate Fast (F) / Slow (S)
BJVr- 1		X	X			X			1.0	S
2		X	X			X			1.0	S
3		X	X			X			1.0 - 1.5	S
4		X	X			X			1.0 - 1.5	S
BJVr- 5	X							2.0-4.0		F
6	X		X			X		3.0-5.0		F
7	X		X			X[2]		2.0-3.0		F
8	X		X			X[2]		1.0-1.5		F
9	X		X				X[2]	4.0-4.5		F
10	X		X			X		3.0-5.0		F
11	X		X			X		3.0-4.0		F
12	X		X			X		4.0		F
Controls:										
CB 81	X				X		X		1.0 - 1.5	S
R 302		X			X		X		1.0	S
Totals	34*	4	21	4	13	18	20	30	8	F = 30
Percent	89.5	10.5	55.3	10.5	34.2	47.4	52.6	78.9	21.1	S = 8

[1] Ipil-ipil isolates BJL1-8, BJL1-9, BJL1-12 and BJL1-15 were also tested but they did not give characteristic reactions of rhizobia.

[2] Formed clear serum zone on the surface.

[3] All isolates exhibited characteristic white colony growth and did not appreciably absorb the dye.

x = The type of reaction that occurred with the indicated isolate.

Table 2. Mean effect of rhizobial inoculation on the nodule number and weight of mungbean grown in a growth chamber for 6 weeks.[1]

Treatment/Isolate	Nodule Number (no/3 plants)	Nodule dry weight (mg/3 plants)
Uninoculated	0	0
BJVr- 1	47 bc	9.5 a
2	36 bcd	11.9 a
3	45 bc	9.7 a
4	52 ab	10.5 a
5	25 d	4.9 b
6	6 e	3.9 b
7	33 cd	10.0 a
8	43 bc	11.8 a
9	7 e	2.0 b
10	26 d	5.2 b
11	7 e	2.5 b
12	45 bc	9.0 a
R-302 (Control)	63 a	12.6 a

[1] Means followed by the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

BJVr-12. The rest of the isolates produced lower nodule dry weights. Under screenhouse conditions, however, nodulation and dry matter production failed to show any significant differences among isolates, strain R-32, N control (30 kg N/ha rate) and uninoculated control (Table 3). Likewise, the amount of nitrogen fixed did not differ significantly except for isolates BJVr-6 and BJVr-4. It appears therefore that the native rhizobia present in the soil (uninoculated control) had the same nodulating ability as the 12 isolates and they were as effective as the 10 isolates. Although isolates BJVr-6 and BJVr-4 were able to fix significantly higher amounts of nitrogen, they did not produce the higher nodule dry weight. Higher nodulating ability did not consequently mean effective nitrogen fixation. In terms of the controls used, the result revealed no significant differences among the five control treatments included in the experiment.

Among the ipil-ipil rhizobial isolates, the number of nodules produced was comparable with that of the control strain CB 81, except isolates BJLI-1 and BJLI-2 (Table 4). Isolate BJLI-30 formed the

highest number of nodules/3 plants. In terms of nodule weight, only 17 were comparable with CB 81 including the two isolates forming the lowest number of nodules. The remaining nine isolates produced lower nodule yield. The results of the first screenhouse experiment involving 14 ipil-ipil rhizobial isolates revealed no significant differences among isolates, CB 81 and uninoculated control in terms of nodulation and dry matter production (Table 5). Although the native rhizobia in the soil (uninoculated control) formed the lowest number of nodules and dry matter production, these were not significantly different from those of the isolates. Thus, they seemed to have the same infective ability as the isolates. The nitrogenase activity measured by acetylene reduction assay (ARA) and the amount of N fixed in the plants revealed that the isolates did not differ with CB 81.

In the second experiment involving 12 ipil-ipil rhizobial isolates, the nodule yield did not differ among isolates and the same strain CB 81. However, five isolates had significantly higher nodule yield than the uninoculated control (Table 6). The nitrogenase activity did not show significant differences among CB 81 and BJLI-6, BJLI-18, BJLI-20, BJLI-22, BJLI-25, BJLI-26, BJLI-27, BJLI-19 and BJLI-24. The rest (3) exhibited lower nitrogenase activity. In terms of the dry matter yield, no significant difference was observed between the isolates and CB 81. Likewise, the amount of nitrogen fixed failed to show any significant differences.

Seven mungbean and 19 ipil-ipil rhizobial isolates were selected for biochemical characterization. The test for tolerance to NaCl failed to show much variation among the isolates since 50% did not tolerate even 1% NaCl while the other 50% tolerated 1% with isolates BJLI-14, BJLI-30, BJVr4-7 and BJVr-12 tolerating even 5% NaCl (Table 7). In terms of pH tolerance, the isolates exhibited varied responses to pH ranging from 3.5 to 9.5. Four groups of reactions were observed. The four isolates most tolerant to NaCl including isolate BJLI-27 and BJVr-8 constituted the group with the widest pH tolerance range of 4.5 -9.5.

Six others constituted the group tolerating pH 5.0-9.5 while eight grew in the alkaline pH but not in extreme acidic pH. On the other hand, the last group grew towards the acidic pH but not in alkaline pH.

The determination of natural resistance to antibiotics such as streptomycin, penicillin G and tetracycline was also conducted at 50 and 100 ug/ml levels (Table 7). The same 4 isolates which tolerated high concentration of NaCl and widest pH range were also resistant to streptomycin and penicillin G but sensitive to tetracycline. The rest of the isolates were sensitive to the three antibiotics except mungbean rhizobial isolates BJVr-1, BJVr-2, BJVr-3 and BJVr-4 which

Table 3. Mean effect of rhizobial inoculation on the nodulation, dry matter production and nitrogen fixation of mungbean grown in the screenhouse for 6 weeks (3rd batch screenhouse experiment.[1/]

Treatment/ Isolate	Nodule No. (No./3 plants)	Nodule dry weight (mg/ 3 plants)	Dry Matter production (g/3 plants)	N_2 Fixed (mg/3 plants)
BJVr- 1	92 abc	97.6 abc	3.8 b	2.9 bc
2	98 ab	105.6 abc	5.1 a	22.5 abc
3	121 a	110.7 abc	3.9 b	12.4 bc
4	70 bcd	92.0 bc	5.2 a	31.3 ab
5	78 bcd	90.2 bc	4.6 ab	16.4 abc
6	64 bcd	104.1 abc	5.2 a	42.3 a
7	51 cd	135.8 abc	4.4 ab	7.0 bc
8	50 cd	85.2 c	3.7 b	0 c
9	59 bcd	166.4 a	5.0 a	10.4 bc
10	69 bcd	111.2 abc	3.9 b	0 c
11	38 d	80.9 c	4.4 ab	2.2 bc
12	60 bcd	162.5 ab	4.3 ab	2.9 bc
R-302 (Control strain)	94 ab	110.7 abc	3.7 b	0 c
30 kg N/ha	70 bcd	116.1 abc	4.3 ab	1.0 c
Uninoculated control	77 bcd	142.5 abc	4.7 ab	–
R-302 (US)[2/]	57 bcd	101.2 abc	3.7 b	0 c
Uninoculated (Us)[2/]	65 bcd	125.7 abc	3.7 b	0 c

[1/]Means followed by the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

[2/]Unameliorated soil (US) was used to determine whether Annam clay soil can subsequently be used for mungbean inoculation without further ammendment.

Table 4. Mean effect of rhizobial inoculation on the nodule number and dry weight of ipil-ipil grown in a growth chamber for 9 weeks.[1/]

Treatment/Isolate	Nodule Number (No/3 plants)	Nodule dry weight (mg/3 plants)
BJLI- 1	6 d	9.6 abcde
BJL1- 2	6 cd	10.6 abcde
3	13 abcd	9.9 abcde
4	15 abc	13.8 abcd
5	12 bcd	14.4 abc
6	16 ab	10.6 abcde
7	14 abcd	11.5 abcde
10	15 abcd	15.3 ab
11	8 bcd	8.6 bcde
13	9 bcd	12.3 abcde
14	12 abcd	10.0 abcde
16	13 abcd	10.3 abcde
17	11 bcd	9.6 abcde
18	15 abc	13.2 abcd
19	10 bcd	12.9 abcde
20	10 bcd	9.5 abcde
21	12 bcd	8.6 bcde
22	15 abcd	14.0 abcd
23	12 bcd	12.1 abcde
24	10 bcd	5.9 e
25	15 abcd	6.7 de
26	12 abcd	6.6 de
27	11 bcd	8.7 bcde
28	9 bcd	5.9 e
29	11 bcd	7.6 cde
30	21 e	8.6 bcde
CB 81 (Control Strain)	16 ab	16.8 a

[1/]Means followed by the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

Table 5. Mean effect of rhizobial inoculation on the nodulation, nitrogenase activity of the nodules, dry matter production and nitrogen fixation of ipil-ipil grown in the screenhouse for 12 weeks (first batch screenhouse experiment).[1/]

Treatment/ Isolate	Nodule Number (no/2 plants)	Nodule weight (mg/2 plants)	Nitrogenase Activity (nMC_2H_4/hr/ mg nodule)	Dry matter production (g/2 plants)	ΔN_2 Fixed (mg/2 plants)
BJL1- 2	52 b	50.0 b	21.2 abc	6.1 abc	42.7 ab
3	78 ab	128.2 ab	16.2 abc	7.2 abc 1	53.7 ab
4	108 ab	152.7 ab	11.8 ab	8.6 a	67.7 ab
5	41 b	79.8 ab	15.9 abc	8.6 a	75.5 ab
11	58 b	51.3 b	18.2 abc	6.9 abc	60.2 ab
13	69 b	72.0 ab	21.4 ab	4.9 bc	22.6 b
14	81 ab	117.6 ab	21.2 abc	7.8 ab	65.8 ab
16	61 b	67.6 ab	20.9 abc	6.0 abc	40.9 ab
17	147 a	185.3 a	7.0 c	6.1 abc	40.8 ab
21	103 ab	118.9 ab	18.2 abc	6.7 abc	47.0 ab
23	69 b	105.1 ab	13.6 abc	8.9 a	36.3 a
28	69 b	124.8 ab	12.0 abc	8.1 ab	76.1 a
29	95 ab	113.7 ab	8.6 bc	6.2 abc	40.2 ab
30	64 b	119.7 ab	23.2 a	7.1 abc	64.1 ab
CB 81 (control strain)	78 ab	105.2 ab	11.0 abc	7.3 abc	56.5 ab
Uninoc. control	36 b	43.5 b	13.8 abc	4.2 c	0

[1/]Means followed by the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

Table 6. Means effect of rhizobial inoculation on the nodulation, nitrogenase activity of the nodules, dry matter production and nitrogen fixation of ipil-ipil grown in the screenhouse for 12 weeks (second batch screenhouse experiment).[1]

Treatment/ Isolate	Nodule Number (no/2 plants)	Nodule weight (mg/2 plants)	Nitrogenase Activity (nMC_2H_4 hr/ mg nodule)	Dry matter Production (g/2 plants)	ΔN_2 Fixed (mg/2 plants)
BJL1- 1	29 ab	79.9 ab	85.2 cd	7.6 abcd	102.1 abc
6	33 ab	150.5 a	133.7 abc	8.7 abc	112.8 abc
7	31 ab	111.7 ab	85.9 cd	7.6 abcd	97.6 abc
10	34 ab	132.8 a	66.3 d	8.8 abc	134.3 a
18	23 abc	142.2 a	136.5 ab	7.2 abcd	94.1 abc
19	32 ab	131.9 a	90.3 bcd	5.8 cd	73.7 c
20	30 ab	109.0 ab	159.0 a	6.2 cd	87.5 bc
22	25 abc	79.9 ab	143.7 a	5.2 d	67.9 c
24	12 bc	84.1 ab	90.5 bcd	10.4 a	120.4 a
25	30 ab	118.3 ab	124.4 abc	9.6 ab	105.6 abc
26	25 abc	86.5 ab	166.1 a	9.6 ab	112.1 ab
27	39 a	127.3 a	136.1 ab	6.6 bcd	81.8 bc
CB 81 (control)	27 abc	129. 1 a	137.4 ab	7.6 abcd	100.7 abc
Uninoculated	8 c	38.9 b	73.6 d	1.2 e	–

[1] Means followed by the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

Table 7. Biochemical characteristics of 26 selected ipil-ipil and mungbean rhizobial isolates incubated for 3-7 days at 28°C[1/]

Isolate	NaCl Tolerance					pH Tolerance								Antibiotic resistance					
														Strepto-mycin (ug/ml)		Pen. G. (ug/ml)		Tetracyc-line (ug/ml)	
	1%	2%	3%	4%	5%	3.5	4.0	4.5	5.0	7.0	8.0	9.0	9.5	50	100	50	100	50	100
BJL1- 3	±	−	−	−	−	−	−	−	−	+	+	±	−	−	−	−	−	−	−
4	+	−	−	−	−	−	−	−	−	+	±	−	−	−	−	−	−	−	−
5	±	−	−	−	−	−	−	−	−	+	±	−	−	−	−	−	−	−	−
6	+	−	−	−	−	−	−	−	−	+	±	−	−	−	−	−	−	−	−
7	±	−	−	−	−	−	−	−	−	+	±	±	+	−	−	−	−	−	−
10	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−
11	+	−	−	−	−	−	−	−		+	+	+	+	−	−	−	−	−	−
13	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
14	+	+	+	±	±	−	−	+	+	+	+	+	+	+	±	+	+	−	−
16	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
17	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
18	−	−	−	−	−	−	−	+	+	+	±	−	−	−	−	−	−	−	−
19	−	−	−	−	−	−	−	+	+	+	±	−	−	−	−	−	−	−	−
21	−	−	−	−	−	−	−	+	+	+	±	−	−	−	−	−	−	−	−
22	+	−	−	−	−	−	−	±	+	+	±	±	±	−	−	−	−	−	−
23	−	−	−	−	−	−	−	+	+	+	±	±	−	−	−	−	−	−	−
27	+	−	−	−	−	−	+	+	+	+	±	±	+	−	−	−	−	−	−
28	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
30	+	+	+	+	+	−	+	+	+	+	+	+	+	±	±	+	+	−	−

Table 7. Biochemical characteristics of 26 selected ipil-ipil and mungbean rhizobial isolates incubated for 3-7 days at 28°C.[1/]

BJVr-	1	−	−	−	−	−	−	−	−	+	+	+	+	±	−	−	−	−	+	−
	2	−	−	−	−	−	−	−	−	+	+	+	+	±	−	−	−	−	+	−
	3	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	—	+	−
	4	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	+	−
	7	+	+	+	±	±	−	−	±	+	+	+	+	+	+	±	+	+	−	−
	8	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	−
	12	+	+	+	±	±	−	−	+	+	+	+	+	+	+	±	+	+	−	−
CB	81	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
R-	302	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	−

[1/]Test for catalase (positive for all isolates), hypertrophy initiating ability (negative for all isolates), and tyrosinase test (negative for all isolates) were also conducted. The hypertrophy initiating ability was done by using the carrot disc assay technique. All observations were based on two replicates.

+ = Approximately the same growth as in the control medium ± = Less growth than in the control medium — = No growth

tolerated 50 ug/ml level of tetracycline but not 100 ug/ml. Furthermore, the isolates were tested for catalase (positive for all isolates) and tyrosinase (negative for all isolates). Hypertrophy initiating ability test failed to show gall production on carrot discs indicating that the isolates belonged to *Rhizobium* or *Bradyrhizobium* but not *Agrobacterium.* Finally, further tests such as carbohydrate utilization, nitrate reduction and others are under way to fully characterize/classify the isolates.

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STUDY 2. STUDIES ON CONDITIONS FOR MASS PRODUCTION OF RHIZOBIAL INOCULANTS

OPTIMIZATION STUDIES ON SOME PHYSICO-CHEMICAL VARIABLES AFFECTING THE PRODUCTION OF RHIZOBIAL INOCULANTS AND EXPLORATION OF ALTERNATIVE APPROACHES FOR COST REDUCTION DURING MASS-INOCULANT PRODUCTION

ABSTRACT

Optimization studies were conducted to determine the best pH, temperature and oxygen supply rates necessary for the growth of promising rhizobial strains from agro-forest legumes. Results revealed that BGs-5 could grow well at pH 6.0-8.0 but not at pH 4.0-5.0 using the standard medium YEMB. The best growth of the strain was observed at pH 7.0-8.0. In the case of the slow-growing strain BEs-4, a wider range of pH tolerance was observed, i.e., pH 5.0-8.0. Best growth was observed at pH 7.0-8.0 but the strain failed to grow at pH 4.0.

Growth behaviors of the three strains on two temperature settings, viz., 30-32°C, and 33-35°C were determined. Results showed that although all strains could grow relatively well on both temperature settings, the best growth was attained at 30-32°C.

All data necessary to compute for the oxygen-transfer coefficient (Kd) of BGs-5, BAp-21, and BEs-4 were collected. These included the best working volume in a 500-ml Sakaguchi flask and oscillation rate. Results revealed that the strains had almost the same growth behavior in three working volumes, viz., 50, 70, and 100 ml. However, to ensure maximum growth, it was desirable to use a working volume of 100 ml.

Results of the growth behavior study using three strains at three oscillation rates, viz., 70 osc./min, 90 osc./min, and 120 osc./min revealed that BGs-5 and BEs-4 could grow equally well in all these oscillation rates. However, the growth of strain BAp-21 varied with the rate of oscillation. The best growth of the said strain was observed at 120 osc./min. This observation revealed that the amount of dissolved O_2 in the medium was critical for BAp-21 but not for either BGs-5 or BEs-4.

The ability of strains BGs-5 and BEs-4 to utilize three local table sugars, i.e., refined sugar (RS), medium-refined sugar (MRS), and unrefined sugar (URS) was determined. These alternative carbon sources were used instead of mannitol (1:1 substitution rate) in the preparation of the growth medium. Results revealed that strain BGs-5 could grow well on both URS and RS while BEs-4 preferred MRS and URS as alternative carbon sources. This means that it may be possible to use URS which costs ₱5.56/kg as an alternative carbon source for mannitol which costs ₱600.00/lb.

The growth of strains BGs-5, BEs-4, and BAp-21 in YEMB formulated by using tap water was determined in order to explore the possibility of utilizing undistilled water in the preparation of YEMB. Results revealed that while BGs-5 and BAp-21 could grow well in the medium with tap water, strain BEs-4 failed to grow. This indicates the inability of the latter to tolerate high salt concentration in tap water.

INTRODUCTION

The ultimate goal of Biological Nitrogen Fixation (BNF) research is the production of high-quality inoculants which could be distributed to the farmers at a reasonable cost. The production of inoculants involves the continuous selection of superior strains, mass production of these strains in a suitable growth medium under optimum culture conditions, incorporation of the cultured cells into a suitable carrier, and storage of carriers under conditions which will ensure maximum survival of cells.

Optimization studies play a vital role in any R & D project. Before using big fermentors, it is essential to understand the growth behavior of the promising strains under shake-flask conditions. Usually, the most critical physico-chemical factors are temperature, pH and dissolved oxygen concentration.

The ability of rhizobial strains to tolerate certain temperature ranges have already been studied by some workers. In 1982, Boonkerd and Weaver (1) reported a decrease of two log values in two rhizobial strains when the inoculants were kept at 35°C after 45 days of incubation. For all strains tested, 46°C was found to be lethal. Higashi, et al. in 1983 (2) also reported a 50% loss of infectivity of a rhizobial strain when the cells were incubated at 37°C without shaking for 7 days.

Another factor affecting growth is the pH of the medium. Various strains of rhizobia have different adaptation to the pH of the substrate (3, 4, and 5). Although most rhizobial strains grow at pH values ranging from 5.0-7.0, some are able to tolerate low pH values ranging from 4.5-3.5 (6 and 7). Hartel in 1983 (8) observed that most of the 20 strains he tested could grow even at pH 3.6 but they preferred pH 4.5. Munns and Keyser in 1979 (9) however, reported that low pH generally increased lag time and slowed growth of most of the cowpea strains he studied. Fifty percent of these strains stopped growing under acidic conditions.

Since rhizobia are strictly aerobic organisms, they require a considerable amount of oxygen for growth. Vigorous aeration of broth cultures must be supplied for rapid growth and higher cell yields (10). Although some strains may not require high amounts of dissolved oxygen, infectivity and survival might be affected by low concentration of available oxygen in stationary cultures. This might be the reason why Higashi et al. in 1983 (2) observed the loss of infectivity of surviving cells of *Rhizobium trifolii* when the latter was grown in broth medium without shaking for seven days.

Besides the technical aspect of mass production, researchers should not lose sight of its economic aspect. There should be a con-

tinuous search for inexpensive and indigenous growth media. Although some researchers reported the possibility of using coconut water as growth medium (11 and 12), problems in handling and transport of this local substrate from the processing plant to the mass-inoculant plant may effect the feasibility of its utilization. Another alternative however, is to use local table sugar as substitute for mannitol which costs as much as P600.00 per lb. Most recently, Padilla and Takahashi in 1985 (13) reported the successful use of the Japanese table sugar from sugar beet in growing rhizobial strains from agro-forest legumes. Studies conducted by BIOTECH scientists also revealed that sucrose, in the form of locally refined table sugar could be used as substitute for mannitol in growing isolates from food legumes (14). Other researchers also suggested the use of sucrose instead of mannitol because the former is cheap, readily available and growth of the isolates are not negatively affected (15 and 16). In fact, Iswaran in 1973 (17) reported that *R. japonicum,* an unusually slow-growing strain in YEMB could grow quickly if sucrose is used instead of mannitol.

The cost of using distilled water for making the growth medium is also an economic constraint. In the Philippines where most of the reservoirs supply hard water, it could be very expensive to use filters or water-distillation apparatus to remove water impurities ranging from mineral elements to colloidal particles. If local rhizobial isolates could tolerate high salt concentration in undistilled tap water, so much could be saved in the process of production. Yelton in 1983 (18) reported the ability of a fast-growing strain of *R. japonicum* to tolerate high concentration of salts. However, the growth of a slow-growing strain was affected negatively by the high salt concentration.

This study was undertaken to determine the optimum pH, temperature, and oxygen requirement necessary for the growth of promising rhizobial strains from agro-forest legumes and to determine the possibility of using modified YEMB as growth medium for these isolates.

MATERIALS AND METHODS

Optimization of the physico-chemical factors — temperature, pH, and oxygen-requirement — affecting the growth of the strains was done using rhizobial strains, BGs-5, BAp-21, and BEs-4. Fresh cultures of these strains were prepared by streaking a loopful of the stock cultures on YEMA slants contained in flat bottles. The slant cultures were incubated inside a 30°C incubator until maximum growth is obtained. Starter cultures for all experiments were prepared by flooding the freshly prepared slant cultures of each strain with

100 ml sterile distilled water. The resulting cell suspension was properly mixed by hand-shaking.

Optimum temperature requirement was determined using shake-flask experiments. Flat-shouldered flasks containing 100 ml of YEMB were inoculated with 1% starter cultures of BGs-5, BAp-21, and BEs-4. Two temperature settings, i.e. 30-32°C and 33-35°C were used in two separate batches due to lack of shaker-incubators. Each sample was replicated twice. Cell population was determined daily by direct microscopic counting for four consecutive days. The growth behavior of the strains at the two temperature settings were compared.

Determination of optimum pH necessary for the growth of BGs-5 and BEs-4 was conducted using pH levels, pH 4.0, 5.0, 6.0, 7.0, and 8.0. Adjustment of pH was done by the addition of either 1 N NaOH or 0.1 N HCl. One percent (v/v) of the starter cultures of BGs-5 and BEs-4 were inoculated separately onto the prepared broth media with different pH levels contained in flat-shouldered flasks. The inoculated cultures were dispensed into sterile test-tubes at a rate of 10 ml/tube. Each sample was replicated eight times. The tubes were incubated on a rotary shaker at room temperature (28-32°C). Daily sampling for optical density measurement and direct microscopic counting were done for four consecutive days. For the optical density measurement, an electrophotometer with a wavelength of 420 nm was used.

All information necessary to compute the oxygen transfer rate coefficient (Kd) were gathered. For the first part of the experiment, the most suitable working volume relative to the size of the shaking vessel which should be used for growing strains BGs-5, BAp-21, and BEs-4 was determined. Three working volumes of YEMB, viz., 50, 70 and 100 ml were tested using a 500-ml flat-shouldered flask. Each flask was inoculated with 1% (v/v) of the starter cultures of BGs-5, BAp-21, or BEs-4. Each sample was replicated twice. Cell population was determined daily by direct microscopic counting for four consecutive days.

The next variable tested was the oscillation rate. Three oscillation rates, i.e. 70 osc/min, 90 osc/min, and 120 osc/min were used in three separate batches. One percent (v/v) of the starter culture of BGs-5, BAp-21, or BEs-4 was inoculated separately into 100 ml YEMB contained in 500 ml flat-shouldered flasks. The broth cultures were incubated on a shaker-incubator at different oscillation rates mentioned earlier. Cell population was determined daily by the direct microscopic counting technique. The growth of the strains in the different oscillation rates were compared.

The ability of two rhizobial strains viz., BGs-5 and BEs-4, to utilize locally available table sugars was determined. Different grades of table sugars (sucrose) i.e. refined (RS), medium refined (MRS), and unrefined (URS) were substituted for YEMB at a rate of 10 g/l. The

standard YEMB was used as control. The broth media contained in flat-shouldered flasks weree inoculated with 1% (v/v) of the starter broth of BGs-5 and BEs-4 in separate batches. The inoculated broth were dispensed into empty sterile test tubes at a rate of 10 ml/tube. Each sample was replicated eight times. Cell population was determined daily for four consecutive days using the surface plating technique. Growth of the strains in these different types of table sugars was compared.

The possibility of using BIOTECH tap water instead of distilled water in constituting YEMB was also explored. One percent (v/v) of the starter broth of strains BGs-5, BAp-21, and BEs-4 was inoculated separately into YEMB with tap water or YEMB with distilled water contained in flat-shouldered flasks. The inoculated broth cultures were dispensed into empty sterile test tubes at a rate of 10 ml/tube. The tubes were incubated on a rotary shaker at room temperature (28-33°C). Each sample was replicated twice. Population determination was conducted daily for four days using the direct microscopic counting.

RESULTS AND DISCUSSION

Optimum temperature requirement

The growth behavior of isolates BGs-5, BAp-21, and BEs-4 at temperature settings 30-32°C and 33-35°C are shown in Figs. 1, 2, and 3. Apparently, isolate BGs-5 has a longer lag period when grown at 33-35°C. At a lower temperature, i.e., 30-32°C, the lag phase of the isolate is presumed to be shorter. Cell population was also higher at 30-32°C than at 33-35°C. The logarithmic growth of the isolate occurred earlier between 0-24 hrs at 30-32°C, and only after 24 hrs at 33-35°C.

In the case of BAp-21, much higher cell population was observed at 30-32°C than at 33-35°C. For both temperature settings, a lag phase of 24 hrs was observed before the population entered the logarithmic phase. The stationary phase was earlier attained at 33-35°C (i.e., 48 hrs) then at 30-32°C (i.e., 72 hrs.).

Among the three strains tested, BEs-4 appeared to show the slightest difference in growth behavior at 30-32°C and 33-35°C. The same lag period was observed using the two temperature settings. Although slightly higher cell populations were observed at 30-32°C, the population started to level-off after 72 hrs. At 33-35°C, however, the population continued to increase even at 96 hrs.

In the over-all analysis, all strains were able to grow at both temperature settings. However, better growth of these strains was observed at 30-32°C than at 33-35°C.

Optimum pH requirement

The growth behavior of BGs-5 in YEMB at different pH levels is shown in Figs. 4 and 5. Results showed that the best growth of the isolate was at pH levels 4.0 and 5.0. At pH 6.0, a moderate growth was observed. Since BGs-5 can tolerate pH 6.0 but not pH 4.0 and 5.0, it may not be effective under acidic soil conditions.

Strain BEs-4, however, had a wider range of pH tolerance (pH 5.0-8.0) as indicated by both optical density measurements and direct cell counts (Figs. 6 and 7). At pH 4.0, however, the said strain failed to grow. It was further observed that at pH 5.0-8.0, the cell population started to enter the stationary phase at 72 hrs. However, at pH 4.0, the cell population remained almost constant from 0 hr up to the last sampling period, i.e., 96 hrs.

Oxygen requirement

The first step towards determining oxygen requirement of the organism in shake-cultures is the selection of a suitable working volume relative to the size of the shaking vessel or flasks. This working volume is an important factor in the computation of the oxygen-transfer rate coefficient (Kd).

The growth behavior of BGs-5 in YEMB using three working volumes, viz., 50 ml, 70 ml, and 100 ml is shown in Fig. 8. Results showed that the strain could grow equally well using all three working volumes. Logarithmic phase was obtained shortly after inoculation of the growth media (i.e., 0-24 hrs). After 48 hrs however, the population of BGs-5 varied slightly with the working volumes used. Higher cell population was attained in 100 ml than in 70 ml and 50 ml working volumes, respectively.

The same trend of growth behavior using the same working volumes was observed in BEs-4 (Fig. 9). The strain attained the logarithmic phase starting from 24 hrs of incubation in all three working volumes used. Although after 72 hrs the cell population was slightly higher at 70 ml than at 50 and 100 ml, the growth curves in all working volumes were relatively the same.

In the case of BAp-21, better growth was observed at 100 ml working volume than at 50 ml and 70 ml (Fig. 10). The logarithmic phase was attained starting from 24 hrs using all three working volumes. Higher cell population, however, was attained at 100 ml than at 50 ml and 70 ml after 48 hrs up to the end of the incubation period.

These results showed that although strains BGs-5, BEs-4 and BAp-21 could grow relatively well on all the working volumes used, it is desirable to use 100 ml for the succeeding part of the experiment.

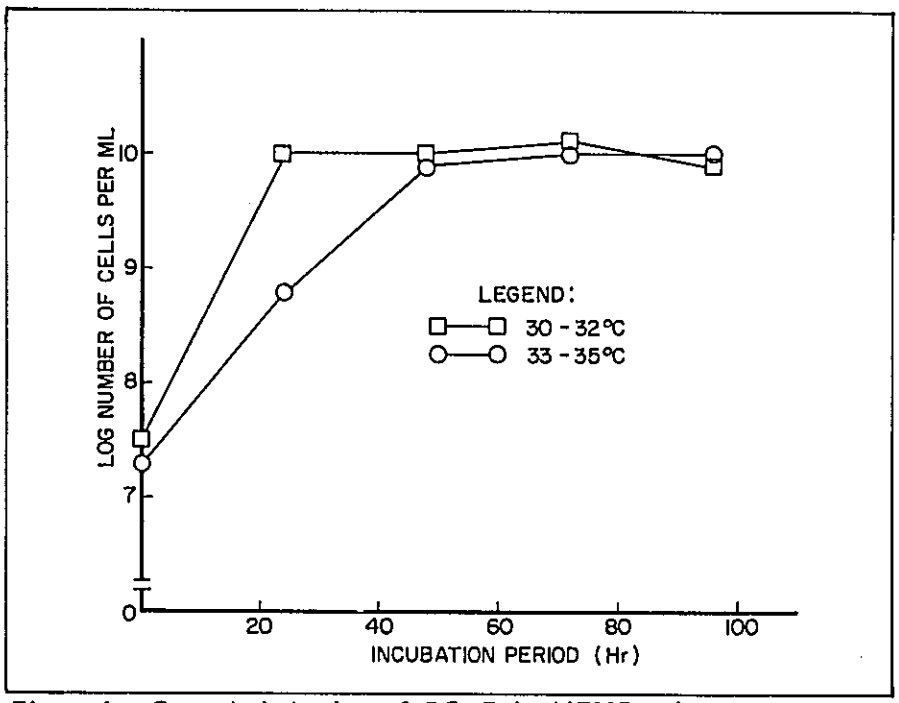

Figure 1. Growth behavior of BGs-5 in YEMB using two temperature settings (30-32°C and 33-35°C).

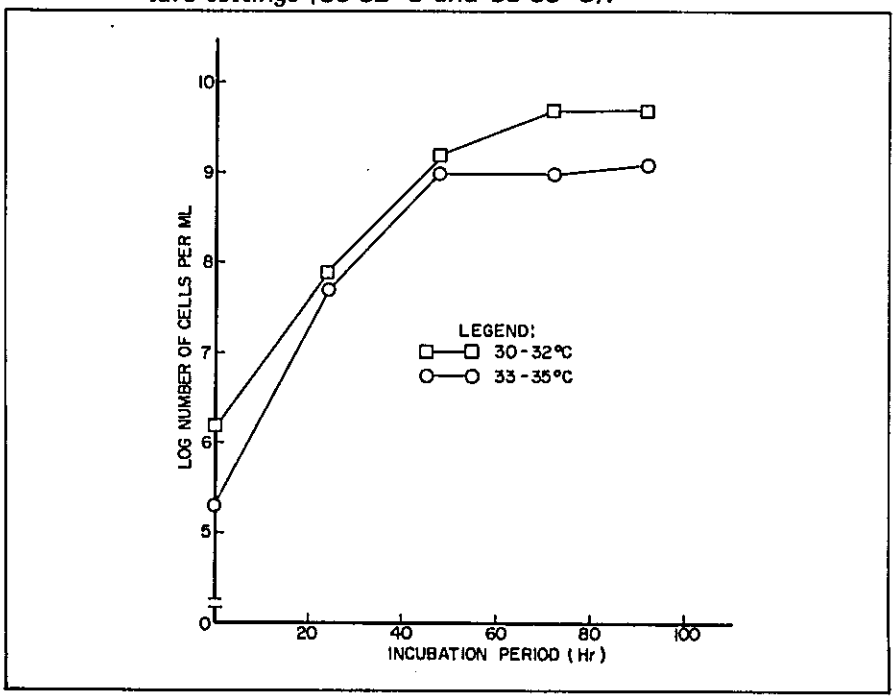

Figure 2. Growth behavior of BAp-21 in YEMB using two temperature settings (30-32°C and 33-35°C).

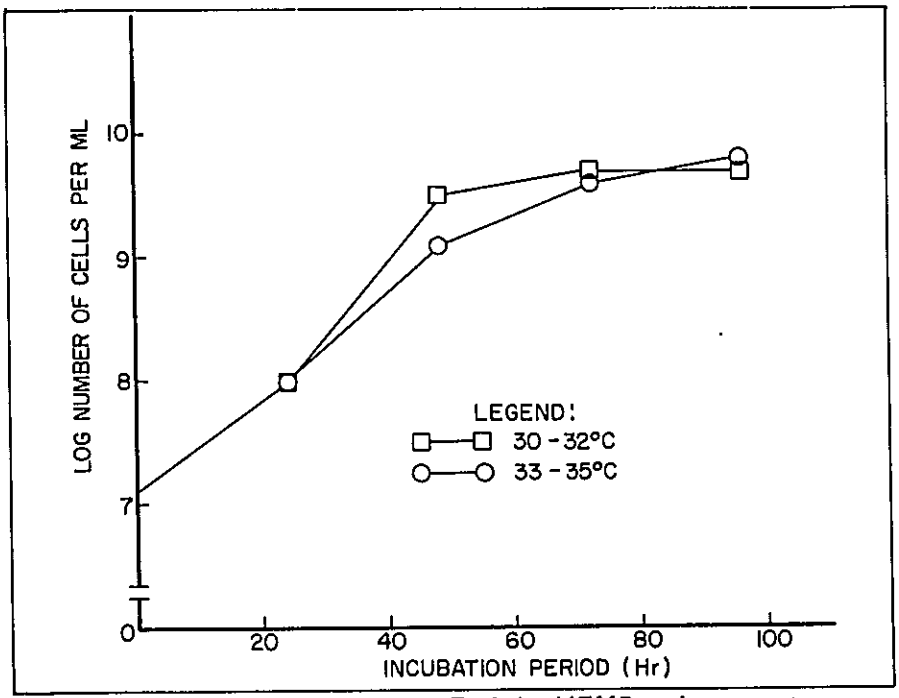

Figure 3. Growth behavior of BEs-4 in YEMB using two temperature settings (30-32°C and 33-35°C).

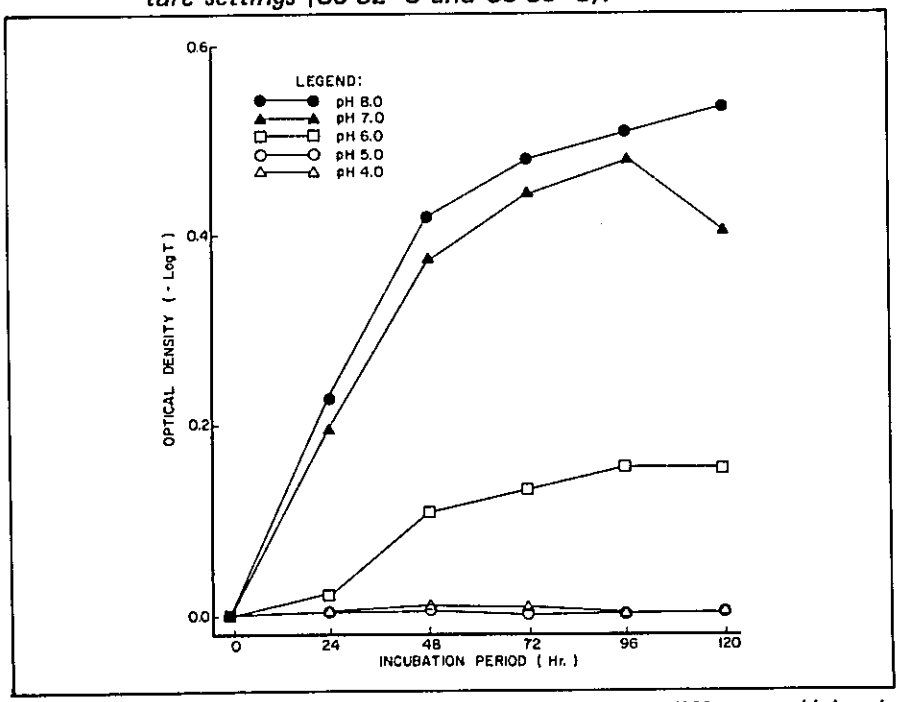

Figure 4. Growth behavior of BGs-5 in YEMB at different pH levels as determined by optical density measurement (— log T).

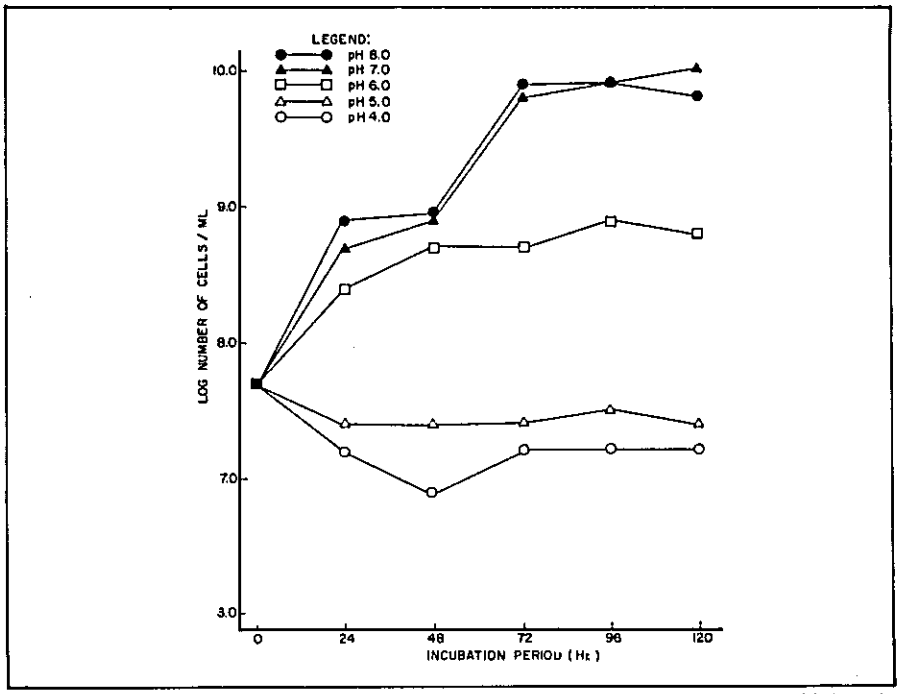

Figure 5. Growth behavior of BGs-5 in YEMB at different pH levels as determined by direct microscopic counting.

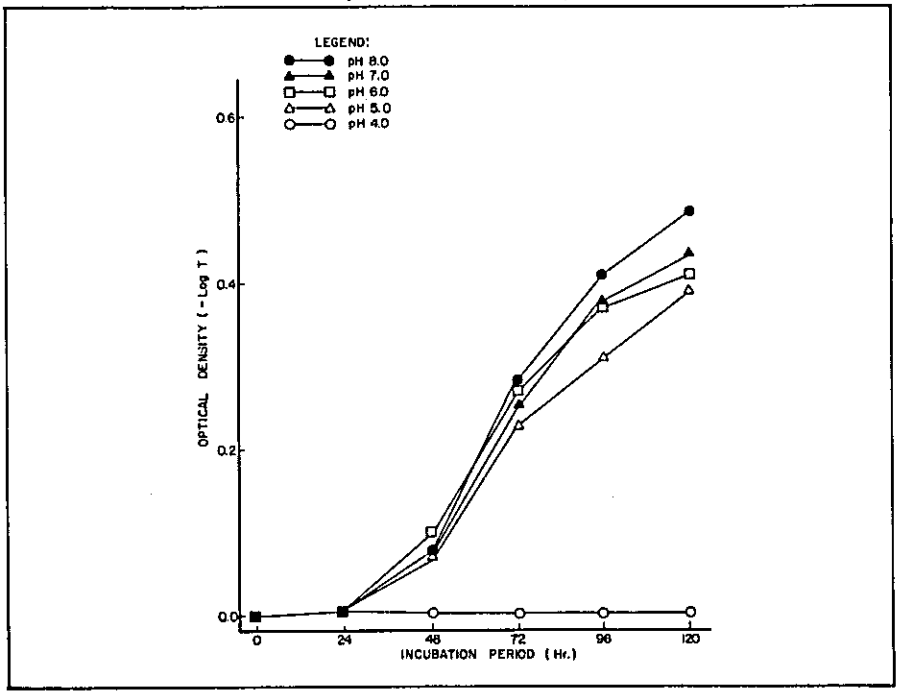

Figure 6. Growth behavior of BEs-4 in YEMB at different pH levels as determined by optical density measurement (– log T).

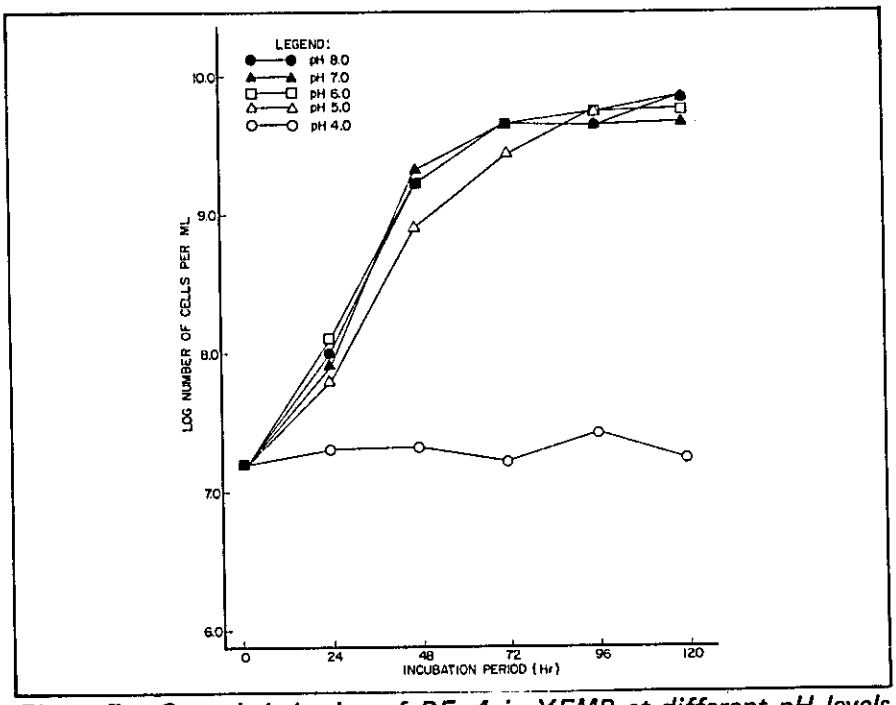

Figure 7. Growth behavior of BEs-4 in YEMB at different pH levels as determined by direct microscopic counting.

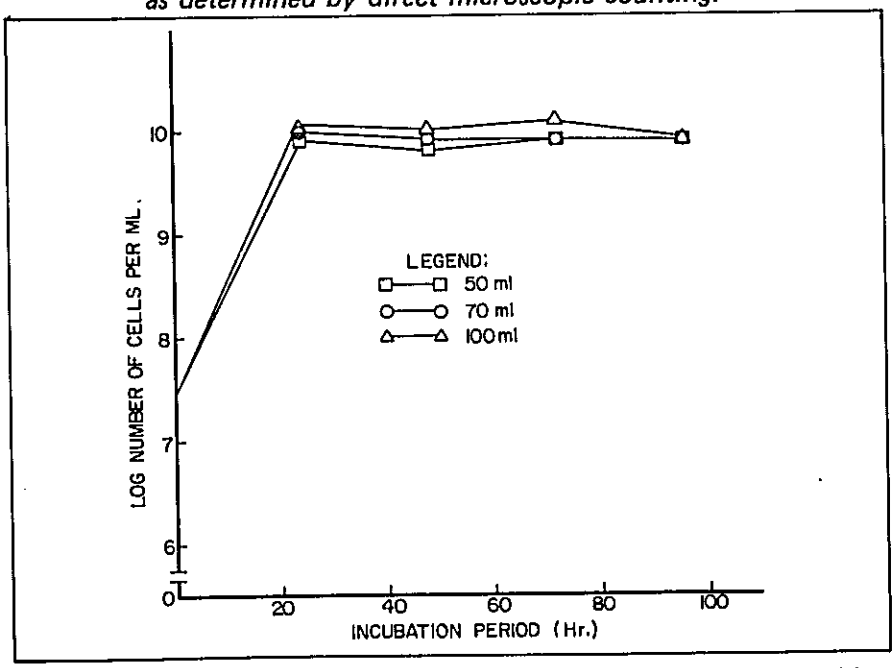

Figure 8. Growth behavior of BGs-5 in YEMB using three working volumes (50, 70 and 100 mL) in Sakaguchi flask (shake-flask experiment) at 30-32° C.

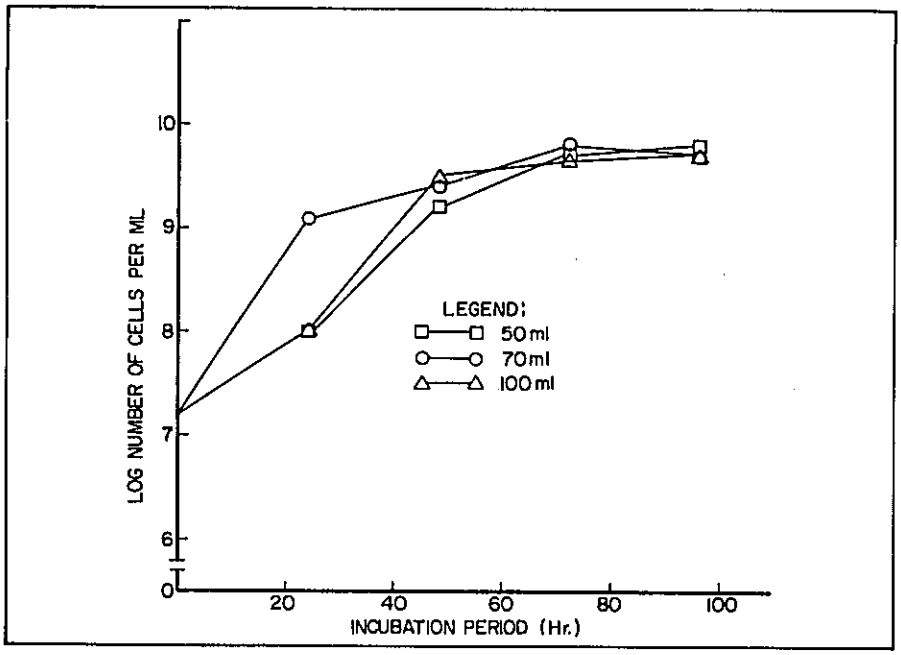

Figure 9. Growth behavior of BEs-4 in YEMB using three working volumes (50, 70 and 100 ml) in Sakaguchi flasks (Shake-flask experiment) at 30-32°C.

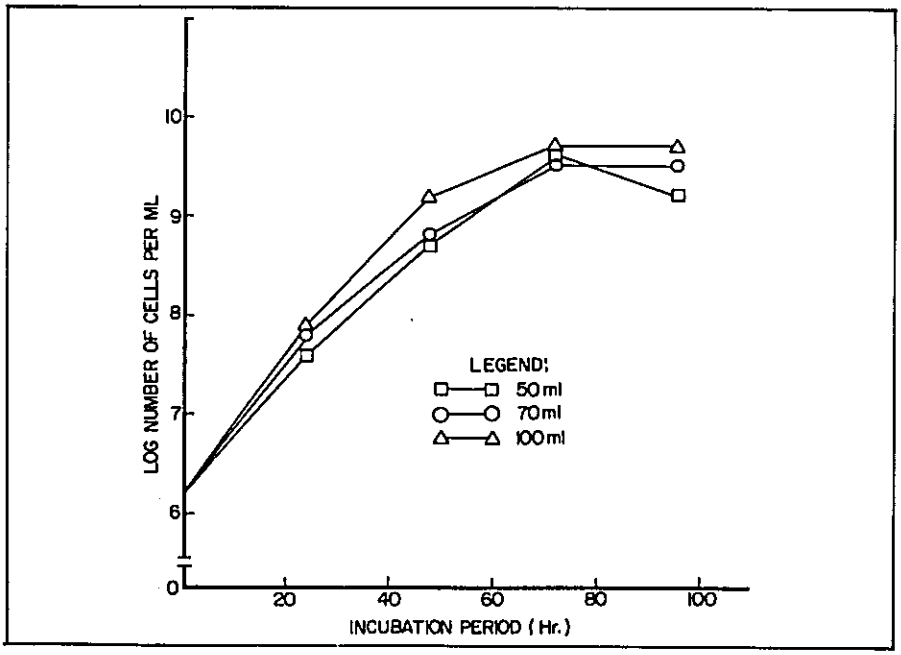

Figure 10. Growth behavior of BAp-21 in YEMB using three working volumes (50, 70 and 100 ml) in Sakaguchi flask (Shake-flask experiment) at 30-32°C.

The next step towards determining oxygen requirement is the selection of an oscillation rate which could provide the best growth condition for the organism.

For strain BGs-5, results showed that the growth behavior was relatively the same using three oscillation rates per min., viz., 70, 90, and 120 (Fig. 11). Logarithmic phase in all these oscillation rates occurred shortly after the initial period of incubation and maximum growth was attained at approximately 24 hrs. Although a slightly higher cell population was attained using 120 osc/min, the trends were similar in the case of 70 osc/min and 90 osc/min.

The same result was observed for strain BEs-4 (Fig. 12). The growth behavior of this organism in all three oscillation rates was the same. The logarithmic phase was attained shortly after 24 hrs and the maximum cell population was reached at 72 hrs.

In the case of BAp-21, differences in growth behavior varied with the three oscillation rates (Fig. 13). A lag period of 24 hrs was observed when the organism was grown at 120 osc/min. Logarithmic phase occurred starting from 24 hrs of incubation up to 72 hrs. When the oscillation rate was decreased to 90 osc/min, the lag phase was observed to be longer, i.e., from 0-48 hrs, afterwhich, the population entered the logarithmic phase. Poor growth of the strain was observed when the oscillation rate was further decreased to 70 osc/min.

In the over-all analysis, strains BGs-5 and BEs-4 are not fastidious insofar as oxygen requirement is concerned. Although both strains required oxygen for growth as all aerobes do, the amount of oxygen needed was not very critical. In the case of BAp-21, however, the amount of dissolved oxygen in the medium could be a critical factor for growth. This is based on the observation that at slower oscillation rates, i.e., 70-90 osc/min, poor growth was observed and the lag phase was longer.

Growth behavior of promising strains in modified YEMB

The current price of YEMB is estimated to be approximately ₱7.00/liter. The most expensive ingredient of the medium is mannitol which costs around ₱1333.00/kg. This carbon source is being incorporated in the growth medium at a rate of 10 g/liter. If this component of YEMB could be substituted, the cost of the medium will go down, and consequently, the production cost will be lowered. However, since organisms are nutritionally different from each other, it is necessary to test the growth of the different strains in the modified medium. This is to make sure that growth behavior is not affected by the substitution process.

In an effort to find a substitute carbon source for mannitol, growth behavior of strain BGs-5 (fast-grower) and BEs-4 (slow-grower) were determined using three types of local table sugar, viz., refined sugar (RS), medium-refined sugar (MRS), and unrefined sugar

(URS) as carbon sources. The results of the experiment are shown in Figs. 14 and 15.

Based on these results, growth of BGs-5 in mannitol, URS and RS were comparable. Although the same trend of growth was also observed in MRS, the cell population slightly decreased especially at 48 hrs. The logarithmic phase in all kinds of sugar occurred shortly after the initial period of incubation (i.e., 0-24 hrs). However, the maximum population was attained earlier in YEMB (i.e., 24 hrs) than in either URS or RS (i.e., 48 hrs).

In the case of BEs-4, growth behavior in mannitol, MRS and URS were comparable. Although the same trend of growth was observed in RS the cell population after 72-96 hrs was comparatively lower than in mannitol, URS and MRS. The organisms showed a lag phase of 48 hrs in all sugars before the population entered the logarithmic phase.

These results show that the two organisms are different inasfar as utilization of the carbon source is concerned. Strain BGs-5 preferred RS and URS than MRS as alternative carbon sources. On the other hand, strain BEs-4, which is a slow-growing strain, preferred both MRS and URS than RS as alternative carbon sources. Apparently, some constituents of MRS and URS are not present in RS. Perhaps, these constituents were lost in the process of sugar refining.

In general, URS could be a good substitute for mannitol in growing both BGs-5 and BEs-4. Since URS costs only ₱5.00/kg or approximately ₱2.50/lb., so much could be saved in the production process.

The growth behavior of three rhizobial strains, viz., BGs-5, BAp-21 and BEs-4, in YEMB with tap water was determined to explore the possibility of substituting distilled water in the preparation of the medium. Results of the experiments showed that strains BGs-5 and BAp-21 could grow relatively well in both tap water and distilled water (Figs. 16 and 17). The phase of growth of these strains were not affected when tap water was used. In fact, slightly higher cell populations (which may not be significant) could be observed in tap water than in distilled water. Apparently, both BGs-5 and BAp-21 were able to tolerate the high mineral salts present in undistilled tap water.

Strain BEs-4, however, showed a different kind of growth behavior when grown in undistilled tap water (Fig. 18). Although the isolate assumed a normal growth behavior in distilled water, it failed to grow in tap water. This probably indicates that the strain could not tolerate high levels of mineral salts present in undistilled tap water. The inability of the slow-growing strains to tolerate high salt concentrations was also reported by Yelton in 1983.

In general, tap water could be used in preparing growth medium for BGs-5 and BAp-21. However, it cannot be used to substitute for distilled water in the case of BEs-4.

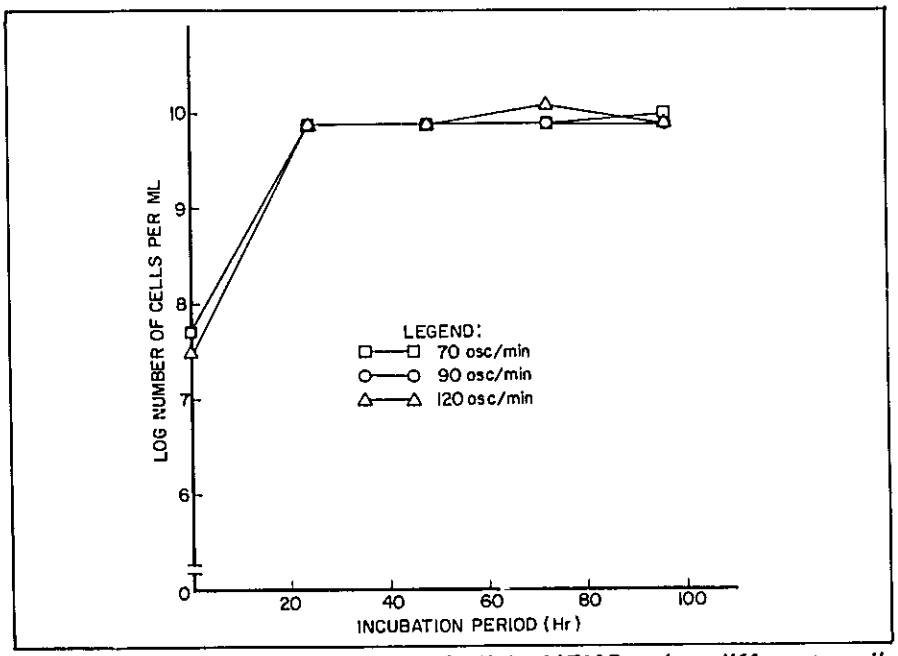

Figure 11. Growth behavior of BGs-5 in YEMB using different oscillations (70, 90 and 120 osc/ min.), shake-flask experiment with a working volume of 100 ml at 30-32° C.

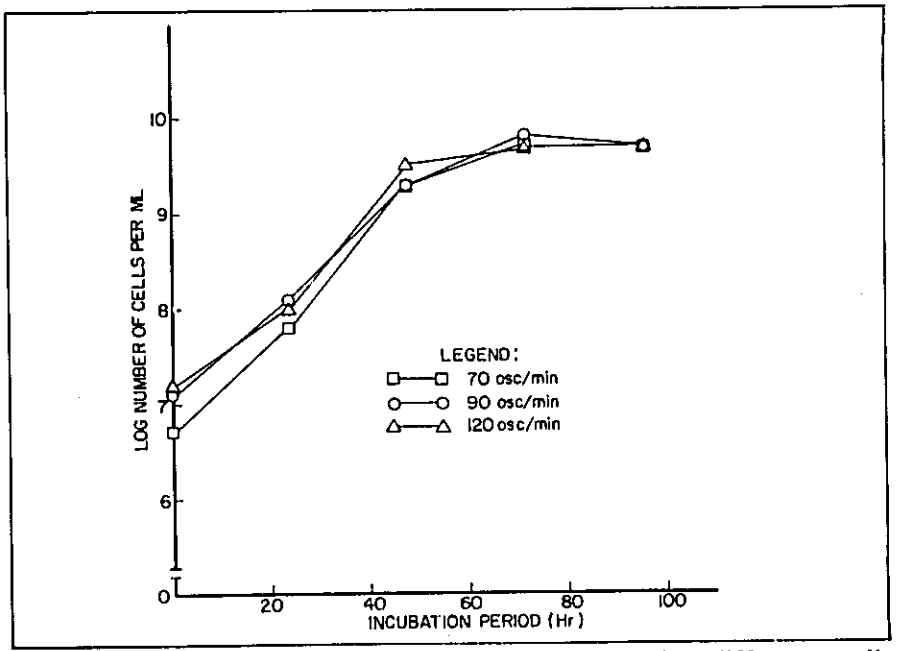

Figure 12. Growth behavior of BGs-4 in YEMB using different oscillations (70, 90 and 120 osc/min.), shake-flask experiment 30-32° C.

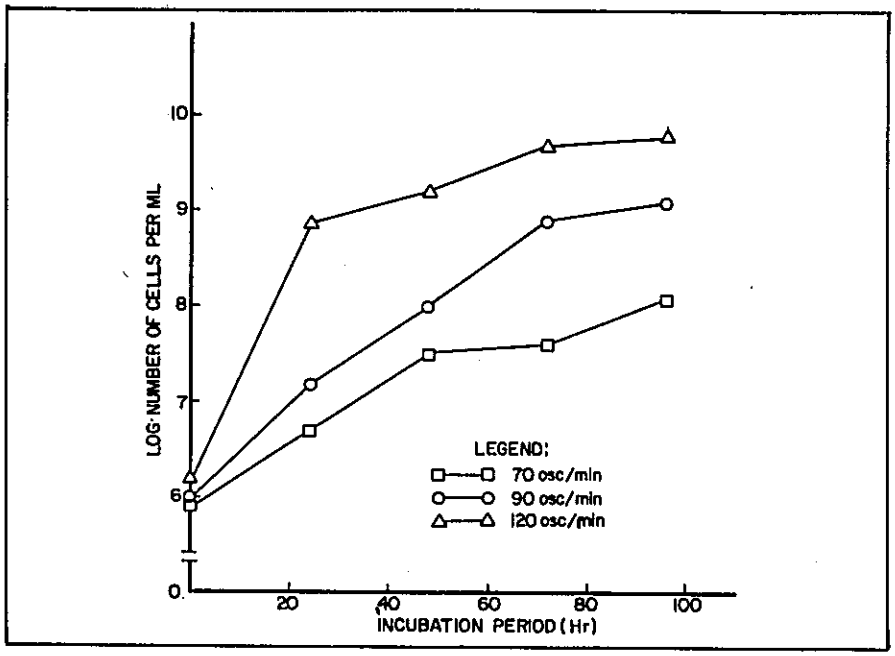

Figure 13. Growth behavior of BAp-21 in YEMB using oscillations (70, 90 and 120 osc/min.), shake-flask experiment with working volume of 100 ml at 30-32°C.

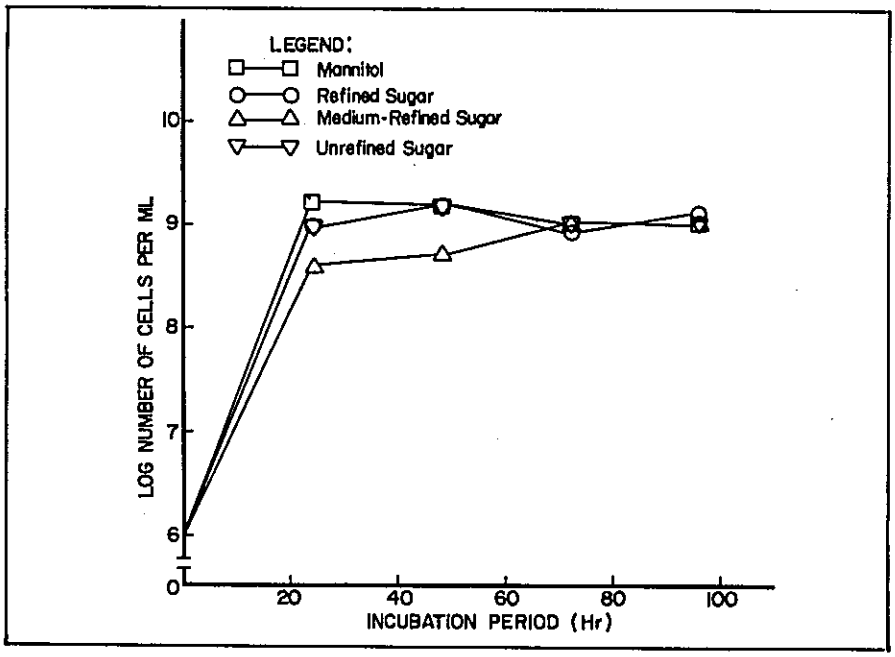

Figure 14. Growth behavior of BGs-5 in modified YEMB using different types of table sugar as substitute for mannitol.

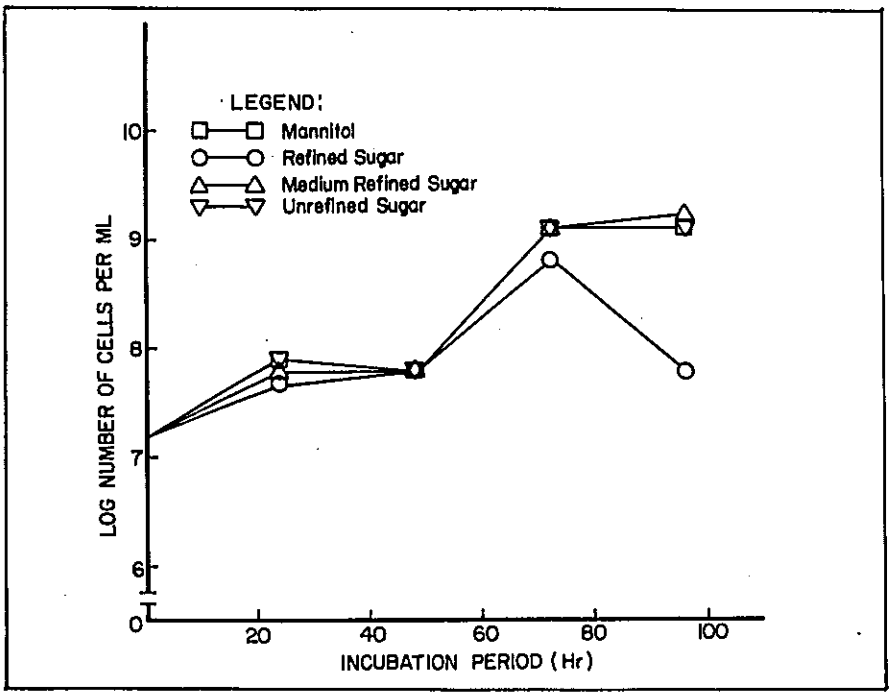

Figure 15. Growth behavior of BEs-4 in modified YEMB using different types of table sugar as substitute for mannitol.

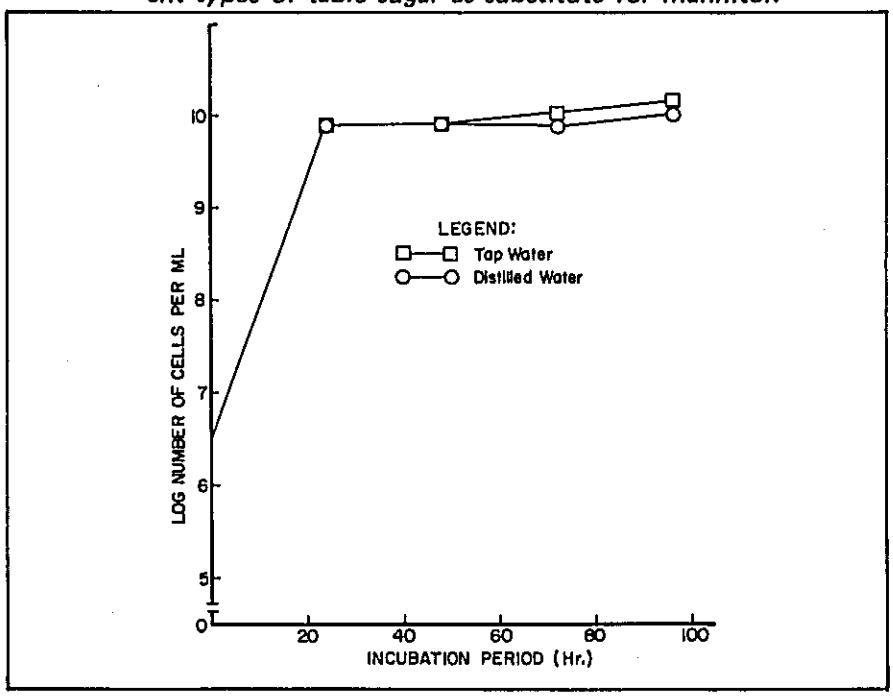

Figure 16. Growth behavior of BGs-5 in YEMB using tap water and distilled water.

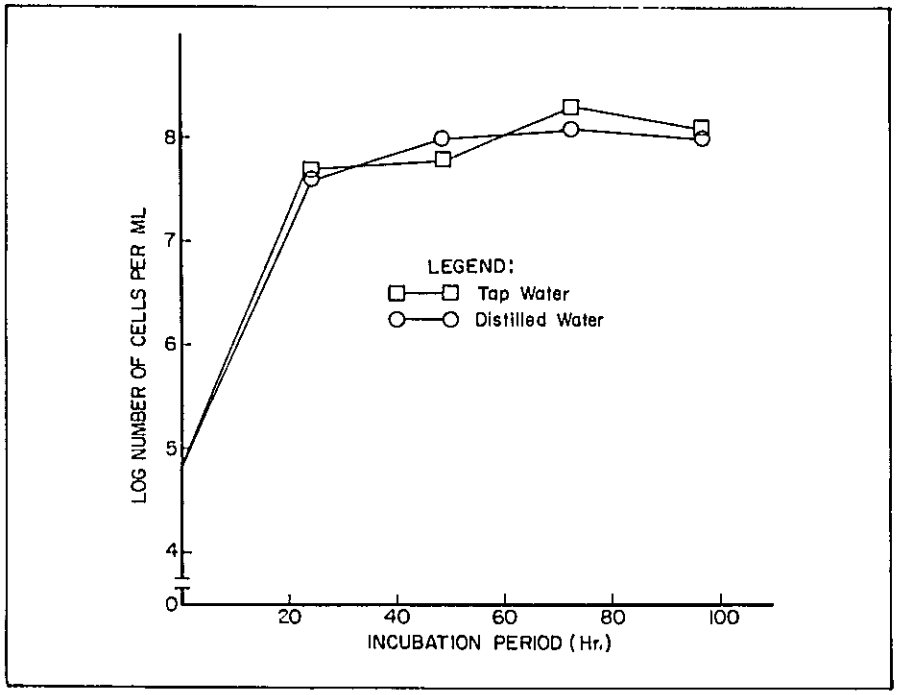

Figure 17. Growth behavior of BAp-21 in YEMB using tap water and distilled water.

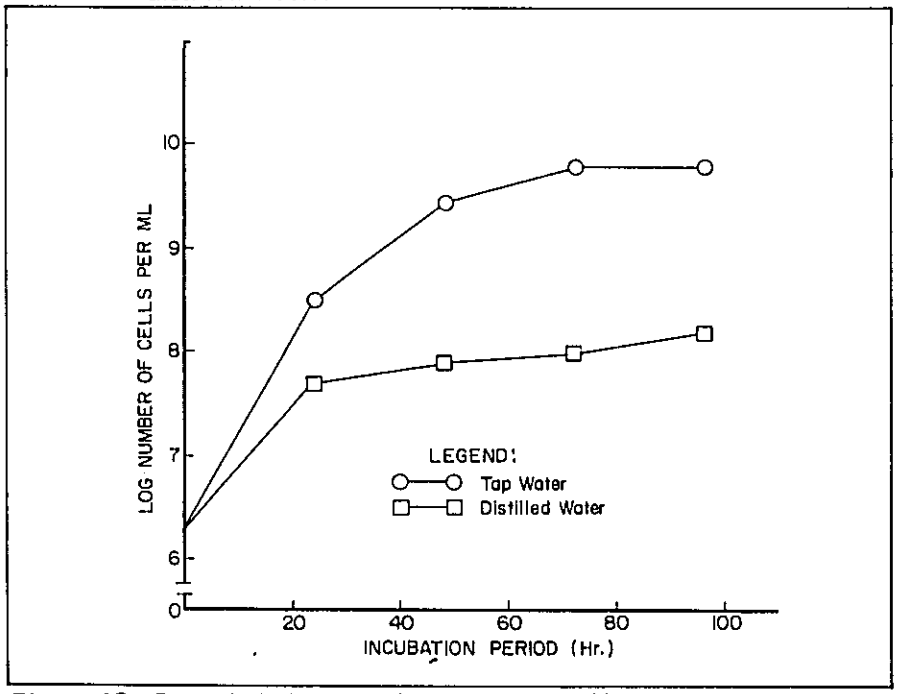

Figure 18. Growth behavior of BEs-4 for YEMB using tap water and distilled water.

Figure 19: Determination of rhizobial population by plate-counting.

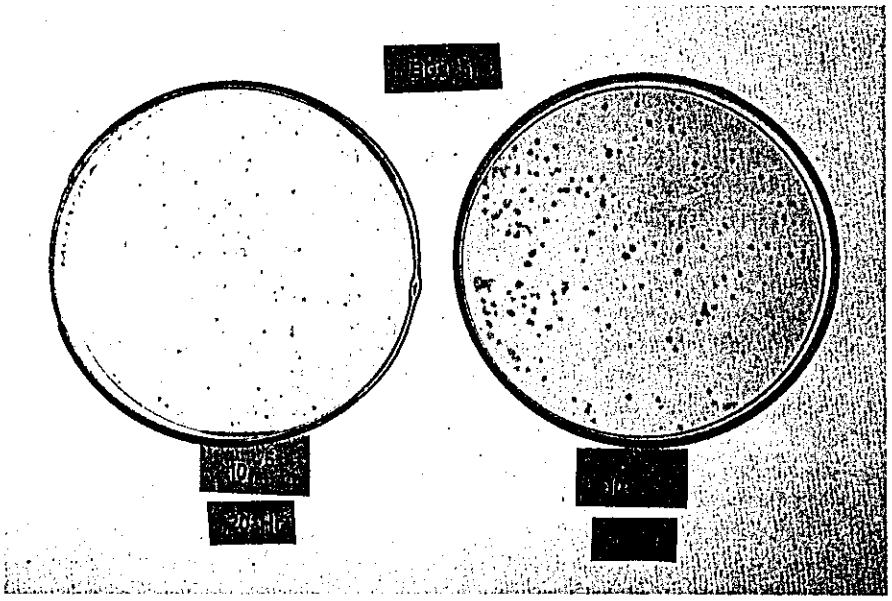

Figure 20: Colonies of strain BGs-5 on YEMA plates.

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STUDY 3: DEVELOPMENT OF ASSAY TECHNIQUES FOR NITROGEN FIXATION MEASUREMENTS

EXPERIMENT 1. MEASUREMENT OF EFFECTIVENESS OF RHIZOBIAL STRAINS OF CENTROSEMA BY ACETYLENE REDUCTION ASSAY AND TOTAL NITROGEN ANALYSIS

ABSTRACT

The acetylene reduction assay and total nitrogen analysis were used in evaluating the effectiveness of seven promising rhizobial strains of Centrosema under field conditions. These seven strains (BCp 3, CB 1923, CIAT 590, CIAT 1780, CIAT 3101, CIAT 3714 and CIAT 3334) were compared against application of 30 kg N/ha as urea and uninoculated control. Strain CIAT 3334 and CIAT 590 fixed the highest amounts of nitrogen while strains CB 1923 and CIAT 3714 were ineffective. No significant difference was observed in nitrogenase activities of different strains including the native strains that infected the control plants and plants fertilized with nitrogen.

INTRODUCTION

The acetylene reduction assay (ARA) is a measure of nitrogenase activity. This technique is a simple and inexpensive rapid-scan method for detecting potential N_2-fixing microorganisms. In this study the acetylene reduction assay was compared with total nitrogen analysis to determine the effectiveness of some *Centrosema-Rhizobium* associations under adverse soil conditions.

MATERIALS AND METHODS

The experimental site was in Barrio Puting Lupa, Calamba, Laguna. It is located at the footslope of Mt. Makiling at an elevation of about 390 to 440 meters above sea level. The grassland vegetation is dominated by *Imperata cylindrica* and *Saccharum spontaneum*. The climate is Type I with almost 94% of the total annual rainfall occurring during the months of May to October. The soil is classified as Macolod series. It has hilly to mountainous relief. External drainage is good to excessive while internal drainage is fair to good. The soil has kaolinitic and x-ray amorphous clay mineralogy that is noted for high phosphorus fixing capacity (Magcale, 1982). The soil has about 4.65% organic matter, 1.63 ppm of available Bray P2 phosphorus and 1.58 me/100 g of exchangeable potassium.

Planting was done on September 18, 1985. The rhizobial strains tested were: CB 1923 from CSIRO, Brisbane, Australia; BCp 3 (BIOTECH *Centrosema pubescens* 3) and five strains from CIAT (strain number 590, 1780, 3101, 3334 and 3714). A 30 kg nitrogen treat-

ment in the form of urea and an uninoculated control were also included. Each treatment had two square meters area with 36 plants. The treatments were replicated four times in randomized complete block (RCB). The approximate plant population was 270,000 plants per hectare. The distance of planting was 14 cm between hills and 50 cm between rows. Each hill had two plants. *Centrosema pubescens* Benth. cv. "Common" was used. The seeds were scarified with concentrated sulfuric acid for ten minutes, washed with water and air-dried. Neutral gum arabic was used as sticker at the rate of 0.8 m/16 gram seeds. Each inoculant strain was inoculated individually at the rate of 10^6 cells/seed. After inoculation, lime and powdered ordinary superphosphate were used for seed pelleting.

The plants were harvested on January 9, 1986, about 16 weeks after planting. The nodules were subjected to acetylene reduction assay using Shimadzu GC-7A gas chromatograph. The nodule counts and nodule dry weight were determined. The shoots were oven dried at 80°C for 48 hours in a forced draft oven and the dry weight determined. Total nitrogen analysis was done using the microkjeldahl method. The delta (N) nitrogen fixed was determined by taking the difference between total nitrogen of inoculated plants and control plants.

RESULTS AND DISCUSSION

The mean nodule number, nodule dry weight and nitrogenase activity of different centrosema-rhizobia associations are presented in Table 1. Rhizobial strain CIAT 590 produced the highest number of nodules which was significantly higher than strains CB 1923 and the native strain that infected the nitrogen fertilized plants. On the other hand, strain CIAT 590 was not significantly different from the other strains including the control in terms of nodule number. Rhizobial strain CIAT 3101 produced the highest nodule dry weight which was significantly higher than the native strain in nitrogen fertilized plants but was not significantly different from the strains tested. The acetylene reduction assay showed that rhizobial strain CIAT 3110 had the highest nitrogenase activity on a per plant basis, but the activities of all strains were not significantly different from each other, except strain CIAT 1780. The nitrogenase activity in nmoles C_2H_4 per milligram nodule was highest in the native strain that infected the nitrogen fertilized plants followed by strain CB 1923. The addition of urea depressed nodulation but it did not affect the nitrogenase activity.

The dry matter yields of uninoculated plants, fertilized plants, and those inoculated with strain CB 1923 were significantly lower

Table. 1 Mean nodule number, nodule dry weight and nitrogenase activity of different centrosema-rhizobia associations 16 weeks after planting in Puting Lupa, Calamba, Laguna (1985).

Treatment	Nodule Number	Nodule Dry Weight (mg/8 plants)	Nitrogenase Activity (nmoles C_2H_4/ hr/6 plants)	Activity (nmoles C_2H_4/ mg nodules)
Uninoculated	42 abc	118.33 ab	1892 ab	15.11 ab
30 kg N/ha	30 bc	86.43 b	1785 ab	20.54 a
Inoculated:				
BCp 3	31 abc	122.63 ab	1675 ab	14.21 ab
CB 1923	72 c	117.20 ab	2230 ab	18.59 ab
CIAT 590	61 a	194.03 ab	2148 ab	13.22 ab
CIAT 1780	35 abc	99.87 b	1047 b	10.28 b
CIAT 3101	59 ab	267.73 a	3767 a	14.72 ab
CIAT 3714	36 abc	193.03 ab	3100 ab	15.35 ab
CIAT 3334	50 abc	194.17 ab	1900 ab	12.52 ab

Means followed by the same letter(s) are not significantly different at 5% level based on Duncan Multiple Range Test.

than plants inoculated with strains BCp 3, CIAT 590, CIAT 1780, CIAT 3101, CIAT 3714 and CIAT 3334 (Fig. 2 and Table 2). The latter strain CIAT 3334 produced the highest dry matter yield. The dry matter yields obtained from the above mentioned strains were not significantly different from each other. Strain CIAT 3334 caused the highest total nitrogen uptake, followed by strain CIAT 590 and strain CIAT 1780. The total nitrogen uptake of the control plants, fertilized plants, and those inoculated with strains BCp 3, CB 1923, CIAT 3101, and CIAT 3714 were significantly lower than plants inoculated with strain CIAT 3334. Nitrogen fixation was highest with strains CIAT 3334 and CIAT 590 and was significantly higher with strain CB 1923 and CIAT 3714. The least effective strains were CB 1923 and CIAT 3714. The most effective strains were CIAT 3334 and CIAT 590.

Table 2. Mean effect of rhizobial inoculation on dry matter yield, total nitrogen and nitrogen fixed of centrosema 16 weeks after planting in Puting Lupa, Calamba, Laguna (1985).

Treatment	Dry Matter Yield (g/8 plants)	Total Nitrogen (mg/8 plants)	Δ Nitrogen Fixed (mg/8 plants)
Uninoculated	2.33 c	12.33 d	–
30 kg N/ha	3.00 bc	75.24 dc	–
Inoculated:			
BCp 3	4.33 abc	127.31 bcd	64.98 abc
CB 1923	2.40 c	68.31 bcd	8.42 c
CIAT 590	5.23 ab	165.65 ab	109.32 ab
CIAT 1780	4.96 ab	148.78 abc	81.45 abc
CIAT 3101	4.63 ab	132.39 bcd	70.57 abc
CIAT 3714	3.73 abc	91.81 bcd	29.49 bc
CIAT 3334	6.56 a	207.92 a	145.59 a

Mean followed with the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

EXPERIMENT 2. MEASUREMENT OF EFFECTIVENESS OF RHIZOBIAL STRAINS OF IPIL-IPIL BY ACETYLENE REDUCTION ASSAY AND TOTAL NITROGEN ANALYSIS

ABSTRACT

The acetylene reduction assay was used in evaluating the effectiveness of nine promising rhizobial strains of ipil-ipil under adverse soil condition of Mount Makiling grassland. These nine strains (L6, L32, L46, BL1 49, BL1 56, BL1 62, BL1 80, CB 81 and TAL 1145) were compared against application of 30 kg N/ha (as urea) and uninoculated control. Strain BL1 80 was the most effective while strain BL 1 56 was the poorest. Direct relationship was observed between nitrogenase activity and nitrogen fixation.

MATERIALS AND METHODS

The experiment was laid out adjacent to the area of Experiment I in Barrio Puting Lupa, Calamba, Laguna.

Nine promising rhizobial strains that have been screened from previously conducted growth chamber, screenhouse and field experiments were tested in the area. These strains were *Rhizobium* sp. L6, L32, L46, BL1 49, BL1 56, BL1 62, BL1 80, CB 81 and TAL 1145. An uninoculated control and a nitrogen treatment were also included. Nitrogen in the form of urea was applied at 30 kg/ha. Ipil-ipil variety "K8" was planted. The seeds were scarified with concentrated sulfuric acid for five minutes, washed with tap water and soaked overnight to select highly viable ones. Each rhizobial strain was inoculated individually at the rate of 10^6 cells/seed before planting. Twelve grams ordinary superphosphate and 10 grams calcium carbonate per hill were applied basal one month before planting.

Each treatment had 12 hills in a plot with three plots serving as replication in a complete randomized block design. The distance of planting was one meter between rows and 90 centimeters between hills. Four seeds were sown per hill. The plants were harvested 16 weeks after planting. The nodules were collected and acetylene reduction assay was conducted. Shoots and nodule dry weights were determined after oven drying at 80°C for 48 hours in a forced draft oven. Total nitrogen was determined by microkjeldahl method. Nitrogen fixed was determined by taking the difference between total nitrogen of inoculated plants and control plants.

RESULTS AND DISCUSSION

The nodulation of ipil-ipil was highest with *Rhizobium* sp. BL1 80 and lowest with the nitrogen fertilized plants. However there were no significant differences in nodule number in all treatments (Table

1). It was noted that adding fertilizer nitrogen as urea depressed nodulation and consequently decreased nodule dry weight. Strain BL1 80 and native strain that infected the control plants gave the highest nodule weight but the differences were not significant over that of other strains. There were no statistical differences in nitrogenase activity between strains in terms of nmole of ethylene produced per milligram nodule although strain L6 had the highest activity. The nitrogenase activity on a per plant basis was highest with strain BLI 80, followed by strains BLI 49, L6, and the native strains that infected the control plants. The nitrogenase activities of these four strains were not significantly different. Strains TAL 1145, CB 81, BL1 62, BL1 56, L46, L32 and the native strains in nitrogen fertilized plots caused low nitrogenase activities which were significantly different from strain BL1 80.

Table 1. Mean nodule number, nodule dry weight and nitrogenase activity of different ipil-ipil rhizobia-associations 16 weeks after planting in Puting Lupa, Calamba, Laguna (1985).

Treatment	Nodule Number	Nodule Dry Weight (mg/4 plants)	Nitrogenase (nmoles C_2H_4/hr/ mg nodules	Activity (nmoles C_2H_4/ 4 plants)
Uninoculated	126 a	0.59 a	14.43 a	8106 ab
30 kg N/ha	66 a	0.26 a	28.68 a	6604 b
Inoculated:				
L 6	118 a	0.40 a	25.77 a	10291 ab
L32	118 a	0.46 a	22.91 a	6828 b
L46	128 a	0.38 a	17.03 a	5671 b
BL1 49	155 a	0.57 a	22.46 a	11444 ab
BL1 56	101 a	0.31 a	13.25 a	3934 b
BL1 62	109 a	0.38 a	10.15 a	3848 b
BL1 80	200 a	0.59 a	22.77 a	15394 a
CB 81	140 a	0.32 a	18.84 a	6584 b
TAL 1145	114 a	0.32 a	18.44 a	5796 b

Means followed by the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

The dry matter yield, nitrogen yield, and nitrogen fixed were highest in plants inoculated with *Rhizobium* sp. BL1 80 (Table 2). Strains TAL 1145 and BL1 80 had almost similar dry matter yields but these two were not significantly different from the other strains including the control and the fertilized plants. No benefit was realized from the applying nitrogen as shown by lower dry matter yield of the fertilized plots. The nitrogen yield of the uninoculated plants and plants inoculated with strain BL1 56 were significantly lower than plants inoculated with strain BL1 80. It was noted that strain BL1 56 had lower nitrogen yield than control plants. Nitrogen fixed was highest with strain BL1 80 and was significantly different from BL1 56 and BL1 46 but was not significantly different from that of the strains tested. Based on all parameters, it was strain BL1 80 that was the most effective while strains BL1 56 and BL1 46 were the poorest. These observations were similar to the results obtained in strain evaluation experiment in ipil-ipil conducted in Pantabangan watershed (BIOTECH-JICA Joint Study Project, 1985 Annual Technical Report).

Table 2. Mean effect of rhizobial inoculation on dry matter yield, total nitrogen fixed of ipil-ipil 16 weeks after planting in Puting Lupa, Calamba, Laguna.

Treatment	Dry Matter Yield (g/8 plants)	Total Nitrogen (mg/8 plants)	Nitrogen Fixed (mg/8 plants)
Uninoculated	7.26 ab	179.67 bc	–
30 kg N/ha	7.93 ab	240.67 ab	
Inoculated:			
L 6	8.90 a	258.00	78.33 ab
L32	8.33 a	219.33 abc	39.66 abc
L46	7.23 ab	192.67 abc	19.00 bc
BL1 49	8.13 ab	230.67 ab	51.00 abc
BL1 56	5.80 b	138.67 c	0.00 c
BL1 62	7.66 ab	203.67 abc	34.33 abc
BL1 80	9.33 a	270.33 a	90.66 a
CB 81	8.47 a	240.50 ab	70.25 ab
TAL 1145	9.35 a	254.50 ab	74.50 ab

Means followed by the same letter (s) are not significantly different at 5% level based on Duncan's Multiple Range Test.

REFERENCES

1. JICA-BIOTECH JOINT STUDY PROJECT. 1985. Annual Technical Report, 1985.
2. MAGCALE, D. B. 1982. The effect of lime on nutrient status of two grassland soils grown to *Imperata cylindrica* (L.) P. Beauv. and *Themeda triandra* Forsk. Unpublished M. S. thesis. UPLB.

APPENDIX

List of Exchange Scientists

JICA-BIOTECH JOINT STUDY PROJECT ON BIOFUELS

STUDY 1.

Philippine counterpart: NESTOR R. APUYA
Period of stay in Japan: Nov. 28, 1984 – Oct. 1, 1985
Place of training: University of Tokyo
Activities: Isolation of heat tolerant alcohol yeast from soils collected in the Philippines, and training of cell fusion technique to give alcohol tolerance.

Japanese counterpart: TAKASHI YAMAKAWA
Period of stay in BIOTECH: a) Mar. 19, 1985 – Apr. 14, 1985
b) Aug. 6, 1985 – Aug. 19, 1985
Activities: a) Collection of soils, fruits and flowers from different points in Luzon and the Visayas for isolation of heat and acid tolerant alcohol yeast.
b) Demonstration on techniques of fusing yeast protoplasts for genetic improvement of thermotolerant and alcohol tolerant yeasts.

STUDY 2.

Japanese counterpart: YOSHIKI TANI
Period of stay in BIOTECH: Aug. 6, 1985 – Aug. 19, 1985
Activities: a) Screening of 200 fungal strains for growth and digestivity of raw starch on agar plate incubated at 35°C.
b) Determination of glucoamylase activity of crude enzyme produced by potential strains cultivated in liquid medium containing 10% raw starch and incubated at 35°C in a reciprocal shaker.

Philippine counterpart: MARIA AUSSIELITA L. LIT
Period of stay in Japan: Sept. 29, 1986-Jun. 30, 1987
Place of training: Kyoto University

Activities: Screening, selection and optimization of cultural conditions for the production of raw starch digestive-glucoamylase.

STUDY 3.

Philippine counterpart: ELIZABETH C. BUGANTE
Period of stay in Japan: Sept. 12, 1985 – Jun. 11, 1986
Place of training: Osaka City University
Activities: Purification of methane bacteria accumulated in the Philippines, and training of fundamental techniques on maintenance of culture and analysis of fermented broth.

Japanese counterpart: SUSUMU OI
Period of stay in BIOTECH: Mar. 20, 1985 – Apr. 14, 1985; Jul. 22, 1986 – Sept. 1, 1986

Activities: Collection of mud and soil samples and accumulation of methane bacteria.
Set-up thermophilic fermentation of slop wastes.

Japanese counterpart: KIYOMOTO UEDA
Period of stay in BIOTECH: Jan. 16, 1985 – Oct. 31, 1987
Activities: Isolation of agar bacteria capable of decolorizing molasses slop pigments, and development of continuous decolorization system using immobilized bacterial cell.

JICA-BIOTECH JOINT STUDY PROJECT ON NITROGEN FIXATION

STUDY 1.

Philippine counterpart: BAYANI ESPIRITU
Period of stay in Japan: Nov. 28, 1984 – Oct. 1, 1985
Place of training: The University of Tsukuba
Activity: Training on isolation and genetic improvement of rhizobia.

Philippine counterpart: ROSARIO G. MONSALUD
Period of stay in Japan: Sept. 29, 1986 – Jun. 30, 1987
Place of training: Obihiro University
Activity: Training on classification of *Rhizobium* sp.

Japanese counterpart: SHIRO HIGASHI
Period of stay in BIOTECH: Mar. 12, 1985 – Apr. 11, 1985
Activities: Isolation of rhizobia from root nodules of legumes plasmid and bacteriophage isolation, electrophoresis experiments and demonstration of techniques.

Japanese counterpart: YOSUKE MINO
Period of stay in BIOTECH: Oct. 21, 1985 – Jan. 10, 1986; Nov. 1, 1986 – Jan. 7, 1987.
Activities: Collection of nodules particularly in the Mindanao area and demonstration of some serological techniques.
Installation of water purifier and deionizer. Characterization of nitrogen-fixing rhizobial isolates from root nodules.

STUDY 2.

Philippine counterpart: VIRGINIA PADILLA
Period of stay in Japan: May 23, 1985 – Mar. 4, 1986
Place of training: Tokachi Agricultural Cooperatives, Hokkaido
Activities: Studies on appropriate conditions for mass production of rhizobial inoculants.

Japanese counterpart: TOSHIKAZU TAKAHASHI
Period of stay in BIOTECH: Oct. 21, 1985 – Nov. 30, 1985; Nov. 29, 1986 – Dec. 7, 1986
Activities: Collection of nodules particularly in the Mindanao area and demonstration of some techniques on growth curve determination.
Installation of water purifier and dionizer. Consultation with nitrogen fixation pilot plant crew.

STUDY 3.

Japanese counterpart: TOMIO YOSHIDA
Period of stay in BIOTECH: Feb. 25, 1985 – Mar. 17, 1985
Activities: Assessment of existing conditions in N-fixation laboratory of BIOTECH.

LIST OF EQUIPMENT, CHEMICALS AND GLASSWARE DONATED BY JICA

(May 28, 1987)

Equipment Donated at the Beginning of Joint-Study Project

1985 Fiscal Year

1.	"NISSAN Pick-Up 2500 Diesel D/Cabin Model: UJLY 720 TFC, and Spare Parts	¥ 2,208,002
2.	"SHIMAZU" Gas Chromatograph GC-7APTF	¥ 1,597,000
3.	and Optical Accessories	
3.	Digital pH Controller LM-4HC	¥ 700,000
4.	Antifoam Control Equipment LM-2A	¥ 540,000
5.	Dissolved Oxygen Measurement and Control Equipment LM-6D	¥ 330,000
6.	Reciprocal Shaking Incubator TLR-3 and 100 pcs Flasks	¥ 2,907,875
7.	Mini Jar-Fermentor MD 300-5L 2 pcs	¥ 1,646,000
8.	Autoclave	
9.	Glove box PV-W	
10.	Auto Dispenser	
11.	Biological Microscope NIKKON and Accessories	¥ 3,457,000
12.	Potable Gaschromatigraph detector	
13.	Roller Pump PR-5 with Tube (10 M)	¥ 141,400
14.	Air Current Meter 4 pcs	¥ 36,000
15.	Chromatopac SHIMAZU C-R3A	¥ 490,000
16.	Micro Tube Pump MP32 (4 sets) with Tube	¥ 638,471

Additional Donation

1986 Fiscal Year

17.	High-Speed Cooling Centrifuge SCR 20B (20,000 rpm 45170 X g)	¥ 2,173,285
18.	Angle Rotor RPR16 1 set	¥ 340,000
19.	Continuity Rotor RPRC13 1 set	¥ 432,500
20.	Collector Assy for RPRC13 1 set	¥ 237,000
21.	Bottle Assy 2 sets	¥ 46,350
22.	Victor Video Camera, Recorder TV with Video Tape and Adaptor for Microscope	¥ 905,519
23.	High Purity Water Housing with Accessories	¥ 2,357,176
	TOTAL	¥ 21,183,578

Equipment, Chemicals and Glasswares Carried by Short Time Experts

1984 Fiscal Year

Electrophoresis System "MUPID"	¥	140,000
Transilluminator TM-15	¥	340,000
Voltage Regulator	¥	190,000
Electronic Cooling Electrophoresis TC-3	¥	510,000
COD Reflux Distilation Apparatus	¥	500,000
Chemicals, Glasswares and Others	¥	2,616,455
TOTAL	¥	4,296,455

1985 Fiscal Year

Dispenser 2 pcs	¥	94,000
Tool Set	¥	70,000
Centrifuge No. KS500PV and Accessories	¥	404,000
Shaker Personal-JR	¥	303,000
Hot Plate	¥	35,000
Pipetman 7 pcs	¥	259,500
Magnetic Stirrer 2 sets	¥	64,000
Floppy DISK DRIVE "SHIMAZU"	¥	545,900
Angle Rotor RPR18-3 1 set	¥	247,000
Almighty Tool Set	¥	19,500
Simple Official Examination Set for Soil	¥	34,900
Photometer, ANA-1	¥	120,000
Pipet Aid with Standard Accessories	¥	110,000
Colony Counter	¥	25,500
pH Meter L-7LC	¥	87,000
Luxmeter ANA-500	¥	21,500
Bacterium Counter	¥	44,000
Balance EW-300A with Trans	¥	76,000
Chemicals, Glasswares and Others	¥	3,683,406
TOTAL	¥	6,244,206

1986 Fiscal Year

Incubator 2 sets	¥	358,000
Culster "SHIBATA-1602-4000" 2 sets	¥	152,000
Stirring Set 2 sets	¥	54,000
Magnetic Stirrer MB-10A 2 sets	¥	160,000
Culture Jar	¥	63,000
pH Meter "HRIBA" M8	¥	150,000
Thermoregular TP-100	¥	91,800
Pump Aspit Model: Q1	¥	68,000
Pen Recorder	¥	212,000
Electric Furnace MCK-2020	¥	,251,500
SLAB Gel Electrophoresis Apparatus 1 set	¥	60,800
Vacuum Pump ODF-50W 1 set	¥	80,800
Fixing Table 2 pcs	¥	80,000
Electric Clipper 220V 1 set	¥	35,000
Dilutor 1 box	¥	33,000
Eppendorf Multipette No. 4980	¥	45,000
Electrophoresis Apparatus 1 set	¥	40,000
Chemicals, Glasswares and Others	¥	1,474,591
TOTAL	¥	3,409,491

1987 Fiscal Year

Autoclave Parts 1 set	TOTAL ¥	190,825
	GRAND TOTAL ¥	14,140,977

LOCAL EXPENSES

Fiscal Year

1984	(Sept. to Mar. '85)	₱	96,154.06
1985		₱	809,166.43
1986		₱	801,750.17
1987	(Apr. to Oct.)	₱	565,800.00
	GRAND TOTAL	₱	2,272,820.50

Technical Reviewers: Dr. William G. Padolina
Dr. Kiyomoto Ueda

Editor and
Layout Designer: Mariechel J. Navarro

Artist and
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JICA - BIOTECH
joint study project

JAPAN INTERNATIONAL
COOPERATION AGENCY

NATIONAL INSTITUTES OF BIOTECHNOLOGY
AND APPLIED MICROBIOLOGY

University of the Philippines at Los Baños
College, Laguna, Philippines