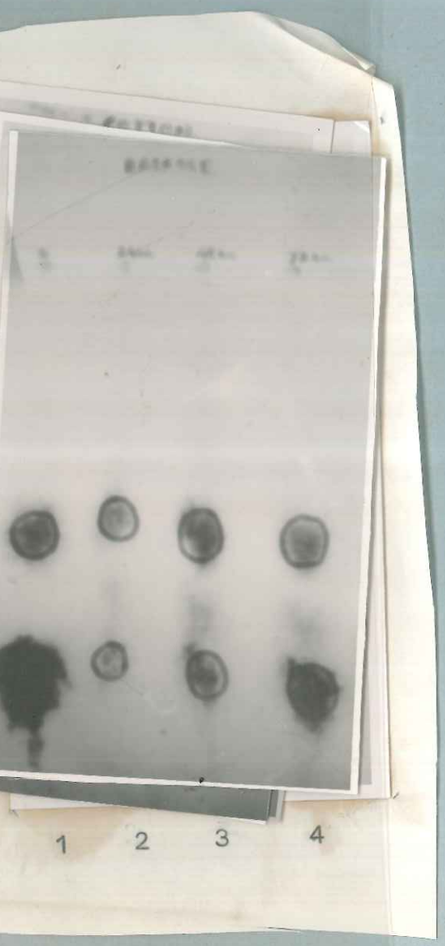
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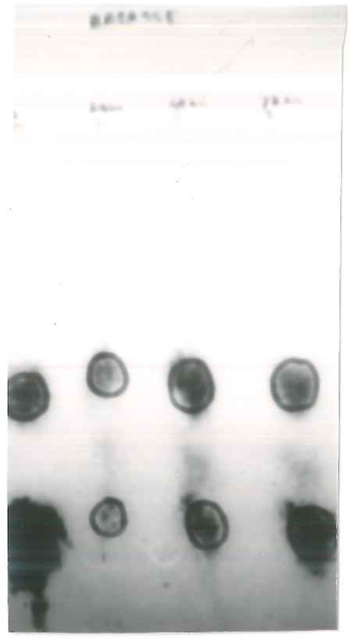
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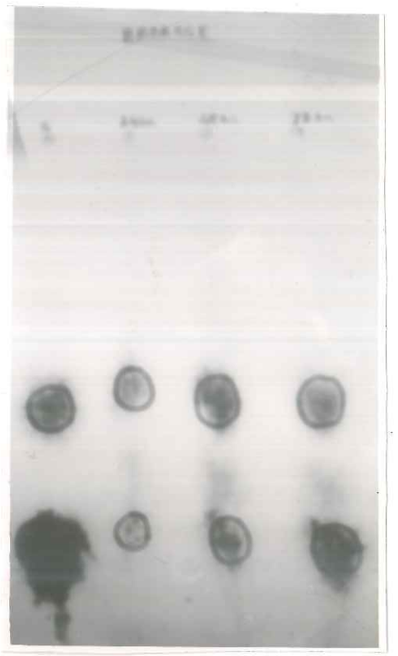
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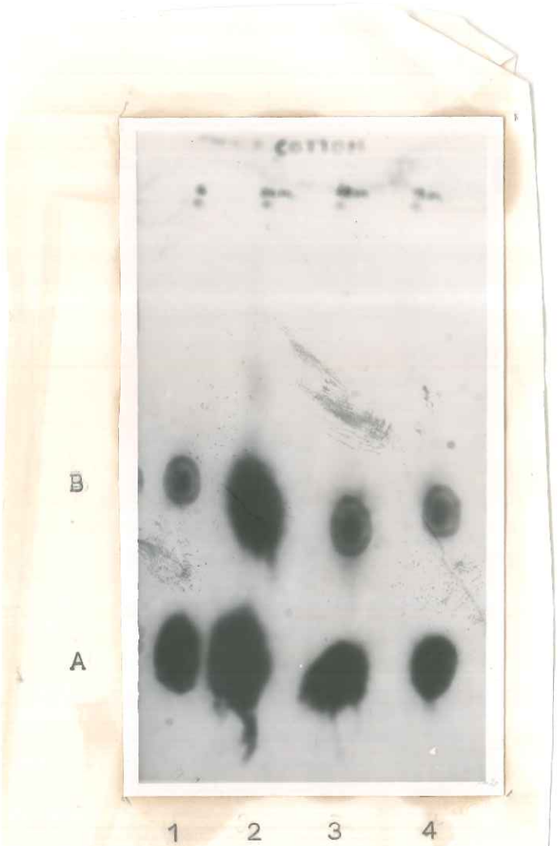
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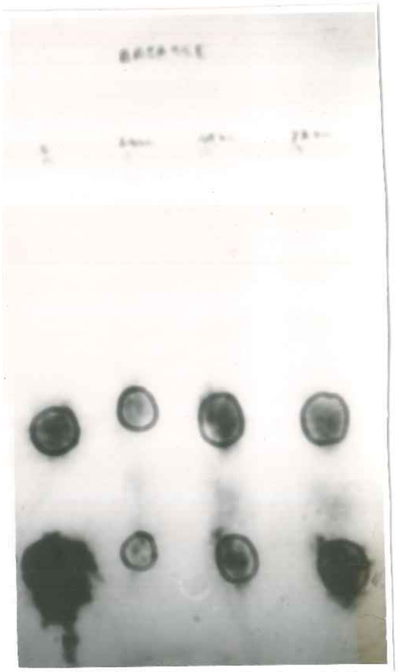
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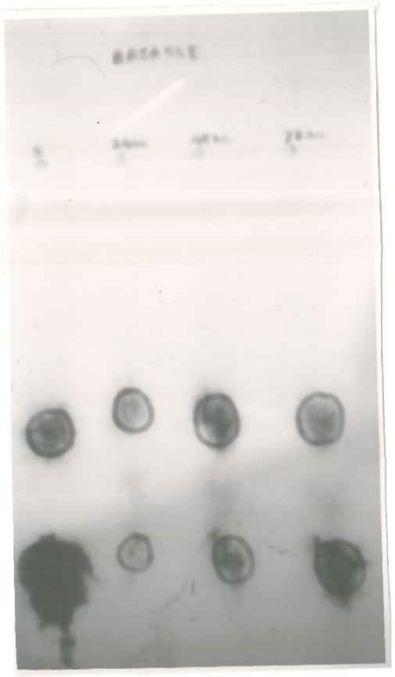
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STUDIES ON CELLULASE

A THESIS

SUBMITTED TO THE

UNIVERSITY OF POONA

FOR THE DEGREE OF

MASTER OF SCIENCE

(Partly by Papers and Partly by Research)

by

Mrs. Ulka S. Puntambekar

Division of Biochemistry

NATIONAL CHEMICAL LABORATORY

PUNE - 411 008

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U.S. Puntambekar

March 1981. (Mrs. U.S. Puntambekar)

C O N T E N T S

CHAPTER I

INTRODUCTION

The need for utilizing renewable resources as a means of combating the increasing demands for food and energy has been well realised in recent years. Petroleum which is being consumed at an alarmingly rapid rate is limited in its supply and is not renewable. Alternate energy resources to petroleum are being investigated and among them attention is getting increasingly focussed on cellulose which is the most abundant renewable resource produced by photosynthesis. Besides energy, nutrition is also of great importance, particularly of protein nutrition of man and his domesticated animals. It has been realised that with the population explosion taking place, future demands for protein cannot be met solely through conventional protein sources such as milk, pulses, meat etc. Microbial protein for which the name single cell protein (SCP) has been given is expected to play a greater and more significant role in the protein nutrition of man and animals. Among the substrates for bioconversion, cellulose is being considered as a potential raw material. Another important aspect is the utilization of the renewable cellulosic raw materials as the feed stock for generating basic ingredients of the chemical industry, which are presently being obtained from petrochemical sources (e.g. ethylene, ethanol etc.).

The utilization of cellulosic materials for generating food is well exemplified by the technology of

mushroom cultivation. Mushrooms have been used as a human food from times immemorial. Mushrooms belong to the class Basidiomycetes, having umbrella shaped reproductive structure (carpophore or basidiocarp) with gills on the lower surface. These fungi are capable of growing on lignocellulosic wastes forming fruiting bodies which are rich in protein and this fungal protein is acceptable in human nutrition (Han, 1978).

Vedas - the most ancient sacred literature of Hindus, refer to mushrooms (Purkayastha, 1976). Agaricus campestris (a common mushroom) was familiar to Romans and Greeks. In China, a mushroom Cortinellus berkeleyanus has been cultivated for several centuries on a large scale (Bhattacharjee, 1970). The paddy straw mushroom, Volvariella volvacea, has been cultivated in large quantities in South China, Philippines, Malaysia, Indonesia, Thialand and Burma (Chang, 1974). Other commonly cultivated basidiomycetes include Lentinus edodes (Shiitake), Pleurotus ostreatus (Oyster mushroom), which produce edible fruit bodies (Han, 1978).

The mushroom Agaricus bisporus, most commonly cultivated in Western Countries, is grown on horse manure compost and crop residues such as corn cobs, stalks and straw (Han 1978).

The paddy straw mushroom Volvariella volvacea is known to grow on rice straw. Other kinds of materials

such as residues of tapioca manufacture, wheat straw, sorghum, Bombax malabaricum and cotton waste are used successfully for the cultivation of this mushroom.

The other common edible mushroom Lentinus edodes (Shiitake) grows on wood. The logs cut from hard wood trees such as oak, chestnut are used for preparing the bed in which holes are drilled and the inoculum is placed inside. The fungus Pleurotus ostreatus is cultivated in a similar way as Shiitake on beech trunks and logs of deciduous trees (Hayes and Nair, 1975).

The main feature in the cultivation of Volvariella, Lentinus or Pleurotus is that, no extra nitrogen needs to be added to the substrate whereas in the cultivation of Agaricus horse manure is an essential additive, which is chemically complex (Chang-Ho and Yee, 1977).

The genus Volvariella belongs to the family Amanitaceae of the order Agaricales (Singer, 1975). The fungus is saprophytic on cellulose-rich paddy straw, and is commonly known as 'paddy straw' mushroom (Rangaswami, 1976). Shaffer (1957) has discussed in detail the validity of the generic name Volvariella in preference to the more popular name Volvaria.

The common substrate for the cultivation of Volvariella is paddy straw. In China, for several centuries, the mushroom V. volvacea has been grown on paddy straw (Chang, 1974). As it was grown under natural

conditions the yield of mushroom was very poor. Other sources of straw such as wheat and sorghum were found inferior as the mushroom yields were comparatively less (Singer, 1961; Gupta et al, 1970). It was successfully cultivated on Bombax malabaricum in Indo-China by Sallet (1936). The mushroom was also grown on other materials such as water-hyacinth Eichhornia crassipes, (Cheng and Mok, 1971); oil palm bunch waste and pericarp waste, (Naidu, 1971; Chen and Graham, 1973); banana leaves and sawdust (Chua and Ho, 1973). Their mean yields were comparatively low varying from 1.5 to 2.5%; whereas by the use of paddy straw the yield was reported to be from 4.5 to 14.7%.

Chang (1974) grew V. volvacea on cellulosic materials such as cotton waste compost. The compost consisted of first grade cotton waste, rice or wheat bran and calcium carbonate to maintain the pH at the required level. The compost was prepared by soaking this mixture into water and fermented for four days and then it was subsequently steamed at 80°C temperature, cooled at 30°C to 34°C and made ready for spawning. When the mushroom was grown on this compost at 30°C to 40°C, the fruiting bodies were picked up within 10 to 15 days after spawning and the yield was about 25 to 35% which was higher than the yield from traditionally used paddy straw.

The physiology of growth in V. diplasia was studied by Rangaswami (1956). Starch and peptone were recorded

as the best carbon and nitrogen sources, respectively. The ambient temperature of about 35°C and pH 5.0 to 6.0, gave optimum growth of the fungus. Ghosh and Sengupta (1977) grew V. volvacea under submerged conditions and observed that the fungus utilizes soluble starch in preference to amylum, dextrin or glucose, whereas lignin or cellulose were poorly utilized. Potassium nitrate was found to be a suitable nitrogen source. Chandra and Purkayastha (1975) found that the optimum growth of V. volvacea was at pH 5.5 and 30°C. In physiological studies of V. volvacea on hemicellulose and cellulose Chang-Ho and Yee (1977), observed that it utilizes both cellulose and hemicellulose efficiently in the presence of asparagine. The straw on which the mushroom Volvariella is commercially cultivated consists mainly of cellulose (37%), hemicellulose (21%), lignin (8%) and about 4.5% of crude protein (Han, 1978; Mishra, 1980).

One of the earlier attempts at microbiological conversion of inorganic nitrogen and carbohydrate materials such as straw, sawdust or plant residues to protein was probably that of Robertson (1920). In the same year Pringsheim and Lichtenstein (1920) reported on the feeding of animals with Aspergillus fumigatus grown on straw and inorganic nitrogen. During World War II, Germany developed a process to produce food yeast, Candida utilis (Torula) from sugars obtained through acid hydrolysis of woods (Han, 1978). In the United States

during World War II investigations were carried out for the production of mushroom mycelium by submerged fermentation instead of compost or manure beds (Litchfield, 1968). Several other authors (Block, 1960; Gilbert and Robinson, 1957; Robinson and Davidson, 1959) extended these studies. Recently SCP production has been studied with different types of microorganisms such as bacteria, yeasts, actinomycetes and filamentous fungi by utilizing different cellulosic wastes. A bacterium Cellulomonas sp., has been reported to produce protein from sugar-cane bagasse, (Callihan and Dunlap, 1969). Chahal and Gray (1970), have grown some fungi imperfecti on low lignin pulps, with the required level of nitrogen substrate to produce a biomass with 23% crude protein. Paper mill waste cellulose has been converted by Myrothecium verrucaria to SCP (Updegraff, 1971), where the final product contained 10% crude protein. Crawford et al (1973) grew a thermophilic actinomycete on pulping fines, a cellulosic waste of the paper industry. The resulting biomass product was found to contain 30% protein. The white rot fungus, Sporotrichum pulverulentum has also been grown on waste mechanical fibres from a newsprint mill by Eriksson and Larsson (1975) and the culture produced had about 30% mycelial protein. Peitersen (1975) reported on the production of cellulase enzymes and protein from barley straw by growing Trichoderma viride. The protein content of the product was about 21-26%. A thermotolerent cellulolytic fungus,

Chaetomium cellulolyticum has also been used for SCP production by utilizing various insoluble cellulosic materials such as paper-pulp, wood, sawdust and wheat straw (Moo-Young et al; 1977; Chahal et al, 1977). This process of bioconversion has been developed on a large scale and details of the fermentation reported to as the "Waterloo process" have been published (Moo-Young et al, 1979). The nutritive quality of SCP depends on the amino acid composition of the protein. The pattern of amino acids in several SCPs is reasonably good when compared to high quality proteins such as those of egg and milk, (Miller, 1968). According to Hayes (1975), the mushroom protein has high digestibility and in its overall quality it is intermediate between low grade vegetable and high grade meat protein. Bano (1976), has given the composition of essential amino acids in a few mushrooms which shows that mushrooms contain most of the essential amino acids e.g. V. diplasia contains the following amino acids, in g/100 g. of protein: Isoleucine 5.5; leucine 3.5; lysine 2.68; phenylalanine 4.89; tyrosine 1.6; Cystine 2.3; methionine 0.89; threonine 4.2; tryptophan 1.1; valine 6.8; arginine 4.09; histidine 2.09.

Mushrooms are rich in vitamins. Orton et al (1922) found Agaricus campestris to be a good source of water soluble vitamins. Filios and Esselen (1946) reported in canned and cooked mushroom the presence of vitamins such

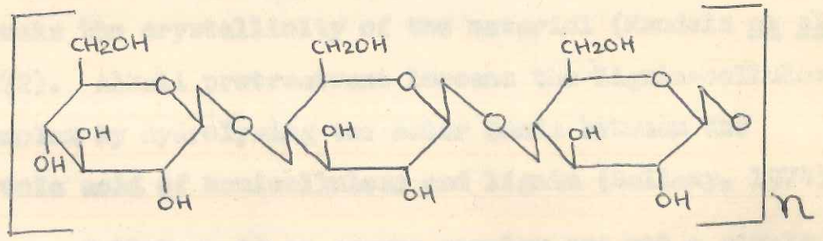
as riboflavin, niacin, pantothenic acid and biotin. Esselen and Fellers (1946) have reported the presence of folic acid in mushroom. In addition to the B group vitamins, mushrooms are also reported to contain Vitamin D and ergosterol (Bano, 1976).

There are two possibilities of converting cellulose to SCP (i) directly growing cellulolytic organisms on cellulosic substrates with the resulting biomass serving as the source of microbial protein and (ii) production of cellulases which can be further used for enzymatic hydrolysis of cellulosic materials in order to produce fermentable sugars. The sugars so produced can be used as substrates for cultivation of yeasts and other accepted microorganisms to produce SCP. (Ghose and Kostick, 1969).

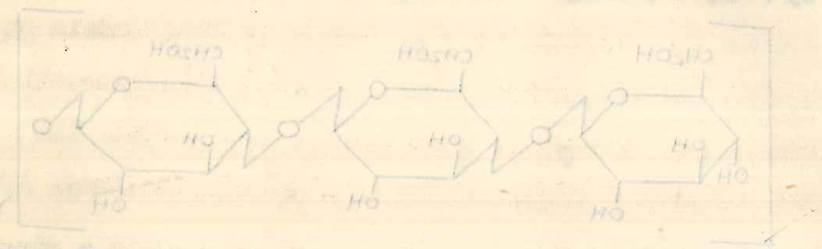
The history of research on cellulase at U.S. Army Natick Development Center has been discussed by Reese (1976). Among several microorganisms isolated from military materials undergoing extensive rotting under humid tropical conditions, a strain of Trichoderma was identified as the most potent cellulolytic culture. Extensive investigations on this strain, which has been recently named Trichoderma reesei have been published (Mandels and Sternberg, 1976; Mandels and Andreotti, 1978; Dewey and Mandels, 1980). From the point of enhancing cellulase activity as well as productivity to develop a viable technology, the culture has been

subjected to mutation studies and many promising mutants have been isolated (Montenecourt and Eveleigh, 1977 (a); 1977 (b)). Since cellulose is an abundant, renewable carbohydrate resource, which can be hydrolysed enzymatically to glucose, which in turn can be converted to alcohol by fermentation, there has been tremendous increase in the research inputs to develop process for high cellulase production.

Cellulose is a polymer of glucose units connected by β-1, 4 linkages such as,



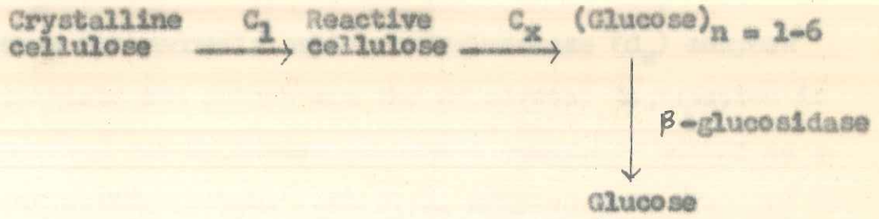
Under natural conditions, cellulose most commonly occurs in association with hemicellulose and lignin in the waste materials such as crop residues and forest wastes. Hemicellulose is a polymer of pentoses (xylose and arabinose), hexoses (mannose) and a number of sugar acids. Lignin is a high molecular weight phenolic compound which is generally resistant to microbial degradation. The white rot fungi have been shown to possess enzymes for breakdown of the lignin polymer and recently even bacteria and actinomycetes have been shown to possess the capacity to attack lignin, (Crawford and Crawford, 1980). Cotton fiber is an example where the cellulose occurs in almost pure state.



The hydrolysis of cellulose is difficult due to the high level of crystallinity associated with native cellulose and also because the cellulose in most instances is found in association with lignin which appears to form a mechanical barrier preventing the accessibility to the cellulose for the hydrolytic agents (Tsao et al, 1978). For the rapid enzymatic hydrolysis of cellulose, a physical or chemical pretreatment has been found to be necessary. Ball-milling reduces the size of the particles thereby increasing the surface area and also breaks the crystallinity of the material (Mandels et al, 1972). Alkali pretreatment loosens the lignin-cellulose complex by hydrolysing the ester bonds between the uronic acid of hemicellulose and lignin (Bellamy, 1974).

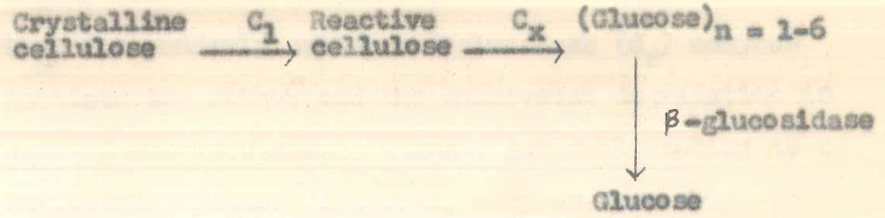
Cellulase is an enzyme complex and not a single homogeneous enzyme. In recent years the three major components of microbial cellulase have been identified as endoglucanase, exoglucanase and β-glucosidase and the mechanism of their action in cellulose degradation has been extensively reviewed (Wood and McCrae, 1979).

Reese et al (1950) have suggested the sequence of enzymatic reactions involved in the degradation of native cellulose as follows :



The enzyme C_1 was believed to act first on crystalline cellulose modifying it in such a way that subsequent enzymes can hydrolyze the product. It has little or no effect on soluble derivatives of cellulose such as carboxymethyl cellulose (CMC). The enzyme C_x randomly hydrolyzes celluloses pretreated to decrease their crystallinity as well as soluble cellulose derivatives and β-1,4 oligomers of glucose. β-glucosidase converts low molecular weight oligomers such as cellobiose and cellotriose to glucose.

The $C_1 - C_x$ hypothesis suggested by Reese was questioned by several workers after detailed studies on purified enzymes isolated from the culture filtrates of a number of cellulolytic fungi. Wood and McCrae (1972); Eriksson and Pettersson (1975); Berghem and Pettersson (1973) suggested that C_1 is an exoglucanase, in some cases it is specifically cellobiohydrolase, which successively removes cellobiose unit from the non-reducing end of the cellulose chain. CMCase, an endo β-1, 4 glucanase, attacks randomly the cellulose chain. Wood and McCrae (1979) have discussed the enzyme system comprising endo and exo-glucanases, involved in the



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degradation of crystalline cellulose. They suggested that the randomly acting endoglucanase (C_x) enzymes initiate the attack and the subsequent degradation is carried out by the exoglucanase generally acting as a cellobiohydrolase. The C_1-C_x enzymes were found to be synergistic in action. Neither C_1 nor C_x is able to degrade cellulose separately (Sadana et al, 1979a).

Many fungi and bacteria are reported to have the ability to secrete extracellular cellulases in sufficient quantity to merit consideration as a source of enzyme for saccherification of cellulosic substrates (Enari and Markkanen, 1977). These include filamentous fungi such as T. lignorum, T. koningii, T. reesei, Penicillium funiculosum, P. iriensis, Myrothecium verrucuria, Fusarium solani and Chaetomium thermophile var. dissitum. More recently certain bacterial strains such as species of Cellulomonas have been studied and mutants have been isolated which can degrade cotton wool (Choi et al, 1978) and cellulose. Saccharification using cellulases of these mutants have also been studied (Gray et al, 1980). The cellulolytic enzymes of a white rot fungi such as Sporotrichum pulverulentum and Polyporus adustus have also been studied (Eriksson and Pettersson, 1975; Eriksson, 1975). The other basidiomycetes reported to have significant extracellular cellulolytic activity are Irpex lacteus (Kanda et al, 1976); Pellicularia filamentosa (Mizukoshi et al, 1977); Poria placenta

(Highly, 1977); Poronia oedipus (Denison and Kohn, 1977); and Sclerotium rolfsii (Sadana et al, 1979). Krishnamohan (1975) reported C_1 and C_x cellulase activities during the growth of Volvariella esculanta and V. diplasia grown on paddy straw. Ramaswamy and Kandaswamy (1976) have shown that, the mushroom Podoxis pistillaris when grown on Czapek's synthetic medium with 3% cellulose, produces C_x and C_1 activity.

Pleurotus flabellatus have been reported to exhibit cellulase and hemicellulase activity (Rajarathnam et al, 1979).

Cellulase preparations from T. reesei have been used to convert newsprint cellulase to glucose on a pilot plant scale (Nystrom and Andren, 1976). Bastawde et al (1977) have studied the cellulolytic enzymes of a Penicillium strain for the saccharification of cellulose. Alkali-treated wood, cotton and bagasse were hydrolysed at a rapid rate by the Penicillium enzyme. Sadana et al (1979a) have obtained high saccharification values using a basidiomycete culture filtrate.

Present investigation :

In the present work cellulolytic enzyme production has been studied by cultivation of Volvariella diplasia in submerged culture using pure cellulosic substrates and under experimentally defined conditions of pH,

nutrient composition etc. Optimization of conditions both for bicmass production and extracellular enzyme production have been investigated. Saccharification of cellulosic substrates using the extracellular enzyme produced by the culture has also been studied.

CHAPTER II

MATERIALS AND METHODS

Chemicals and Reagents :

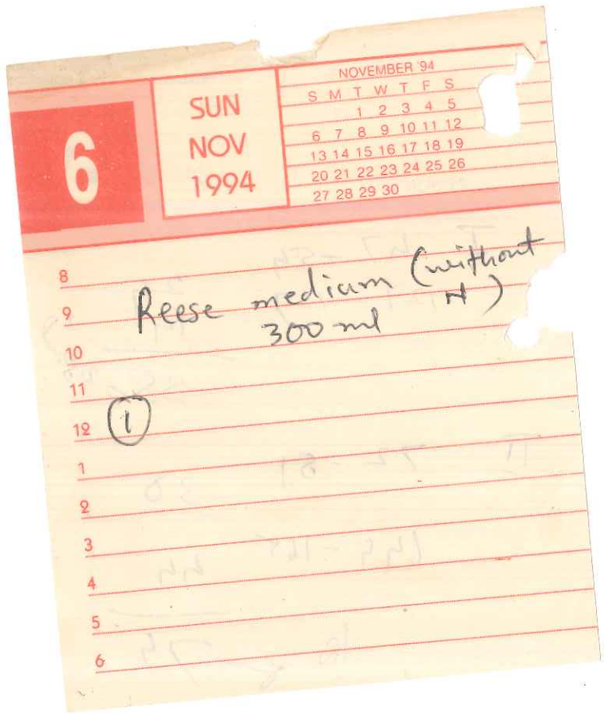
All the inorganic salts as well as carbohydrates used in the fermentation experiments were of analytical grade (B.D.H. or Sara-Merck). All the B vitamins used were obtained from B.D.H. Laboratories, excepting Vit B_{12} which was obtained from Themis Pharmaceuticals, Bombay. Ascorbic acid was obtained from Sarabhai Chemicals. Bacto peptone and Bacto yeast extract (Difco Laboratories, U.S.A.) were used as nutrient supplements in most of the experiments. The cellulose used was cellulose powder 123 obtained from Carl Schleicher and Schull, West Germany. For analysis the following chemicals and reagents were used and the source from which they were obtained is indicated in bracket. Carboxymethyl cellulose (Sigma); Bovine serum albumin (Sigma); Dinitrosalicylic acid (E. Merck); Avicel P.H. 101 (Honeywell and Stein, England); Anthrone (Loba-Chem. Industries, Bombay). Wheat bran, rice bran, rice straw, bagasse were purchased from the local market. Mesta wood (Hibiscus cannabinus) was obtained from the Forest Department, Chandrapur.

Organism : Volvariella volvacea (NCIM 1125) and V. diplasia (NCIM 1126) which are maintained in the National Collection of Industrial Microorganisms, National Chemical Laboratory, Poona, were used in preliminary studies. Detailed studies were carried out with V. diplasia only.

Maintenance : V. diplasia was maintained by periodic subculture on potato dextrose agar medium supplemented with 0.01% yeast extract and grown at 28°C for 8 to 10 days. It was observed that good mycelial growth accompanied by development of reddish brown sclerotic bodies took place during this period. Stock cultures were refrigerated at 5-10°C and also preserved under paraffin oil. Subcultures were made at bimonthly intervals for stock culture maintenance.

Media and Growth Conditions :

The basal medium employed in these studies was formulated according to Mandels and Weber (1969) with modification and had the following ingredients in g/l: KH_2PO_4 2.0; $(NH_4)_2 HPO_4$ 4.2; $MgSO_4.7H_2O$ 0.3; Urea 0.3; $CaCl_2.2H_2O$ 0.3. Trace elements in mg/l : $FeSO_4.7H_2O$ 5.0; $MnSO_4 . H_2O$ 1.56; $ZnSO_4.7H_2O$ 1.4 and $CoCl_2.2H_2O$ 2.0. It was observed that supplementation of the basal medium with peptone (0.25 g/l) and yeast extract (0.1 g/l) was helpful in stimulating the growth and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hence these ingredients were routinely included in all the studies unless mentioned otherwise. Thus the total nitrogen of the medium was 107 mg per 100 ml. Tween-80 at a concentration of 0.03% was also used in most of the experiments. The initial pH of the medium was adjusted to 5.4 with 1N HCl or 1N NaOH. Hundred ml of medium was dispensed in 500 ml Erlenmeyer flasks and 0.5 g/100 ml cellulose powder was added to each flask and it was autoclaved at 121°C for 20 min.

The different variations, modifications and supplements added to the basal medium in the case of different experiments are indicated at the appropriate places in the text.

Inoculum Development and Fermentation :

For all experiments 7-10 days old slant cultures were used as the starting material and the inoculum developed by the following procedure.

A one cm square of actively growing mycelial disc was transferred to 10 ml of potato dextrose broth in a large test tube and incubated at 28°C in an inclined position. It was observed that rapid mycelial growth took place growing on the thin layer of the liquid medium. After 7 days the liquid portion was aseptically removed and the mycelium carefully transferred to the experimental flasks. The dry weight of the mycelium per tube was determined and found to average 30 to 40 mg. The

experimental flasks were kept for incubation at 30°C on a rotary shaker at 220 rev/min for 15 days. Experimental flasks were run in duplicate and wherever the results were in agreement, the result is presented as a mean value and the mean of the results recorded.

Methods of Analysis

The flasks were harvested after 15 days by filtering the biomass on filter paper in a Buchner funnel. The biomass was repeatedly washed with distilled water to make it free of media constituents, and dried to constant weight at 50°-60°C. The clear culture filtrate was used to study extracellular enzyme activity.

Determination of Protein :

Crude Protein : The crude protein of the biomass (N x 6.25) was determined by Kjeldahl's method.

Soluble Protein : Soluble protein of the filtrate was determined by using the method of Lowry et al (1951) at 500 nm.

Determination of nitrogen in the filtrate :

Residual ammonia nitrogen of the filtrate was determined by using Kjeldahl's method where the sample without digestion was taken for estimation.

Inorganic nitrate was determined by the brucine method (Benjamin and Bridgeton, 1944).

Determination of cellulose :

Cellulose in the biomass was determined by the anthrone method (Updegraff, 1969).

Enzyme activities :

CMCase and filter paper activities were determined by the 3-5, dinitrosalicylic acid (DNSA) method of Mandels and Weber (1969).

Carboxymethyl cellulase (CMCase) activity :

0.5 ml of enzyme (suitably diluted to give approximately 0.5 mg glucose equivalent of reducing sugar) was taken in a test tube and 0.5 ml of 1% carboxymethyl cellulose (CMC) prepared in 0.05 M sodium acetate buffer (pH 4.8) was added and incubated at 50°C for 30 min. The reaction was stopped by adding 1 ml of 1% DNSA solution and the sample was heated to 100°C in a boiling water bath for 5 minutes. The tubes were cooled and the total volume was made to 12 ml and readings were taken, at 540 nm. The unit of enzyme activity is defined as the amount of enzyme which produces 0.5 mg of reducing sugar per 30 min at 50°C.

Filter paper activity (FP ase) :

1.0 ml of enzyme (suitably diluted to give about 0.5 mg equivalent of glucose) was added to 1.0 ml of 0.05 M acetate buffer at pH 4.8. To this mixture 50 mg (rolled 1 cm x 6 cm strip) of Whatman No.1 filter paper

was added and incubated for 1 hour at 50°C. The liberated reducing sugar was measured by the DNSA method at 540 nm. The unit of enzyme activity is defined as the amount of enzyme which produces 0.5 mg of reducing sugar per 60 min at 50°C.

β-glucosidase activity :

The activity was determined by using p-nitrophenyl-β-D glucoside (PNPG) according to the method of Berghem and Pettersson (1973). To 0.1 ml of diluted enzyme, 0.1 ml of PNPG (3 mg/ml of PNPG in 0.05 M acetate buffer at pH 4.8) and 0.9 ml of 0.05 M acetate buffer (pH 4.8) were added and kept at 40°C for 30 min. 2 ml of 1 M Na_2CO_3 solution was then added and the volume was made upto 13 ml. Readings were taken at 420 nm. The unit of enzyme activity is defined as the amount of enzyme which produces 1 μ mole of p-nitrophenol per min at 40°C.

CMCase activity at different pH levels

1% CMC in 0.05 M citrate or Tris-glycine buffer of required pH was prepared and the activity was determined as above.

Saccharification :

The percentage saccharification was determined as described by Mandels et al (1974). Enzymatic hydrolysis of untreated and alkali-treated cellulosic materials was carried out under defined conditions to evaluate the

eznymatic saccharification. Pretreatment of the substrate with alkali was carried out by incubation with 5 N NaOH overnight at room temperature followed by thorough washing to remove the alkali. The substrate was stored moist without drying until use in saccharification studies. For saccharification 100 mg (dry wt.) of substrate was taken in stoppered test tube and 10 filter paper units of an enzyme preparation concentrated 10 fold by ultrafiltration was added along with 0.1 ml of 0.5 M acetate buffer at pH 4.8 and incubated at 50°C for 24, 48 and 72 h. It was then boiled for 10 min and reducing sugars in an aliquot were determined by the DNSA method.

The end products of the saccharification were identified by paper chromatography using n-Butanol : Pyridine : water, system as described by Trevelyan et al (1950).

n-Butanol : Pyridine : water
46 : 36 : 19

CHAPTER III

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

SECTION I

GROWTH EXPERIMENTS

For the study of the growth of V. diplasia, the culture was grown in modified Mandels and Weber (1969) medium (without cellulose) as mentioned under Materials and Methods. The utilization of different carbon sources, nitrogen sources and the effect of trace elements and vitamins on growth and biomass production in submerged culture was studied. In all experiments excepting the studies on carbohydrate utilization, glucose at 0.5% level was used as the carbon source. The fermentation was carried out under surface as well as submerged condition, but no significant difference was observed, thus all the experiments were carried out under submerged condition. The initial pH was adjusted to 5.4 after sterilization and the experimental flasks were incubated at 28°-30°C on a rotary shaker at 220 rev/min for 15 days. The pH was periodically adjusted back to 5.0 during the fermentation, particularly where acid formation and pH drop were significant.

The growth was assessed on the basis of dry weight of biomass. The percentage of protein formed on the basis of total carbon source supplied and of the carbon source utilized was also determined. A correction for the dry weight of inoculum added and the nitrogen contributed by inoculum was also made.

Crude protein in the biomass was calculated as follows :

Protein of biomass = Kjeldahl N x 6.25

Total protein = protein of biomass + soluble protein in the medium.

$$\text{Percent protein conversion} = \frac{\text{total protein} \times 100}{\text{initial carbon source}}$$

$$\text{Percent protein conversion on utilized carbon source} = \frac{\text{total protein} \times 100}{\text{initial carbon source} - \text{residual carbon source.}}$$

Section I - A

Effect of different concentrations of glucose on biomass production

The effect of different concentrations of glucose on growth of the fungus was studied by using 1 to 5% of glucose concentrations in the basal medium. The results of the experiment are presented in Table 1.(Fig.1)

It was observed that with the increase in the concentration of glucose the growth of the organism was reduced. The same result was obtained when the percentage of glucose converted to protein was determined. It was also found that only about 0.4-0.7 g of glucose was utilized in 15 days.

TABLE 1 : EFFECT OF GLUCOSE CONCENTRATION ON GROWTH

Concentration of glucose %	Final pH	Dry wt. of biomass mg.	Residual N from filtrate mg	N of biomass mg	Crude protein of biomass mg	Soluble protein of filtrate mg	Total protein mg	Residual glucose mg	Protein conversion on basis of total glucose %
0.5	3.5	216	83.1	5.78	36.1	18.0	54.1	0.0	10.8
1.0	3.7	208	85.9	6.52	40.8	19.7	60.5	0.6	6.0
2.0	3.5	194	73.6	5.6	35.0	22.6	57.6	1.5	2.9
3.0	3.7	173	73.5	5.6	35.0	23.6	58.6	2.5	2.0
4.0	3.9	167	72.5	5.5	34.3	23.3	57.6	3.45	1.5
5.0	3.7	154	83.6	5.3	33.1	23.4	56.5	4.3	1.1

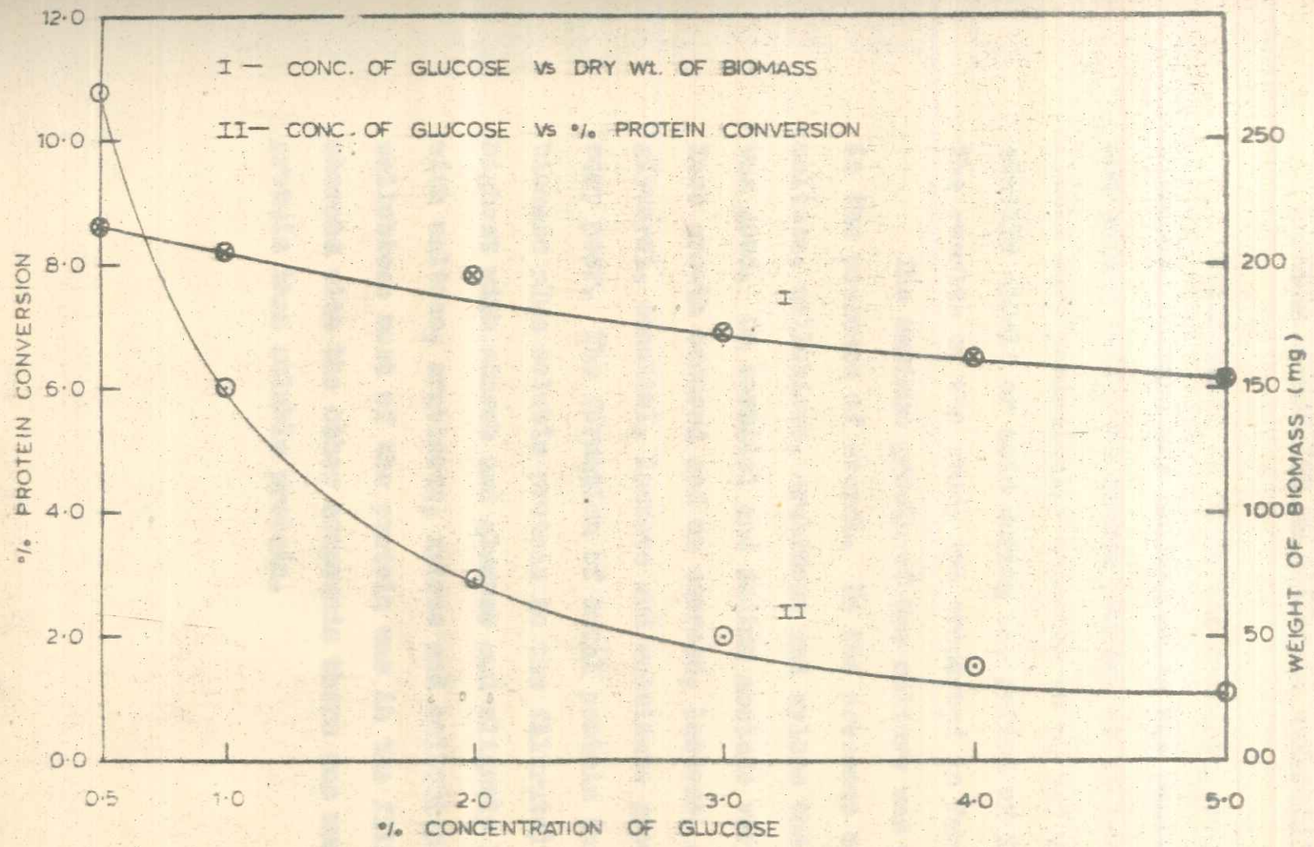


FIG. 1 : EFFECT OF GLUCOSE CONCENTRATION ON GROWTH

Section I - B

Biomass production on different carbohydrate

Utilization of different carbohydrates in submerged culture was studied using the basal medium and adding different carbon sources at 1% level. The flasks were periodically adjusted to pH 5.5 with sterile alkali or acid during the period of fermentation. The results of the study are presented in Table 2.

The maximum growth of the culture was observed in the presence of starch. In the presence of glucose, maltose, cellobiose, arabinose and xylose the growth was good. On sorbitol and sodium acetate very much less growth occured and on sucrose, lactose, galactase, glycerol, mannitol, lactate and succinate growth was very poor. The formation of total protein (protein of biomass plus soluble protein in the filtrate) was also highest with starch and glucose and slightly lesser with maltose, arabinose, xylose and cellobiose. With cellobiose most of the protein was in the filtrate, whereas with the other compounds there was more biomass protein than soluble protein.

TABLE 2 : GROWTH IN PRESENCE OF DIFFERENT CARBON SOURCES

Carbon source	Final pH	Dry wt. of biomass	Residual nitrogen in filtrate	Nitrogen of biomass	Crude protein of biomass N x 6.25	Soluble protein of the filtrate	Total protein	Protein conversion on basis of total carbon source
		mg	mg	mg	mg	mg	mg	%
Arabinose	3.7	172	91.9	5.4	33.8	21.0	54.8	5.5
Xylose	3.5	157	93.0	4.7	29.2	22.1	51.3	5.1
Glucose	3.4	208	85.8	7.6	47.8	22.8	70.6	7.0
Galactose	5.9	31	-	-	-	-	-	-
Sucrose	6.0	51	-	-	-	-	-	-
Maltose	5.1	181	88.3	5.3	33.4	21.6	55.0	5.5
Cellobiose	3.8	166	92.1	1.3	8.0	22.4	30.4	3.0
Starch	3.5	238	76.4	9.4	59.0	20.0	79.0	7.9
Glycerol	6.0	82	-	-	-	-	-	-
Sorbitol	6.0	140	100.6	1.4	8.9	9.6	18.5	1.9
Mannitol	6.0	63	-	-	-	-	-	-
Na-acetate	7.8	103	-	-	-	-	-	-
Na-lactate	5.9	30	-	-	-	-	-	-
Succinic acid	5.9	60	-	-	-	-	-	-

(Wherever the dry weight of biomass is less than 100 mg protein has not been determined).

SECTION I - C

Effect of different nitrogen sources on growth

In a nitrogen free basal medium supplemented with 0.5% glucose, the utilization of different inorganic and organic nitrogen sources were studied. The nitrogen level was adjusted to give 40-45 mg per 100 ml.

The different nitrogen sources used and their results obtained are given in Table 3.

Of the different nitrogen sources tested urea was utilized best on the basis of weight of mycelium as well as percentage of total protein formed. With di-ammonium hydrogen phosphate growth and mycelial protein was 76% of that of urea. Ammonium sulfate, potassium nitrate and ammonium nitrate were also utilized but growth and protein conversion were from 50 to 60% as compared with urea. Sodium nitrate and ammonium chloride were poorly utilized.

TABLE 3 : EFFECT OF NITROGEN SOURCES ON GROWTH

Nitrogen source	Concentration of nitrogen mg/100 ml	Final pH	Dry wt. of biomass mg	Residual nitrogen in filtrate mg/100 ml	Nitrogen of biomass mg	Crude protein of biomass Nx6.25 mg	Soluble protein of filtrate mg/100 ml	Total protein mg	Protein conversion on basis of total carbon source %
$(NH_4)_2SO_4$	43	3.1	107	35	1.99	12.4	12.9	25.3	5.0
$(NH_4)_2HPO_4$	40	3.5	136	30	2.5	15.6	15.7	31.3	6.2
KNO_3	40	4.6	101	33	1.87	11.7	11.7	23.4	4.7
NH_4NO_3	44	3.4	87	37	1.6	10.0	10.4	20.4	4.1
$NaNO_3$	43	5.1	28	ND	ND	ND	ND	ND	ND
NH_4Cl	40	4.9	35	ND	ND	ND	ND	ND	ND
Urea	42	6.7	178	29	3.31	20.7	19.8	40.5	8.1

(ND - Not determined)

SECTION I - D

Effect of different trace elements on growth

Four trace elements viz. Fe^{++}, Mn^{++}, Zn^{++} and Co^{++} were tested for their effect on the growth of *V. diplasia*. Basal medium was prepared omitting one trace element, at a time and was compared with controls with all the trace elements. One set of experimental flasks were also inoculated in which no trace element were added. The results are presented in Table 4.

It was observed that, in the presence of all the four trace elements maximum growth was obtained. In the absence of all the four trace elements growth was reduced by more than 40%, as indicated by the decrease in dry weight of biomass and percentage protein conversion. In the absence of one of the trace elements the dry weight of biomass was reduced by 17-28% and percentage protein conversion by 23-36%.

TABLE 4 : EFFECT OF DIFFERENT TRACE ELEMENTS ON GROWTH

Trace elements added	Final pH	Dry wt. of biomass	Residual nitrogen of filtrate	Nitrogen of biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Protein conversion on basis of total glucose
		mg	mg	mg	mg	mg	mg	mg
Zn^{++},Mn^{++},Co^{++}	5.1	156	91.1	4.00	25.0	14.0	39.0	7.8
Fe^{++},Mn^{++},Co^{++}	5.3	162	94.2	3.34	20.9	14.8	35.7	7.0
Fe^{++},Zn^{++},Co^{++}	5.1	175	93.0	3.90	24.3	16.2	40.5	8.1
Fe^{++},Zn^{++},Mn^{++}	4.6	181	89.5	4.34	27.1	15.2	42.3	8.4
Fe^{++}, Zn^{++}, Mn^{++}, Co^{++}	3.4	216	83.1	5.78	36.1	18.0	54.1	10.8
None	5.3	133	96.1	3.00	19.1	12.0	31.1	6.3

SECTION I - E

Effect of various vitamins on growth

Basal medium without yeast extract was used to observe the influence of various added vitamins on the growth of the mold.

Ascorbic acid, pyridoxine and thiamine were added at a concentration of 500 μg/l, while Biotin, riboflavin, folic acid and Vit. B_{12} were added at a concentration of 10 μg/l.

The results are presented in Table 5.

It was observed that there is a slight enhancement in growth in the presence of thiamine and to a smaller extent in the presence of biotin. Riboflavin and folic acid appeared to be inhibitory at the levels added and also showed lesser biomass protein content and the other Vitamins had no significant effect.

TABLE 5 : EFFECT OF VARIOUS VITAMINS ON GROWTH

Vitamin	Final pH	Dry wt. of bio-mass	Residual nitrogen of filtrate	Nitrogen of biomass	Crude protein of biomass N x 6.25	Soluble protein of filtrate	Total protein	Protein conversion on basis of total glucose
		mg	mg	mg	mg	mg	mg	%
Ascorbic acid	4.9	204	88.1	5.6	34.8	15.6	50.4	10.0
Biotin	4.7	227	88.0	5.8	36.0	20.0	56.0	11.2
Pyridoxine	4.7	188	93.2	4.8	30.0	12.4	42.4	8.5
Riboflavin	4.9	152	90.5	4.2	26.1	14.4	40.5	8.1
Thiamine	4.7	240	87.4	6.4	40.0	20.0	60.0	12.0
Folic acid	4.9	150	91.6	2.6	16.2	17.4	33.6	6.7
Vit. B_{12}	4.9	217	88.3	5.6	35.0	19.2	54.2	10.8
Control (without vitamin)	4.4	209	88.6	6.1	38.0	14.4	52.4	10.5

SECTION II

SINGLE CELL PROTEIN PRODUCTION

For the production of single cell protein (SCP) from cellulose, the culture was grown in basal medium with 0.5% cellulose powder as carbon source at initial pH 5.4 at 30°C under submerged condition on a rotary shaker at 220 rpm for 15 days. The amount of growth was difficult to assess from dry weight of biomass, as the biomass contained mycelium as well as unutilized substrate. Percentage protein content of the biomass calculated on the basis of initial substrate concentration as well as substrate utilized gave a satisfactory measure of evaluating the growth. The description for calculation of total protein and percentage protein formed is given in Section I.

For the total nitrogen of the biomass a correction was made for the nitrogen contributed by the inoculum.

All the experiments were carried out in duplicate and the mean data is presented.

SECTION II - A

Effect of different inorganic nitrogen sources on SCP production

The effect of adding different inorganic nitrogen sources to $(NH_4)_2HPO_4$ free basal medium at equivalent and 2 to 3 times higher concentrations on SCP production was studied.

Table 6 shows the different nitrogen sources added and their results obtained.

In the presence of diammonium hydrogen phosphate maximum protein was formed especially at 0.42% $(NH_4)_2HPO_4$. Potassium nitrate and ammonium nitrate also gave high protein conversion.

Ammonium sulfate at 0.14% was nearly as effective as the nitrates but at higher concentration it was inhibitary. Ammonium chloride was the least effective nitrogen source.

TABLE 6

EFFECT OF DIFFERENT INORGANIC NITROGEN SOURCES

Nitrogen source	Concentration of N source mg/100	pH	Wt. of biomass	Residual N of filtrate	N of biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Residual cellulose	Protein conversion on basis of total cellulose	Protein conversion on basis of utilized cellulose
%	ml		mg	mg	mg	mg	mg	mg	mg	%	%
$(NH_4)_2SO_4$ 0.14	48	5.7	226	34.5	4.65	29.1	20.0	49.1	29.7	9.8	10.4
" 0.28	78	4.6	215	66.2	4.84	30.3	9.4	39.7	21.5	7.9	8.3
" 0.7	168	5.0	256	150.1	3.8	23.8	28.0	51.8	48.6	7.9	8.3
$(NH_4)_2HPO_4$											
" 0.14	47.7	5.2	264	29.9	6.1	38.1	19.8	57.9	28.1	11.6	12.3
" 0.28	77.4	5.3	254	53.4	5.0	31.3	16.8	48.1	22.0	11.6	12.2
" 0.42	107.1	5.2	256	81.8	6.6	41.3	29.4	70.7	14.0	12.1	12.4
KNO_3 0.23	57.8	5.2	240	39.2	5.9	37.0	16.8	53.8	24.0	10.8	11.3
NH_4NO_3 0.18	81.0	5.6	251	67.0	5.2	32.8	17.2	50.0	45.6	10.0	11.0
NH_4Cl 0.12	49.7	5.2	274	33.3	4.9	30.6	9.3	39.9	100.2	7.9	9.9

SECTION II - B

Effect of different amounts of cellulose powder

The culture was grown in basal medium with different amounts of cellulose powder from 0.5 to 5.0%. The effect of different concentrations of cellulose powder is given in Table 7. (Fig.2)

As the amount of cellulose powder was increased the percent conversion of cellulose to protein was less.

TABLE 7

EFFECT OF DIFFERENT CONCENTRATIONS OF CELLULOSE POWDER

Concentration of cellulose powder	Final pH	Dry wt. of biomass	Residual N of filtrate	N of biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Residual cellulose	Protein conversion	
									on basis of total cellulose	on basis of utilized cellulose
%		g	mg	mg	mg	mg	mg	g	%	%
0.5	5.3	0.240	88.4	6.6	41.3	19.4	60.7	0.014	12.1	12.4
1.0	5.2	0.494	78.4	8.2	81.3	16.2	97.5	0.110	9.8	10.9
2.0	4.6	1.622	68.6	20.4	128.0	13.8	141.8	0.56	7.1	9.8
3.0	4.5	2.560	59.0	23.0	144.0	11.8	155.8	1.01	5.2	7.8
4.0	4.5	3.450	69.6	30.4	190.0	18.8	208.8	1.21	5.2	7.5
5.0	4.4	4.450	61.0	33.8	211.2	14.0	225.2	1.8	4.5	7.0

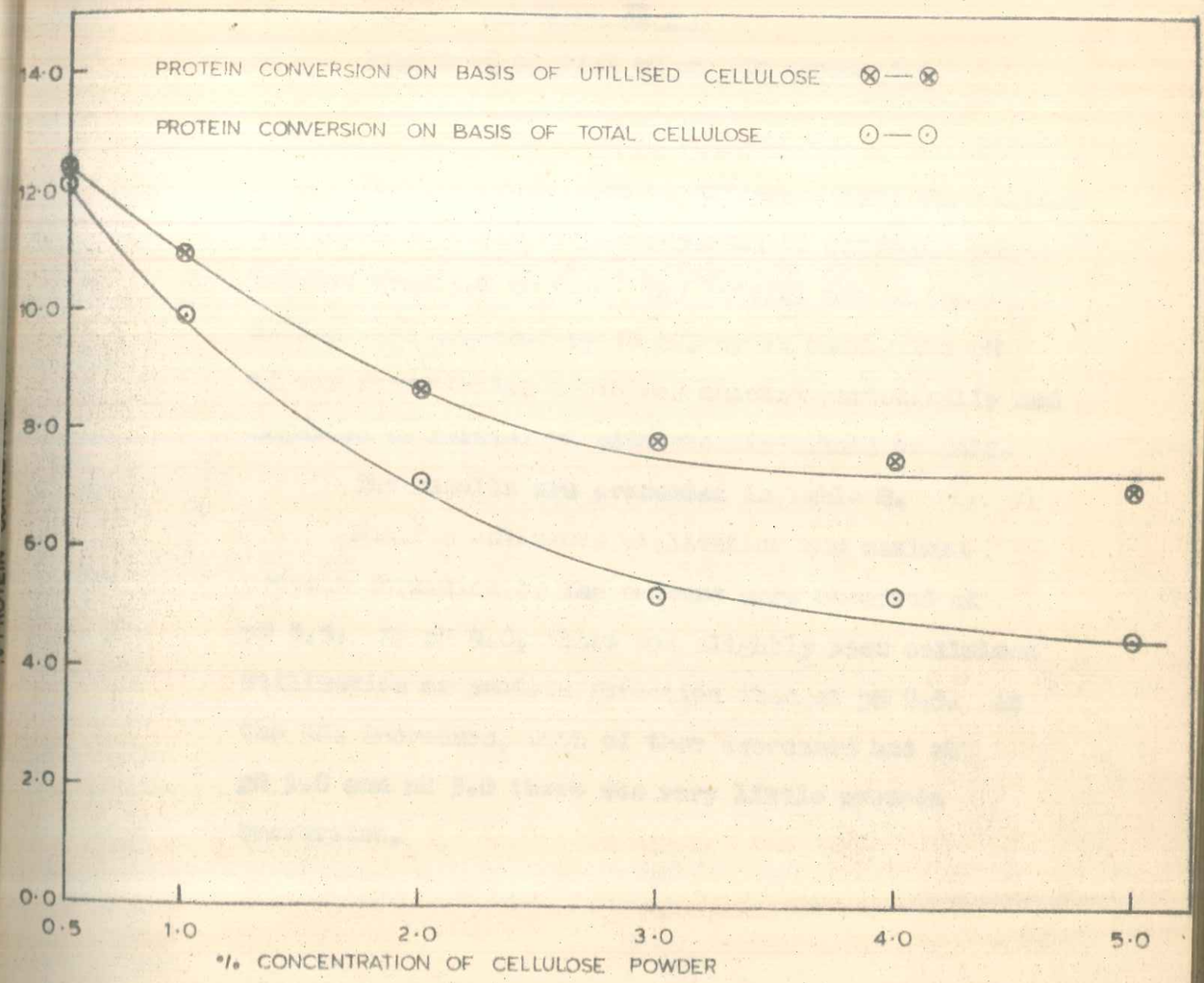


FIG. 2 : EFFECT OF DIFFERENT LEVELS OF CELLULOSE POWDER ON PROTEIN FORMATION

SECTION II - C

Effect of initial pH on SCP production

For SCP production at different pHs, the culture was grown in basal medium adjusted to different pH levels, from 3.0 to 9.0. The initial pHs of the medium were adjusted by 1N HCl or 1N NaOH. The pH of the fermentation broth was checked periodically and adjusted to initial pH with sterile alkali or acid.

The results are presented in Table 8. (Fig. 3)

Maximum cellulose utilization and maximum protein formation by the culture were observed at pH 5.5. At pH 4.0, there was slightly less cellulose utilization or protein formation than at pH 5.5. As the pHs increased, both of them decreased and at pH 9.0 and pH 3.0 there was very little protein conversion.

TABLE 8

EFFECT OF pH ON SCP PRODUCTION

Initial pH	Final pH	Dry wt. of biomass	Residual nitrogen of filtrate	Nitrogen of biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Residual cellulose	Protein conversion	
									on basis of total cellulose powder	on basis of utilized cellulose powder
		mg	mg	mg	mg	mg	mg	mg	%	%
3.0	3.3	471	101.3	1.4	8.8	1.2	10.0	321.3	2.0	5.6
4.0	5.0	251	89.1	5.1	32.0	9.6	41.6	55.3	8.3	9.3
5.5	5.2	240	85.4	6.6	41.3	9.4	50.7	24.4	10.1	10.8
6.0	5.5	300	92.0	4.2	26.4	4.8	31.2	132.0	6.2	8.5
7.0	6.8	330	90.7	4.2	26.4	3.4	29.8	132.0	5.9	8.1
8.0	7.2	400	96.8	2.6	16.0	4.2	20.2	212.0	4.0	7.0
9.0	8.0	460	103.0	0.93	5.8	1.9	7.7	309.0	1.5	3.9

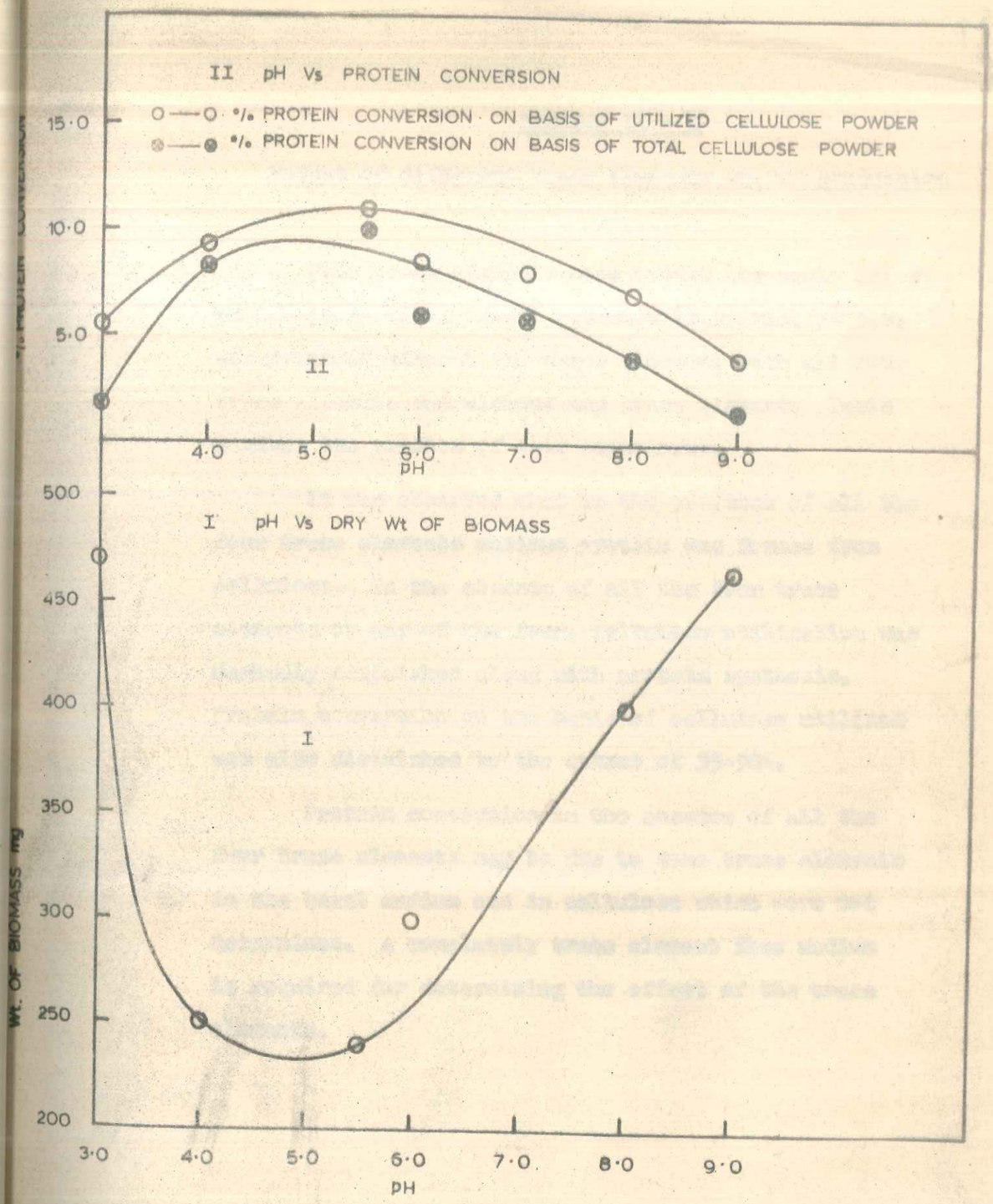


FIG. 3 : EFFECT OF pH ON SCP PRODUCTION

SECTION II - D

Effect of different trace elements on SCP production

Four trace elements were tested for their effect on SCP production. Basal medium with initial pH 5.4, was prepared without one trace element, with all four trace elements and without any trace element. Table 9 gives the results of this experiment.

It was observed that in the presence of all the four trace elements maximum protein was formed from cellulose. In the absence of all the four trace elements or any of the four, cellulose utilization was markedly diminished along with protein synthesis. Protein conversion on the basis of cellulose utilized was also diminished to the extent of 35-50%.

Protein conversion in the absence of all the four trace elements may be due to some trace elements in the basal medium and in cellulose which were not determined. A completely trace element free medium is required for determining the effect of the trace elements.

TABLE 9

EFFECT OF DIFFERENT TRACE ELEMENTS ON SCP PRODUCTION

Trace elements added	Final pH	Dry wt. of biomass	Residual nitrogen of filtrate	Nitrogen of biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Residual cellulose	Protein conversion on basis of total cellulose powder	Protein conversion on basis of utilized cellulose powder
		mg	mg	mg	mg	mg	mg	mg	%	%
Mn^{++}, Zn^{++} and Co^{++}	6.0	303	95.2	2.7	17.0	5.0	22.0	160.0	4.4	6.4
Fe^{++}, Mn^{++} and Co^{++}	5.9	305	92.3	2.7	18.5	6.6	25.1	185.0	5.0	7.9
Fe^{++}, Zn^{++} and Co^{++}	6.0	319	95.6	1.9	11.6	10.0	21.6	192.0	4.3	7.0
Fe^{++}, Zn^{++} and Mn^{++}	5.4	294	89.2	3.8	23.9	8.2	32.1	12.0	6.4	8.2
Fe^{++}, Mn^{++}, Zn^{+} and Co^{++}	5.2	266	83.4	6.6	41.3	17.0	58.3	34.4	11.7	12.5
None	6.0	460	96.1	2.3	14.6	4.6	19.2	200.0	3.8	6.0

SECTION II - E

Effect of various vitamins on SCP production

Basal medium without yeast extract was used to observe the influence of various vitamins on SCP production by the organism. Initial pH of the medium was adjusted to 5.4. Ascorbic acid, pyridoxine and thiamine were added at a concentration of 500 µg/l. Biotin, riboflavin, folic acid and Vit. B_{12} were added at a concentration of 10 µg/l. Table 10 presents the effect of various vitamins on SCP production.

It was observed that in the presence of thiamine and biotin, there was a slight enhancement in SCP production. Folic acid to a lesser extent reduced cellulose utilization and protein formation.

TABLE 10

EFFECT OF VARIOUS VITAMINS ON SCP PRODUCTION

Vitamin	Final pH	Dry wt. of biomass	Residual nitrogen of filtrate	N of the biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Residual cellulose	Protein conversion on basis total cellulose powder	Protein conversion on basis of utilized cellulose
		mg	mg	mg	mg	mg	mg	mg	%	%
Ascorbic acid	4.9	244	88.7	4.9	30.9	19.2	50.1	40.6	10.0	10.9
Biotin	4.7	274	83.4	6.3	39.6	15.0	54.6	47.6	10.9	12.0
Pyridoxine	4.9	283	90.1	4.7	29.3	11.6	40.9	100.5	8.1	10.2
Riboflavin	4.9	291	89.0	5.3	33.1	13.6	46.7	40.2	9.2	10.1
Thiamine	5.2	266	82.5	6.1	38.0	16.6	54.6	33.6	10.9	11.7
Folic acid	5.7	271	92.1	3.7	23.0	9.2	32.2	110.1	6.4	8.3
Vit B_{12}	5.3	243	90.2	4.9	30.5	12.8	43.3	60.8	8.7	10.1
Control (without vitamin)	5.2	248	88.5	4.9	30.6	14.6	45.2	257.2	9.0	10.2

SECTION II - F

The effect of time on SCP production

For studying the optimum period for SCP production, the culture was grown in the basal medium at an initial pH of 5.4. The flasks were harvested at two days intervals and their protein and residual cellulose were determined. The results are given in Table 11.(Fig. 4)

Cellulose was utilized progressively till the 15th day and total protein increased till the 15th day. There was a decrease in protein content when the culture was kept for an additional 5 or 10 days. The optimum period of growth was 15 days.

TABLE 11

STUDY OF OPTIMUM PERIOD FOR SCP PRODUCTION

Period (days)	pH	Wt. of biomass	Residual N of filtrate	N of the biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Residual cellulose	Protein conversion	
									on basis of total cellulose powder	on basis of utilized cellulose powder
		mg	mg	mg	mg	mg	mg	mg	%	%
0	5.4	530	107.0	-	-	6.0	-	500	-	-
2	5.4	513	101.3	0.3	2.0	12.0	14.0	453	2.8	-
4	5.5	492	96.8	0.99	6.2	11.4	17.6	400.9	3.5	-
6	5.5	458	93.2	1.6	9.9	13.6	23.5	346.0	4.7	9.5
8	4.6	415	92.7	2.05	12.8	13.8	26.6	302.2	5.3	13.4
10	4.7	333	90.4	3.3	20.9	20.8	41.7	159.1	8.3	12.2
12	5.0	280	88.2	4.8	30.1	25.0	55.1	58.0	11.0	12.5
15	5.2	266	85.7	6.6	41.3	29.4	70.7	34.4	14.1	15.2
20	5.6	228	85.0	5.6	10.0	35.0	45.0	34.2	9.0	9.7
25	5.6	230	85.6	5.7	8.9	35.4	44.3	32.9	8.9	9.5

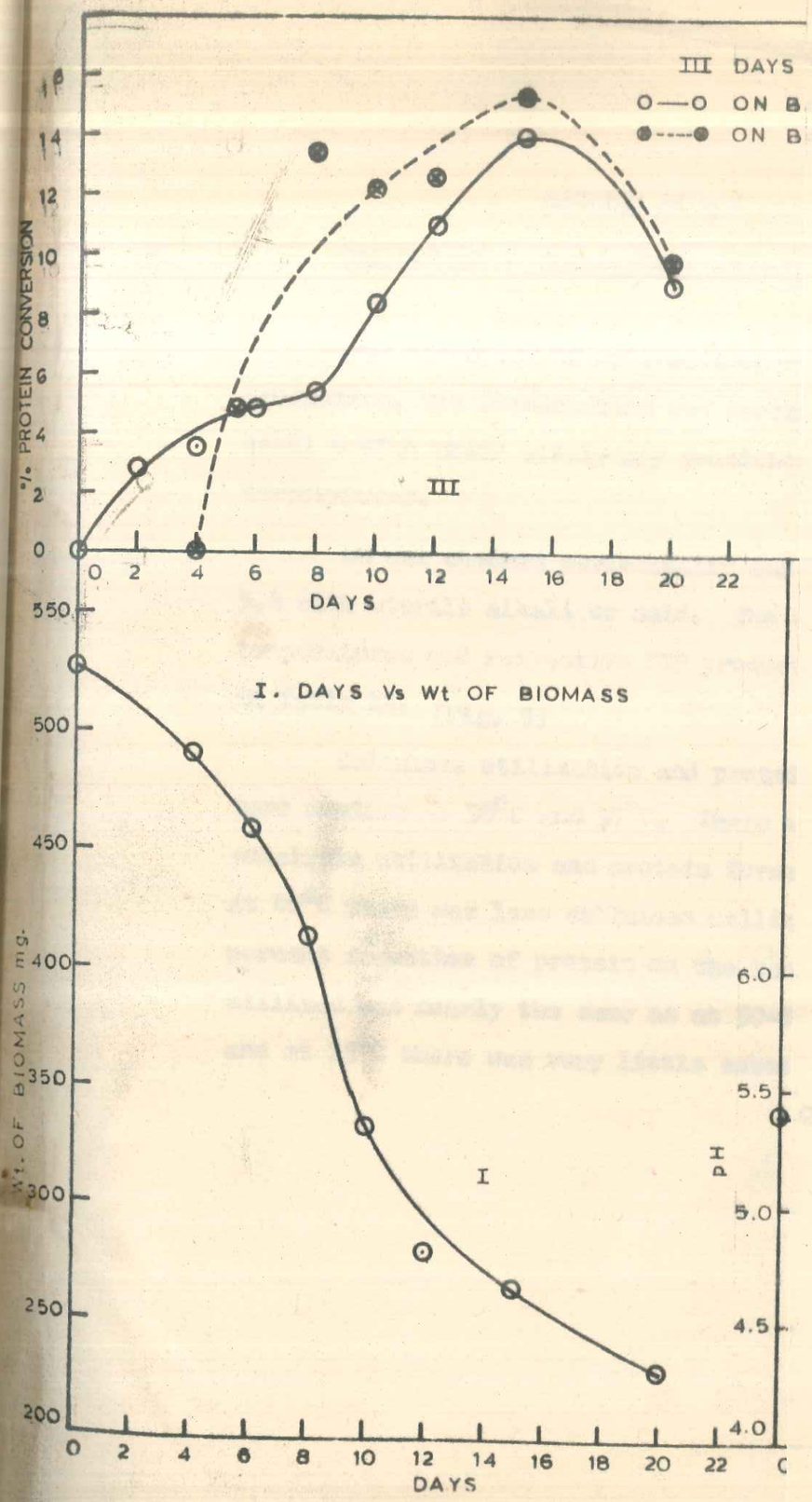


FIG. 4: EFFECT OF TIME ON SCP P

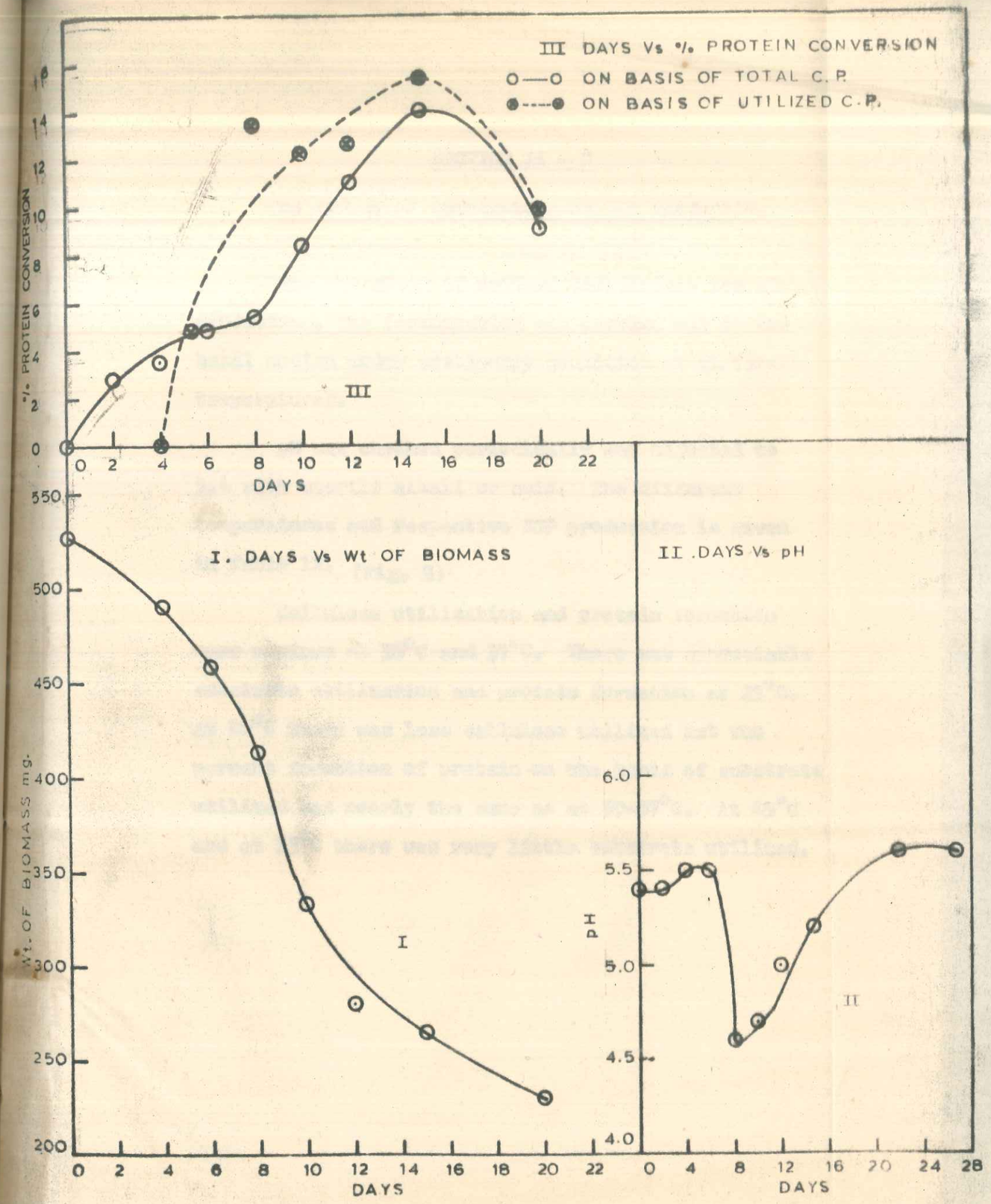


FIG. 4: EFFECT OF TIME ON SCP PRODUCTION

SECTION II - G

The effect of temperature on SCP production

For the study of optimum temperature for SCP production, the fermentation was carried out in the basal medium under stationary condition at different temperatures.

pH was checked periodically and adjusted to 5.4 with sterile alkali or acid. The different temperatures and respective SCP production is given in Table 12. (Fig. 5)

Cellulose utilization and protein formation were maximum at 30°C and 37°C. There was appreciable substrate utilization and protein formation at 25°C. At 40°C there was less cellulose utilized but the percent formation of protein on the basis of substrate utilized was nearly the same as at 30-37°C. At 45°C and at 15°C there was very little substrate utilized.

TABLE 12

EFFECT OF TEMPERATURE ON SCP PRODUCTION

Temperature	pH	Dry wt. of biomass	Residual N of filtrate	N of the biomass	Crude protein of biomass Nx6.25	Soluble protein of filtrate	Total protein	Residual cellulose	Protein conversion	
									on basis of total cellulose powder	on basis of utilized cellulose powder
°C		mg	mg	mg	mg	mg	mg	mg	%	%
15	5.7	494	98.1	0.5	3.2	10.2	13.4	431.1	2.7	-
25	5.8	333	90.0	5.1	32.0	11.9	43.9	92.0	8.9	10.7
30	5.3	266	87.4	5.2	32.3	27.4	59.7	34.1	11.8	12.6
37	5.3	250	87.5	5.7	35.4	23.6	59.0	18.0	11.6	12.2
40	5.5	415	93.0	3.2	20.0	5.2	25.2	272.8	5.0	11.0
45	5.5	513	102.8	-	-	12.0	-	480.0	-	-

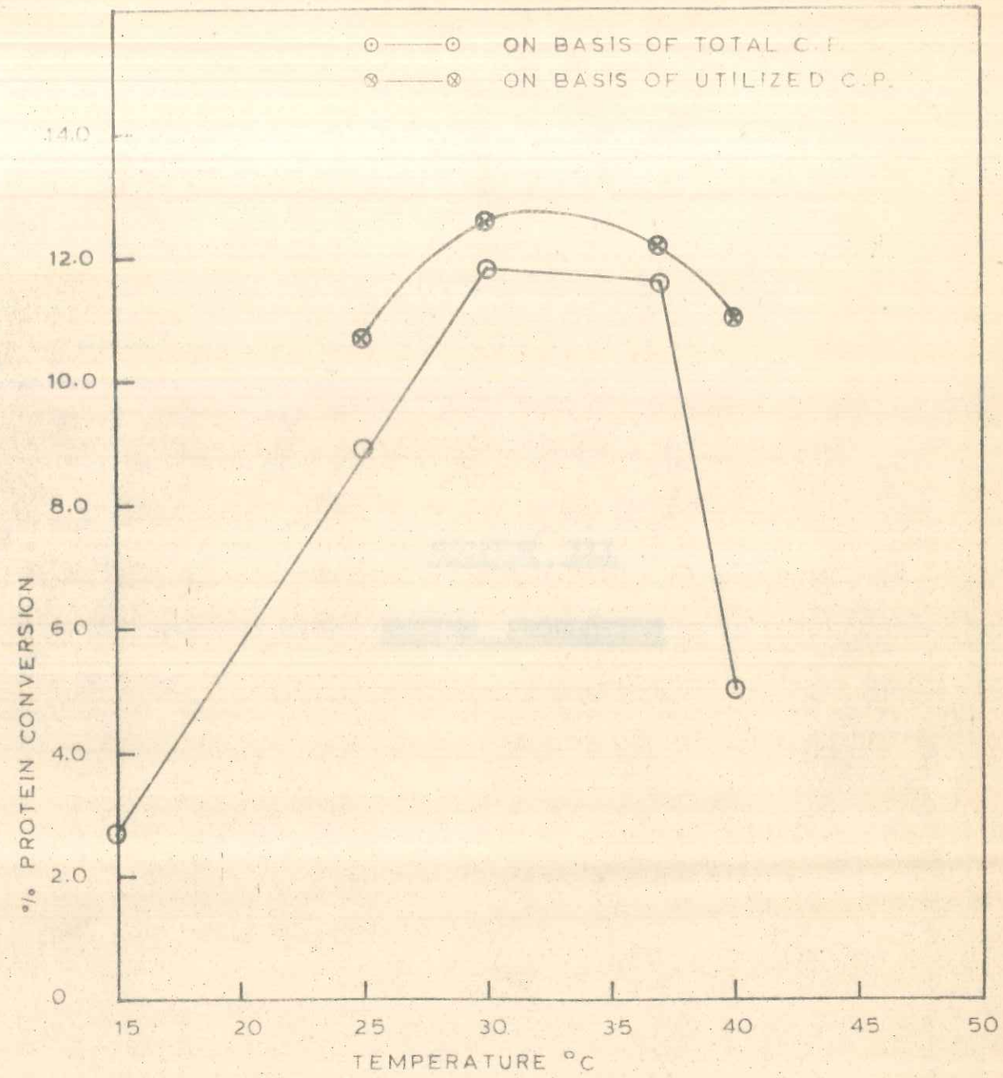


FIG. 5. EFFECT OF TEMPERATURE ON SCP PRODUCTION

SECTION III

ENZYME PRODUCTION

CELLULOLYTIC ACTIVITY OF V. VOLVACEA AND V. DIPLASIA

V. volvacea and V. diplasia were grown in $(NH_4)_2HPO_4$ free basal medium with 0.14% $(NH_4)_2SO_4$, at an initial pH of 5.4 for 15 days in shake flasks and enzyme activity (CMCase) was determined.

V. volvacea showed 3.5 units/ml of CMCase activity, whereas V. diplasia showed 6.0 units/ml of CMCase activity.

For further experimental work V. diplasia was selected, since it gave higher activity than the other culture.

SECTION III - B

EFFECT OF DIFFERENT INORGANIC NITROGEN SOURCES ON ENZYME PRODUCTION

The effect of adding different inorganic nitrogen sources to $(NH_4)_2HPO_4$ free basal medium at equivalent and at 2-3 times higher concentrations on cellulase production was studied. The initial pH was 5.4 and the fermentation was carried out for 15 days, under shaking condition. The pH was periodically adjusted to 5.4 with sterile alkali or acid. Results are given in Table 13.

TABLE 13

EFFECT OF DIFFERENT INORGANIC NITROGEN SOURCES ON ENZYME PRODUCTION

Nitrogen source	Concentration %	Concentration of nitrogen mg/100 ml	Final pH	CMCase activity units/ml
$(NH_4)_2SO_4$	0.14	48	5.8	6.0
"	0.28	78	4.6	3.0
"	0.70	168	5.0	3.0
$(NH_4)_2HPO_4$	0.14	47.7	5.2	5.0
"	0.28	77.4	5.3	5.0
"	0.42	107.1	5.3	6.0
KNO_3	0.23	57.8	5.2	2.5
NH_4NO_3	0.18	81.0	5.7	5.0
NH_4Cl	0.12	49.7	5.4	0.8

with higher concentration of ammonium sulfate enzyme production was decreased whereas with higher concentration of diammonium hydrogen phosphate enzyme production was increased. These results are similar to those observed on protein production. Ammonium nitrate was observed to be as effective for enzyme production as ammonium sulfate or phosphate. Potassium nitrate gave a lower yield at least at the concentration at which it was tested. Ammonium chloride gave very little enzyme production and also very little protein formation as shown in the previous section.

For further experimental work, diammonium hydrogen phosphate was used as the nitrogen source.

SECTION III - C

EFFECT OF DIFFERENT INDUCERS ON ENZYME PRODUCTION

Basal medium (without cellulose) with 1% concentration of different untreated and alkali treated (AT) substrates at initial pH 5.4 was used for the study of effect on enzyme production by the organism. The amount of alkali treated substrate taken was calculated on the basis of the dry weight determined with aliquots.

For alkali treatment of substrate it was autoclaved with 1N NaOH for 121°C for 20 min. Then the autoclaved substrate was thoroughly washed and stored in water without allowing it to dry.

The different cellulosic sources used and the respective enzyme activities obtained are presented in Table 14.

TABLE 14

EFFECT OF INDUCERS ON ENZYME PRODUCTION

Cellulosic source	Final pH	CMCase activity units/ml
Cellulose powder	5.1	4.8
*AT cellulose powder	4.0	2.0
Carboxymethyl cellulose	6.1	1.8
Avicel	5.2	3.2
Filter paper (Whatman No.1)	4.6	1.6
*AT Filter paper	4.0	1.9
Cotton	4.2	1.0
*AT Cotton	4.8	5.6
Wheat bran	6.4	1.5
Rice bran	6.3	0.5
Bagasse	6.0	0.0
*AT bagasse	4.4	1.3
Straw	6.0	1.0
*AT straw	4.4	1.0
Mesta wood	5.9	0.0
*AT mesta wood	4.3	1.3

(*AT - Alkali treated)

Alkali treated cotton and cellulose powder gave maximum enzyme production. Untreated wood and bagasse did not show any enzyme production. Moderate activity was given by the crystalline cellulose Avicel. All other substrates induced enzyme activity though in much smaller amounts.

SECTION III - D

EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME PRODUCTION

The culture was grown in the basal medium with different amounts of cellulose powder. Cellulose powder was used at 0.5% to 5.0% levels. The initial pH of the medium was 5.4. Table 15 shows the different cellulose powder concentrations and the enzyme activities obtained.

TABLE 15

EFFECT OF DIFFERENT LEVELS OF CELLULOSE POWDER ON ENZYME PRODUCTION

Cellulose powder %	Final pH	CMCase activity units/ml
0.5	5.3	6.0
1.0	5.2	4.8
2.0	4.6	3.2
3.0	4.5	1.5
4.0	4.5	0.0
5.0	4.4	0.0

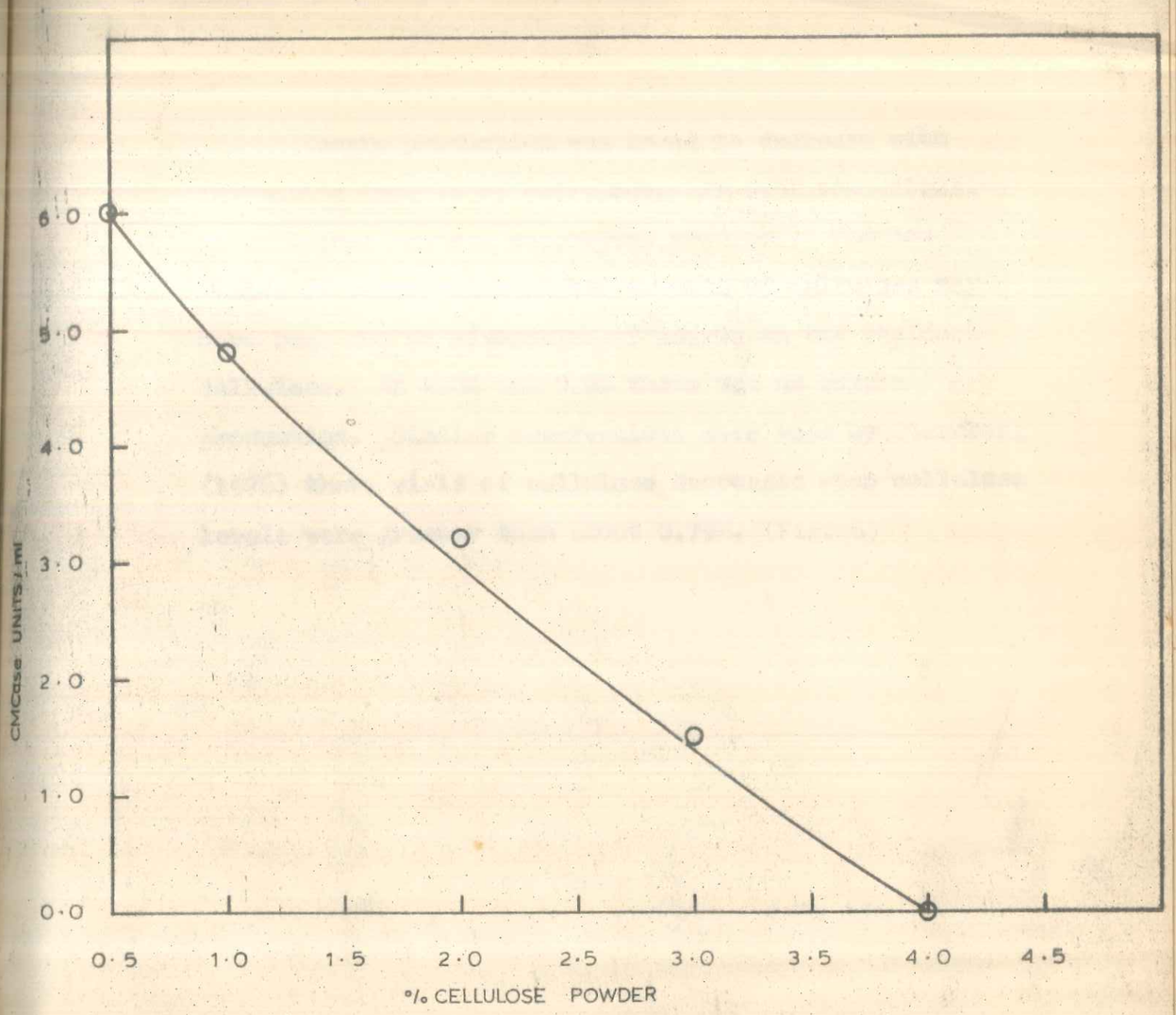


FIG. 6: EFFECT OF DIFFERENT LEVELS OF CELLULOSE POWDER ON ENZYME PRODUCTION.

Enzyme production was found to decrease with increasing amounts of cellulose. At 0.5% concentration of cellulose maximum enzyme was produced. The low yields of enzyme with higher amounts of cellulose may have been due to adsorption of enzyme on the residual cellulose. At 4.0% and 5.0% there was no enzyme production. Similar observations were made by Sternberg (1976) that, yield of cellulase decreases when cellulose levels were greater than about 0.75%. (Fig. 6)

SECTION III - E

EFFECT OF pH OF FERMENTATION ON ENZYME PRODUCTION

Basal medium with different initial pHs was used to observe the effect of different pHs on enzyme production. The initial pH of the basal medium was adjusted within the range of 3.0 to 9.0, and enzyme production was determined. Table 16 shows the enzyme activities at different pHs. (Fig. 7)

TABLE 16

EFFECT OF INITIAL pHs ON ENZYME PRODUCTION

Initial pH	Final pH	CMCase activity units/ml.
3.0	3.3	0.0
4.0	5.0	4.0
5.5	5.2	6.0
6.0	5.5	4.8
7.0	6.8	2.0
8.0	7.2	0.4
9.0	8.9	0.0

For enzyme production pH 5.5 was optimum. The pH range for enzyme production was 4.0 to 8.0 and there was no enzyme production at pH 3.0 or pH 9.0.

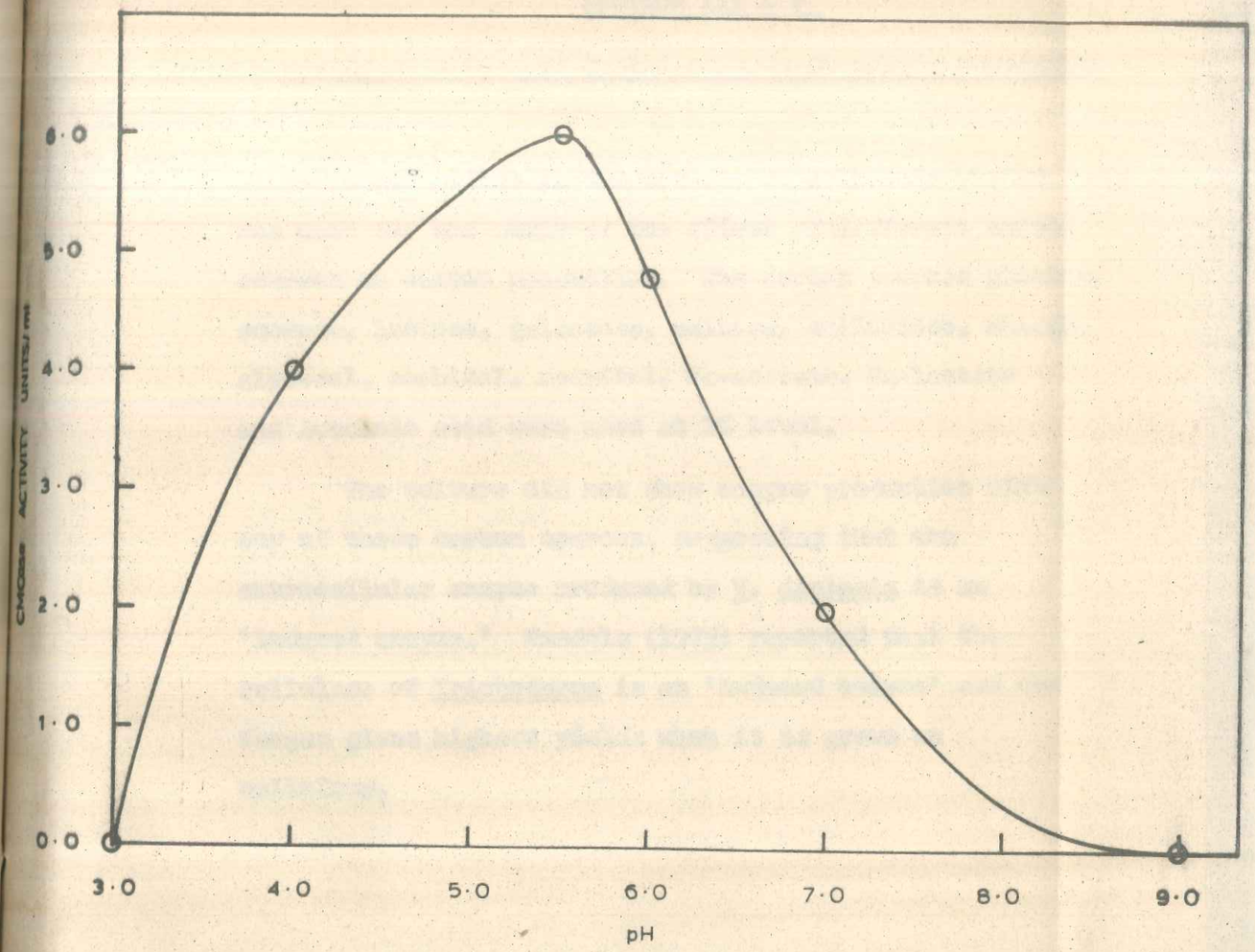


FIG. 7: EFFECT OF pH ON ENZYME PRODUCTION

SECTION III - F

EFFECT OF DIFFERENT CARBON SOURCES ON ENZYME PRODUCTION

Basal medium in the absence of cellulose powder was used for the study of the effect of different carbon sources on enzyme production. The carbon sources glucose, sucrose, lactose, galactose, maltose, cellobiose, starch, glycerol, sorbitol, mannitol, Na-acetate, Na-lactate and succinic acid were used at 1% level.

The culture did not show enzyme production with any of these carbon sources, suggesting that the extracellular enzyme produced by *V. diplasia* is an 'induced enzyme.' Mandels (1975) reported that the cellulase of *Trichoderma* is an 'induced enzyme' and the fungus gives highest yields when it is grown on cellulose.

SECTION III - G

EFFECT OF DIFFERENT TRACE ELEMENTS ON ENZYME PRODUCTION

Four trace elements were tested for their effect on enzyme production. Basal medium was prepared without one trace element, with all four trace elements and without any trace element at initial pH 5.4. Table 17 presents the results.

TABLE 17

EFFECT OF DIFFERENT TRACE ELEMENTS ON ENZYME PRODUCTION

Trace elements added	Final pH	CMCase activity units/ml
Mn^{++}, Zn^{++} and Co^{++}	6.0	3.2
Fe^{++}, Mn^{++} and Co^{++}	5.9	3.2
Fe^{++}, Zn^{++} and Co^{++}	6.0	3.2
Fe^{++}, Zn^{++} and Mn^{++}	5.9	4.4
Fe^{++}, Mn^{++}, Zn^{++} and Co^{++}	5.2	5.6
None	6.0	3.0

Deletion of any of the four trace elements viz. Fe^{++}, Zn^{++}, Mn^{++} and Co^{++} led to decrease in enzyme production. Though the absence of Co^{++} reduced enzyme production least. In the absence of all four elements the reduction in yield was not greater than in the absence of only one metal.

SECTION III - H

EFFECT OF VITAMINS ON ENZYME PRODUCTION

Basal medium without yeast extract was used to observe the effect of vitamins on enzyme production. Ascorbic acid, pyridoxine and thiamine were added at a concentration of 500 µg/l. Biotin, riboflavin, folic acid and Vit. B_{12} were added at a concentration of 10 µg/l. Table 18 shows the various vitamins tested and their respective enzyme activities obtained.

TABLE 18

EFFECT OF VITAMINS ON ENZYME PRODUCTION

Vitamin source	Final pH	CMCase activity units/ml
Ascorbic acid	4.9	4.0
Biotin	4.7	4.5
Folic acid	5.7	1.3
Pyridoxine	4.9	4.4
Riboflavin	4.9	3.5
Thiamine	5.2	4.3
Vit.B_{12}	5.3	3.5
Control (without vitamin)	5.2	4.3

It was observed that there is no enhancement in enzyme production by the addition of any vitamin. The reason for a marked decrease in enzyme activity due to the addition of folic acid is not known.

SECTION III - I

STUDY OF OPTIMUM PERIOD FOR ENZYME PRODUCTION

For studying the optimum period of enzyme production the culture was grown in the basal medium at pH 5.4 and the flasks were harvested at two day intervals and the enzyme activity was determined. The results are presented in Table 19. (Fig. 8)

TABLE 19

OPTIMUM PERIOD FOR ENZYME PRODUCTION

Period (Days)	Final pH	CMCase activity units/ml
0	5.4	0.0
2	5.4	0.0
4	5.5	0.0
6	5.5	0.5
8	4.6	1.0
10	4.7	2.0
12	5.0	4.8
15	5.2	5.2
20	5.6	2.7
25	5.6	0.0

Enzyme production was noticeable only on the 6th day. It increased progressively and maximum enzyme was produced between the 12th and 15th days. After the 15th day enzyme activity decreased and was negligible on the 25th day.

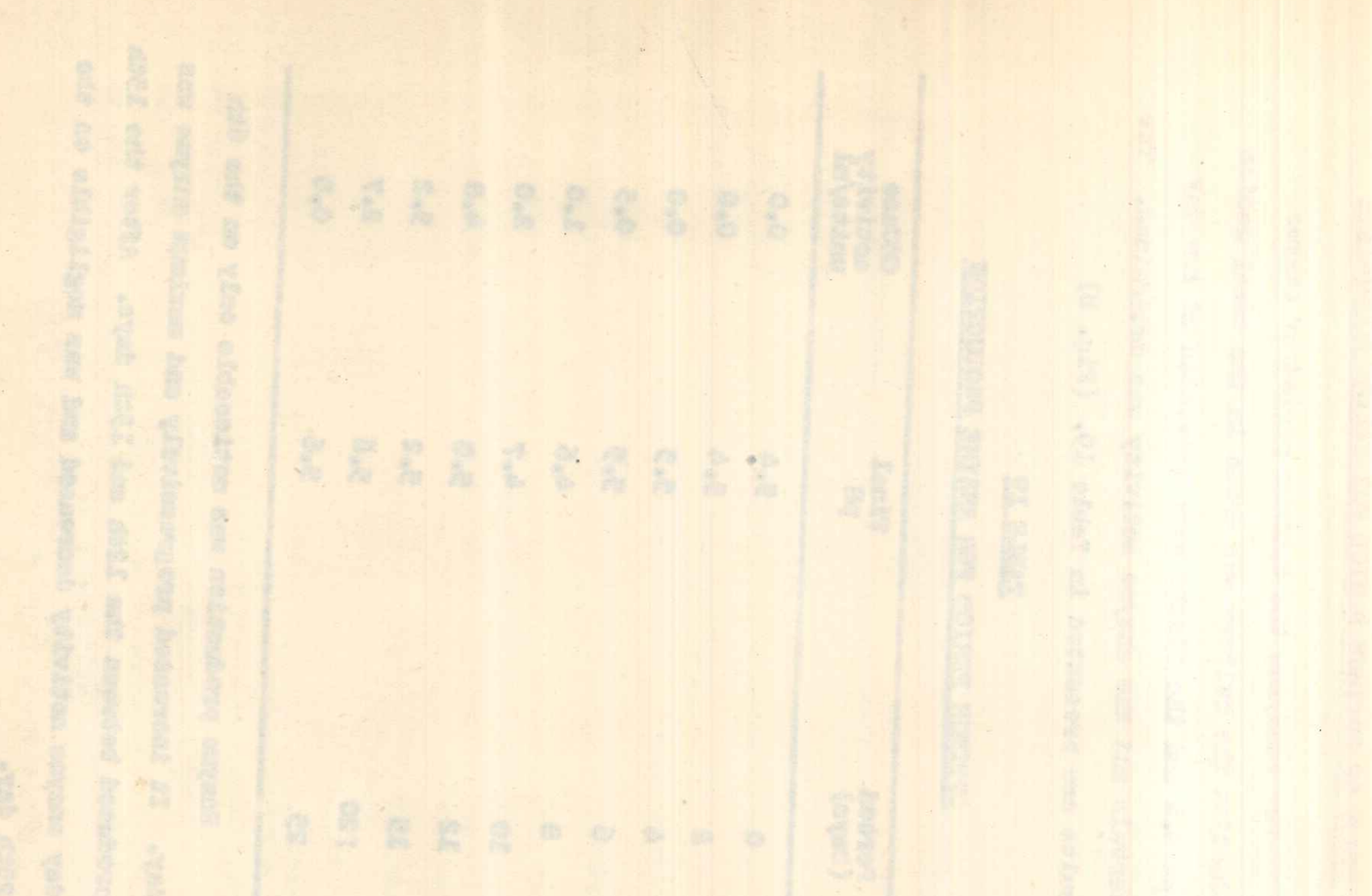


FIG. 8: EFFECT OF TIME ON ENZYME PRODUCTION AND pH.

SECTION III - J

STUDY OF OPTIMUM TEMPERATURE FOR ENZYME PRODUCTION

For the study of optimum temperature for enzyme production, the fermentation was carried out in the basal medium at the stationary state at different temperatures, at pH 5.4 and for 15 days. Table 20 gives the enzyme production at different temperatures.

TABLE 20

EFFECT OF TEMPERATURE ON ENZYME PRODUCTION

Temperature °C	Final pH	CMCase activity units/ml
15	5.7	0.0
25	5.8	1.6
28	5.2	5.2
37	5.1	4.0
40	5.5	0.5
45	5.5	0.0

For enzyme production the optimum temperature was 28°C. There was no enzyme activity at 15°C or 45°C and there was a sharp decrease in activity above 37°C. (Fig. 9)

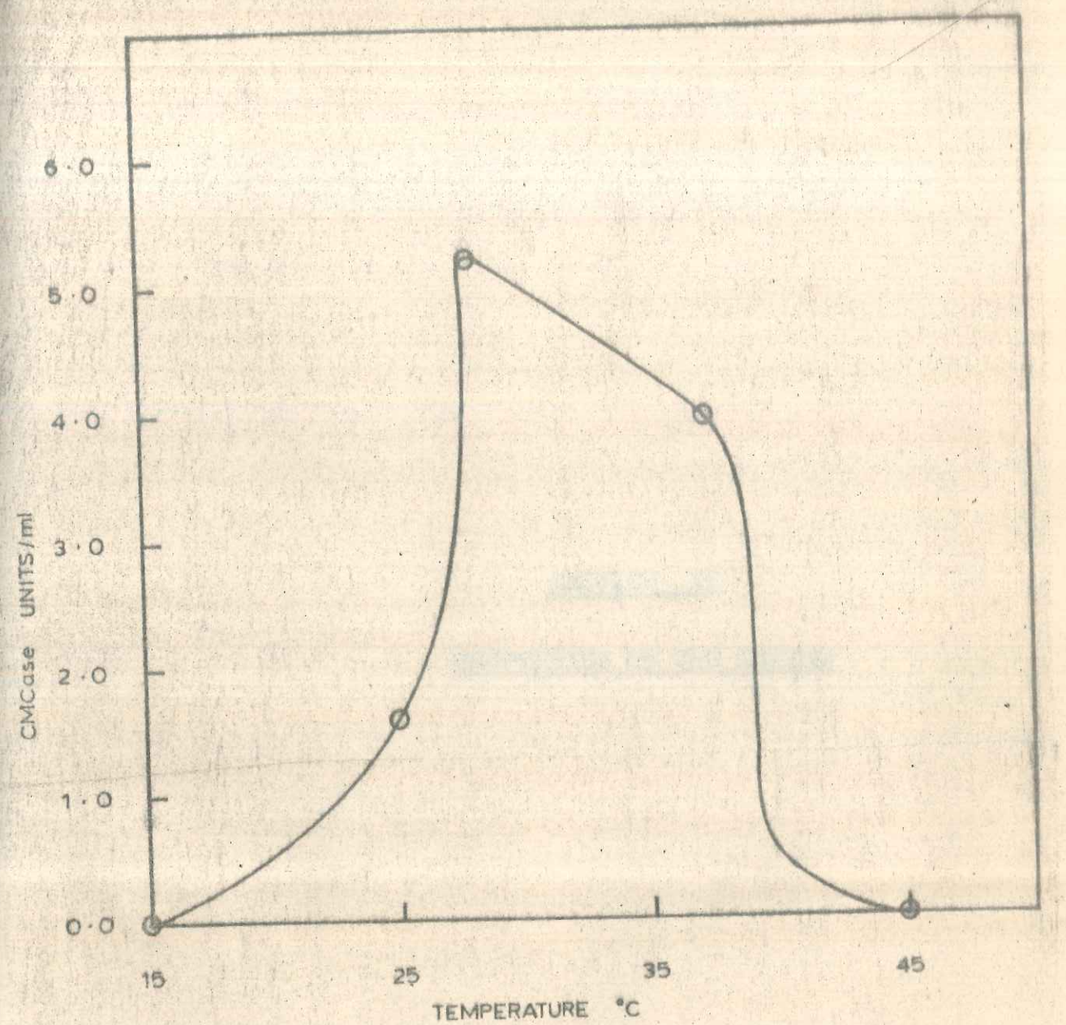


FIG.9: EFFECT OF TEMPERATURE ON ENZYME PRODUCTION.

SECTION IV

PROPERTIES OF THE ENZYME

SECTION IV - A

STUDIES OF THE PROPERTIES OF THE CELLULASE OF VOLVARIELLA

To study the properties of the extracellular enzyme of V. diplasia, enzyme was first concentrated ten fold by ultra filtration with filter UM-10, since the activity of the fermentation broth was too low for several experiments.

The enzyme was dialysed through a dialysis membrane in 0.25 M Tris-glycine buffer (pH 8.3) with three changes at 4^{o}-8^{o}C. There was no loss in enzyme activity on dialysis.

Results on the cellulase activity determined before and after concentration of the enzyme is presented in Table 21.

TABLE 21

DETERMINATION OF CELLULASE ACTIVITY BEFORE AND AFTER CONCENTRATION OF ENZYME

Enzyme	Enzyme Activity			
	Before concentration		After concentration	
	Units/ml	Units/ 230 ml	Units/ml	Units 23 ml
CMCase (units/30 min)	6.0	1380.0	60.0	1380.0
F.P. activity (units/60 min)	ND	-	15.1	347.3
β-glucosidase (pNPGase) (units/min)	ND	-	0.01	0.23

(ND - not determined)

There was no loss in enzyme activity (CMCase) during ultra filtration. The ratio of CMCase to filter paper activity was high and comparable to that with the T. viride enzyme.

SECTION IV - B

EFFECT OF pH ON ENZYME ACTIVITY (CMCase)

To study the effect of different pHs, the pH of the enzyme (10 fold concentrated) was adjusted to different values (2.5 - 10.0) with buffer as stated in "Materials and Methods" and the CMCase activity was determined at 50°C for 30 min. Table 22 gives the CMCase activity tested at different pHs. (Fig. 10)

TABLE 22

EFFECT OF pH ON ENZYME ACTIVITY

pH	CMCase activity units/ml
2.5	0
3.2	19
3.5	35
4.2	50
4.8	60
5.0	55
5.2	50
5.5	45
6.2	36
6.5	30
7.2	24
7.6	20
8.0	11
8.4	8
9.0	5
10.0	0

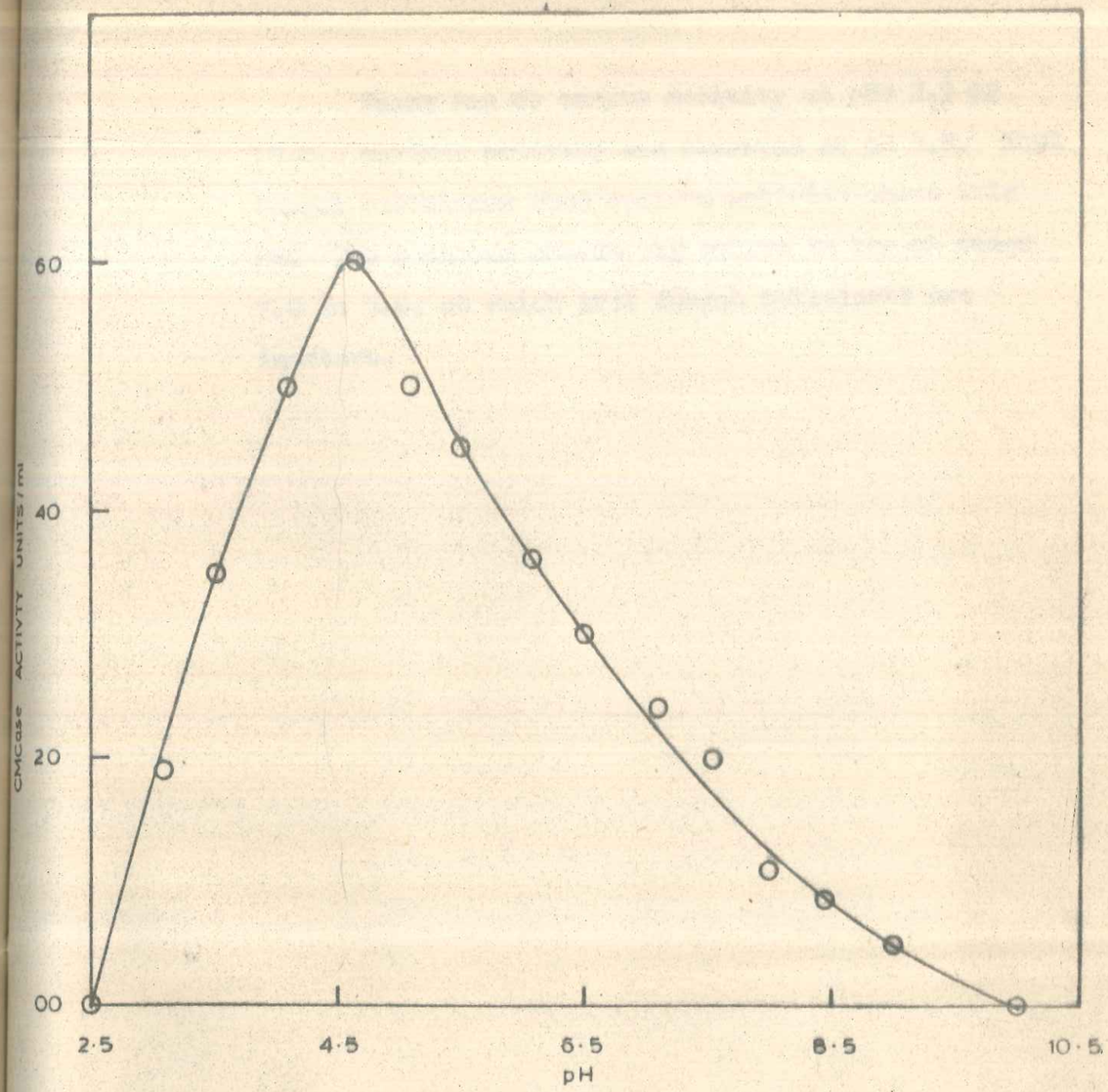


FIG. 10: EFFECT OF pH ON ENZYME ACTIVITY.

There was no enzyme activity at pHs 2.5 or 10.0. Maximum activity was observed at pH 4.8. Most fungal cellulases show optimum activity about this pH. The mushroom enzyme was active in the pH range 7.0 to 9.0, at which most fungal cellulases are inactive.

SECTION IV - C

EFFECT OF TEMPERATURE ON ENZYME ACTIVITY

CMCase activity was determined at different temperatures for 30 min. The activity was determined at pH 4.8. The different temperatures and the respective activities obtained are given in Table 23 (Fig. 11).

TABLE 23

EFFECT OF TEMPERATURE ON CMCase ACTIVITY

Temperature ^{o}C	CMCase activity units/ml
0	0.0
15	8.0
25	20.0
30	26.0
35	37.0
42	50.0
50	60.0
55	45.0
60	42.0
70	15.0
80	0.0

The enzyme showed maximum activity at 50°C. It was active even at 70°C but not at 80°C. The temperature range for its activity was 15°C - 70°C.

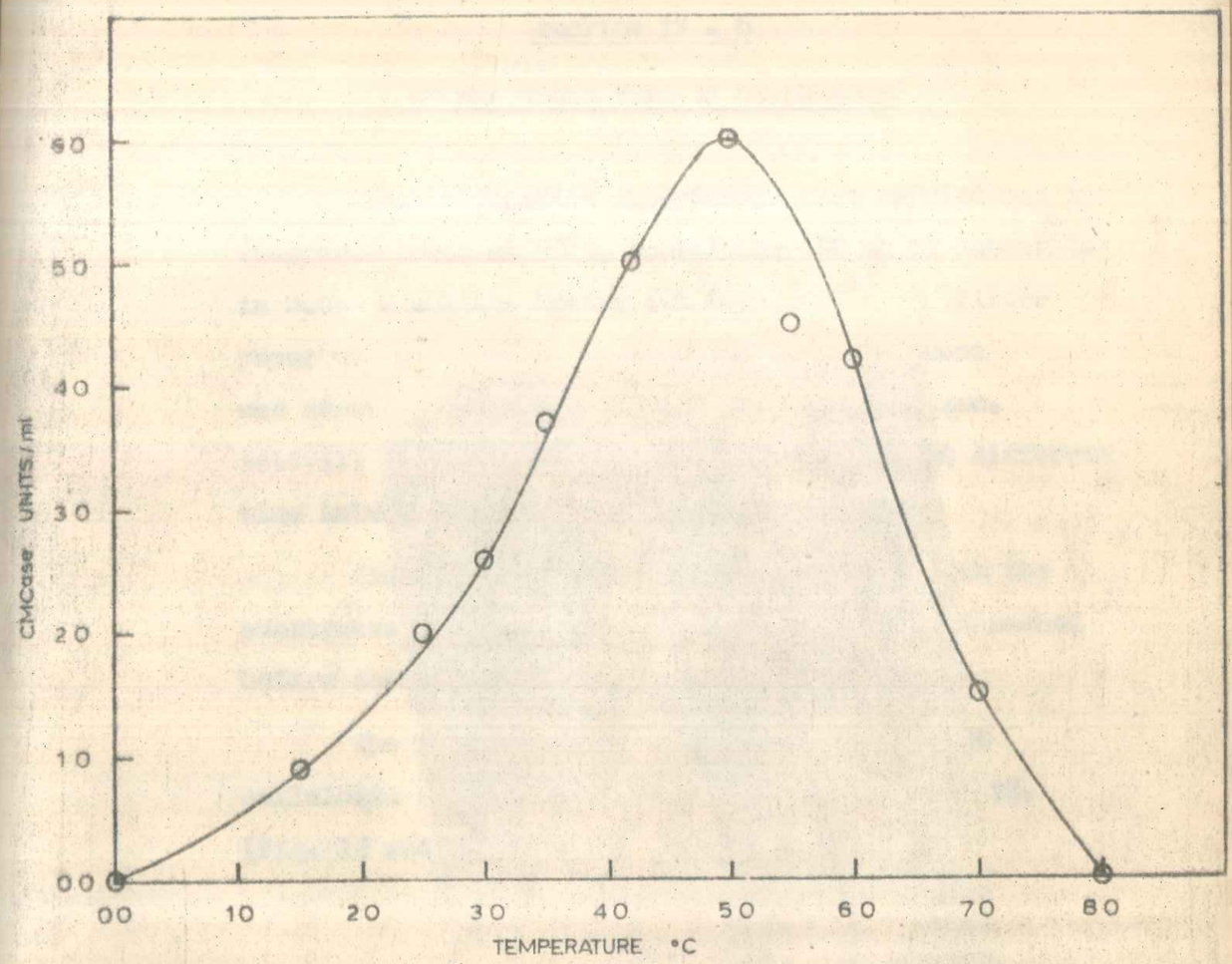


FIG. 11: EFFECT OF TEMPERATURE ON ENZYME ACTIVITY.

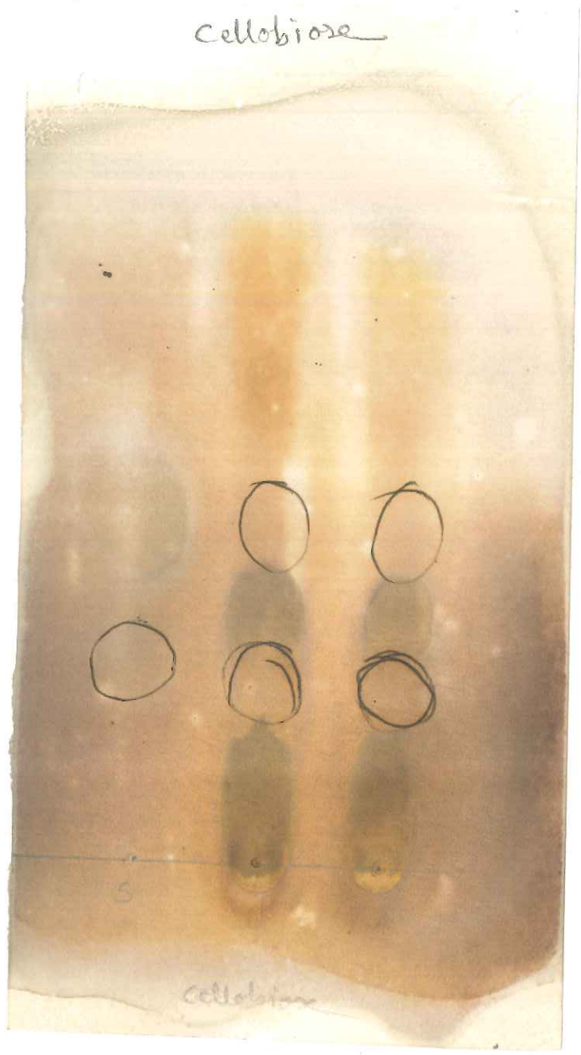
SECTION IV - D

ENZYMATIC HYDROLYSIS OF CELLULOSES

Saccharification experiments were carried out in stoppered tubes at 50°C, containing 100 mg of substrate in 0.05 M acetate buffer (pH 4.8), using 10 filter paper units of the concentrated enzyme. Toluene was added to lessen the growth of microorganisms. Activity was determined by the DNSA reagent at different time intervals.

Similar experiments were carried out with the substrates which were treated with 5 N NaOH and washed before saccharification as stated in "Methods".

The percent saccharification of different cellulosic substrates are given in Table 24 and 25. (Fig. 12 and 13)


Cellobiose
S
Cellobiose

Fusion of cells, organelles or cell nuclei with different properties & of different origin, which results in the formation of new cell systems, represents another type of gene manipulation which can be used to produce cells with the desired properties. The electrically induced fusion & entrapment of membrane - impermeable substances & genes in cells provide a new tool for the production of a wide range of cells with manipulated functions which could be used for the

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(1) ← Van veen et al (1988)

Hille et al (1983 ——→ (5)

TABLE 24

ENZYMATIC HYDROLYSIS OF UNTREATED CELLULOSIC SUBSTRATES

Substrate	% Saccharification		
	24 h	48 h	72 h
Cellulose powder	3.4	10.6	11.6
Absorbent cotton	2.0	5.0	5.8
Filter paper (Whatman No.1)	3.8	6.4	7.1
Rice straw	6.3	7.4	8.0
Bagasse	2.8	3.5	4.4
Mesta wood	1.8	1.9	2.2
Avicel	3.7	4.7	8.9

% saccharification = Reducing sugar mg/ml × 1·8

$$= \frac{\text{reducing sugar}}{\text{amount of substrate}} \times \frac{162}{180} \times 100$$

Mandels et al. (1974)

Biotechn. Bioeng 16, 1471

TABLE 25

ENZYMATIC HYDROLYSIS OF ALKALI TREATED CELLULOSIC SUBSTRATES

Alkali treated substrate	% Saccharification			
	24 h	48 h	72 h	96 h
Cellulose powder	7.0	15.2	18.1	23.8
Cotton	12.0	15.9	20.0	27.9
Filter paper	13.8	16.3	20.0	25.3
Rice straw	11.3	18.3	18.8	19.5
Bagasse	10.8	11.3	13.8	12.5
Mesta wood	6.9	11.6	14.8	12.5

Alkali treated substrates gave markedly higher saccharification compared to untreated substrates as in the case of other cellulases. But the V. diplasia enzyme showed significant activity with untreated substrates which are generally difficult to hydrolyze with most enzymes, such as Avicel and cotton.

The end products of the saccharification of AT cellulose powder, AT cotton, AT straw and AT bagasse were determined by paper chromatography and the reducing sugars were found to be cellobiose and glucose. In the case of cotton and cellulose powder for the 24 h hydrolysis

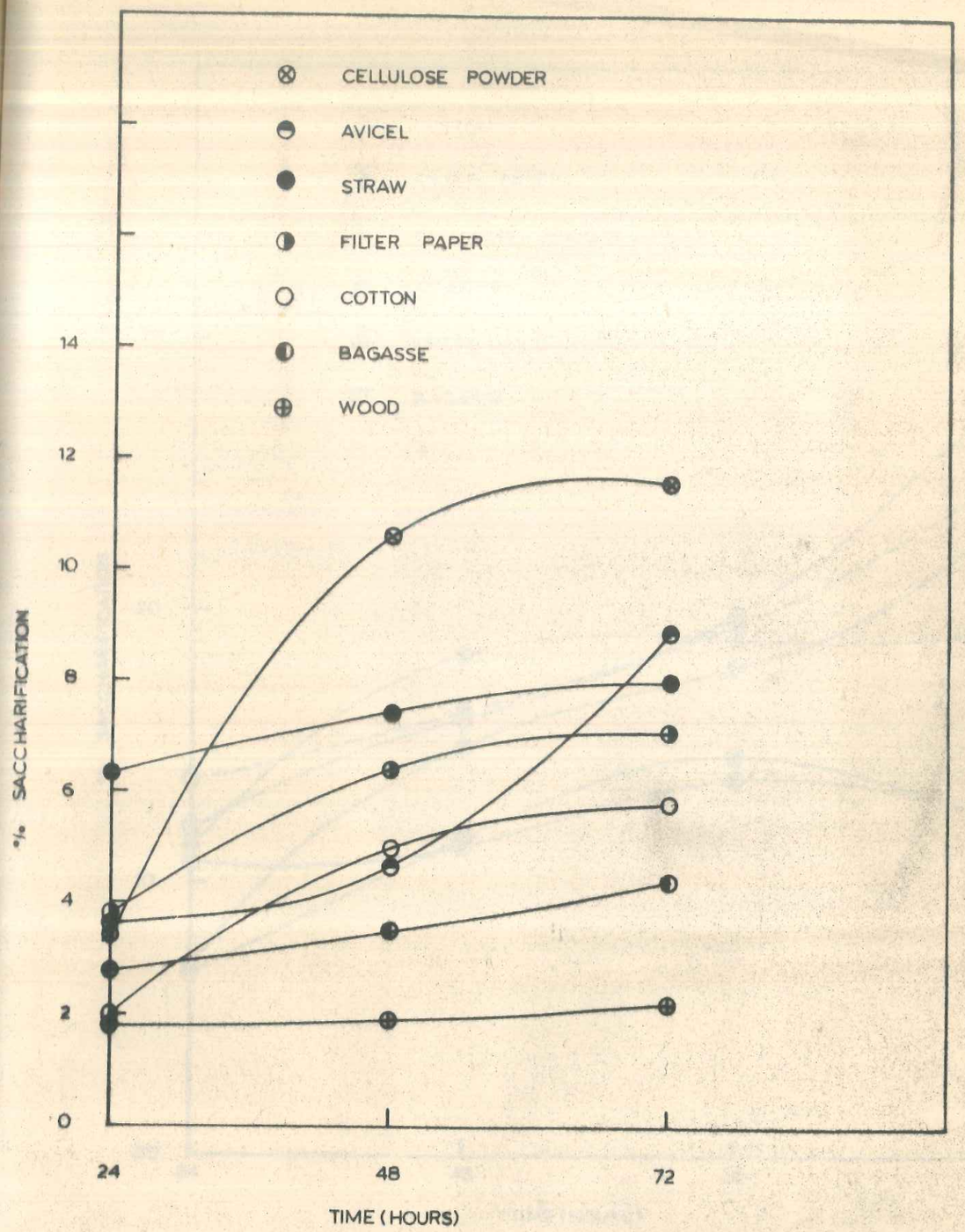


FIG. 12: ENZYMATIC HYDROLYSIS OF UNTREATED CELLULOSIC SUBSTRATES

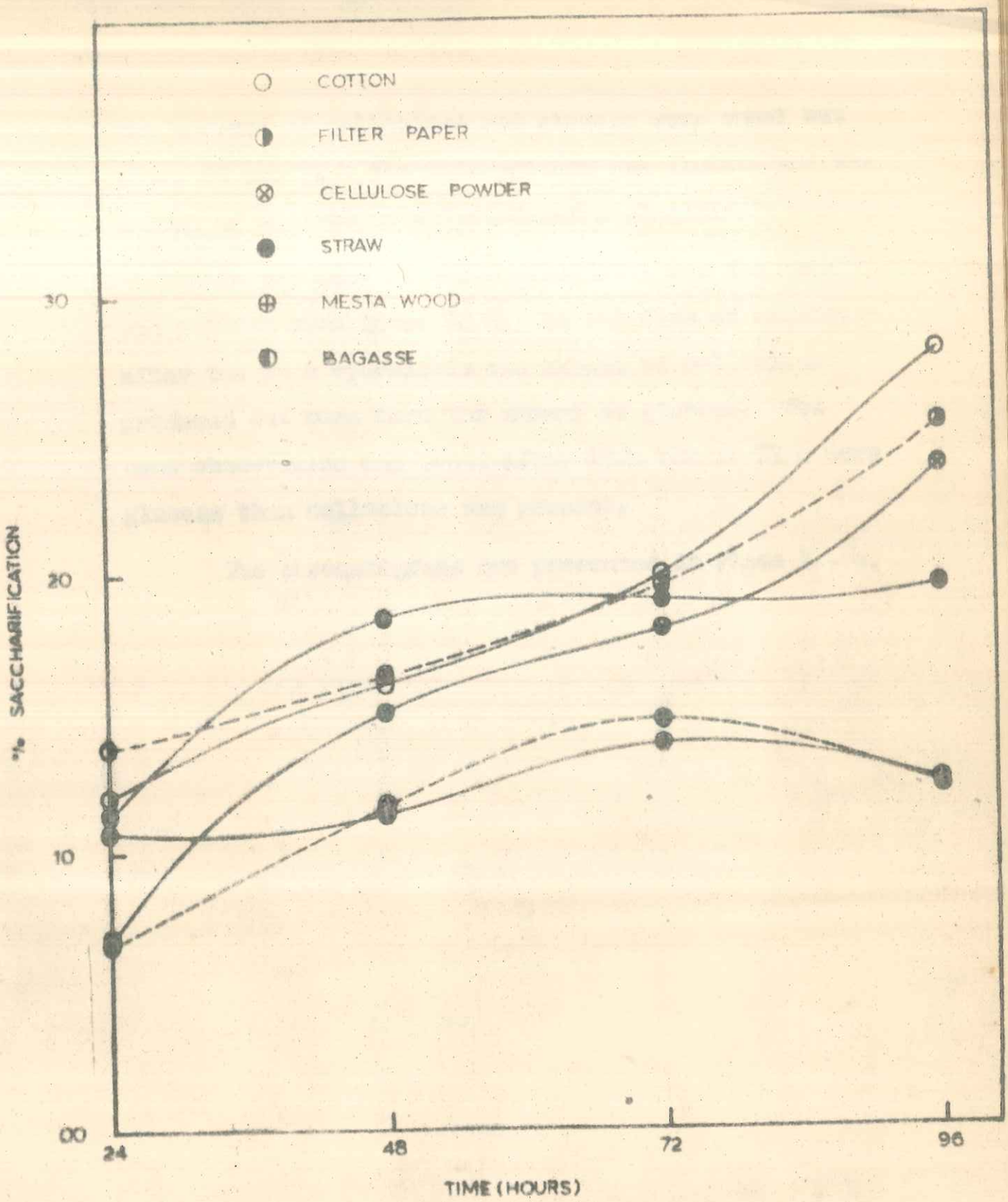


FIG. 13: ENZYMATIC HYDROLYSIS OF ALKALI TREATED CELLULOSIC SUBSTRATES

the amounts of cellobiose and glucose were equal but after 48 and 72 h the main product was glucose and the amount of cellobiose detected was negligible.

In the case of straw cellobiose was the main end product even after 72 h. In the case of bagasse after the 24 h hydrolysis the amount of cellobiose produced was more than the amount of glucose. The same observation was found after 48 h but at 72 h more glucose than cellobiose was present.

The chromatograms are presented in Plate 1 - 4.

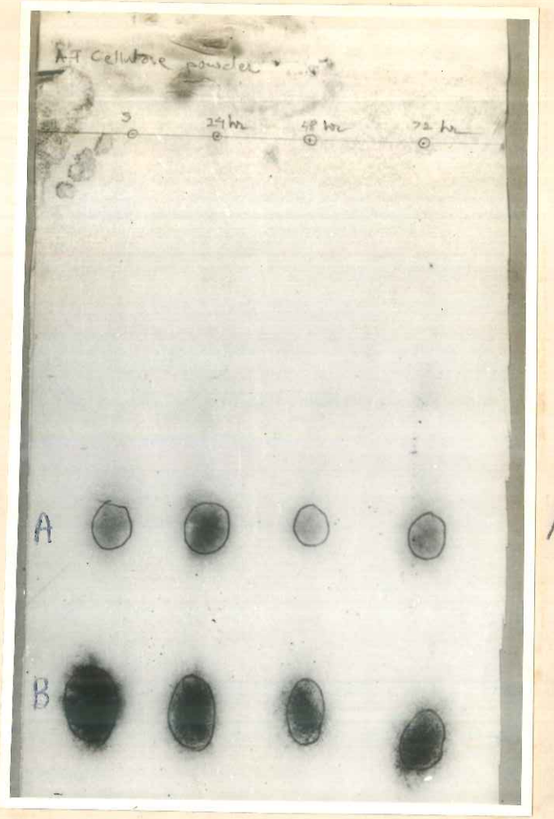
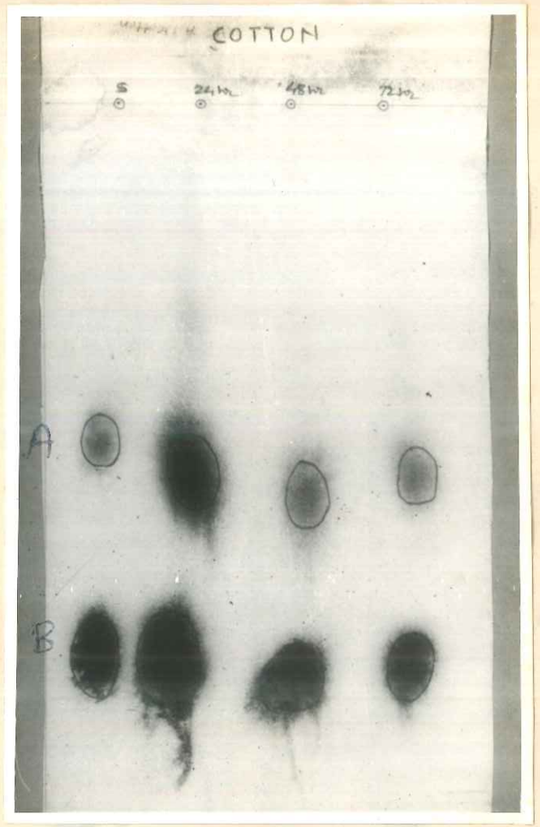


Plate 1 : End products from hydrolysis of Alkali treated cellulose powder.

A = Cellobiose

B = Glucose



A

B

Plate 2 : End products from hydrolysis of Alkali treated cotton.

A = Cellobiose

B = Glucose

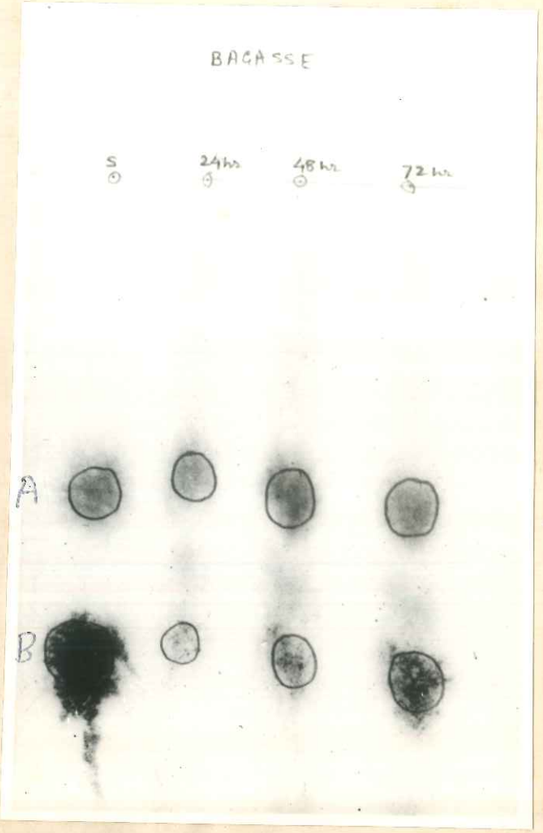


Plate 3 : End products from hydrolysis of Alkali treated bagasse.

A = Cellobiose

B = Glucose

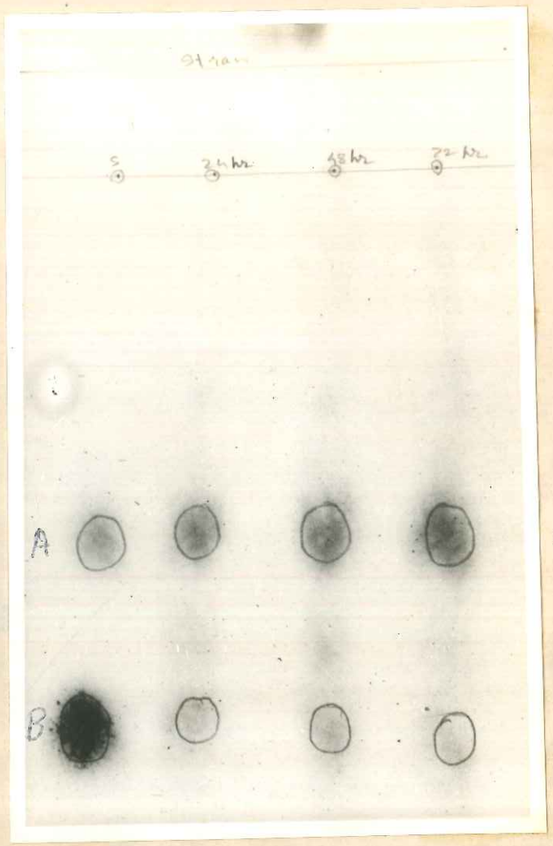


Plate 4 : End products from hydrolysis of Alkali treated straw.

A = Cellobiose

B = Glucose

CHAPTER IV

GENERAL DISCUSSION AND SUMMARY

General Discussion

Mushrooms have been used as a source of food from times immemorial, for their flavour. They are rich in proteins and vitamins. Commercially they have been cultivated on cellulose-rich agricultural wastes. In the case ofthe most extensively cultivated Agaricus campestris, the mold cannot metabolise cellulose effectively and the nutrients for the growth are provided by the breakdown of cellulose by a mixed microflora activated during composting. Species of Volvariella are extensively cultivated on paddy straw without any prior composting, indicating ability to degrade the carbohydrate components of the straw substrate. However, in uncontrolled non-sterile fermentation, the supporting role of associated microflora in the breakdown of cellulose cannot be ruled out. In an interesting study on the cultivation of Volvariella on cotton waste, Chang (1974) obtained good growth of the mushroom on cotton waste compost in which thermophilic actinomycetes are involved in composting and reported them to have a stimulatory influence on the growth of the mushroom.

Very few studies are available where evaluation of growth of Volvariella species has been carried out on pure cellulosic substrates in defined media under submerged culture conditions. Production of cellulolytic enzyme in submerged culture has also not been studied

or reported. Krishnamohan (1975) reported cellulase production by V. diplasia during the cultivation of paddy straw, but details of enzyme activity have not been published. Rangaswami (1956) reported poor utilization of cellulose by V. diplasia. Ghosh and Sengupta (1976), obtained very poor growth on carboxymethyl cellulose with V. volvacea and reported that lignin and cellulose are poorly utilized.

In the present study, when the mushrooms V. volvacea and V. diplasia were screened for determining the cellulose degrading ability in shake flasks by using pure ∝-cellulose as the substrate, it was observed that both the species were able to degrade the cellulose. There was evidence of extracellular cellulase activity. The activities, however, were low and only endoglucanase (carboxymethyl cellulase) activity was detectable. Evaluation of cellulose degradation and extracellular cellulase activity were carried out under defined conditions in submerged culture.

From the point of view of SCP production ammonium sulfate, di-ammonium hydrogen phosphate, ammonium nitrate and potassium nitrate showed comparable results and the net protein conversion based on cellulose utilized ranged from 8.5 - 12.5%. Ghosh and Sengupta (1976) observed maximal mycelial growth of V. volvacea in synthetic medium under submerged condition when

potassium nitrate was used as a nitrogen source in a starch containing medium. For enzyme production ammonium sulfate, di-ammonium hydrogen phosphate and ammonium nitrate were found to be the more suitable nitrogen sources. Potassium nitrate gave lower enzyme yield.

Among the various carbon sources tested for the growth of V. diplasia (Rangaswami, 1956) and V. volvacea (Ghosh and Sengupta, 1976), starch was reported to be utilized preferentially by the cultures. The formation of biomass and total protein by V. diplasia in the present study was also maximum with starch and glucose, and relatively less with maltose, arabinose, xylose and cellobiose. No cellulase activity was detected in any of the cultures grown on the different carbohydrates excepting cellulose indicating the induced nature of the enzyme. Similar observations have been made for cellulase induction in fungi, such as Trichoderma (Mandels, 1975).

In the presence of thiamine and biotin there was slight increase in growth and SCP production. Chandra and Purkayastha (1977) found increased biomass weight when V. volcacea was grown in thiamine supplemented media while no significant enhancement in enzyme production was observed by addition of vitamins. A slight inhibition in enzyme production as well as of

biomass protein was observed when folic acid was added to the medium.

In the present study, in shake flasks, levels higher than 0.5% of cellulose were found to be less favourable, for enzyme production. Similar observations were made by Sternberg (1976), who showed that in Trichoderma shake flask studies the activity was highest when 0.75% cellulose was used and was substantially less when 2.0% cellulose was used. However, in recent years high cellulase activity has been obtained in instrument controlled fermentors using cellulose levels upto 8% and hyper cellulolytic mutants of Trichoderma reesei (Sternberg and Dorval, 1979).

The extracellular enzyme produced by V. diplasia after a 10-fold concentration by ultra filtration showed high ratio of CMCase activity to filter paper activity (60:15). β-glucosidase (PNPGase) activity, however, was very low in the Volvariella culture filtrate.

Maximum endoglucanase (CMCase) activity was found at pH 4.8 and 50°C, which is similar to that observed in most fungal cellulases. The enzyme was about 50% active at pH 7.2 and only 20% active at pH 8.4.

Alkali treated substrates were saccharified to a greater extent compared to untreated substrates, as in the case of most cellulase preparations. An interesting feature of the V. diplasia enzyme was its ability

to attack crystalline cellulose such as cotton and Avicel resulting in significant saccharification. This quality of the enzyme makes the fungus an interesting material for further studies aimed at improving the cellulase yields.

Different types of microorganisms such as bacteria, yeast, actinomycetes and higher fungi have been used for SCP production from cellulosic wastes, by several workers (Callihan and Dunlap, 1969; Peppler, 1970; Crawford et al, 1973 and Peitersen, 1975). The white rot fungus Sporotrichum pulverulentum has been reported to produce about 30% crude biomass protein from paper mill wastes (Eriksson and Larsson, 1975).

The nutritive quality of SCP depends on the amino acid composition of the protein and the pattern of amino acids in several SCP preparations is reasonably good and comparable with high quality proteins such as those of egg and milk. The nutritive quality of cellulosic waste like straw is less, as rice straw contains about 4.5% of protein (Han, 1978). V. diplasia when grown on pure cellulose was found to produce about 12-15% crude biomass protein. Because Volvariella species are nutritionally accepted, a process developed for rapid bioconversion of cellulosic wastes to microbial protein employing Volvariella would be more

readily accepted from the nutritional and toxicological aspect. While at present Volvariella species are cultivated for their edible fruit bodies, it may become possible through further research to develop a rapid fermentation technology for mass production of Volvariella mycelium in submerged culture by growth on suitably pretreated cellulose-rich agricultural wastes. While there are not many reports published on the use of basidiomycetes grown on cellulosic wastes in submerged culture, the work of Torev (1973) in Bulgaria in which a process using white rot fungi for microbial conversion of cellulose to SCP is a worthwhile lead which could be followed up in many developing countries for supplementing their protein needs, especially for animal nutrition.

Microbiologically, selection of rapidly growing clones better adapted to cellulose utilization could be achieved in the case of V. diplasia through mutation. The preliminary results obtained in these basic studies of the cultures could be developed further in identifying the nutritional factors and fermentation conditions for developing a technology for SCP production possible based on Volvariella species.

Summary

The edible mushroom Volvariella diplasia was studied for its growth requirements, biomass production on cellulose and cellulase formation in submerged culture in nutritionally defined medium. Saccharification of cellulosic substrates by the enzyme andsome of its physical properties were studied.

Growth requirements

1) It was observed that with increasing concentration of glucose, growth of the organism was reduced and about 400-700 mg of glucose in 100 ml was utilized within 15 days.

2) Maximum growth as well as biomass protein was obtained with starch and glucose and slightly less with cellobiose, maltose, arabinose and xylose.

3) Among different nitrogen sources tested urea gave maximum dry weight of mycelium as well as biomass protein and di-ammonium hydrogen phosphate gave about 75% growth and biomass protein as compared to urea. Ammonium sulfate, potassium nitrate and ammonium nitrate were also utilized approximately to give about 50-60% of growth and mycelial protein obtained with urea. Poor growth was observed with sodium nitrate and ammonium chloride.

4) Maximum growth was observed where all the four trace

elements, Fe^{++}, Zn^{++}, Mn^{++} and Co^{++}, were present. Omission of one ofthe trace elements as well as total omission of all of them resulted in reduced biomass weight and protein formation.

5) Thiamine showed stimulation of growth, whereas riboflavin and folic acid appeared to have a slight inhibitary effect on mycelial yields.

SCP production

1) Maximum biomass was obtained with 0.5% cellulose in the medium. With higher levels growth as well as biomass protein were reduced.

2) Maximum biomass protein was obtained in the presence of di-ammonium hydrogen phosphate, potassium nitrate and ammonium nitrate. Ammonium chloride was found as the least effective nitrogen source.

3) Maximum protein was observed in the presence of the four trace elements, Fe^{++}, Mn^{++}, Zn^{++}, and Co^{++}. In the absence of any one or all the four trace elements, protein synthesis was decreased by about 35-50%.

4) In the presence of thiamine and biotin slight enhancement in biomass protein production was observed, while folic acid reduced protein formation.

5) Maximum cellulose utilization and protein formation were observed at pH 5.5 while at pH 3.0 and 9.0 very low protein yield was observed.

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5) Maximum cellulose utilization and protein formation were observed at pH 5.5 while at pH 3.0 and 9.0 very low protein yield was observed.

6) Cellulose utilization and biomass protein formation were maximum at 30°-37°C. At 45°C and 15°C there was very little substrate utilized.

7) The optimum period for cellulose utilization and biomass protein formation from cellulose was 15 days.

Enzyme production

1) V. diplasia (6.0 units/ml) gave more CMCase activity than V. volvacea (3.5 units/ml).

2) Enzyme production was found to decrease with increasing amounts of cellulose and at 0.5% cellulose level maximum enzyme was produced.

3) For enzyme production ammonium sulfate, di-ammonium hydrogen phosphate and ammonium nitrate were found to be more suitable, while with potassium nitrate the yield of enzyme was low.

4) No enzyme was produced in the presence of carbon sources other than cellulose.

5) Deletion of Fe^{++}, Zn^{++}, Mn^{++} or all of the trace elements resulted in about 40% reduction in enzyme production; deletion of Co^{++} gave only 20% reduction.

6) Vitamins B_1, B_2, B_6, Biotin and ascorbic acid had no effect on enzyme production. Addition of folic acid caused a 70% reduction in enzyme production.

7) Enzyme production was observed between pH 4.0 to 9.0 with a maximum at pH 5.5..

8) The optimum temperature for enzyme production was 28°C.

9) Enzyme production was increased progressively from the 6th day and maximum enzyme was produced between the 12th and 15th days.

Enzyme properties

1) Maximum CMCase activity was observed at pH 4.8 and 50°C.

2) Alkali treated substrates showed higher percentage of saccharification than the untreated substrates.

3) Alkali treated cotton, filter paper and cellulose were saccharified to the maximum extent.

4) Enzyme showed significant saccharification with crystalline cellulose such as Avicel and cotton.

5) The reducing sugars produced by enzymatic hydrolysis were found to be cellobiose and glucose.

CHAPTER V

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