UTILIZATION OF BAGASSE FOR THE
PRODUCTION OF SINGLE CELL PROTEIN

A THESIS
SUBMITTED TO THE KURUKSHETRA UNIVERSITY
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY

By
SATYENDER KUMAR GARG

DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(Indian Council of Agricultural Research)
KARNAL—132 001 (HARYANA)
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DEDICATED TO THE MEMORY OF MY LATE
FATHER SHRI KALYAN MAL GARG WHO WAS SNATCHED AWAY BY THE CUE OF DESTINY ON FEBRUARY 8, 1979
I certify that the work reported in this Thesis entitled
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was carried out by SHRI SATYENDER KUMAR GARG, under my guidance,
for the requirements of degree of DOCTOR OF PHILOSOPHY in Dairy
Microbiology, in the Faculty of Dairying, Animal Husbandry and
Agriculture, Kurukshetra University, Kurukshetra.

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CHAPTER I

INTRODUCTION
INTRODUCTION

A rapid rise in population and pace of industrialization have increased organic waste-output to a point where natural reclamation pathways are rendered inadequate. Hence, new methods of waste reclamation and reuse have to be attempted.

Cellulose is most abundant in agro-industrial wastes and since, it presents a serious pollution problem, the development of a process, which converts these cellulosic wastes into valuable products viz., food, feed and fuel, which are in short supply, will have immense economic and environmental importance. The annual production of cellulose in the form of municipal trash, agricultural residues, industrial sludges and forest residues is about 100 billion tonnes, which is approximately 140 pounds per day for each and every living human being on this planet.

Although cellulose is generally utilized by cattle as a source of carbohydrate, it can also be converted to protein by microorganisms and additional protein, which is in short supply can be made available for animal nutrition. Increasing needs for food and animal feed and the constant demand for finding new ways of waste utilization create strong incentive for the development of fermented products rich in carbohydrates, lipids, minerals and vitamins, in addition to proteins containing high amounts of essential amino acids.
Now-a-days, the primary concern of nutritionists has been protein rather than energy sources, due to shortage of protein in diet. Production of microbial proteins from cellulosic substrates is being given ever increasing importance. Such proteins are referred to as Single Cell Protein (SCP). The term single cell protein was coined at Massachusetts Institute of Technology by Professor J.L. Wilson in the year 1966, to represent the cells of algae, bacteria, yeasts and fungi grown for their protein contents. This terminology has certain psychological value, since any unpleasant connotations with terms such as bacterial, fungal or microbial proteins are avoided.

The biological degradation of cellulose has been of paramount importance in the activities of the living system. If there was no biological degradation of cellulosic substances, the earth would be covered with the masses of dead vegetation. Obviously strong cellulolytic activities do exist in nature. Such activities are mainly due to the cellulases produced by microorganisms. Even though many animals and plants are known to have cellulases, they do not play an important role in the natural degradation of cellulose. Hence, the researchers concentrated their attention on the microbial cellulases due to their ease of production and high cellulase activity. Among the cellulolytic microorganisms, fungi have been the subject of most intensive study, because of their higher activity and the large amount of biomass obtained.

India is one of the largest sugarcane growing country in the world and produces sugarcane bagasse, a by-product of sugar industry to the tune of 20 million tonnes annually. Most of this bagasse is
being burnt away as fuel by the producing units. However, there is
scope for alternative uses of bagasse such as production of cellulase
enzyme, furfural extraction and single cell protein production.

More than half of the dry weight of bagasse and most of other
agro-wastes consists of cellulose and hemicellulose and the rest
comprises of lignin, nitrogenous compounds and ash. Due to the
lignification and the crystalline nature of these cellulosic materials,
the cellulose becomes inaccessible for the cellulase enzyme activity.

Recently, cellulase enzyme has assumed greater importance due
to its potential use in modifying low grade roughages, production
of syrups and power alcohol, in addition to other applications like
single cell protein production.

Direct burning of cellulosic materials such as wood, straw and
sugarcane bagasse is one of the conventional methods used to generate
heat and power. However, if it is chosen to convert these annually
replenishable resources to other energy and protein-rich products
that can be stored, shipped and used anywhere, then it is highly
desirable to hydrolyse the cellulose to its primary glucose molecules.
It is estimated that, from one ton of waste paper, half a ton of
glucose can be produced. The hydrolysis of cellulose to glucose
and subsequent utilisation of glucose as a source of fuels, food,
chemicals, vitamins, single cell protein and a multitude of other
useful products, opens new vistas in the field of energy, food
and chemicals to augment and conserve current world energy and
protein sources. The glucose can also be used as a source of
feedstock to make solvents, plastics and other chemicals now made
from petroleum and can be fermented to produce fuel alcohol.

According to World Health Organisation estimates, approximately two billion people suffer from malnutrition in different regions of the world due to their substandard diet. The fact that agricultural land cannot be increased indefinitely and an increase in food and feed production has its maximum limits, which adds to the complexity of the problem. Consequently, a large population in the underdeveloped world have fallen prey to protein deficiency. In order to guard against such a situation, some alternative protein foods and feeds other than the ones obtained from land cultivation, must necessarily be harnessed. The increasing world demand for food and feed has spurred the search for non-conventional protein sources to supplement the available protein. Among the novel ideas proposed, the extraction of leaf protein and production of microbial protein, could be of immense help in solving problems of malnutrition. SCP produced by microbial fermentation from agro-waste substrates, is a high quality protein and can, therefore, be very safely used as the milk replacer for animal kids and in the concentrate mixture for grown-up animals, without any adverse effect on growth and nutrient utilization. The single cell protein can be as much utilized by pre-ruminants during their early stage of growth as, it can be by fully developed ruminants.

Due to the deficiency of protein in diet and the abundance and scope of sugarcane bagasse utilization, the present study has been attempted to produce single cell protein and cellulase enzymes through bioconversion of sugarcane bagasse with an efficient cellulolytic mold.
Hence, the following studies were conducted:

i) Isolation and characterization of cellulolytic molds and their screening for cellulolytic activity for single cell protein production,

ii) Production of single cell protein and cellulase enzyme by optimizing the cultural and nutritional conditions for the most efficient cellulolytic mold,

iii) Evaluation of the quality of product by *in vitro* rumen digestibility and its amino acid analysis and

iv) Characterization of partially purified mold cellulase enzyme.
CHAPTER II

**********

REVIEW OF LITERATURE

**********
2.1.0. BACKGROUND

The processes involving the natural ecosystem, recycle wastes through the food chains in the biological transformation of their various mineral components viz., carbon, nitrogen, and other elements into plant, animal and microbial biomass, so that very little recalcitrant accumulation occurs. Why should it be necessary then to introduce man-made upgrading processes to channel the nutrients in other directions and deprive the natural cycles of their full compliment of nutrients? The answer lies with man's ability to control and accelerate natural processes of food and feed production, by increasing their efficiency (Davis, 1974).

The life-supporting resources of the earth and its atmosphere are being depleted and polluted at an alarming rate, while the population increases ever more quickly. The use of the earth's ecosystem by man has resulted in the absolutely predictable problem of too much waste and too few resources. The historical role of industry has been one of processing raw materials into usable consumer goods. The inevitable fate of these goods has been to become wastes. Nature is then left with the task of reconverting these wastes into resources (Birch et al., 1976). Increasing population and industrialization have increased waste output to a level, where natural reclamation pathways cannot keep up. The biosynthetic pathways of nature are overloaded.
it is very essential to develop the new methods of waste reclamation and reuse (Gaden and Humphrey, 1977).

In parallel with the industrial waste problems, but occurring more recently, is the increased production of agricultural wastes, due to intensification of crop and animal production. The by-products from cereal production are causing a serious pollution problem. Cellulose occurs as the structural substance of most plants. It is thus present as the component in all agricultural products and arises as waste in such materials like bagasse, coffee grounds, straw, etc. The utilization of cellulose for the production of SCP, especially for use as cattle feed, is of considerable importance in view of the shortage of protein (Han et al., 1971; Tannenbaum, 1971). Man for centuries has used microorganisms as food and feed. In Germany, during the World Wars, new plants were set up for producing food and feed yeasts rich in protein and fat from molasses and other substrates (Kühberg, 1972; Sobkowski, 1976).

The main incentive for the widespread research on the utilization of microorganisms as food and fodder is provided by authoritative prognoses concerning world food supplies and population increase. The significance of dietary proteins, during infancy for resistance towards infections (Sorimach, 1964) and for physical growth and mental development (Finick, 1969), is now specifically recognized. National and international bodies have expressed their concern over the scarcity of high-quality proteins in certain regions and the global availability of proteins in the future. Apart from the non-utilitarian motives
for microbial cell production, there is a strong commercial interest based on the opinion that microbial products will be able to compete economically with the conventional high protein supplements used in livestock feeding and, at a later stage, with the traditional protein of human diet.

Production of single cell protein from different substrates and their nutritive evaluation as feed and food have received greater attention from the forties of the present century and have been reviewed (Almqvist, 1951; Christensen and Daly, 1951; Thakker, 1954; Baran, 1968; Metales and Tannenbaum, 1968; Bhattacharjee, 1970; Degley, 1971; Kirk, 1971; Klug and Markovetz, 1971; Cooney and Levine, 1972; Kihlberg, 1972; Davis, 1974; Tannenbaum and Wang, 1975; Birch et al., 1976; Gaden and Humphrey, 1977; Litchfield, 1977; Han, 1978; Scott, 1978; Ryu and Mandels, 1980; Kalra, 1981).

The development of upgrading methods for the recycling of agricultural wastes as a microbial animal feed component could help to alleviate the waste problems of high density stocking and cropping, so that the process developed is quick and economically viable.

2.2.0. FACTORS INFLUENCING SINGLE CELL PROTEIN PRODUCTION

Production of SCP (microbial biomass) is influenced by four important factors:

A. Correct choice of microorganisms,

B. Selection of substrate, which is cheaper and available easily on a large scale,

C. Pretreatment of substrate and

D. Cultural and nutritional aspects of fermentation,
2.2.1.0. Choice of Microorganisms:

Microbes as a result of their smallness in size, can double in their mass in as little as half an hour. Thus, the short generation time could be harnessed to high productivity under correct manipulation. There are three groups of microorganisms viz., algae, bacteria and fungi (yeast and mold), which can be used for biomass production. The suitable criteria for the selection of microorganisms in large scale biomass production are: (i) growth rate; (ii) yield; (iii) protein content of the biomass; (iv) efficiency of fermentation; (v) nutritive value; (vi) freedom from toxicity and (vii) acceptability as food and feed.

2.2.1.1. Algae:

Lagoon culture of algae, using either sewage or nitrogen or phosphorus-rich waste water as primary substrates, could produce higher dry weight yields of superior protein quality than could higher plants under similar climatic conditions.

Hindak and Tribil (1968) and Ionescu (1970) investigated the biomass production by different algae. It was observed that with urea and nitrate nitrogen sources, *Brasona harlowi*, *Hormidium flaccidum*, *Dichotrix* sp., and *Stichococcus* sp., gave biomass production and protein content in the range of 0.84-47.8 g/m²/day and 21.58-51.95 % respectively, using different levels of CO₂. Priestley (1976) reported that the protein content of *Scenedesmus obliquus*, *Chlorella pyrenoidosa*, *C. vulgaris*, *Chlorella rheinhardtii*, *Hormidium* sp., *Dichotrix* sp., and *Brasona gigas* was in the range of 36.4-60.0 %.
2.2.1.2. Bacteria

Han and Srinivasa (1969) isolated a cellulose degrading *Cellulomonas*. The organism decomposed various cellulosic materials with varying degree depending upon the nature of the cellulose. Dunlap et al. (1970) reported that *Cellulomonas* grown on purified wood pulp were golden-yellow, while those grown on bagasse were deep-yellow brown. The cellulase excreted into the menstrum by *Cellulomonas*, hydrolysed various cellulosic materials producing cellobiose as the final breakdown product. When sugarcane bagasse was treated with hot-alkali, the organism could decompose up to 90% of the initial substrate within 5 days (Han et al., 1971).

Thayer et al. (1974) evaluated the potential for the conversion of brush mesquite (*Prosopis* sp.) into either a complete animal feed or a protein supplement. Several species of bacteria, which could use extremely hard mesquite wood as their sole source of carbon, were isolated by enrichment culture techniques and evaluated for SCP production, cellulase activity etc. The results indicated that a high-energy, high protein, complete cattle feed or an excellent protein supplement could be produced from mesquite wood by bacterial fermentation.

Han (1975) reported that during fermentation of rice straw with *Cellulomonas* sp., and *Mesorhizobium fasciculare*, 75% of the rice straw substrate was digested and 18.6% of the utilized substrate was recovered as microbial protein. The microbial cell fraction was 37% protein and 5% crude fiber and the residue was 12% protein and 45% crude fiber.
Tewari and Chahal (1977 a,b) isolated some cellulolytic
Bacillus spp., and Pseudomonas spp., from soils and studied their
growth on wheat straw cellulose, modified filter paper, carboxymethyl
cellulose and cellulose powder.

Crawford et al. (1973) reported that the cellulolytic
thermophilic actinomycetes, Thermomonospora fusca grown on pulping
fines (a cellulosic waste of paper industry), yielded the biomass
containing about 30% microbial protein. The aerobic thermophiles
efficiently degraded cellulosic wastes for the production of SCP
(Bellamy, 1974; Humphrey et al., 1977; Rao and Bhala, 1978).

2.2.1.5. Molds:

Several investigations revealed that different cellulolytic
molds produced SCP from waste cellulose and fungal mycelia were
acceptable source of edible protein (Mary and James, 1969; Rade et al.,
1972; Solomon, 1973). The two possibilities of converting cellulose
to microbial protein are: (i) growing the microorganisms on cellulosic
substrates for microbial protein production and (ii) producing
extracellular cellulase enzyme, which can be used for the saccharification
of cellulosic substrates prior to the cultivation of microorganisms.

Among fungi, some molds are known to have high cellulolytic
activity. Hence, the use of molds in processes for the production
of SCP, sugars and liquid fuels from cellulosic wastes attracted
considerable attention. Unfortunately, it is not possible to measure
directly the fungal biomass on cellulosic materials, because the
mycelia can not be separated from the undigested solid cellulosic substrate matrix. The cell concentration is usually estimated by measuring the nitrogen or protein content of the fermentation solids. When molds are grown on cellulosic materials, their enzymes hydrolyze cellulose to oligosaccharides, such as cellotetrasaccharides, cellotriose, cellobiose and to glucose, that are utilized by the microorganisms for their growth. Most of the fungi possessed a wide range of enzyme systems and were capable of utilizing the more complex mixtures of organic compounds (Gray, 1972; Seal and Leggins, 1976). Molds could be easily harvested by simple harvesting methods, which were not applicable to bacteria or yeasts (Worgan, 1968). Although fungi were found to be slow growing and to yield biomass with lower protein content, several fungi grew rapidly and gave yields of protein equivalent to those of food yeast (Delaney and Worgan, 1970). Worgan (1972) reported 50% protein in some of the Fusarium spp. In general, from 100 g of carbohydrate substrates, the maximum practical yield of true protein that can be obtained by bacteria, yeast and mold is about 25 g.

Sennelli et al. (1975) studied three thermophilic cellulolytic molds viz., Chaetomium thermophilum var. asamophilum, Saccharothrix thermophile and Thermoascus aurantiacus for cellulose degradation and microbial protein production. It was found that addition of an organic carbon and nitrogen source to cellulose mineral medium, markedly increased the rate of cellulose degradation and S. thermophile gave the highest rate of substrate utilization. Oshel and Hawksworth (1976) isolated a thermotolerant, cellulose utilizing mold.


*Chasmania cellulolytica* from decomposing straw during cultivation of mushrooms.

Chahal and Gray (1969, 1969, 1970) reported that some cellulolytic molds viz., *Myrothecium verrucaria*, *Chasmania globosa*, *Phialophora australis* and *Trichoderma* sp., grew well on wood pulp and produced good amounts of protein rich in essential amino acids.

Updegraff (1971) used *Myrothecium* sp., a highly cellulolytic mold for SCP production by growing directly on waste paper substrates after ball milling. Eight molds viz., *Trichoderma koningii* 1M1 - 193763, *Amerinillus terreus* 1M1 -183764, 1M1 -183765, *A. candidus*, *Verticillium* sp., *Drechslera hawaiiensis*, *Curvularia* sp., and *Helmintosporium* sp., were isolated from the marine environments and tested for their ability to degrade cellulose as a sole source of carbon (D'Souza and Freitas, 1976).

Four cellulolytic molds viz., *Fusilospora* sp., *Myrothecium verrucaria*, *Amerinillus terreus* and *Geosaphe* sp., were grown on sugar beet pulp for SCP production. *M. verrucaria* yielded maximum crude protein (35.6%) in biomass (Gupta et al., 1977). Hoo-Young et al. (1978) studied the growth of *Chasmania cellulolytica* in slurry fermentation system using variously pretreated saw dusts from hard wood as substrates. Disaccharide growth pattern was observed, suggesting an initial utilization of soluble hemicellulose sugars followed by utilization of insoluble cellulose. Pauwet et al. (1978) fermented the treated saw dust solids by *Chasmania cellulolytica* for 48 hr and
obtained a product containing 12.5% crude protein.

Sood et al. (1974) screened eleven cellulolytic molds viz.,
*Aspergillus niger, A. nigerius luchmensis, Alternaria tenuis, Trichoderma viride, Fusarium solani red strain, white strain, E. rosea, Fusarium sp., P. oryzae, Penicillium sp.* and *Pythium irregulare* on wheat straw substrate for SCP production.

Peterson (1975) fermented barley straw with *Trichoderma viride* and obtained a fermented product containing 13-24% protein. Han and Anderson (1975) observed that, there was five fold increase in protein of rye straw, when it was fermented with *Trichoderma viride*.

Singh et al. (1976) studied the growth of six molds viz.,
*Cochliobolus versicolor, Chaetomium globosum, Myrothecium verrucaria, Penicillium funiculosum, Alternaria tenuis* and *A. microspora* on wheat straw and ground nut hull substrates. The maximum protein was produced by *E. globosum* (102.39 mg) and minimum by *P. verrucaria* (39.4 mg) per g wheat straw substrate. Wheat straw was utilized better as carbon source than ground nut hulls. Rao (1977) isolated a cellulolytic *Aspergillus* sp. from soil. In surface fermentation with the mold, the protein level of rice bran increased from 15% to 25% in six days.

Geethadevi et al. (1978) isolated different cellulolytic molds viz., *Aspergillus, Fusarium, Penicillium, Trichoderma* and *Trametes*. The molds were evaluated for cellulase and protein production using alkali-treated rice straw. The crude protein content of biomass from fermented alkali-treated rice straw substrate was in the range of 7.11-41.04%.
The ability of five strains of *Aspergillus niger*, one strain each of *Penicillium chrysogenum*, *Fusarium sp.*, and a basidiomycetous fungus to produce microbial protein on three different pretreated cellulosic substrates viz., rice straw, bagasse and ground nut shells was studied. *P. chrysogenum* was found to be the best cellulolytic organism on alkali-treated rice straw substrate producing maximum protein (Biteram et al., 1978).

Araujo and D'Mora (1978) reported on the SCP production by marine molds viz., *Aspergillus terreus* Thom pale strain, *A. terreus* Thom, *Verticillium sp.*, and *Penicillium sp.* with untreated and treated rice straw and rice hull substrates. The protein content of biomass in shake flask increased from 2.95 to 44%, while in stationary cultures, it increased to 32%. In case of rice hulls, the biomass had 30% crude protein in shake flask and 20% in stationary cultures. The maximum substrate utilization of 85 and 65% and biomass protein content of 44 and 29.56% was obtained on rice straw and hull substrates, respectively with *A. terreus* Thom pale.

Singh et al. (1978) reported the efficiency of cellulose utilization in corn stalk by nine cellulolytic molds viz., *Mucor circinelloides*, *Ghataéisia elahena*, *Alternaria alternata*, *Aspergillus niger* I, II, *A. terreus*, *Penicillium funiculosum*, *Aspergillus aquaeus* and *Piranius sp.* The highest biomass crude protein content (16.99%) with *C. elahena*, and the lowest biomass crude protein content (7.71%) with *I. funiculsum* were obtained. There was nine fold increase in biomass protein content as compared to control, when *C. elahena* was grown on corn stalk substrate.
Chahal et al. (1977) reported that the percentage of protein produced by *Trichoderma longibrachiatum* on sugarcane bagasse substrate was found to be less than the protein produced by *Chaetomium cellulolyticum* on treated wheat straw and saw dust substrates. Joshi and Parhad (1978) fermented sugarcane bagasse with *Trichoderma sp.* for microbial protein production. Though the system was not sterilized and closed, the mold culture predominated during the experimental period. Sharma et al. (1978) studied the effect of various growth conditions by fermenting sugarcane bagasse substrate with *Chaetomium globosum* on the production of SCP. The mold increased the biomass protein content to a range of 17.5-23.75% by various treatments.

Sidhu (1978) studied the biodegradation of sugarcane bagasse by *Trichoderma longibrachiatum* and observed that it was comparable with *Chaetomium cellulolyticum* (ACC-32519) on the basis of production of SCP and cellulase enzyme with carboxymethyl cellulose substrate. Sidhu and Sardana (1979, 1980) tested the cellulolytic activity and SCP production by *Aspergillus fumigatus, A. niger, A. diploid, A. terreus, Penicillium sp.*, *Trichoderma longibrachiatum* and *Aspergillus* on sugarcane bagasse substrate. They fermented sugarcane bagasse cellulose with *Trichoderma longibrachiatum* and deduced that holocellulose was the best substrate for SCP production.
Chahal and Fang (1978) found *Chaetomium cellulolyticum* to be a fast growing organism at 1.0% cellulose concentration with a protein synthesis rate of 0.09 hr⁻¹. The rate of protein synthesis was increased more than three folds (0.3 hr⁻¹), when 1.0% additional substrate was added immediately after the complete conversion of the first batch of substrate. The growth behaviour of *C. cellulolyticum* on cellulose indicated that it might be the most suitable organism for cyclic batch or continuous fermentation of cellulose for microbial protein production. Chahal and Dhalwal (1973) studied SCP production using cellulosic substrates as carbon sources with three isolates of *Piriformis solani*, one each of *S. bataticola*, *Trichoderma lixorum* and *Chaetomium globosum*.

Rogers et al. (1972) and Petersson (1975 b) obtained biomass protein ranging from 13 to 21.26% by using various cultures including *Trichoderma viride* and different cellulosic substrates. Cabrera et al. (1974) used the agro-wastes for the production of fungal protein by growing *Aspergillus oryzae*, which could be used as animal feed supplement. Han et al. (1976) reported on the scope of using *Aureobasidium pullulans* for SCP production by growing on cellulosic agro-wastes. Dakhur et al. (1979) screened fifty isolates of molds for SCP production and observed that *Aspergillus* spp., gave low yield of mycelial matter (5.01 to 7.94 g/litre) with comparatively low protein content (9.26-14.79%). However, two isolates of * Fusarium* spp., (one parasitic and other asympothetic) gave about 16.5 g/litre dry mycelium containing about 35% protein. *Sclerotium rolfsii* yielded 12 g dry mycelium/litre with 8.8% protein.
Deshpande et al. (1978) screened several high yielding edible mushrooms for growth on cellulosic materials, as a source of low cost protein for use as cattle feed. Eriksson and Larsson (1975) cultivated a white rot fungus, i.e., *Sporotrichum pulverulentum* on different cellulosic and lignocellulosic waste fibers from the sulphite mill in continuous studies for the conversion of waste into protein.

2.2.1.4. Mixed Cultures

The rate of cellulose degradation in pure culture studies is usually extremely slow compared to that found in nature. This is partly due to the fact that, a single organism is used in the laboratory, while the natural phenomenon is a result of the action of mixed cultures. The rate of degradation of cellulose and the production of cell crops and other useful by-products are all limited by the organisms' innate physiological capacity. This limitation may be overcome by employing two or more organisms in the fermentation process by their synergistic or symbiotic associations.

By establishing the symbiosis between *Cellulomonas* sp., and other cellobiose utilising organisms, Han et al. (1971) made the following observations: (i) elimination of yeast extract, which was not only expensive as a nutrient but also increased the chance of contamination; (ii) no requirement of heat sterilization, since a very limited number of organisms grew in cellulose-mineral media; (iii) maximum bacterial growth by removing the accumulated cellobiose or other growth limiting by-products; (iv) improvement in the quality of protein by modifying the amino acid composition and (v) recovery of useful by-products other than the microbial protein from
cellulose fermentation.

Han et al. (1971) isolated a cellobiose utilizing bacterium, A. faecalis and grew it symbiotically with the Cellulomonas on cellulose substrate. The growth studies of the mixed cultures showed almost five-fold increase in cell density and growth rate, compared to that of Cellulomonas alone. The symbiotic fermentation was scaled up in a pilot plant and similar results were obtained. In an effort to establish symbiotic fermentation between other organisms, a dozen different cellobiose metabolizing yeasts were grown with Cellulomonas. Most of the yeasts grew better with the Cellulomonas than by themselves. However, none showed as good a growth as the mixed cultures of Cellulomonas and A. faecalis (Yu et al., 1976).

Culture combinations of S. cellulovorans and Candida utilis (Hy and Eriksson, 1975) and T. viride and Candida utilis (Eriksson, 1975 a) grew better on cellulosic wastes, as the symbiotic partners for the production of SCP. The first organism being cellulytic and second non-cellulolytic, the former degraded the cellulose and the latter grew at the expense of degradation product and hence, increased cell mass. Peitersson (1975 b) observed an overall increase in protein and cellulase enzyme production, when barley straw was fermented with mixed cultures of T. viride and Saccharomyces cerevisiae or Candida utilis.

2.2.2.0. Selection of Substrates;

The various parameters which are considered important for substrate choice are: (1) abundant availability of raw materials with
respect to quantity and distribution in time; (ii) price; (iii) freedom from undue toxicity; (iv) carbohydrate content; (v) supplementary nutrient requirement and (vi) advantage of utilization of the substrate by selected microorganisms for biomass production.

Besides, other economic factors are also important for a number of waste processing proposals, with a view towards generating an unified approach to the problem (Davis, 1974).

The concept of producing food from waste has become of great interest within the last few years as a result of increasingly frequent food shortages and price rises. Direct human food production by fermentation of waste materials is most unlikely at present due to the problems of meeting safety requirements. However, feed production is more likely and the production should focus on schemes for fermentation of wastes to animal feed ingredients (Birch et al., 1976).

With the exception of algae, all the organisms, which have been used or proposed for the manufacture of microbial protein, require complex carbon sources. Until the introduction of hydrocarbons, a decade ago, virtually all commercial production of SCP were based on carbohydrates. Now, in addition to these glucose-yielding materials, substrates in use or under consideration include alkanes ranging from methane to middle-distillate materials, lower alcohols (from methanol to isopropanol) and various organic acids (Caden and Humphrey, 1977).

2.2.2.1.0. The Carbohydrates:

Carbohydrate substrates are grouped into two major classes: saccharides and polysaccharides.
2.2.2.1.1.6. **Saccharide Substrates:**

The saccharides include the simpler five- and six-carbon sugars, which are assimilable by many microorganisms. Most organisms of interest for microbial protein production utilize a group of disaccharides, such as sucrose, maltose, and lactose, because they possess the enzymatic components necessary to first hydrolyze them to their monosaccharide constituents. **Examples of raw materials used, containing saccharide substrates are molasses, whey and sulphite waste liquor.**

2.2.2.1.1.7. **Molasses:**

Molasses from beet, cane, citrus and corn is the most abundant and readily available saccharide substrate for microbial protein production (Epinoza et al., 1977). Cane and beet molasses require pretreatment before they can be used for microbial protein production. Simple chemical treatments are used to reduce the mineral content and to remove excess suspended organic matter. Beet molasses should also be heated and aerated to remove 30%. In practice, a dry cell yield of 25% based on weight of molasses is obtained. Since the microbial cells are approximately half protein, about 7-8 kg of molasses will be required per kg protein production (Davis, 1974).

2.2.2.1.1.8. **Whey:**

Whey obtained from cheese-factories can be a potential raw material for microbial protein production. It contains about 4 to 5% lactose. Pilot studies with yeasts and molds indicated
that a yield of about 20% (kg dry cells/kg lactose) could be realized as a relatively high protein (60%) product. The steady increase in dairy product consumption plus growing restrictions on direct disposal of this high BOD material to the environment have made feasible proposition of utilizing the whey as a SCP substrate. However, the commercial cheese production has been limited to the most highly developed economies, where many protein sources compete. Furthermore, recent developments in separation techniques have made it possible to fractionate whey directly into a series of relatively pure and saleable products (Bernstein et al., 1977). So, the availability of whey as substrate for SCP is limited due to the above mentioned utilities.

2.2.2.1.2.1. Polysaccharides:

The polysaccharide substrates for microbial protein production are mostly agricultural wastes or by-products. These polysaccharides are abundant, theoretically inexhaustible and cheap. Their utilization for SCP production could partly relieve disposal problems (Davis, 1974).

Gregory et al. (1976) developed a cheap and low technology process for the conversion of cassava, an important tropical root crop, into microbial protein for use as an animal feed. The prototype culture for this study was a strain of Aspergillus niger L-21. It was proved to be possible not only to produce fungal biomass from cassava extract, but also from fermentation medium containing whole cassava mash.
Cellulosic raw materials, on the other hand, are reported to be available in abundance and cheap at the point of origin. Barley straw (Peitersson, 1975 b), wheat straw (Wood et al., 1974), rice straw and hulls (Rao, 1977; Aranjo and D'Souza, 1978), corn stalk (Singh et al., 1978), sugarcane bagasse (Sidhu, 1978; Sidhu and Sandhu, 1979), mesquite wood and saw dust (Chakal and Gray, 1968, 1969, 1970; Hoc-Young et al., 1978) have been used as microbial substrates. About 90% of the cellulosic wastes are derived from agricultural sources and rest from urban and industrial wastes. Most of the sugarcane bagasse is being burnt away by the producing units as a cheap fuel. The heat value per kg of bagasse is much lower than that of oil or coal, such a value implied paying a value of nothing more than 10 paisa per kg. Hence, alternative uses of bagasse such as production of SCP, attracted the attention of researchers to reduce its fuel value (Agarwal, 1974).

Considerable attention is directed towards the development of animal feed from bagasse and other agricultural waste materials. Even though bagasse contains enough cellulose to make it as an excellent source of energy for ruminants, it is a poor quality feed in its natural state. The main shortcomings of bagasse as animal feed are its (i) low digestibility; (ii) low protein content; (iii) poor palatability and (iv) bulkiness. The amount of crude protein in sugarcane bagasse is approximately 2.0 to 2.5%. Any crop residue with less than 8.0% crude protein is considered inadequate for all classes of cattle feed. Any agro-waste without supplementation can not sustain the nitrogen balance in an animal. The digestion coefficient increased as
the proportion of nitrogen concentrate was increased (Clawson et al., 1970).

2.2.2.2. Hydrocarbons

Hydrocarbons are utilized as energy sources by some microorganisms. A wide variety of yeast and bacteria alone or in mixed culture, grow rapidly on hydrocarbons. Their general preference is for straight chain types (Alkanes) in the range of \( C_{10} - C_{20} \). Liquid hydrocarbon of smaller chain lengths appeared to be toxic, while larger ones were so insoluble as to be only slowly utilized (Withfield, 1977).

Hydrocarbons are available in large quantities at one location and are independent of agricultural and climatic factors. The recovery of SCP from hydrocarbon substrates was higher, in general, than those for carbohydrate processes (Davis, 1974; Birch et al., 1976).

The production of SCP from cellulosic substrates was more economical than the production of protein from hydrocarbons or molasses, which were either highly priced or available only in limited quantities or required for other purposes, such as alcohol production and might leave toxic residues in the protein (Sista and Brivestava, 1978).

Commoner (1976) reported that the yeast grown on crude oil contained a large amount of original paraffin of the class \( C_{18} - C_{32} \), which were absorbed into the tissues of the feeding animals. Yeasts grown on hydrocarbons also contained carcinogens absorbed from the
crude oil, which were ingested by the feeding animal. He concluded that multigeneration feeding trials would be necessary for evaluating the nutritional value of SCP obtained from hydrocarbons, even though, it would increase production cost considerably.

2.2.3. **Pre-Treatment of Substrate**

The growth of microorganisms on native cellulose was sparse but on denatured or pretreated cellulose, growth was abundant (Johpande, 1970; Pan et al., 1980). Therefore, proper pretreatment of substrate before microbial fermentation would add to the success of the SCP production from cellulosic substrates. Although various forms of pretreatments of cellulosic materials were proposed, their effectiveness varied depending on the substrate (Han et al., 1971).

The cell wall of bagasse, straw and other agro-waste comprised mainly of lignin, cellulose and hemicellulose. The cellulose and hemicellulose contents were easily attacked by cellulolytic microorganisms and decomposed (Jolain et al., 1965). However, the lignocellulose complex, present in these materials are difficult to digest (Feist et al., 1970; Kirk, 1971; Sidal and Sandin, 1980). Lignin acted as a physical barrier and allowed the microbial breakdown of these compounds due to its physical and chemical association with the cell wall polysaccharides. Digestibility of lignocellulosic material was inversely correlated to the amount of lignin present in the substrates (Baker, 1973; Cross et al., 1974; Han et al., 1975).

The straw could also be treated by the simultaneous application of cellulase and lignase. Lignin decomposing enzymes (polyphenol oxidase)
have been reported to be present in fungi belonging to basidioscetes. These microorganisms have been frequently used to improve the digestibility of various lignocellulosic materials (Higuchi, 1971; Eriksson and Larsson, 1975).

The chemical and/or physical pretreatment of cellulosic agro-waste was necessary in order to facilitate the degradation of cellulose by microorganisms (Han et al., 1971; Han and Anderson, 1974; Kelsay and Shahzad, 1980). The cellulose fiber was delignified, depolymerized and swollen by alkali treatment, thus making the substrate more susceptible to enzymatic hydrolysis.

The effect of chemical, physical and enzymatic treatments of rice straw, rye grass straw and sugarcane bagasse on the microbial digestibility of cellulose was investigated (Han and Callahan, 1974; Han and Anderson, 1975; Yu et al., 1976; Grant et al., 1978).

The commercialization of the process of converting plant biomass and municipal wastes to glucose by chemical or enzymatic hydrolysis was hampered by the crystallinity of the cellulose microfibrils, that were aggregated and embedded within the lignified cell wall matrix (Brandt, 1976; Cowling and Kirk, 1976 and Mandels, 1976). The separation of the elementary cellulosic microfibrils could be achieved by a variety of chemical and mechanical pretreatments such as steaming (Hoese et al., 1977), delignification (Millett et al., 1976) and partial swelling by sodium hydroxide or ammonia (Mandels et al., 1974) and solvent extraction (Ladiesh et al., 1978). The crystalline cellulose was difficult to penetrate by solvents, enzymes or chemical
agents, whereas, the amorphous cellulose was easily penetrated.
There was a linear relationship between crystallinity index and the
digestibility of cellulosic substrates (Baker et al., 1959; Segal et al.,
1959; Han, 1979).

2.2.4. Cultural and Nutritional Aspects of Fermentation:

The fermentation of cellulosic substrates by microorganisms is
influenced by various cultural factors viz., hydrogen ion concentration,
temperature, aeration, agitation and size of inoculum. The optimum pH
and temperature for fungal cellulases and crude protein production
varies from species to species. In most of the cases, the optimum pH
and temperature range from 3.0 to 6.0 and 25 to 30°C, respectively.
Chahal and Dhalwal (1973) reported maximum crude protein production
at pH 4.0, when Trichoderma lignorum was grown on wood pulp substrate.
Dhillon and Chahal (1978) reported that pH 4.5 was most suitable for
Fusarium sp., Mucor hirae sp., Cochliobolus sp., and Rhizoctonia sp.,
pH 5.0 for Aspergillus terreus, A. niger and Penicillium sp. and pH 4.0
for Geotrichum sp. Maximum cellulase production by molds was reported
at a pH range of 3.5 – 6.0 (Jin, 1951; Spalding, 1963; Ueal, 1968;
Kawi, 1980).

Romanelli et al. (1975) investigated the optimum temperature
for the growth of Thermus aurantiacus. Sporotrichum thermophile
and Chaetomium thermophile var. thermophile grown on cellulosic substrates.
T. aurantiacus had the highest growth temperature range (46–51°C),
whereas, S. thermophile had the broadest range over which good growth
occurred (36–45°C). Optimum temperatures for the three organisms viz.,
T. aurantiacus, S. thermophile and C. thermophile were 40, 40 and 47°C,
respectively. Chehal and Hawksworth (1976) reported 37°C as an optimum temperature for the growth of Chaetomium cellulolyticum and 40°C was observed to be inhibitory.

Andreotti et al. (1977) studied the production of SCP and cellulase by Trichoderma viride 34-9414 in a laboratory fermentor. The maximum yield of cellulase enzyme on 1.7% cellulose substrate was obtained at 27°C using 2.7% inoculum of 5 days old culture. The maximum biomass recovery was obtained in two days and represented a 54% yield of consumed substrate. The cellulose was consumed in 3 days, but the maximum cellulase and extracellular protein were not achieved until 7 days.

Sitaran et al. (1978) studied the effect of physical conditions like pH and substrate concentration on protein production by Aspergillus niger, Penicillium chrysogenum, Pestalotia sp. and a Basidiomycete. Maximum protein was not achieved until 7 days. Philon et al. (1981) reported 37°C as an optimum temperature for the growth of Chaetomium cellulolyticum for SCP production. The cultures were incubated for 5 days.

The conversion of cellulose to SCP was more efficient under aerobic than under anaerobic conditions (Usami, 1968; Lavi, 1970). Sharma et al. (1978) reported that, out of 5, 10, 15 and 20% (v/v) of the inoculum used for SCP production, maximum protein was obtained with 20% inoculum after 72 hr.

A large number of inorganic nitrogen (ammonium and non-ammonium) and organic nitrogen sources are utilized by the microbes
for their growth. Organic nitrogen sources are preferentially utilized over inorganic nitrogen sources. The choice of nitrogen source utilization varies from species to species.

Hutton *et al.* (1965) reported that cattle urine could be effectively utilized as a nitrogen source by the microorganisms, because of higher urea content and other constituents present in it.

Chahal and Gray (1969) reported that urea was the best nitrogen source for protein production by *Aurantiosporium ventricariae* on wood pulp substrate, while all other ammonium or nitrate sources were poor. The inhibitory effect of high urea concentration might be attributed to the increase in pH during accumulation of high concentration of ammonia released with the degradation of urea by the enzyme urease. The pH also steadily increased with increase in nitrogen concentration. The inhibitory effects of ammonium salts on fungi were reported (Pratt, 1924; Neal *et al.*, 1932; Neal and Emerson, 1936; Keit and Palmier, 1937; Sakense and Bangawa, 1943; Macblis, 1953; Butt *et al.*, 1961).

Chahal and Gray (1970) reported similar trend with different nitrogen concentration in case of *Phomopsis solani*, *Chaetomium globosum*, *Trichoderma sp.*, and *Aurantiosporium ventricariae*. They reported that *P. solani* could withstand a high concentration of nitrogen in the form of urea, compared to other test organisms. The variation in the tolerance to high doses of urea nitrogen by different isolates of *P. solani* might be due to strain variation (Chahal and Manshi, 1970; Chahal and Shalival, 1973). When the effect of various nitrogen sources was studied on SCP production using *Phomopsis solani* and *Trichoderma liemorum* on wood pulp substrate, the maximum utilization of substrate was obtained with urea nitrogen followed by potassium nitrate.
On the other hand, production of CMCase was maximum with KNO$_3$ followed by NH$_4$Cl and urea was the poorest in CMCase production. The CMCase production was reported to be not related to utilization of substrate and protein production (Chahal and Dhalwal, 1975). Chahal and Haskeworth (1976) reported that among the various nitrogen sources investigated, KNO$_3$ yielded the maximum growth rate of Chactonium cellulolyticum.

Rhodes and Fletcher (1977) reported that cornsteep liquor, a by-product of starch industry, could be used as an excellent nitrogen supplement for the growth of molds for SCP production. Besides nitrogen, the cornsteep liquor also contained balanced source of carbon, sulphur, mineral salts, amino acids and growth factors, which might certainly accelerate cellulose utilization and protein production by fungi.

Sharma et al. (1978) reported that out of different nitrogen sources tried, potassium nitrate supported highest total protein production as well as cellulolytic activity by Chactonium cellulolyticum. Nitrate nitrogen was also reported to be an excellent source for the growth of molds (Lilly and Barnett, 1951; Hasegawa et al., 1954; Zeltin, 1970; Dhillon and Chahal, 1978; Dhillon et al., 1981).

2.3.0. CELLULASE ENZYME PRODUCTION AND ACTION

The biopolymer cellulose has been considered as an inexhaustible and easily available energy source. Because of the current concern over energy and food shortages, much effort has been made for the recovery and reuse of waste cellulose. One of the processes for
cellulosic waste reuse is its hydrolysis, which converts cellulosic materials into glucose. The utilization of cellulose depends upon its conversion to glucose, which might be regarded as the basic substrate for various products like SCP as food, feed, ethanol, butanol, as well as other functional products.

Acid hydrolysis of cellulose was attempted, but the high costs as well as environmental problems made this process uneconomical. Therefore, much effort had been undertaken to make cellulose economically hydrolysable under mild conditions (Reese et al., 1972; Ladisch, 1978). Increasing knowledge of enzymes and their mode of action as well as recent progress in their industrial applications, greatly expanded the prospects for enzymatic processes. Wilks and Mitra (1975) developed an enzymatic process for the production of sugar from waste cellulose. In spite of the findings of new and active enzyme preparations, the enzymatic saccharification of cellulose so far could not compete with the conversion of starch to glucose by microbial enzymes.

Although the enzymatic saccharification of cellulose has been studied, further understanding of the enzymatic hydrolysis is required for the purpose of developing a practical process of cellulose utilization. One of the least known aspects of this mechanism is the influence of the major structural features of cellulosic materials on the rate of enzymatic hydrolysis. Native cellulose is water insoluble because of complex structure. Thus, its susceptibility to hydrolytic enzyme attack depends significantly on its structural features.
The major structural features of cellulose material that determine its susceptibility to enzymatic degradation include (i) the degree of water swelling; (ii) the crystallinity; (iii) the molecular arrangement; (iv) the content of associated material such as lignin and (v) the capillary structure of cellulose fibers.

2.3.1. Structure of Cellulose:

Cellulose is a large polymer molecule made up of D-glucose elements joined by \( \beta-1, 4 \)-glucosidic bonds and its structural formula is presented in Fig.1 (Markram, 1967). The extent of polymerisation varies from source to source. The long polymer molecule is variously folded, bundled and stabilised by hydrogen bonding (Sanby, 1969). The degree of order created within the bundle determines whether it is crystalline or amorphous cellulose. Cotton comprises of almost pure cellulose and is, therefore, of a relatively crystalline form termed \( \alpha \)-cellulose. Wood on the other hand, comprises of a mixture of \( \beta \)-cellulose and \( \alpha \)-cellulose. In the natural state, the cellulose fibrils are associated with other materials such as lignin and hemicellulose in a complex heterogeneous structure. Acid treatment hydrolysates the cellulose and liberates the cellobiose, cellotriose and cellotetraose constituents and finally produces glucose. The enzymatic hydrolysis of cellulose yields glucose with little cellobiose (Birch et al., 1976).

2.3.2.0. Cellulases:

Cellulase, a multicomponent enzyme system, degrades the cellulose in a stepwise manner into its ultimate component monosaccharide unit,
FIG. 1. MOLECULAR STRUCTURE OF CELLULOSE POLYMER
glucose. The process of degradation of cellulose is, however, interfered by crystallinity and lignification of substrate. The enzyme is considered to be under catabolite repression control. In recent years, the enzyme assumed greater importance due to its potential use in modifying low grade roughages, production of syrups, various chemicals and other applications, from cellulosic wastes.

2.3.2.1. Mechanism of Cellulase Enzyme Action

A simple schematic diagram of the possible mechanism of enzymatic cleavage is shown in Fig. 2 (Birch et al., 1976). During the final stage of hydrolysis, β-1-4-glucosidas or celllobiase acts on celllobiase to produce two glucose molecules. The other group of enzymes exp-β-1, 4-glucanase, hydrolyses the reactive cellulose into celllobiase and glucose at the loose chain ends, that exists in its structure. Reduction of the polymer length is carried out by endo-β-1, 4-glucanase, which hydrolyses the bonds in the middle of the polymer chain. The hypothetical step between reactive and crystalline cellulose has been partly elucidated. Ekman (1975) proposed that the opening-up of the crystalline structure was assisted by an oxidative step, which introduced carboxylic acid groups into its structure.

Reese et al. (1950) broadly classified the cellulase complex into C₁, Cx and β-1, 4-glucosidas. Existence of these subdivisions was based on the fact that many enzyme preparations were found to be capable of hydrolyzing simple cellulosic materials such as carboxymethyl cellulose (CMC), but only a few cellulase enzyme preparations had extra components (C₁), which attacked the native crystalline materials.
FIG. 2. ENZYMATIC DEGRADATION OF CRYSTALLINE CELLULOSE
Microorganisms possessing component C₁ were said to be the true and completely cellulolytic, whereas, the other organisms that did not possess C₁ were pseudocellulolytic (Wood, 1969).

2.3.2.2.0. Occurrence of Cellulases:

Cellulases enzymes have been reported to be present in animals, plants as well as in microorganisms.

2.3.2.2.1. Animal Origin:

Cellulases have been reported to be produced by molluscs, echinoderms, protozoans, nematodes and arthropods (Gascoigne and Gascoigne, 1960). However, it was not fully understood, as to whether the animal or the microorganisms present in its intestine were responsible for the elaboration of these enzymes. Lasker (1957) reported that the silver fish (Ctenopharyngodon linnati) maintained its cellulolytic activity even when grown under sterile conditions. Cellulases of some herbivorous animals have been reported to be produced by the microorganisms inhabiting their rumen or gut.

2.3.2.2.2. Plant Origin:

The cellulases from plant materials, like seeds, stems and leaves were studied by Reese and Mandels (1963). The extracts of seeds of vegetable marrow, kohlrabi (Brassica oleracea), the stem and leaves of Oxytis sp., and dandelion (Taraxacum officinale) exhibited maximum cellulolytic activity. In general, plants produced incomplete cellulase system, which could not hydrolyze highly organized cellulose like cotton. Chi-cellulose was however, degraded by sap of
bean leaves, fruits of vegetable marrow and extracts from tobacco plants, but the activity of the enzyme was extremely low.

2.3.2.2.1  Microbial Origin

Even though many animals and plants possess cellulases, they normally do not degrade the natural cellulose in the ecosystem. Hence, investigators of cellulases have been concentrating their attention on the cellulases produced by microorganisms, because of ease of production and high cellulase activity. Bacteria and fungi, the two main groups of microbes degrade most of the celluloses, though species of algae, yeasts and mycobacteria occasionally elaborate the enzyme. Both anaerobic and aerobic species of bacteria belonging chiefly to Cellulomonas and Cellvibrio genera, were associated in cellulolytic break down (Norkrans, 1967). Some bacteria belonging to genera Sporocytophaga and Cytophaga, also exhibited appreciable cellulolytic activity.

Cellulases from the rumen of ruminants and the gut of other herbivorous animals are of microbial origin. Bacteroides succinogenes, Atrivibrio fibrillosa, Ruminococcus flavefaciens, Ruminococcus albus, Clostridium and Cellulomonas constituted the cellulolytic bacteria in the rumen (Bryant, 1959). Hungate (1966) isolated the cellulolytic Clostridium lachaei and Cellulomonas fimii. Amongst protozoa inhabiting the rumen of cattle, Dialonemia was reported to be able to partially digest large particles of cellulose.
Although many molds possess cellulolytic activity, *Trichoderma viride* (Selby and Neilland, 1967; Halliwell and Riz, 1971; Bergman and Pettersson, 1971; Gose and Patnaik, 1973; Mandels et al., 1974; Lyons, 1976; Syström and Andren, 1976), *Sporotrichum pulverulentum* (Mandels and Seppe, 1964; Wood and McCrae, 1972), *Fusarium solani* (Wood and McCrae, 1972; Wood and Phillips, 1969), *Penicillium triplex* (Moretti et al., 1972) and *L. Numerolomus* (Betrabet et al., 1974) have been reported to produce high levels of enzyme capable of degrading extensively the insoluble cellulose to soluble sugars.

Agarwal et al. (1962) studied the cellulolytic enzymes from *Chaeotomus cellulosylicum*. Both intracellular and extracellular enzymes active on carboxymethyl cellulose (CMC), cellulose powder and cellubiose were elaborated by the fungus. The extracellular enzyme was more active at earlier stages of growth, when the fungus was not well acclimatized on filter paper. However, after prolonged acclimatization, the activity was fairly uniform.

Chahal and associates extensively surveyed the possibilities of utilizing various agro-wastes for the production of cellulase enzyme and microbial protein. Out of various cellulolytic fungi screened for their potential to synthesize cellulase enzyme, *Asperillus terreus* and *Fusarium sp.* were found to yield good amount of cellulase enzyme (Chahal, 1966; Chahal et al., 1969; Chahal and Anilwal, 1973).

The extent of cellulase production and its activity was influenced by different factors viz., carbon (Pettersson, 1975 A.D.).
nitrogen (Chang and Umasi, 1969), phosphorous sources (Nisizawa et al., 1971), pH (Gupta et al., 1972; Meghna and Pettersson, 1973; Sternberg, 1976) and aeration (Mandela et al., 1975).

Coutts and Smith (1976) reported maximum production of C\textsubscript{4} and C\textsubscript{x} cellulases by *Sapromycetum thermophila* on Solka floc substrate at 45°C in 2 to 4 days, when Na\textsubscript{3}D\textsubscript{2}O or urea was used as a source of nitrogen. Under these conditions, cellulolytic activity of culture filtrates appeared to be similar to that reported for *Trichoderma viride* grown under optimum cultural and nutritional conditions.

Among the aspergilli tested, cellulolytic activity of *A. niger* was highest (Sellars et al., 1976; Flannigan and Sagar, 1977; Flannigan and Sellars, 1977).

Two isolates of *Trichoderma sp* causin pink rot of *Malus sylvestris* fruits were found to secrete C\textsubscript{x} enzyme on synthetic media. There was a relationship between the age of the culture and enzyme secretion. However, no correlation could be drawn between dry weight yield and enzymatic activity (Nasima and Agarwal, 1977). In the screening test for cellulase producers from 257 strains of microorganisms, 5 strains of *Aspergillus* sp., 2 strains of *Trichoderma* sp., and 4 strains of *Pellisularia* sp., were found to produce more cellulase in their culture filtrates. Of these cellulase producing fungi, *E. filamentosus* isolated from diseased cucumber seedling showed high cellulase activity, comparable to that of *E. viridae* (Misukoshi et al., 1977).

Rao (1977) studied the cellulolytic activity of *Aspergillus* sp. The organism was found to be truly cellulolytic and addition of rice bran to the growth medium enhanced C\textsubscript{x}, Pase, C\textsubscript{1} and β-glucosidase
activities by 2.8, 3.5, 2.0 and 7.0 fold, respectively. D’Souza and Furtado (1977) studied the cellulase producing ability of *Aspergillus terreus*. The enzyme activity was reported to be highest on the 7th day of fermentation using alkali-treated rice straw substrates. Geethadevi et al. (1978) reported slightly higher cellulase activity in *Aspergillus niger* 59-2M-1 and *Trametes* sp. 10-a, compared to cultures of *Trichoderma viride* 39-9123 and *Aspergillus terreus* -6365, when grown on alkali-treated rice straw. Sadha et al. (1977) studied the effect of cultural conditions for maximising the cellulase synthesis by *Aspergillus terreus* and *Trametes* sp.

Luo (1978) studied the ability of *Trichoderma aspergillum* to synthesise extracellular cellulase in stationary liquid medium. He suggested that the mold was able to synthesise Cx, but not C1, enzymes. The optimum temperature for the synthesis of Cx enzyme was found to be 45°C. The cellulolytic activity of fungi viz., *Aspergillus, Humicola, Monosporium, Pencillium*, *Rhizopus* and *Stachybotrys* isolated from soil and decomposing pieces of plant materials was observed to depend upon the type of organism, the nature of substrate and temperature. The cellulase produced in the presence of cellulose powder was active against carboxymethyl cellulose and vice versa (Lal and Mishra, 1978). Mutant strains of *Trichoderma viride* not producing cellulase were induced to produce cellulase (Nevvalainen and Palva, 1978).

Herr et al. (1978) tested five strains of fungi viz., *Aspergillus niger, Lentinus tetybus, Hypothecium verrucaria, Trichoderma koninckii* and *Trichoderma limproris*, for the production of cellulolytic
enzymes. The most active strains belonging to the genera of *Trichoderma*, *Amorphillus* and *Fusarium*, also secreting high activities of β-glucosidase, were grown in a bioreactor under defined conditions. Depending upon the strain, this procedure resulted in a manifold increase in cellulytic activities.

Sidhu and Sandhu (1979) studied the cellulytic nature of different fungi as compared to *Chromobium cellulolyticum*. In each fungus, with an increase in cellulase content, there was a proportional increase in dry weight, but when different fungi were compared, the amount of cellulase did not correspond to an equal amount of mycelial dry weight, indicating differences in specific activity of different cellulases. *C. cellulolyticum* had a high mycelial dry weight, although it did not show high cellulase activity as compared to other fungi. Thus, enzyme activity alone could not be taken as a criterion for assessing the cellulytic ability. This was further supported by the finding with *Amorphillus flavus*, which although showed high cellulase activity, recorded poor growth. On the other hand, *A. niger* showed very little cellulase activity but produced more cellular growth. Maximum cellulase activity was expressed in 5 days of growth in all fungi except *Trichoderma longibrachiatum*. *Trichoderma viride* mutants QU-9125 and QU-9414 produced cellulases suitable for practical applications (Vohra et al., 1980).

Sidhu and Sandhu (1980) reported the highest cellulase activity of *Trichoderma longibrachiatum* on cellulosic bagasse and control, moderate activity on holocellulose and least on untreated
baggasse. Depending on the availability of the substrate, the mold
produced the cellulase enzyme, which was of adaptive nature and
inducible (Cooper and Wood, 1975; Pennelli and Cervone, 1977).

2.3.2.3. Assay of Enzyme Activity

The assay systems of the cellulase enzyme activity could not be
precise due to the following two factors: (i) the synergistic action of
different component units of the enzyme, the activity being greatly
influenced by the proportion of different components, which might vary
and (ii) the substrates used for the assay of enzyme activity being
dependent on crystallinity and lignification of cellulose. Any meaningful
assay would, therefore, require a substrate, which could be an insoluble
cellulosic material, not too easily hydrolysed, but be capable of hydrolysis
within reasonably short time for standardization.

Mandel and Weber (1969) recommended filter paper as the substrate,
because this substrate was neither too susceptible nor too resistant
and was also readily available. If carboxymethyl cellulose (CMC) was
used as the substrate, endo-$\beta$-glucanase activity of the cellulase
complex could be assayed. However, rate of CMC hydrolysis was affected
by both chain length and degree of substitution.

Pettersson (1975) recommended celloctetraose as the substrate,
because this substrate was acted upon by all known members of cellulase
complex. Halliwell (1975) decided cotton as the substrate, because only
a complete cellulase would hydrolyse it. Unfortunately, celloctetraose
was not commercially available and cotton was so slowly hydrolysed,
that any meaningful assay required 24 hr.
Thus, the assay methods for cellulase enzyme were usually based on the measurement of the quantity of reducing sugar (measured using the dinitrosalicylic acid method) produced under standard conditions from a variety of substrates (Summer and Somers, 1944). Filter paper was considered to be a test substrate for C₄ type activity (Mandels and Weber, 1969), whereas, carboxymethyl cellulose was used for assaying the levels of C₅ activity (Horton and Leen, 1966; Hulme and Stranks, 1970). A viscometric method was suggested in the case of C₄-C₅-cellulose (Almin and Eriksson, 1967).

2.3.2.4.0. Properties of Cellulase Enzymes

2.3.2.4.1. Effect of Temperature and Heat Treatment

Cellulases from different sources showed differences in optimum temperatures for their activity and heat stability. Ikeda et al. (1967) reported the optimum temperature of incubation at 47°C for two fractions of Aspergillus niger cellulase, which were not active toward cellobiose and at 65°C for the third fraction. Hurst et al. (1977) observed maximum activity of CMCase from A. niger cellulase at 45°C. The C₄ component of Trichoderma viride cellulase was found to tolerate heat up to 150 seconds at 100°C and lose 50% activity toward cotton fiber substrate (Selby and Waltland, 1967). Okada (1976) reported an optimum temperature of 50°C for "cellulase III", a component of the enzyme from T. viride, which retained 40% of the original CMCase activity after heating to 100°C for 10 min. The optimum temperature for filter paper degrading activity of cellulase from Pellucidaria filamentosus was 65°C (Misukoshi, 1977).
2.3.2.4.2. Effect of pH on Cellulase Activity and Stability

Cellulases from different sources exhibited wide range of pH optima. Different pH optima were obtained with different substrates for the enzyme from the same source. Clarke and Stone (1965) reported an optimum pH range of 4.5-6.0 for Aspergillus niger cellulase with cellulose dextrin sulphate as the substrate. The CMCase from A. niger had optimum activity at pH range of 3.5-4.0 and was stable at 25°C over a pH range of 1 to 9 (Hurst et al., 1977). Okada (1976) reported an optimum pH range of 4.5-5.0 for cellulase III from T. viride cellulase. It was stable over the pH range of 4.5-7.5 at 4°C for 24 hr. Shoemaker and Brown (1978) observed a similar pH optima of 4.0-4.5 for endoglucanases II, III and IV from T. viride and all the components were labile at pH values greater than 8.0. The pH optimum for the filter paper degrading activity of Pseudomonas cellulosa was 5.0 (Mishkoshi, 1977).

2.3.2.4.3. Effect of Substrate Concentration

Clarke and Stone (1965) reported a K_m value of 0.25% for A. niger cellulase with cellulose dextrin sulphate as the substrate. Okada (1976) observed that the K_m for oligosaccharides of "cellulase III" from T. viride decreased proportionately with increase in chain length of the substrate. He reported K_m values for G_3, G_4, G_5, G_6 (oligosaccharides) and CMC to be 2.95, 0.67, 0.45 mM and 0.54%, respectively. Hurst et al. (1977) reported a K_m value of 3.3-9.2 mg CMC/0.01 mg protein and V_max of 46.2-60.9 units/mg protein/min for the cellulase enzyme from Aspergillus niger. Shoemaker and Brown (1978) determined the K_m values for three endo-glucanases of T. viride cellulase as
0.25-10 mM for cellobiose, 0.25-10 mM for cellotriose and 1-5 mM for cellotetraose, respectively.

2.3.2.4.4. Effect of Modulators:

Cellulase production is inhibited by its own products viz., cellobiose and glucose and the degree of inhibition varies with the source from which cellulase is derived.

Cellulose itself was recognized as the best inducer for the complete cellulase complex and other inducers included sophorose and lactose (Wandels et al., 1962; Misizawa, 1971; Wandels, 1975). Cellulose cellobiose and lactose were effective only at high concentrations, while sophorose was active only at very low concentration (Loewenberg and Chapman, 1977; Sternberg and Wandels, 1979).

Misizawa et al. (1972) reported that the catabolite repression of biosynthesis of inducible cellulase enzyme occurred at the translation level based on their studies with actinomycin D and pyrromycin. When glucose was pulse-fed to the culture during progressive cellulase biosynthesis, it ceased immediately, presumably due to catabolite repression, until glucose was exhausted or its residual concentration fell below a critical level (Pettersson, 1977; Gallo et al., 1979).

Harr (1980) demonstrated glucose to be the most potent inhibitor of total hydrolysis of cellulose. The addition of glucose to the enzyme-substrate solution at zero time, completely stopped its own formation and cellobiose and reducing groups (oligosaccharides) accumulated. By removing glucose through an ultrafiltration device, about 90% saccharification of cellulose to glucose was achieved in 48 hr without any accumulation of cellobiose.
Natural inhibitors of \textit{T. viride} cellulase, produced by plants, mainly belonged to phenolics, tannins and polymeric leucoanthocyanines (Manels and Reese, 1965). It was presumed that these inhibitors acted in the natural host resistance against fungal cellulase action.

The hydrolysis of celloolactose by \textit{A. niger} was reported to be inhibited by \( \text{Hg}^{2+} \) (1.0 mM), phenylmercuric nitrate (0.7 mM) and 10 mM iodine (Clarke and Stone, 1965). Cellulase from \textit{T. viride} was inhibited by cellubiose and methocel and inactivated by some protein reactants such as halogens, heavy metals and detergents (Manels and Reese, 1965). Other inorganic ions such as \( \text{Mn}^{2+} \), \( \text{Zn}^{2+} \), sodium borate, \( \text{Na}_2 \text{SO}_4 \), \( \text{NaCl} \), \( \text{KCl} \), \( \text{CaCl}_2 \), \( \text{Na}^+ \), \( \text{Hg}^{2+} \), \( \text{Ca}^{2+} \), were also found to be inhibitors of \textit{T. viride} cellulase (Katz and Reese, 1968; Ghose, 1969; Ghose and Keatrick, 1969; Manels and Weber, 1969). The "cellulase III" of commercial cellulase preparation produced by \textit{T. viride} was reported to be completely inactivated by 1.0 mM \( \text{Hg}^{2+} \) and partially by 1.0 mM \( \text{Ag}^+ \) and \( \text{Cu}^{2+} \) (Okada, 1976). The inhibition of cellulase by heavy metals including \( \text{Cu}^{2+} \) and \( \text{Hg}^{2+} \) was observed to be reversed by cysteine (Pal and Basu, 1962).

Recent studies on enzyme regulation revealed the complexity of the problem of cellulase production. Cellulose was a poor choice as a substrate and inducer for the production of cellulase. Therefore, a combination of a non-repressive carbon source and a gratuitous inducer, a sophorose derivative seemed to be a viable alternate inducer. Production of constitutive mutants requiring no inducer to form cellulase could be a possible solution. Other than merely using non-repressive carbon
sources, the organism could be mutated for insensitivity to catabolite repression. With respect to genetic engineering, it was attempted to transfer the cellulase genes from \textit{P. viride} or any other good cellulase producers into \textit{E. coli} or some other plasmid bearing host, where the gene could be amplified hundreds of time. Further, mutation studies on cellulase production would offer enormous avenues for increasing enzyme yields (Jenian, 1976).

2.4.0. \textbf{ENZYMIC SACCARIFICATION OF CELLULOSE}

A schematic diagram of the process of microbial enzymatic conversion of waste cellulose to glucose and other by-products is shown in Fig. 3 (Spam, 1976). The first step in the above scheme constituted the production of the enzyme by the microorganism. This was accomplished by growing the organism in a culture medium containing shredded cellulose and various nutrient salts. Following its growth, the microbial culture was centrifuged or filtered. The clear straw coloured supernatant or filtrate contained the enzyme, which was used in the saccharification vessel. Prior to its use, the enzyme broth was assayed for cellulase activity and the acidity of the reaction mixture was adjusted to the desired pH. The reaction mixture containing milled cellulose and the enzyme solution was incubated for 2 days for the determination of the end product, glucose. The saccharification of cellulose occurred at atmospheric pressure and at a temperature of 50°C. The untreated cellulose and enzyme was recycled back into the vessel, and the crude glucose syrup was filtered and used for the production of chemicals, feedstocks, SCP, fuels, solvents etc.
FIG. 3. BIOCONVERSION OF WASTE CELLULOSE THROUGH ENZYMATIC SACCHARIFICATION
Toyama and Ogawa (1972) studied the feasibility of sugar production from cellulolic substrates by the action of cellulase enzyme produced by \textit{T. viride}. Sugar solutions containing 10-15\% reducing sugar were obtained by incubating delignified cellulolic substrates with 5.0\% (v/v) \textit{T. viride} cellulase for 2 days.

The U.S. Army Fatick Laboratory conducted detailed studies for the conversion of cellulose into glucose (Shose and Kostick, 1969; Handels et al., 1974). Andren et al. (1975) evaluated a variety of cellulolic materials for enzymatic saccharification with \textit{T. viride} cellulase. Fifty per cent conversion of waste paper cellulolic material obtained from municipal trash or high cellulolic agricultural residues was achieved in 6-24 hr, yielding 5-15\% sugar solutions. The culture filtrate of \textit{T. viride} was most commonly used, but other organisms such as \textit{Spomtrichum pulverulentum} (Bk and Eriksson, 1975) and thermophilic actinomycetes were also used for enzymic saccharification of cellulolic materials (Ju and Paulavicius, 1975).

\textit{Trichoderma viride} filtrate contained very low \(\beta\)-glucosidase activity for the saccharification process, and the hydrolysed sugar solutions comprised of glucose and cellobiose in the ratio of 1:3 (Huang, 1975). Furthermore, owing to the low \(\beta\)-glucosidase activities, the cellobiose accumulated, which according to the C\(_4\)-C\(_x\) concept, inhibited the production and activity of C\(_4\) enzyme, resulting in a reduced velocity of hydrolysis (Reese et al., 1950; Bergren et al., 1975; Howell and Stuck, 1975). Therefore, Seeley (1976) proposed a two-step enzyme process, with a \(\beta\)-glucosidase step in addition to the treatment of cellulosic wastes with \textit{T. viride} cellulase. The
two-step enzyme process yielded a sugar solution containing 95% 
D-glucose. Similar results were obtained by supplementing Trichoderma 
cellulase with β-glucosidase from Aspergillus (Katz and Reese, 1969; 
Sternberg et al., 1976). Herr et al. (1978) achieved 47% conversion 
of crystalline cellulose to glucose by Trichoderma cellulase within 
48 hr. Herr (1980) obtained a 4.0% solution of pure glucose from 
1.0% cellulose solution by using culture filtrate of T. viride ITCC-1433 
mutant. Wood pulp and newsprint were hydrolyzed to a much lesser 
extent.

2.5.0. SINGLE CELL PROTEIN AND CELLULASE PRODUCTION ON 
CELLULOSE SUBSTRATES

Vogt and Staffeldt (1977) cultured several species of fungi 
on single substrates of starch, xylan, cellulose, lignin and mixtures 
of two of these substrates and found that cellulolytic activity was 
not positively correlated with cellulase enzyme production.

Pyrothecium sp. gave higher enzyme activity but lesser biomass 
production than Phoma sp. Although Chaetomium cellulolyticum 
yielded more microbial protein from alkali-treated than from untreated 
straw, the cellulase production was more with untreated straw 
substrate. The cellulase enzyme production was not directly 
correlated with protein recovery (Chalal et al., 1977).

2.6.0. SINGLE CELL PROTEIN EVALUATION

2.6.1. As Food and Feed:

The use of SCP products in food and feed increases quanti-
tatively and qualitatively their nutritional value. For use as
human food, the SCP product should have satisfactory nitrogen and protein contents, amino acid profile, lipid, minerals and vitamin contents. In addition, the SCP should have a satisfactory biological value as demonstrated in actual animal feeding studies and in human nutritional trials. From a sensory evaluation point of view, the product should have a satisfactory flavour, aroma, colour and texture. The product should also be free from toxins (Kihlberg, 1972).

For feed applications, SCP products should meet many of the same criteria as those required for human food applications. The nitrogen and protein contents and amino acid profile are the indicators of potential usefulness of SCP product in animal feed applications. Methionine supplementation to SCP product is required to obtain satisfactory feeding performance in non-ruminants. The lysine:arginine ratio has also to be adjusted, because of high lysine content of most of SCP products (Kihlberg, 1972).

The various factors that determine the usefulness of a protein source for man or animal are nutritional value, cost and aesthetic acceptability. The arguments advanced in the literature, in favour of SCP products suggest that by use of microbial protein, one could obviate the inefficiency of conversion of plant protein to animal protein. Gray (1962) reported that theoretically, an acre of corn crop might produce 261 lbs of corn protein and 280 lbs of fungal protein instead of 72 lbs of beef (Fig. 4). Thus, efficiency of microbial conversion was reported to be higher than that of animal conversion.
FIG. 4. EFFICIENCY IN THE PRODUCTION OF PROTEIN FROM ONE ACRE OF CORN DIRECTLY THROUGH FEEDING BEEF ANIMALS AND THROUGH FUNGAL CONVERSION OF CORN STARCH (GRAY, 1962)
2.6.2.0. **Nutritional Aspects of SCP**

The amino acid composition of a protein primarily determines its value as a source of nitrogen for growth and maintenance. Thus, the analysis for the amino acid content gives valuable information about the potential nutritional value of the protein. The composition of amino acid content of yeasts, molds, bacteria, fish meal, and soybean meal was comparable and the SCP contained almost all essential and non-essential amino acids, present in standard conventional proteins (Kihlberg, 1972).

For the microbial protein to be used as feed, it should be evaluated in feeding trials with the animal in question, usually as a substrate for another high protein supplement on top of a cereal basal diet. Since, the product was intended for the food market, the animal tests should be followed by nutritional studies with human beings (Birch et al., 1976).

The nutritional and toxicological value of SCP products has not been so far fully assessed. The content of amino acid is the best criterion for comparing different microorganisms as potential source of protein for livestock. The yeast and mold generally provide better quality SCP on the basis of their amino acid composition. However, long term trials for the application of SCP to animal feedstock should be conducted (Tavernier and Karunajeewa, 1976).

The SCP of algal origin is the poorest source for most of the amino acids. The available reports on the feeding value of algae pertain to non-ruminants such as rats and chicks than for humans.
Supplementation of algal SCP with some essential amino acids induced higher weight gain in rats. The algal SCP containing mixed culture of *Scenedesmus obliquus* and *Chlorella pyrenoidosa*, when supplemented with lysine or *C. pyrenoidosa* alone supplemented with methionine, resulted in higher weight gains than with the crude dried algae alone (Hendley *et al.*, 1956; Lavoille *et al.*, 1962). Nitrogen absorption was reported to be a function of the mechanical damage to which the cells had been subjected (Meffert and Pabat, 1963). Cooked algae for example, were digested better than uncooked (Cook *et al.*, 1963). Thus in general, it appeared that unfractionated protein was not utilised by non-ruminants, although it could be tolerated with any really harmful effects. It was evidenced by Hinz *et al.* (1966) that algae was digested better by cattle (ruminants) than by swine (non-ruminants). For utilizing the algal SCP product adequately, therefore, two alternatives were suggested: (i) fractionation of the raw material, with the attendant increase in cost on protein basis or (ii) introduction of an extra link in the food chain by feeding to ruminants, which could upgrade the protein at the expense of a tenfold loss of energy for each successive link in the chain (Priestley, 1976).

Khor *et al.* (1977) carried out the safety evaluation of *Aspergillus fumigatus* L-21, grown in a cassava carbohydrate and salt medium. The fungal SCP was fed to the male weanling rats at 20, 30 and 40% of replacement of the normal diet for 90 days. The SCP product of *A. fumigatus* L-21 was found to be satisfactory.
2.7.0. **ECONOMIC CONSIDERATION FOR SCP PRODUCTION**

The economics of SCP products was of primary concern in the decision to implement this process. Although, many waste bioconversion systems appeared to have zero or negative costs, there were often hidden costs in additional processing, necessary to prepare the material for fermentation (Kihlborg, 1972; Davis, 1974).

From the point of scientific exploration of new sources of crude protein, in the present context, the question of economics might be considered to be of secondary importance. When the scientific discovery of SCP product manufacture is scaled up industrially, there is bound to be competition with natural substances, where the price would be a decisive factor of selection. However, economics of the SCP process might be considered as one of the key issues determining its utility (Litchfield, 1977). The SCP product at every level of its application as feed or food supplement or as an independent product should compete with the conventional materials. Taunser and Karunajeewa (1976) reported that the use of SCP products for feeding animals had economic advantages over other protein sources.

2.8.0. **UTILITY OF SINGLE CELL PROTEIN PRODUCTS**

The main utility of the fermentation processes for the production of SCP should be for use as human food and cattle feed.

A. **ADVANTAGES**: The industrial production of SCP should prove to be economical over the conventional plant or animal proteins. Then only, the SCP products would be preferentially utilized. The other advantages of microbial protein (SCP) production are given below—
(i) Microorganisms possess very short generation time and thus provide a rapid mass increase.

(ii) Microorganisms can be easily modified genetically.

(iii) SCP products have high nutritive value as compared to vegetable proteins due to the availability of essential amino acids in a balanced way.

(iv) The protein content of microbes is high. Most microorganisms contain about 7 to 12% nitrogen on dry weight basis.

(v) Microbial process can use waste materials as raw organic substrate, which are locally available in large quantities, such as cellulose, whey, petroleum or natural gas, and which will be readily available for a long time to come, thus minimizing the chances of environmental pollution.

(vi) The production of microbial protein can be carried out in continuous culture, independent of vagaries of climate, weather and with only a small land area and water requirement.

(vii) The waste disposal problems are limited, as compared to other food production processes.

(viii) A fermentation plant occupies a small area.

B. DISADVANTAGES—

(i) Toxin production by some microorganisms e.g. Asperillus flavus and A. parasiticus.

(ii) The physiological properties of food produced by fermentation may be unsuitable for its direct consumption.

(iii) Aesthetic acceptability of the new SCP product.

(iv) High nucleic acid content present in microbial cells.
Nucleic Acid Content of SCP Products: The presence of high nucleic acid content of SCP (especially yeast proteins) reduces its protein value. Dietary nucleic acids are depolymerized by nucleases present in the pancreatic juice and then converted to nucleosides by intestinal enzymes prior to absorption. The purine bases (adenine and guanine) are metabolized to uric acid. An increased consumption of purines thus results in a higher uric acid level in plasma and an increased excretion of uric acid in the urine. Since, uric acid is sparingly soluble, it is expected that an increased plasma uric acid concentration may result in the precipitation of urate in tissues and joints, analogous to the conditions in gout. Stones may also be formed in the kidneys and bladder (Thilberg, 1972).

Human beings and other higher primates lack in the enzyme uricase (urease oxidase), which oxidizes uric acid to allantoin. In contrast, other mammals possess uricase and are able to convert uric acid into soluble metabolite, allantoin, that can be excreted easily. When untreated SCP was used as a fodder, the high purine content did not produce any ill effect on the feeding animal (Davis, 1974; Birch et al., 1976).

For enhancing the protein value of SCP products as human food, their nucleic acid content should be reduced. Hence, a process for reducing the nucleic acid content of *Saccharomyces cerevisiae* and *Bacillus subtilis* was developed and the finished SCP product was edible with high protein content (Massachusetts Institute of Technology, 1977). This process initially included heat shocking of cells for a time period, which activated the nuclease enzyme at an
optimum temperature. So, the nucleic acids were degraded without allowing proteins to break or leak out of the cells. Then, the treated cells were incubated at a temperature lower than the heat shock temperature under conditions in which the cells remained intact and the nucleic acid fragments permeated through the cell wall.
CHAPTER III

MATERIALS AND METHODS
3.1.0. MATERIALS

3.1.1. Microorganisms:

Cellulolytic mold cultures of *Aspergillus niger*, *Alternaria tenuissima*, and *Fusarium oxysporum* from the Department of Pathology, Indian Agricultural Research Institute, New Delhi, *Trichoderma viride* TVL and 64Q1 from National Dairy Research Institute, Karnal, and two cultures of *Aspergillus terrus* 1 and *Bacillus subtilis* var. *mucilaginosus* 24-1224 from the Department of Microbiology, Panjab University, Chandigarh were obtained.

3.1.2. Substrate and Chemicals:

Sugarcane bagasse was prepared by crushing the cane between electric crushers to extract the cane juice and then pulverizing the residue using a mechanical grinder to 40 mesh size at Cooperative Sugar Mill, Karnal.

Carboxymethyl cellulose was obtained from Sigma, U.S.A. All other chemicals used were of analytical grade and obtained from BDH, Sigma, etc.

Cornsteep liquor was obtained from the Department of Dairy Microbiology and cattle urine from Animal Nutrition Division, National Dairy Research Institute, Karnal.
3.2.0. METHODS

3.2.1. Preparation of Alkali-Treated Bagasse

One part of dry sugarcane bagasse powder was pretreated with twenty parts of 4.0% (w/v) sodium hydroxide solution by autoclaving for 30 min at 121°C and filtered. The recovered bagasse residue was washed free of alkali with distilled water till neutrality. The pretreated bagasse was dried in an oven at 60°C and ground to 40 mesh size and was incorporated into basal medium as sole carbon source.

3.2.2. Isolation and Characterization of Cellulolytic Molds

The cellulolytic molds were isolated by enrichment culture technique. Samples of decaying plant materials such as leaves, wood, straw, bagasse and also soil in the vicinity of decaying matter were collected and added to enrichment flasks having sterilized alkali-treated bagasse-Czapek's basal medium (Haynes et al., 1955) containing (per litre): sodium nitrate, 2.5 g; potassium chloride, 0.5 g; magnesium sulphate, 0.5 g; ferrous sulphate, 0.01 g; zinc sulphate 0.01 g; copper sulphate, 0.005 g; KH₂PO₄, 1.0 g; alkali-treated bagasse, 10.0 g; distilled water 1000 ml and pH adjusted to 5.5. After incubating for 7 days on a rotary shaker, a small sample of this broth was transferred to fresh enrichment medium in flasks. After 4 to 5 such serial transfers, a dilute suspension of the broth from last flask was prepared and plated out on sterilized Potato Dextrose Agar containing (per litre): boiled potato extract from 200 g potato filtered through muslin cloth; glucose, 30 g; agar, 1.5 g; and distilled water made up to
1000 ml. The desired pH of 4.5 was adjusted after sterilization by adding 1.0 ml of 10% tartaric acid to 100 ml medium. After incubating the PDA plates for 2 to 4 days at 29 ± 1°C, individual mold colonies were obtained. The mold colonies were further purified by subsequent plating on PDA agar. The isolated pure mold cultures were identified by studying their cultural, morphological and biochemical characteristics (Clements and Shear, 1954; Raper and Fennel, 1955) and maintained on soil-sand mixture and filter paper slants.

3.2.3.0. Maintenance of Cultures

3.2.3.1. Soil-Sand Cultures:

Equal amounts of garden soil and fine sand were taken, autoclaved for 30 min and kept in an oven at 160°C for 30 min alternately. This treatment was repeated 4-5 times to ensure complete sterility. After cooling to ambient temperature, the soil-sand mixture was inoculated aseptically with the spores of molds. Using this technique, fungal cultures can be maintained for several years at room temperature without losing any enzymic activity.

3.2.3.2. Filter Paper Slants:

The pure fungal cultures were also maintained on the Czapek's basal agar slants (Haynes at al., 1955) containing filter paper as a sole carbon source. The cultures were incubated at 29 ± 1°C for 7 to 15 days and stored at 4°C.
3.2.4.0. Screening of Molds for SCP Production

3.2.4.1. Cultivation of Molds

The sterilized Czapek's mineral medium containing either 1.0% carboxymethyl cellulose or untreated bagasse or alkali-treated bagasse was inoculated with 4% (v/v) inoculum of all the mold cultures individually (in some cases mixed cultures) and was incubated on a rotary shaker at 28 ± 1°C for 7 days.

3.2.4.2. Harvesting

After 7 days fermentation, the contents were harvested by filtering the biomass through Whatman 6.1 filter paper. The fungal biomass with residual bagasse substrate was washed repeatedly with distilled water to remove any amount of nitrogen adhering to the biomass from the medium. The filter papers and their contents were dried in an oven at 60°C for constant weight and the contents were analyzed for their nitrogen content by Kjeldahl's method (AOAC, 1975).

3.2.5.0. Effect of Cultural Factors on Cellulase and SCP Production

After screening various molds for their cellulolytic activity, the most efficient mold, Aspergillus terreus \(^{1b}\), was selected and used further.

3.2.5.1. Hydrogen Ion Concentration

Basal medium was adjusted to different hydrogen ion concentrations viz., 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0 and 8.0 before
autoclaving. The above basal medium in 50 ml quantities in 250 ml Erlenmeyer flasks containing 1/4 treated bagasse was sterilized, inoculated with 4% inoculum and incubated at 29 ± 1°C under continuous shaking on a rotary shaker for 7 days.

3.2.5.2. Temperature:

The basal medium in 50 ml quantities adjusted to pH 4.0 in 250 ml Erlenmeyer flasks containing 1/4 treated bagasse was sterilized, inoculated with 4% inoculum and incubated at 20, 25, 30, 35 and 40°C under continuous shaking on a rotary shaker for 7 days.

3.2.5.3. Shaking and Aeration:

The basal medium in 50 ml quantities adjusted to pH 4.0 in 250 ml Erlenmeyer flasks containing 1/4 treated bagasse was sterilized, added with 4% inoculum and incubated at 29 ± 1°C under continuous shaking, intermittent shaking and still culture conditions for 7 days.

The basal medium in 50, 100, and 150 ml in 250 ml Erlenmeyer flasks (1/5, 2/5 and 3/5, v/v) containing 1/4 treated bagasse was sterilized, inoculated with 5% inoculum and incubated at 29 ± 1°C under continuous shaking on a rotary shaker for 7 days.

3.2.5.4. Size of Inoculum:

The basal medium in 50 ml quantities in 250 ml Erlenmeyer flasks containing 1/4 treated bagasse was sterilized, inoculated with 1 to 6% (v/v) seven days old mold cultures and incubated at 29 ± 1°C under continuous shaking on a rotary shaker for 7 days.
The experiment on effect of cultural factors was conducted in two sets. The cultures of first set were harvested as described earlier and analyzed for their biomass nitrogen content. The contents of second flask were centrifuged at 10,000 rpm for 15 min and the supernatant was used for assaying the cellulase (CMCase and FPase) activity.

3.2.6.0. Effect of Nutritional Factors on Cellulase and FP Production:

3.2.6.1. Bagasse Substrate Concentration:

The treated sugarcane bagasse was charged at the rate of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0% (w/v) in Casper's basal medium. The basal medium in 50 ml quantities in 250 ml Erlemeyer flasks containing the substrate was sterilized, inoculated with 5% (v/v) inoculum of A. terreus and incubated at 28 ± 1°C under continuous shaking on a rotary shaker for 7 days.

3.2.6.2. Nitrogen Sources:

The effect of various inorganic and organic nitrogen sources viz., ammonium sulphate, ammonium nitrate, ammonium iron sulphate, ammonium chloride, sodium nitrate, cattle urine, urea and comsteep liquor was determined by incorporating the individual nitrogen source at the final concentration of 50, 100, 200, 400, 500 and 600 mg N/litre basal medium.

The basal medium (without the original nitrogen source) in 50 ml quantities in 250 ml Erlemeier flasks containing 1.0% treated sugarcane bagasse substrate was incorporated with varying concentrations
of individual nitrogen sources, inoculated with a week-old 5.0 (v/v) culture of the cellulolytic mould *A. terreus* G1 and incubated under optimum cultural conditions viz., pH 4.0, 28 ± 1°C and continuous shaking on a rotary shaker. After 7 days fermentation, the contents were filtered as described earlier and the crude protein content of the biomass (XP) and the cellulase (CMCase and Fase) activity of the supernatant were determined.

3.2.7.0. **Effect of Incubation Time on SCP Production**

To study the effect of incubation time with optimum cultural conditions viz., pH, temperature, aeration, shaking and dose of inoculum and nutritional factors viz., different nitrogen sources and substrate concentration on microbial protein and cellulase production and on its quality, a modified Caspaks's broth was used containing (per litre): 600 mg N of corn steep liquor; 1.0 g KH₂PO₄; 0.5 g potassium chloride; 0.5 g magnesium sulphate; 10.0 g of either untreated or treated sugarcane bagasse substrate. The modified Caspaks's broth in 50 ml quantity in 250 ml Erlenmeyer flasks was adjusted to pH 4.0, autoclaved, and after cooling, inoculated with 5.0% (v/v) culture of a week-old *A. terreus* G1. The flasks in triplicate, were incubated at 28 ± 1°C on a rotary shaker for 7 days. Two flasks were processed and the biomass was harvested periodically after every 24 hr for 7 days and analysed for its crude protein content, residual cellulose and water soluble carbohydrate. The culture broth in another flask was centrifuged and the supernatant was assayed for the cellulase (CMCase and Fase) activity. The quality of the recovered biomass (XP) was evaluated by in vitro rumen digestibility and by
determining its amino acid content quantitatively.

3.28.0. **Pilot Scale Fermentation of Treated Bagasse Substrate**

3.28.1. **Cultivation**

To investigate the optimum fermentation time in a pilot scale fermentor of 10-litres capacity with optimum cultural and nutritional conditions, a modified Czapek's medium as described earlier was poured aseptically in a sterilized fermentor and was then inoculated aseptically with 5.0% (v/v) inoculum of *A. terreus* CN. The contents were also added with 0.2% (v/v) sterilized antifoaming agent (10% v/v aerocil in liquid paraffin). The fermentor was operated at 28 ± 1°C with a continuous aeration of 2.0 litres air/min. However, the agitator was driven at a speed of 120 rpm at alternate 6 hr interval during 96 hr fermentation.

3.28.2. **Harvesting**

The samples of fermenting slurry were drawn periodically after every 12 hr for 4 days. About 200 ml of each sample was centrifuged at 10,000 rpm for 15 min and the residual solid biomass was mixed with distilled water and filtered through Whatman No. 1 filter paper to harvest fungal biomass along with unfermented bagasse substrate (SCP). The SCP was analyzed for its nitrogen content, water soluble carbohydrate and residual cellulose. The supernatant after centrifugation was used for assaying the cellulase (CMCase and FPase) activity.

3.29.0. **Analytical Determinations**

3.29.1.0. **Nitrogen and Crude Protein**
3.2.9.1.1. **Reagents:**

(i) Mixed indicator solution (0.1% methyl red and 0.2% brown cresol green in ethanol).

(ii) 3/10 sulphuric acid.

(iii) Concentrated sulphuric acid.

(iv) Catalyst mixture: ten parts of potassium sulphate mixed with one part of copper sulphate.

(v) 4.0% boric acid solution in distilled water.

(vi) 40% sodium hydroxide.

3.2.9.1.2. **Procedure:**

Crude protein constitutes a mixture of true protein and non-protein nitrogenous substances and was determined by estimating the total nitrogen of the organic substrate and multiplying the nitrogen content of the biomass by the factor 6.25.

The total nitrogen content of the bagasse and XP was estimated by the conventional Kjeldahl's method, which consists of three steps viz., digestion, distillation and titration. One g of the dried sample was digested with 25 ml concentrated sulphuric acid in the presence of 5.0 g catalyst mixture, converting all the sample nitrogen to ammonium sulphate in the acid mixture until a clear blue colour appeared. The blue acid mixture was made up to 100 ml in a volumetric flask with distilled water and 10 ml of the solution was mixed with 20 ml of 40% sodium hydroxide, distilled with steam and the ammonia liberated was absorbed in 4.0% (w/v) boric acid solution and was
tirated with 0.1 N sulphuric acid to a faint purple end point.

The volume of 0.1 N sulphuric acid used corresponds to the quantity
of ammonia liberated. The nitrogen content is expressed as per cent
(AOAC, 1975).

3.2.9.2.0. **Water Soluble Carbohydrate**

3.2.9.2.1. **Reagents:**

(1) 0.25 N sulphuric acid.

(2) 80% phenol.

(3) Concentrated sulphuric acid.

3.2.9.2.2. **Procedure:**

From the milled and dried sample, 1.0 g was boiled with 100 ml
of 0.25 N sulphuric acid for half an hour. After cooling, it was
filtered and 1.0 ml filtrate was made up to 25 ml with distilled water.
One ml of the diluent was mixed with 0.15 ml of 80% phenol and 5.0 ml
concentrated sulphuric acid. In the presence of phenol sulphuric acid
mixture, sugars give an orange-yellow colour. The intensity of colour
was measured with Elico-Spectrocol at 490 nm using a reagent blank
for setting zero of the instrument and comparing with the sugar standard
curve. Carbohydrates (0.25 g starch and 0.25 g glucose) were dissolved
in one litre distilled water so that 2.0 ml contained 100 μg of
carbohydrate. Aliquots 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of
the above standard solution were taken and the final volume was made up
to 1.0 ml with distilled water along with a blank.

The carbohydrate content of the standard was estimated as given earlier.
and a standard curve was drawn. The water soluble carbohydrate content of the samples was calculated by reading the standard curve and expressed as per cent on dry matter basis (Dubois et al., 1956).

3.2.9.3. Amino Acid Analysis

The dry fungal biomass weighing 0.2 g was hydrolysed with 2.0 ml of 6 N HCl in a freeze-drying vial, which was sealed air tight. The vial was kept in an oven at 110°C for 24 hr to ensure complete hydrolysis (Block, 1951). The contents were then precipitated with 10 ml of 80% ethanol and filtered. The filtrate was concentrated to dryness under vacuum at 40°C. The resulting thin film of hydrolysate was placed in a vacuum desiccator over soda lime for 24 hr. The hydrolysate was then taken up in warm water, filtered, again evaporated to dryness and finally taken up in 1.0 ml HCl buffer of 2.2 pH.

The quantitative estimation of SCP for its amino acid profile was carried out by the method of Moore and Stein (1963). Two hundred and fifty microlitres of standard amino acid (containing 125 mM of each amino acid) and sample hydrolysate was loaded in Beckman Model 116 amino acid analyser and allowed to run against 2.2 pH HCl buffer. The concentration of every amino acid in unknown sample was calculated by comparing its chromatograms with that of standard and expressed as per cent on dry weight basis of protein in SCP.

3.2.9.4.0. Single Cell Protein in viv0 Digestibility

Single cell protein samples were analysed in triplicate for their dry matter digestibility by two stage in viv0 rumen fermentation technique described by Tilley and Terry (1963) and modified by van Soest et al. (1966).
3.29.4.1. **Reagents:**

(i) Trypsinase - a pancreatic digest of casein, USP.

(ii) Sodium sulphide monohydrate.

(iii) Sodium hydroxide - 4.0 g dissolved in 1000 ml of distilled water.

(iv) Cysteine- hydrochloride.

(v) Resazurin - 0.1% (w/v) solution.

(vi) 6 N - hydrochloric acid.

(vii) Toluen.

(viii) Buffer solutions:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium carbonate</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>95.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

(ix) **Macro-Mineral Solution:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium hydrogen phosphate (anhydrous) (\text{Na}_2\text{HPO}_4)</td>
<td>5.7 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (anhydrous) (\text{KH}_2\text{PO}_4)</td>
<td>6.2 g</td>
</tr>
<tr>
<td>Magnesium sulphate (hydrated) (\text{MgSO}_4\cdot7\text{H}_2\text{O})</td>
<td>0.6 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

(x) **Micro-Mineral Solution:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride (\text{CaCl}_2\cdot2\text{H}_2\text{O})</td>
<td>13.2 g</td>
</tr>
<tr>
<td>Manganese chloride (\text{MnCl}_2\cdot4\text{H}_2\text{O})</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Cobalt chloride (\text{CoCl}_2\cdot6\text{H}_2\text{O})</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Ferric chloride (\text{FeCl}_3\cdot6\text{H}_2\text{O})</td>
<td>8.0 g</td>
</tr>
</tbody>
</table>
The above ingredients were added to a volumetric flask and dissolved in a small quantity of distilled water. Finally, it was made up to 100 ml with distilled water.

3.2.9.4.2. Preparation of Buffer-Mineral Solution:

Two g trypsin and 0.1 ml micro-mineral solution were dissolved in 400 ml distilled water by gentle agitation. Then, 200 ml each of buffer and macro-mineral solution and 1.0 ml reassurin solution were added and the contents were thoroughly mixed.

3.2.9.4.3. Reducing Solution:

Six hundred and twenty five mg of cysteine-hydrochloride and 4.0 ml sodium hydroxide solution were dissolved in 95 ml distilled water. Then, 625 mg sodium sulphide monohydrate was added and thoroughly mixed.

3.2.9.4.4. Preparation of Rumen Fluid Inoculum:

The rumen liquor was collected from a fistulated cattle, which was fed on roughage diet, before noon. This liquor was collected in a thermos flask to maintain the temperature and it was filled completely to eliminate any air space. The sample of rumen liquor was immediately brought to the laboratory. The top layer of ingesta was discarded and the rest was mixed in a Waring Blender for 2 min under the atmosphere of carbon dioxide. The blended mass was squeezed through muslin cloth and the filtrate was kept under carbon dioxide.

3.2.9.4.5. Procedure:

(1) First stage: Five hundred mg of dried and milled sample
was taken with 40 ml buffer mineral solution in 100 ml conical flasks. The flasks were stoppered tightly with two pored rubber bungs and assembled in a Scientronic metabolic water bath with a shaker at 40°C. Then, oxygen free carbon dioxide was passed from a CO₂ cylinder at a pressure of about 30-40 cm water column and the Bunsen valves of the flasks were checked. Two ml reducing solution was injected to each flask through the inlet tube with a syringe. The reduction of medium, which was a change from red colour (oxidized) to colourless (reduced) was noticed to ensure complete anaerobiosis. Ten ml of the rumen fluid inoculum was then added with a syringe through the second inlet tube of each fermentation flask. Then, the tubes were sealed and incubated for 40 hr in the Scientronic metabolic water bath with shaking at a rate not to produce splashing. The carbon dioxide pressure was adjusted to 2 cm of water column.

(ii) Second Stage: For the storage of the sample, after first stage fermentation, 1.0 ml toluene was added to each flask and stored in refrigerator by tightly stoppering with rubber bungs. Then, the fresh or stored sample was washed with 50 ml neutral detergent solution into 1000 ml long beaker. After refluxing for half an hour, the solution was filtered in a previously dried and weighed sintered glass crucible (Grade-1) with suction pump. It was washed twice with hot distilled water, twice with acetone and was finally sucked dry. The crucibles with their contents were dried in an oven at 100°C and were weighed to constant weight.
The residue in the crucible was the neutral detergent residue. The dry matter digestibility is expressed as percentage using the formula given below.

**Dry Matter Digestibility (%) = 100 - % Neutral Detergent Residue**

3.2.9.5.0. **Cellulose Estimation**

The residual cellulose content in microbial biomass was determined by the method of Updegraff (1969).

3.2.9.5.1. **Reagents:**

(i) 80% acetic acid,

(ii) Concentrated nitric acid,

(iii) Concentrated sulphuric acid,

(iv) 67% sulphuric acid

(v) Anthrone reagent: It was freshly prepared by dissolving 0.2 g anthrone in 100 ml concentrated sulphuric acid and chilled for about 2 hr in refrigerator prior to use.

(vi) Acetic/Nitric reagent: It was prepared by mixing 150 ml of 80% acetic acid and 15 ml concentrated nitric acid.

3.2.9.5.2. **Procedure:**

The cellulose content of bagasse and BCF was estimated as follows:

Two hundred mg sample was mixed with 10 ml distilled water and homogenised in a Waring Blender. It was centrifuged at 2000-3000 rpm for 5 min and the supernatant was discarded. The solid material was
mixed in 3.0 ml acetic/nitric reagent and mixed thoroughly. The tubes carrying glass marbles on top (to create a refluxing action and to reduce evaporation) was kept in boiling water bath for 30 min. Tubes were again centrifuged for 5 min at high speed and the supernatant was discarded. The sediments were washed thoroughly with 10 ml distilled water. It was centrifuged again and the supernatant was discarded. Then, the sediments were again treated with 10 ml of 67% sulphuric acid in three lots and kept for an hour at ambient temperature. One ml of the above mixture was taken and diluted to 100 ml with distilled water. One ml of above diluent was added with 4.0 ml distilled water and placed in an ice bath for cooling. Ten ml chilled anthrone reagent was added to each tube by layering with pipette. The contents were mixed gently on a Vortex mixer and the tubes were returned to ice bath until all tubes were mixed. These tubes with glass marble on top were placed in boiling water bath for 16 min for colour development. Again the tubes were transferred to ice bath for 2–3 min and then kept at room temperature for 5–10 min. Finally, each tube was read on Gilico-Spectrocol at 620 nm against a reagent blank.

Standard stock solution of cellulose was prepared by dissolving 50 mg pure dried cellulose (dried for 6 hr at 105°C and cooled over anhydrous alumina) in 10 ml 67% sulphuric acid with gentle heating. One ml of the above solution was diluted to 50 ml with distilled water to obtain 100 μg cellulose/ml. Aliquots of 0.5, 1.0 and 1.5 ml of the above standard solution were taken and finally made up to 50 ml with distilled water, so as to give 50, 100 and 150 μg cellulose concentration. The green colour was developed and read as described earlier.
The concentration of cellulose in unknown samples was extrapolated from cellulose standard curve and expressed as percentage on dry weight basis.

3.3.0. Cellulase Enzyme

3.3.1. Preparation of Crude Extract of Cellulase

After fermentation of treated sugarcane bagasse substrate by \textit{Aspergillus terreus}, for 7 days at 23 \pm 1^\circ \text{C}, the liquid portion of the fermentation broth was decanted and centrifuged at 10,000 \text{ rpm} for 15 min to separate out the solids dispersed in it. The supernatant comprising of the crude cellulase was stored at 4^\circ \text{C}.

3.3.2.0. Partial Purification

The crude enzyme solution was first partially purified by ammonium sulphate fractionation followed by desalting through Sephadex G-25.

3.3.2.1. Ammonium Sulphate Fractionation

One hundred ml lots of crude enzyme solution were mixed with solid ammonium sulphate (in 250 ml beakers) with constant stirring, so as to give 10, 20, 30, 40, 50, 60, 70, 80 and 90\% final saturation at 4^\circ \text{C}. Then, solutions were kept undisturbed for 2 hr, centrifuged at 600 \text{ rpm} for 20 min and the precipitate was dissolved in a particular volume of 0.05 M citrate buffer (pH 8.0). All the aliquots were analyzed for their protein content and cellulase activity. The fractions of ammonium sulphate, which showed higher specific activity were pooled and used for further experiments.
3.3.2.2.0. Gel Filtration on Sephadex G-25:

3.3.2.2.1. Preparation of Column:

Dry gel of Sephadex G-25 was soaked in distilled water for 48 hr at room temperature. The water was decanted, fine gel was suspended in eluting buffer (0.05M citrate buffer, pH 4.3) and poured into a glass column (22 x 4 cm). The gel was allowed to settle and the column was flushed with approximately 3-4 volumes of the eluant prior to the application of sample. The uniform packing of the gel was tested by using Blue Dextran-2000.

3.3.2.2.2. Application of Sample:

The column was drained until the buffer surface was in level with the gel surface. Five ml of the above enzyme solution obtained was carefully layered on the surface of the gel. The column was drained slowly until the sample percolated into the top layer of the gel bed. The gel surface and the column wall contacting the sample were washed with a small amount of eluant buffer. Then, column was connected to the eluant buffer reservoir and eluted at the flow rate of 20 ml/hr. Fractions of 5.0 ml each were collected in tubes periodically.

3.3.3.0. Analysis of the Fractions:

All the aliquots free from ammonium sulphate were pooled and the specific activity was determined by measuring the protein content, cellulose enzyme (CMCase and FPase) activity and total recovery of the enzyme was calculated.
3.3.1.3. **Protein Estimation**

Protein content in the enzyme extract was estimated by the method of Lowry *et al.* (1951).

3.3.1.4. **Principle**

Protein reacts with the Folin-Ciocalteu reagent to give a coloured complex. The colour formation is due to the reduction of phosphomolybdate-phosphotungstate salt in the reagent by tyrosine and tryptophan residues present in protein.

3.3.1.5. **Reagents**

(i) Reagent A: 2% sodium carbonate in 0.1 % sodium hydroxide.

(ii) Reagent B: 0.5% copper sulphate (CuSO₄·5H₂O) in 1.0% sodium potassium tartarate.

(iii) Reagent C: Alkaline copper solution was prepared by mixing 50 ml of reagent A with 1.0 ml of reagent B. Reagent C was freshly prepared every time.

(iv) Reagent D: One part of phenol reagent + 2 parts of distilled water.

The phenol reagent (Folin-Ciocalteu reagent) was prepared by refluxing the mixture of 100 g sodium tungstate (Na₂Mo₄·2H₂O), 25 g sodium molybdate (Na₂Mo₄·2H₂O), 700 ml distilled water, 50 ml of 85% phosphoric acid and 100 ml concentrated hydrochloric acid in a 1.5 litre flask for 10 hr. The contents were then cooled and 150 g
lithium sulphate, 50 ml distilled water and few drops of bromine water were added to it. The mixture was boiled for 15 min (without condensing) to evaporate excess bromine. After cooling, the solution was diluted to one litre with distilled water and then filtered.

3.3.1.3. Procedure:

Fifty mg of bovine serum albumin (BSA) was dissolved in 100 ml glass distilled water. Different concentrations of BSA solution (100 to 500 µg) were pipetted in a series of test tubes, in duplicate, with a reagent blank as control. The volume was made up to 0.5 ml with distilled water. Five ml of reagent C was added to each tube with thorough mixing and kept for 10 min at room temperature. Half ml of reagent D was added to every tube and mixed immediately. The mixture was allowed to stand at room temperature for 30 min.

Appropriate aliquots of unknown samples were also processed as described above and colour was developed. The intensity of blue colour was read on aBio-Spectrocol at 660 nm and the concentration of protein in unknown samples was extrapolated with the help of standard curve.

3.3.2.0. Assay of Enzyme Activity:

The cellulase activity of the crude and partially purified enzyme was determined as carboxymethyl cellulase (CMCase) and filter paper enzyme (FPase) activities using carboxymethyl cellulose and filter paper as the substrates, respectively using dinitrophenylhydrazine acid (DNS) method (Miller, 1959).
3.3.3.2.1. Carboxymethyl Cellulase (CMCase or Cx) Activity

Reagents:

(i) 1.0% carboxymethyl cellulose was prepared in 0.05M citrate buffer pH 4.8.
(ii) 0.05M citrate buffer (pH 4.8).
(iii) Dinitrosalicylic acid (DNS) reagent: It was prepared by dissolving 1.0% (w/v) dinitrosalicylic acid, 0.2% (w/v) phenol and 0.05% (w/v) sodium sulphydrate in 1.0% (w/v) sodium hydroxide solution and stored in amber-coloured bottle.

Procedure: The CMCase assay was carried out in a final volume of 5.0 ml with a final concentration of 0.9% of carboxymethyl cellulose. 0.2 ml of enzyme solution was added to 4.5 ml of 1.0% carboxymethyl cellulose solution and 0.3 ml of 0.05M citrate buffer. After incubation of reaction mixture at 50°C for 120 min, 1.0 ml aliquot was assayed as given below:

3.3.3.2.2. Filter Paper Enzyme (Pase or Cx) Activity

Fifty mg of Whatman No.1 filter paper (1x6 cm) was added to a mixture of 0.4 ml enzyme solution and 0.6 ml of citrate buffer (pH 4.8). After incubation of reaction mixture at 50°C for 120 min, 1.0 ml aliquot was assayed.

The assay mixture in 1.0 ml quantity was added to 1.0 ml DNS reagent. The colour was developed by keeping the tubes for 15 min in a boiling water bath and after cooling, diluted to a total volume of 20 ml with distilled water. The intensity of red colour developed
was measured by Bisco-Spectrocol at 550 nm, running a blank
(without substrate) to correct for any reducing sugars present
in the enzyme preparation.

The standard solution was prepared by dissolving 1.0 mg:
glucose/ml distilled water. Aliquots of 0.1, 0.2, 0.3, 0.4, 0.5,
0.6, 0.7 and 0.8 ml were taken in duplicate test tubes and made up
finally to 1.0 ml with distilled water. The red colour was developed
with DNS reagent as described above.

The amount of reducing sugar released in both CMCase and
PFase assays was measured and concentration of glucose was extrapolated
from glucose standard curve.

The enzyme activity is expressed as µM of glucose released/
min/ml of enzyme solution.

3.3.1.3.0. Properties of Cellulase Enzyme

3.3.3.3.1. Effect of Incubation Time:

The reaction mixture containing substrate and partially
purified enzyme solution was incubated for a period of 30, 60, 90,
120 and 150 min and assayed for CMCase and PFase activities.

3.3.3.3.2. Effect of Enzyme Concentration:

Varying concentrations of the enzyme viz., 2.4, 4.8, 9.6,
14.4, 19.2, 24.0 and 29.8 µg of protein were incubated with the
substrate and the activity of the enzyme was determined.
3.3.3.3. Effect of Temperature of Incubation:

The reaction mixture was incubated at temperatures viz., 40, 50, 55, 60, 65, 70 and 75°C and assayed for activity to determine optimum temperature.

3.3.3.3.4. Effect of Heat Treatments:

The enzyme solution was heated in a water bath to temperatures viz., 40, 45, 50, 55, 60, 65, 70 and 75°C for 10 min. Then, the activity of the heat treated enzyme was determined.

3.3.3.3.5. Effect of pH:

The reaction mixtures containing substrate and buffers with varying pH viz., 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 were incubated with enzyme and the activity in each case was determined.

3.3.3.3.6. Effect of Substrate Concentration:

Cellulase was assayed at various substrate concentrations viz., 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) carboxymethyl cellulose and 25, 50, 75, 100, 125 and 150 mg filter paper. The Lineweaver-Burk plots were drawn to determine $K_m$ and $V_{max}$ values of the cellulase enzyme.

3.3.3.3.7. Effect of Metal Ions:

The enzyme was incubated for 10 min with various metal ion solutions viz., $Co^{2+}$, $Mg^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Ag^{+}$ and $Zn^{2+}$ at final concentration of $10^{-4}$, $10^{-3}$ and $10^{-2}$M and assayed for enzyme activity. For the preparation of the above metal ion
solutions, the following salts viz., cobalt sulphate, magnesium sulphate, nickel sulphate, copper sulphate, manganese chloride, ferrous sulphate, mercuric chloride, silver nitrate, zinc sulphate, respectively, were used.

3.3.3.8. Effect of Modulators:

The cellulase was incubated for 1.0 min with various effectors viz., 2-mercaptoethanol, ascorbic acid (vit-C), α-α'-dipyridyl, N-ethyl maleimide, β-hydroxyquinoline, cysteine-hydrochloride, glutathiones, iodoacetamide, sodium diethyl dithio carbonate, thiomersal, ethylene diamine tetracetic acid (EDTA), p-chloro mercuribenzoate (PCMBS), sodium thiosulphate (Na2S2O3), aluminium potassium sulphate (AlKSO4) and sodium tungstate (Na2WO4) at final concentrations of 10^-4, 10^-3 and 10^-2 M and assayed for enzyme activity.

3.4.0. CALCULATIONS AND STATISTICAL ANALYSIS:

3.4.1. Formula Used for Calculating the Yield Mycelium and Undegraded Bagasse Substrate in Biomass (SCP):

ax + by = c(x + y) .......... I

where, (x + y) is the weight of total recovered biomass comprising of mold mycelium (y) and undegraded bagasse substrate (x); a is the crude protein content of bagasse substrate (C.P. content of untreated bagasse was 2.01% and treated bagasse was 3.04%); b is the crude protein content of fungal mycelium only (41.6% for Asperillus terreus IAM); c is the crude protein content (d) of the total biomass recovered.
Illustrations:

Suppose \( x + y = 555 \) mg; \( a = 3.04 \)\( \% \), \( b = 41.64 \)\( \% \), \( c = 19.88 \)\( \% \).

Hence,

\[
ax + by = c (x + y)
\]

\[
= 3.04 x + 41.6 y = 19.88 \times 555
\]

\[x + y = 555\]

By multiplying \((x + y)\) by \(3.04\) and subtracting \(3.04 x + 3.04 y = 11033.4\)

\[
\begin{align*}
3.04 x + 3.04 y &= 11033.4 \\
- &- &- &- &- \\
\therefore 5.56 y &= 9346.20
\end{align*}
\]

\[
\therefore y = 239.89 \text{ mg and } x = 315.11 \text{ mg}
\]

3.4.2. Economic Evaluation of XP Process:

Three days fermentation trial with 1.0% alkali-treated bagasse
substrate in 8 litres modified Czapek's broth in both flask and fermentor
trials are compared below for their cost evaluation.

<table>
<thead>
<tr>
<th>1. Raw materials used</th>
<th>Flask trial (g)</th>
<th>Fermentor trial (l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagasse (150 g)</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Sodium hydroxide (120 g)</td>
<td>7.00</td>
<td>7.00</td>
</tr>
<tr>
<td>Czapek's broth ingredients:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium chloride (4g)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Magnesium sulphate (4g)</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (8g)</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td>Cornsteep liquor (150 g)</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Distilled water (8 litres)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Top water for washing treated bagasse (10 lit) and other purposes, 0.50
0.50

Inoculum of _A. terreus_ 0.50
0.50

### 2. Electricity consumption by Equipments

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Units</th>
<th>Unite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven</td>
<td>18</td>
<td>7.20</td>
</tr>
<tr>
<td>Autoclave</td>
<td>4</td>
<td>1.60</td>
</tr>
<tr>
<td>Shaker</td>
<td>23</td>
<td>9.20</td>
</tr>
<tr>
<td>Fermentor</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>3</td>
<td>1.20</td>
</tr>
</tbody>
</table>

The cost of electricity consumption was taken @ Re 0.40 per unit, as per the rates prevailing for domestic consumption.

### 3. Labour

<table>
<thead>
<tr>
<th>Role</th>
<th>Hr</th>
<th>Inr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab. Attendant</td>
<td>6</td>
<td>6.00</td>
</tr>
<tr>
<td>Lab. Technician</td>
<td>9</td>
<td>40.00</td>
</tr>
<tr>
<td>Supervisor</td>
<td>2</td>
<td>10.00</td>
</tr>
</tbody>
</table>

The pay of labour per month was taken @ Rs 350/-, Rs 800/- and Rs 1200/- for the laboratory attendant, technician and supervisor, respectively.

### 4. Depreciation of Equipment, Glassware and Building (Initial cost)

<table>
<thead>
<tr>
<th>Item</th>
<th>No(s)</th>
<th>Flask trial (Rs)</th>
<th>Fermentor trial (Rs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven</td>
<td>1</td>
<td>1,500</td>
<td>1,500</td>
</tr>
<tr>
<td>Autoclave</td>
<td>1</td>
<td>700</td>
<td>700</td>
</tr>
<tr>
<td>Containers</td>
<td>2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Flasks (500 ml)</td>
<td>80</td>
<td>1,200</td>
<td>-</td>
</tr>
<tr>
<td>Fermentor (10 lit)</td>
<td>1</td>
<td>-</td>
<td>80,000</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>1</td>
<td>25,000</td>
<td>25,000</td>
</tr>
<tr>
<td>Building</td>
<td>1</td>
<td>10,000</td>
<td>10,000</td>
</tr>
</tbody>
</table>

Total: 53,500 1,17,500
Depreciation of equipment and building was calculated on the basis of 10 to 50 years working life for 3 days use.

5. *Breakers of Flasks* (2.5% in flask trial) 20.50 -

6. Interest (on initial investment) for equipment, glasswares and building @ 15% 66.00 145.00

**Production Evaluation**

**SCP:** The SCP (product or residual bagasse and fungal biomass) with 20.57 to 24.54% crude protein was valued on the basis of groundnut cake (animal feed) available @ 0.50 per kg.

Cellulase: The cellulase obtained in SCP process was converted to Sigma cellulase units (Sigma, 1981) and valued @ 5 per 1000 units as per *Aspergillus niger* Grade 1 cellulase (Cat. No. C-7477).

### 3.4.3. Formula Used in Statistical Analysis:

\[
(i) \quad t = \frac{\bar{d} \times \sqrt{n}}{s}
\]

Where, \( s = \sqrt{\frac{\sum d_i^2 - (\sum d_i)^2}{n-1}} \)

and \( \bar{d} = \frac{(\sum d_i)}{n} \)

Where, \( s = \) standard deviation;

\( d_i = \) difference between treated and untreated bagasse parameters;

\( n = \) total number of variables (readings) and

\( \bar{d} = \) mean of differences
The differences between the variables viz., crude protein, protein recovery, CMCase activity, PMase activity and cellulose utilization of untreated and alkali-treated bagasse substrates have been statistically analyzed.

(ii) Correlation Coefficient (r)

\[
r = \frac{\bar{xy} - \frac{\bar{x}\bar{y}}{N}}{\sqrt{\left(\bar{x}^2 - \frac{(\bar{x})^2}{N}\right)\left(\bar{y}^2 - \frac{(\bar{y})^2}{N}\right)}}
\]

The correlation coefficient between the variables viz., time of incubation, crude protein content, protein recovery, CMCase activity, PMase activity and Cellulose utilization for the flask trials of untreated and alkali treated bagasse substrates and the 10 litre fermentor trial have been analyzed.

---------
 .....
  .....
   .....
    .....
     .....

4.1.0. PRE-TREATMENT OF SUGARCANE BAGASSE SUBSTRATE

The chemical composition of sugarcane bagasse before and after hot alkali treatment is presented in Table 1. The treatment of bagasse resulted in dry matter loss of 45%. The cellulose and water soluble carbohydrate contents of untreated bagasse were 30.83 and 11.19%, which after treatment resulted in loss of 7.85 and 67.71%, respectively.

4.2.0. ISOLATION AND IDENTIFICATION OF CELLULOLYTIC MOLDS

From the enrichment cultures, five cellulolytic mold cultures were isolated, purified and characterized (on the basis of their cultural, morphological and biochemical tests) as Aspergillus terreus G1, Aspergillus sydowi G2, G3, G4 and Fusarium sp. G5.

4.3.0. SCREENING OF CELLULOLYTIC MOLDS

The five cultures mentioned above and other mold cultures viz., Aspergillus terreus 1, Aspergillus niger, Trichoderma viride FNL and 6AGM, Myrothecium verrucaria, Fusarium moniliforme G9-1224 and Alternaria tenuis obtained from different laboratories were tested alone and in mixed cultures, for their crude protein content by growing on Czapek’s mineral medium supplemented with 1.0% carboxymethyl cellulose or untreated bagasse or treated bagasse substrates and the results are presented in Tables 2 - 5.
Table 1
Chemical Composition of Sugarcane Bagasse (Before and After Treatment) and Supplements (Dry Matter Basis)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Items</th>
<th>Moisture content (%)</th>
<th>Dry matter recovery (%)</th>
<th>Organic matter (%)</th>
<th>Carbon (%)</th>
<th>Crude protein (%)</th>
<th>Nitrogen (%)</th>
<th>C:N</th>
<th>Cellulose (%)</th>
<th>Water soluble carbohydrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Untreated bagasse</td>
<td>8.7</td>
<td>100.0</td>
<td>93.90</td>
<td>54.47</td>
<td>2.01</td>
<td>0.322</td>
<td>169.21</td>
<td>32.83</td>
<td>11.18</td>
</tr>
<tr>
<td>2.</td>
<td>Hot alkali-treated bagasse</td>
<td>7.5</td>
<td>55.0</td>
<td>97.00</td>
<td>56.73</td>
<td>3.04</td>
<td>0.486</td>
<td>116.71</td>
<td>51.67</td>
<td>6.56</td>
</tr>
<tr>
<td>3.</td>
<td>Per cent loss of bagasse</td>
<td></td>
<td>45.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>constituents after alkali</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Corn steep liquor</td>
<td>24.5</td>
<td>75.5</td>
<td>51.74</td>
<td>30.01</td>
<td>33.11</td>
<td>5.298</td>
<td>5.7:1</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>5.</td>
<td>Cattle urine</td>
<td>92.6</td>
<td>7.4</td>
<td>4.80*</td>
<td>2.78*</td>
<td>-</td>
<td>0.690*</td>
<td>4.6:1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are presented in per cent on fresh weight basis.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Organism</th>
<th>Fungal dry matter (mg/g substrate)</th>
<th>Crude protein (%)</th>
<th>Protein recovery (g/100g CM-cellulose)</th>
<th>Final pH of fermentation broth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aspergillus terreus 1</td>
<td>34</td>
<td>40.02</td>
<td>1.360</td>
<td>6.10</td>
</tr>
<tr>
<td>2.</td>
<td>Aspergillus terreus GH&lt;sub&gt;1&lt;/sub&gt;</td>
<td>50</td>
<td>41.60</td>
<td>2.050</td>
<td>6.10</td>
</tr>
<tr>
<td>3.</td>
<td>Aspergillus niger</td>
<td>52</td>
<td>25.51</td>
<td>1.326</td>
<td>5.92</td>
</tr>
<tr>
<td>4.</td>
<td>Aspergillus aryedi GH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>53</td>
<td>80.54</td>
<td>1.038</td>
<td>5.80</td>
</tr>
<tr>
<td>5.</td>
<td>Aspergillus aryedi GH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>47</td>
<td>25.18</td>
<td>1.183</td>
<td>5.70</td>
</tr>
<tr>
<td>6.</td>
<td>Aspergillus aryedi GH&lt;sub&gt;4&lt;/sub&gt;</td>
<td>50</td>
<td>26.99</td>
<td>1.349</td>
<td>5.82</td>
</tr>
<tr>
<td>7.</td>
<td>Trichoderma viride TVL</td>
<td>51</td>
<td>19.17</td>
<td>0.977</td>
<td>5.82</td>
</tr>
<tr>
<td>8.</td>
<td>Trichoderma viride 6A&lt;sub&gt;3&lt;/sub&gt;</td>
<td>48</td>
<td>22.92</td>
<td>1.100</td>
<td>6.05</td>
</tr>
<tr>
<td>9.</td>
<td>Fusarium sp. GH&lt;sub&gt;5&lt;/sub&gt;</td>
<td>60</td>
<td>24.51</td>
<td>1.590</td>
<td>6.37</td>
</tr>
<tr>
<td>10.</td>
<td>Aspergillus maniliforme G-1224</td>
<td>68</td>
<td>18.60</td>
<td>1.266</td>
<td>5.70</td>
</tr>
<tr>
<td>11.</td>
<td>Hypothecium vernicaria</td>
<td>54</td>
<td>22.42</td>
<td>1.210</td>
<td>5.82</td>
</tr>
<tr>
<td>12.</td>
<td>Alternaria tenuis</td>
<td>74</td>
<td>20.04</td>
<td>1.432</td>
<td>6.00</td>
</tr>
</tbody>
</table>

Initial pH 5.4
<table>
<thead>
<tr>
<th>No.</th>
<th>Organisms</th>
<th>Fungal dry matter (mg/g substrate)</th>
<th>Crude protein (g)</th>
<th>Protein recovery (g/100g CN-cellulose)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>A. terreus</em> + <em>Euphotium verrucaria</em></td>
<td>30</td>
<td>22.10</td>
<td>0.84</td>
<td>5.90</td>
</tr>
<tr>
<td>2.</td>
<td><em>A. terreus</em> + <em>Aspergillus sp. 34</em></td>
<td>40</td>
<td>24.50</td>
<td>0.98</td>
<td>6.05</td>
</tr>
<tr>
<td>3.</td>
<td><em>A. terreus</em> + <em>Alternaria tenius</em></td>
<td>44</td>
<td>31.63</td>
<td>1.26</td>
<td>6.09</td>
</tr>
<tr>
<td>4.</td>
<td><em>Euphotium verrucaria</em> + <em>Aspergillus sp. 34</em></td>
<td>44</td>
<td>22.27</td>
<td>0.98</td>
<td>5.95</td>
</tr>
<tr>
<td>5.</td>
<td><em>Euphotium verrucaria</em> + <em>Alternaria tenius</em></td>
<td>54</td>
<td>18.14</td>
<td>0.98</td>
<td>6.00</td>
</tr>
<tr>
<td>6.</td>
<td><em>Aspergillus sp. 34</em> + <em>Alternaria tenius</em></td>
<td>52</td>
<td>16.15</td>
<td>0.84</td>
<td>5.95</td>
</tr>
<tr>
<td>S. No.</td>
<td>Organism</td>
<td>Biomass dry matter (fungal + mycelium + unutilised bagasse) (g/wt g bagasse)</td>
<td>Crude protein (%)</td>
<td>Protein recovery (g/100g substrate)</td>
<td>Final pH of fermentation broth</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-------------------</td>
<td>-------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>1</td>
<td><em>Aspergillus terreus</em> 1</td>
<td>750</td>
<td>5.10</td>
<td>3.86</td>
<td>6.70</td>
</tr>
<tr>
<td>2</td>
<td><em>Aspergillus terreus G3</em></td>
<td>750</td>
<td>5.40</td>
<td>4.05</td>
<td>6.55</td>
</tr>
<tr>
<td>3</td>
<td><em>Aspergillus niger</em></td>
<td>727</td>
<td>4.18</td>
<td>3.04</td>
<td>6.55</td>
</tr>
<tr>
<td>4</td>
<td><em>Aspergillus sydowi G1</em></td>
<td>749</td>
<td>3.70</td>
<td>2.77</td>
<td>6.55</td>
</tr>
<tr>
<td>5</td>
<td><em>Aspergillus sydowi G2</em></td>
<td>930</td>
<td>2.41</td>
<td>2.24</td>
<td>6.05</td>
</tr>
<tr>
<td>6</td>
<td><em>Aspergillus sydowi G3</em></td>
<td>763</td>
<td>3.00</td>
<td>2.29</td>
<td>6.00</td>
</tr>
<tr>
<td>7</td>
<td><em>Trichoderma viride FVL</em></td>
<td>770</td>
<td>3.84</td>
<td>2.96</td>
<td>6.35</td>
</tr>
<tr>
<td>8</td>
<td><em>Trichoderma viride G1</em></td>
<td>693</td>
<td>4.54</td>
<td>3.17</td>
<td>6.50</td>
</tr>
<tr>
<td>9</td>
<td><em>Aspergillus sp. G3</em></td>
<td>903</td>
<td>3.04</td>
<td>2.74</td>
<td>6.55</td>
</tr>
<tr>
<td>10</td>
<td><em>Aspergillus moniliforme G1-1224</em></td>
<td>901</td>
<td>2.45</td>
<td>2.21</td>
<td>6.20</td>
</tr>
<tr>
<td>11</td>
<td><em>Myrothecium verrucaria</em></td>
<td>702</td>
<td>4.47</td>
<td>3.14</td>
<td>6.05</td>
</tr>
<tr>
<td>12</td>
<td><em>Alternaria tenuissima</em></td>
<td>890</td>
<td>2.84</td>
<td>2.53</td>
<td>6.35</td>
</tr>
<tr>
<td>S. No.</td>
<td>Organism</td>
<td>Biomass dry matter (Fungal mycelium + unutilized bagasse) (g)</td>
<td>Crude protein (g/100g substrate)</td>
<td>Protein recovery (g/100g substrate)</td>
<td>Final pH of fermentation broth</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------</td>
<td>---------------------------------------------------------------</td>
<td>----------------------------------</td>
<td>-------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Asperillus terreus 1</td>
<td>5.39</td>
<td>18.17</td>
<td>9.78</td>
<td>6.30</td>
</tr>
<tr>
<td>2.</td>
<td>Asperillus terreus G1</td>
<td>555</td>
<td>19.88</td>
<td>11.03</td>
<td>6.40</td>
</tr>
<tr>
<td>3.</td>
<td>Asperillus niger</td>
<td>608</td>
<td>9.54</td>
<td>6.05</td>
<td>6.30</td>
</tr>
<tr>
<td>4.</td>
<td>Asperillus sydowi G2</td>
<td>687</td>
<td>8.75</td>
<td>6.01</td>
<td>6.20</td>
</tr>
<tr>
<td>5.</td>
<td>Asperillus sydowi G3</td>
<td>706</td>
<td>7.09</td>
<td>5.57</td>
<td>6.35</td>
</tr>
<tr>
<td>6.</td>
<td>Asperillus sydowi G3</td>
<td>830</td>
<td>5.39</td>
<td>4.47</td>
<td>5.90</td>
</tr>
<tr>
<td>7.</td>
<td>Trichoderma viride TV</td>
<td>702</td>
<td>8.75</td>
<td>6.14</td>
<td>5.60</td>
</tr>
<tr>
<td>8.</td>
<td>Trichoderma viride 6 AGM</td>
<td>725</td>
<td>7.98</td>
<td>5.79</td>
<td>6.00</td>
</tr>
<tr>
<td>9.</td>
<td>Rasamia sp. G1</td>
<td>681</td>
<td>10.93</td>
<td>7.44</td>
<td>6.55</td>
</tr>
<tr>
<td>10.</td>
<td>Rasamia moniliforme G-1224</td>
<td>771</td>
<td>6.23</td>
<td>4.80</td>
<td>6.30</td>
</tr>
<tr>
<td>11.</td>
<td>Myrothecium verrucaria</td>
<td>701</td>
<td>7.65</td>
<td>5.50</td>
<td>6.90</td>
</tr>
<tr>
<td>12.</td>
<td>Alternaria tenuis</td>
<td>797</td>
<td>7.00</td>
<td>5.58</td>
<td>6.25</td>
</tr>
</tbody>
</table>
The biomass production, crude protein content and protein recovery of the different molds grown on carboxymethyl cellulose is presented in Table 2. It was found that *Aspergillus terreus* 1 and G3 had the highest crude protein content of 40.02 and 41.60% as compared to other molds having 18.62 - 26.99% crude protein. But *Alternaria tenius* and *Bacillus moniliforme* G1-1224 gave higher biomass yield of 74 and 69 mg/g substrate as compared to 34 and 50 mg/g substrate by *Aspergillus terreus* 1 and G3, respectively. However, *Aspergillus terreus* G3 gave the highest crude protein recovery of 2.08g/100g carboxymethyl cellulose as compared to other molds.

The effect of combination of mixed mold cultures on SCP production using carboxymethyl cellulose substrate is presented in Table 3. It was observed that the various combinations of individual cultures of *Aspergillus terreus* 1, *Bacillus subtilis* var. *terreus*, *Bacillus sp.* G5  and *Alternaria tenius* did not enhance the protein recovery, biomass yield and crude protein as compared to individual cultures.

The biomass yield, crude protein content and protein recovery of the molds grown on untreated and alkali-treated sugarcane bagasse substrates (the product of residual biomass plus fungal mycelia) are presented in Tables 4 and 5. All the cellulolytic molds had higher crude protein content in biomass with treated bagasse substrate as compared to untreated one. The biomass produced by fermenting with untreated and treated bagasse substrates, *Aspergillus terreus* G3 had the maximum crude protein content of 5.40 and 19.88% and the maximum protein recovery of 4.05 and 11.03g/100g substrate, respectively.
The biomass crude protein per cent with untreated and treated bagasse substrates ranged from 2.41 - 5.40 and 5.39 - 10.39, respectively. The minimum biomass crude protein content was obtained with Aspergillus sydowi G\textsuperscript{4} and G\textsuperscript{6}, on both treated and untreated bagasse substrates. However, lowest protein recovery of 2.21 and 4.47 g/100g substrate was obtained with A. oryzae, G\textsuperscript{2}-1224 and A. sydowi G\textsuperscript{4} with untreated and treated bagasse, respectively.

The change in pH of the culture filtrates after 7 days fermentation of the basal medium supplemented with 1.0% carboxymethyl cellulose, untreated and treated bagasse is also presented in Tables 2 - 5. The initial pH of the fermenting slurry was 5.40 and the pH of the slurry filtrate after 7 days fermentation with different mold cultures changed to 5.70 - 6.37 with carboxymethyl cellulose, 6.20 - 6.85 with untreated bagasse and 5.60 - 6.90 with treated bagasse.

4.4.0. EFFECT OF CULTURAL FACTOR ON SCP AND CELLULASE PRODUCTION

Among the mold isolates tested on cellulose substrates for their biomass yield, crude protein content and protein recovery after 7 days incubation, Aspergillus terreus G\textsuperscript{H} was found to be the most efficient. Hence, A. terreus G\textsuperscript{H} was used for optimizing the cultural conditions for its growth.

4.4.1. EFFECT OF HYDROGEN ION CONCENTRATION

The effect of different hydrogen ion concentrations of the fermenting Casper's broth on the growth of A. terreus G\textsuperscript{H} with treated bagasse substrate on the crude protein content, total protein recovery, biomass
yield and CMCase and TPase activities is presented in Table 6.

It was noted that the highest biomass crude protein content of 20.7% was obtained on the 7th day at pH 4.0, as compared to the protein content of 3.04% in the treated bagasse control. However, the crude protein content of biomass obtained at pH 4.5 to 5.5, was slightly less ranging from 18.2 to 19.1%. The protein recovery was 8.5 to 9.4 g/100 g bagasse at a pH range of 4.0-5.5. The CMCase and TPase activities were maximum 1.4, 0.60 and 0.07 units/ml at pH 4.0, respectively.

4.4.2 Effect of Incubation Temperature:

The effect of incubation temperature on crude protein content and recovery on the 7th day of fermentation is presented in Table 7. At 30°C, the biomass crude protein content of 19.8% was obtained, which was comparable with the crude protein content obtained at 25°C (18.5%). The biomass protein recovery was highest at 25°C (9.8 g/100 g bagasse), but the biomass protein recoveries at 25 and 30°C were comparable (9.8 and 9.2 g/100 g bagasse). The CMCase and TPase activities at 30°C were 0.6 and 0.05 units/ml, respectively.

4.4.3 Effect of Shaking:

The crude protein content in stationary culture was 6.5% only, whereas, higher crude protein content of 19.4% was found in continuously shaken cultures on the 7th day of incubation (Table 8). The crude protein content of intermittently shaken cultures was 15.4%. Even though the biomass crude protein content in intermittently shaken culture was less than continuously shaken culture, the total biomass recovery was comparable (8.7 and 8.8 g/100 g bagasse). The CMCase
Table 6:

Effect of pH on xCP Production and Cellulase Activity by *Aspergillus terrus* GM₁

<table>
<thead>
<tr>
<th>S. No.</th>
<th>pH</th>
<th>Biomass (mycelium + unfermented bagasse) (mg/50 ml broth)</th>
<th>Crude protein (%)</th>
<th>Protein recovery (g/10 g bagasse)</th>
<th>β-casease (units/ml)</th>
<th>β-case (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2.0</td>
<td>330</td>
<td>6.0</td>
<td>4.5</td>
<td>0.4</td>
<td>0.05</td>
</tr>
<tr>
<td>2.</td>
<td>3.0</td>
<td>251</td>
<td>15.4</td>
<td>7.7</td>
<td>0.4</td>
<td>0.05</td>
</tr>
<tr>
<td>3.</td>
<td>3.5</td>
<td>265</td>
<td>16.9</td>
<td>9.0</td>
<td>0.4</td>
<td>0.06</td>
</tr>
<tr>
<td>4.</td>
<td>4.0</td>
<td>221</td>
<td>27.7</td>
<td>9.2</td>
<td>0.6</td>
<td>0.07</td>
</tr>
<tr>
<td>5.</td>
<td>4.5</td>
<td>234</td>
<td>19.1</td>
<td>8.9</td>
<td>0.6</td>
<td>0.06</td>
</tr>
<tr>
<td>6.</td>
<td>5.0</td>
<td>229</td>
<td>18.5</td>
<td>8.5</td>
<td>0.6</td>
<td>0.05</td>
</tr>
<tr>
<td>7.</td>
<td>5.5</td>
<td>258</td>
<td>18.2</td>
<td>9.4</td>
<td>0.4</td>
<td>0.04</td>
</tr>
<tr>
<td>8.</td>
<td>6.0</td>
<td>267</td>
<td>15.4</td>
<td>8.2</td>
<td>0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>9.</td>
<td>7.0</td>
<td>247</td>
<td>11.9</td>
<td>5.9</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>10.</td>
<td>8.0</td>
<td>353</td>
<td>7.5</td>
<td>5.5</td>
<td>0.1</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 7

Effect of Temperature on ACT Production and Cellulase Activity by \textit{A. laguniae} G3

<table>
<thead>
<tr>
<th>S. No</th>
<th>Temperature (°C)</th>
<th>Biomass (mg/mycelium + unfermented bagasse)</th>
<th>Crude Protein Recovery (g/100g bagasse)</th>
<th>CMCase (units/ml)</th>
<th>Pase (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>20</td>
<td>333</td>
<td>9.7</td>
<td>6.5</td>
<td>0.3</td>
</tr>
<tr>
<td>2.</td>
<td>25</td>
<td>265</td>
<td>19.5</td>
<td>9.8</td>
<td>0.5</td>
</tr>
<tr>
<td>3.</td>
<td>30</td>
<td>232</td>
<td>19.8</td>
<td>9.2</td>
<td>0.6</td>
</tr>
<tr>
<td>4.</td>
<td>35</td>
<td>313</td>
<td>8.4</td>
<td>5.3</td>
<td>0.2</td>
</tr>
<tr>
<td>5.</td>
<td>40</td>
<td>358</td>
<td>5.2</td>
<td>3.7</td>
<td>0.2</td>
</tr>
<tr>
<td>S. No.</td>
<td>Extent of shaking</td>
<td>Biomass (mycelium + unfermented bagasse) (mg/50 ml broth)</td>
<td>Crude Protein recovery (g/100g bagasse)</td>
<td>ECase (units/ml)</td>
<td>Fase (units/ml)</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------</td>
<td>----------------------------------------------------------</td>
<td>----------------------------------------</td>
<td>-----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>1.</td>
<td>No shaking</td>
<td>376</td>
<td>6.5</td>
<td>4.9</td>
<td>0.05</td>
</tr>
<tr>
<td>2.</td>
<td>Intermittent shaking</td>
<td>236</td>
<td>15.4</td>
<td>8.8</td>
<td>0.40</td>
</tr>
<tr>
<td>3.</td>
<td>Continuous shaking</td>
<td>224</td>
<td>19.4</td>
<td>8.7</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 8

Effect of Shaking on SCP Production and Cellulase Activity by \( \Delta \) terreus 331.
and $\Phi$ase activity were highest in continuously shaken cultures (0.7 and 0.06 units/ml).

4.4.4. Effect of Fermentation

The crude protein content of biomass was maximum (19.4%) with 1/3 (v/v) broth, whereas, it was reduced to 16.7 and 15.6% in 2/5 and 3/5 (v/v) broth cultures in 250 ml Erlenmeyer flasks during 7 days fermentation (Table 9). As the volume of broth was increased in the flask, there was a considerable decrease in crude protein content of biomass. However, a higher crude protein recovery was obtained in cultures grown with 2/5 (v/v) than 1/5 (v/v) broth cultures. The highest $\Phi$ case activity of 0.7 units/ml was obtained with 1/5 (v/v) broth, whereas, it was less (0.06 units/ml) with 2/5 and 3/5 (v/v) broth cultures. The $\Phi$ase activity was 0.05 units/ml with 1/5 (v/v) broth, whereas, it was 0.04 units/ml with 2/5 and 3/5 (v/v) broth cultures.

4.4.5. Effect of Inoculum Size

The effect of inoculum size (v/v) of a week-old culture of $A.\ indigold$ on crude protein recovery and cellulase production is presented in Table 10. There was a concurrent increase in crude protein content of biomass and protein recovery, when the inoculum size was increased from 1.0 to 6.0%. The crude protein content of biomass was only 13.6% with 1.0% inoculum, whereas, with 5.0% inoculum, it was higher (20.1%). With 6.0% inoculum, the biomass crude protein was 20.2%. The net protein recovery was maximum
Table 9

Effect of Aeration on XT Production and Cellulase Activity by *A. terreus* G-1

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Volume (ml) of broth/250 ml capacity flask</th>
<th>Biomass (mycelium + unfermented protein (mg/50 ml broth))</th>
<th>Crude Protein recovery (%)</th>
<th>Protein (g/100g bagasse)</th>
<th>CMCase (units/ml)</th>
<th>FPase (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>50</td>
<td>224</td>
<td>19.4</td>
<td>8.7</td>
<td>0.7</td>
<td>0.05</td>
</tr>
<tr>
<td>2.</td>
<td>100</td>
<td>270</td>
<td>16.7</td>
<td>9.1</td>
<td>0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>3.</td>
<td>150</td>
<td>279</td>
<td>15.6</td>
<td>8.7</td>
<td>0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>S. No.</td>
<td>Inoculum size</td>
<td>Biomass (mg/50 ml broth)</td>
<td>Crude protein (g/L)</td>
<td>Protein recovery (%)</td>
<td>α-amylase (units/ml)</td>
<td>x-amylase (units/ml)</td>
</tr>
<tr>
<td>--------</td>
<td>---------------</td>
<td>--------------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>1.</td>
<td>1.0</td>
<td>237</td>
<td>13.6</td>
<td>7.8</td>
<td>7.7</td>
<td>0.6</td>
</tr>
<tr>
<td>2.</td>
<td>2.0</td>
<td>296</td>
<td>15.6</td>
<td>9.2</td>
<td>9.1</td>
<td>0.7</td>
</tr>
<tr>
<td>3.</td>
<td>3.0</td>
<td>275</td>
<td>17.0</td>
<td>9.9</td>
<td>9.7</td>
<td>0.7</td>
</tr>
<tr>
<td>4.</td>
<td>4.0</td>
<td>269</td>
<td>20.0</td>
<td>10.9</td>
<td>10.5</td>
<td>0.7</td>
</tr>
<tr>
<td>5.</td>
<td>5.0</td>
<td>277</td>
<td>20.1</td>
<td>11.2</td>
<td>10.8</td>
<td>0.5</td>
</tr>
<tr>
<td>6.</td>
<td>6.0</td>
<td>273</td>
<td>20.2</td>
<td>11.0</td>
<td>10.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>
(10.8g/100g bagasse) with 5.0% inoculum. However, CMCase and BMPase activities were slightly higher (1.6-0.7 and 0.07 units/ml) with 1.0 to 4.0% than 5.0 and 6.0% inoculum.

4.5.0. EFFECT OF NUTRITIONAL FACTORS ON SCP AND CELLULASE PRODUCTION

4.5.1. Charging Rate of Alkali-Treated Bagasse Substrate:

The effect of different levels of treated bagasse substrate (0.5 - 4.0%, w/v) on crude protein content, protein recovery of biomass and cellulase activity is presented in Table 11. At 0.5% substrate concentration, the biomass crude protein slightly increased from initial content of 3.04 to 12.1%. With 1.0% treated bagasse substrate, the crude protein content of 20.0%, protein recovery of 11.5x/10x bagasse, CMCase and BMPase activities of 0.6 and 0.75 units/ml were obtained. There was a concomitant decrease in protein content and cellulase activity of A. niger strain 34 by increasing the charging rate of bagasse from 1.0% level.

4.5.2.0. EFFECT OF NITROGEN SOURCES:

The effect of different nitrogen sources viz., ammonium sulphate, ammonium nitrate, cattle urine, urea, ammonium iron sulphate, ammonium chloride, sodium nitrate and cornsteep liquor 3 250-600 mg /litre broth on biomass yield, crude protein content, protein recovery, CMCase and BMPase activities on the 7th day of fermentation is presented in Table 12 and Figs. 5-1.
Table 11

**Effect of Varying Concentration of Alkali-Treated Bagasse Substrate on Protein and Cellulase Production by A. terreus**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of treated bagasse (%)</th>
<th>Biomass (mycelium + unfermented bagasse) (mg/50 ml broth)</th>
<th>Crude protein recovery (%)</th>
<th>Protein activity (g/10% bagasse)</th>
<th>CMCase activity (units/ml)</th>
<th>FFase activity (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.5</td>
<td>112</td>
<td>12.1</td>
<td>5.4</td>
<td>0.50</td>
<td>0.05</td>
</tr>
<tr>
<td>2.</td>
<td>1.0</td>
<td>288</td>
<td>27.0</td>
<td>11.5</td>
<td>0.60</td>
<td>0.05</td>
</tr>
<tr>
<td>3.</td>
<td>1.5</td>
<td>404</td>
<td>13.1</td>
<td>8.5</td>
<td>0.40</td>
<td>0.04</td>
</tr>
<tr>
<td>4.</td>
<td>2.0</td>
<td>604</td>
<td>10.6</td>
<td>7.3</td>
<td>0.40</td>
<td>0.03</td>
</tr>
<tr>
<td>5.</td>
<td>2.5</td>
<td>890</td>
<td>9.7</td>
<td>6.9</td>
<td>0.40</td>
<td>0.03</td>
</tr>
<tr>
<td>6.</td>
<td>3.0</td>
<td>1053</td>
<td>8.6</td>
<td>6.0</td>
<td>0.40</td>
<td>0.03</td>
</tr>
<tr>
<td>7.</td>
<td>4.0</td>
<td>1368</td>
<td>8.2</td>
<td>5.6</td>
<td>0.39</td>
<td>0.03</td>
</tr>
</tbody>
</table>
4.5.2.1. **Crude Protein Content and Biomass Yield**

There was a gradual increase in biomass crude protein content, when the concentration of ammonium sulphate was increased from 250 to 350 mg N and then declined at 400 mg N followed by a slight decrease up to 600 mg N/litre broth. The highest crude protein content of 9.7% was obtained with ammonium sulphate at a concentration of 350 mg N/litre broth. The biomass yield ranged between 71.2 - 76.0 g/100g bagasse (Table 12).

Addition of ammonium nitrate yielded the biomass crude protein content in the range of 7.1 - 7.8% and the optimum concentration for maximum crude protein production was 400 mg N/litre broth. The biomass yield ranged between 70.2 - 74.4 g/100g bagasse.

The crude protein content of biomass with *A. fumigatus* 3N, was in the range of 11.8 - 17.8%, when the cattle urine concentration of the basal medium was increased from 250-600 mg N/litre broth. The highest crude protein content of 17.8% was obtained with cattle urine nitrogen at a concentration of 600 mg N. The biomass yield ranged between 61.6 - 69.4 g/100g bagasse.

There was a gradual increase in crude protein content from 16.1 to 19.2%, when the urea was increased in the basal medium from 250-550 mg N/litre broth. Then, there was a gradual decrease in crude protein content of biomass with increased concentration of 400-600 mg N. Hence, urea produced a higher crude protein content of 19.2% with an optimum dose of 550 mg N/litre broth. The biomass yield ranged between 57.6 - 65.2 g/100g bagasse (Fig. 5).
FIG. 5. EFFECT OF DIFFERENT NITROGEN SOURCES ON CRUDE PROTEIN CONTENT OF A. terreus GN1 WITH ALKALI TREATED BAGASSE SUBSTRATE.
Table 12

**Effect of Different Nitrogen Sources on Biomass Production by *A. terrae* GN**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of nitrogen source (mg N/lit broth)</th>
<th>Biomass yield with different nitrogen sources (mg /50 ml broth)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ammonium sulphate</td>
<td>Ammonium nitrate</td>
</tr>
<tr>
<td>1.</td>
<td>250</td>
<td>377</td>
</tr>
<tr>
<td>2.</td>
<td>300</td>
<td>365</td>
</tr>
<tr>
<td>3.</td>
<td>350</td>
<td>360</td>
</tr>
<tr>
<td>4.</td>
<td>400</td>
<td>356</td>
</tr>
<tr>
<td>5.</td>
<td>450</td>
<td>358</td>
</tr>
<tr>
<td>6.</td>
<td>500</td>
<td>362</td>
</tr>
<tr>
<td>7.</td>
<td>600</td>
<td>372</td>
</tr>
</tbody>
</table>
With the use of ammonium iron sulphate as nitrogen source, there was a slight increase in crude protein content over control. Further, the crude protein content of biomass decreased by increasing the ammonium iron sulphate concentration from 250-600 mg N/litre broth (Fig. 6). The biomass yield ranged between 77.4 - 87.8 g/100g bagasse.

The crude protein content of biomass with ammonium chloride as a nitrogen source ranged from 6.6 - 7.3% as compared to 3.04% in treated bagasse control. The maximum crude protein content of 7.3% was obtained with 400 mg N/litre broth. The biomass yield ranged between 69.6 - 72.2 g/100g bagasse.

There was an increase in crude protein content of biomass from 13.8 - 20.8% when the concentration of sodium nitrate was increased from 250-500 mg N/litre broth, which was approximately seven folds higher than that of control treated bagasse. However, when the concentration was further increased beyond 500 mg N, there was a steep decline in crude protein content from 20.8 to 13.75%. The maximum crude protein content of 20.8% was obtained with sodium nitrate at a concentration of 500 mg N/litre broth. The biomass yield ranged between 58.0 - 61.4 g/100g bagasse.

With cornsteep liquor supplementation to basal medium as nitrogen source, the maximum crude protein content (32.8%) of biomass was obtained with 600 mg N, which was approximately eleven folds higher to that of control treated bagasse. The biomass yield ranged between 52.2 - 67.4 g/100g bagasse.
FIG. 6. EFFECT OF DIFFERENT NITROGEN SOURCES ON CRUDE PROTEIN CONTENT OF *A. terreus* GN 1 WITH ALKALI-TREATED BAGASSE SUBSTRATE.
4.5.2.2. **Protein Recovery**

The effect of ammonium sulphate, ammonium nitrate, cattle urine and urea nitrogen on protein recovery has been shown in Fig. 7. The higher protein recovery of 7.4 g/100g bagasse was obtained with ammonium sulphate at a concentration of 250 mg N/litre broth. At higher concentration (500 - 600 mg N) of ammonium sulphate, the protein recovery declined and became constant (3.9 g/100g bagasse).

The protein recovery was in the range of 8.0 - 11.5 g/100g bagasse, when the cattle urine nitrogen of the basal medium was increased from 250 - 600 mg N/litre broth. Addition of urea produced a higher protein recovery of 11.5 g/100g bagasse with an optimum dose of 350 mg N/litre broth. There was a decrease in recovery of protein, when urea nitrogen was increased beyond the optimal level.

The effect of ammonium iron sulphate, ammonium chloride, sodium nitrate and cornsteep liquor nitrogen on recovery of crude protein is shown in Fig. 8. With ammonium iron sulphate, there was only a slight increase in protein recovery as compared to control. The decrease in recovery was noted with increase in ammonium iron sulphate concentration from 250 - 600 mg N/litre broth. The higher protein recovery of 5.2g/100g bagasse was obtained with an optimum dose of ammonium chloride at 400 mg N/litre broth. There was high protein recovery ranging from 8.2 - 12.8 g/100g bagasse, which is roughly four times higher than the control, when the concentration of sodium nitrate was increased from 250-500 mg N/litre broth. However, when the nitrogen concentration was further increased beyond 500 mg N, there was a steep decline in recovery from 12.8 to 8.0 g/100g bagasse.
Fig. 7. Effect of different nitrogen sources on protein recovery from A. terreus GN 1 with alkali-treated bagasse substrate.
FIG. 8. EFFECT OF DIFFERENT NITROGEN SOURCES ON PROTEIN RECOVERY FROM A. terreus GN 1 WITH ALKALI TREATED BAGASSE SUBSTRATE.
The maximum protein recovery of 22.2 g/100g bagasse was noted with the optimum cornsteep liquor concentration of 600 mg N/litre broth. With the cornsteep liquor supplementation to basal medium as nitrogen source, the maximum crude protein recovery in biomass obtained was approximately seven and half fold higher to that of control bagasse.

4.5.2.3. Carboxymethyl Cellulase Activity:

The effect of ammonium sulphate, ammonium nitrate, cattle urine and urea on CMCase activity has been shown in Fig. 9. The maximum CMCase activity of 0.2 units/ml with an optimum dose of 350 mg N ammonium sulphate/litre broth was obtained.

A higher CMCase activity of 0.3 units/ml with 250 mg N ammonium nitrate/litre broth was observed. The activity of the enzyme declined further with higher concentrations of ammonium nitrate. The CMCase activity ranged from 0.3 - 0.5 units/ml, when the basal medium was supplemented with cattle urine in the range of 250 - 600 mg N/litre broth. Use of urea nitrogen produced a higher CMCase activity of 0.7 units/ml with an optimum dose of 350 mg N/litre broth. However, the CMCase activity declined up to 0.51 units/ml, when the urea nitrogen concentration was increased up to 600 mg N.

The effect of ammonium iron sulphate, ammonium chloride, sodium nitrate and cornsteep liquor on CMCase activity is shown in Fig.10. The CMCase activity with ammonium iron sulphate, was very low and was in the range of 0.04 - 0.26 units/ml. There was a decrease in activity by increasing the concentration of ammonium iron sulphate from 250 - 600 mg N/litre broth. The CMCase activity
Fig. 9. Effect of different nitrogen sources on carboxymethyl cellulase (CMCase) production by A. terreus GN1 with alkali-treated bagasse substrate.
FIG. 10. EFFECT OF DIFFERENT NITROGEN SOURCES ON CARBOXYMETHYL CELLULASE (CmCase) PRODUCTION BY A. terreus GN 1 WITH ALKALI-TREATED BAGASSE SUBSTRATE.
with all concentrations of ammonium chloride was in the range of 0.45 - 0.5 units/ml. The activity of the enzyme remained constant with all the concentrations of ammonium chloride tested. The increase in the activity of CMCase was in the range of 0.4 - 0.6 units/ml up to 500 mg N of sodium nitrate/litre broth, then, the activity declined to 0.5 units/ml at the concentration of 600 mg of sodium nitrate. The CMCase activity of Aspergillus terreus GM, grown in the medium supplemented with cornsteep liquor nitrogen was in the range of 0.9 - 1.1 units/ml, when the concentration was increased from 250 - 600 mg N/litre broth.

4.5.2.4. Filter Paper Enzyme Activity:

The effect of different nitrogen sources viz., ammonium sulphate, ammonium nitrate, cattle urine and urea on Fase activity is shown in Fig. 11. The Fase activity obtained with ammonium sulphate nitrogen was very low at all the concentrations tried and was in the range of 0.007 - 0.01 units/ml. Maximum Fase activity of 0.01 units/ml was obtained with 0.9 - 400 mg N and then declined (0.007 units/ml) up to 600 mg N/litre broth.

The Fase activity was very low (0.01 unit/ml) at all the concentrations of ammonium nitrate nitrogen tried. The Fase activity with cattle urine was quite high and was in the range of 0.02 - 0.03 units/ml. A higher Fase activity of 0.07 units/ml was obtained with an optimum dose of urea at the concentration of 350 mg N/litre broth.
FIG. 11. EFFECT OF DIFFERENT NITROGEN SOURCES ON FILTER PAPER ENZYME (FPase) PRODUCTION BY A. terreus GN 1 WITH ALKALI-TREATED BAGASSE SUBSTRATE.
The effect of ammonium iron sulphate, ammonium chloride, sodium nitrate and corn steep liquor on PPase activity is shown in Fig. 12. Unlike CMCase activity of \( \text{A. terreus} \), the PPase activity increased from 0.01 - 0.03 units/ml, when the concentration of ammonium iron sulphate nitrogen was increased from 250 - 600 mg N/litre broth. The PPase activity was not appreciably high with ammonium chloride nitrogen source and was in the range of 0.02 - 0.03 units/ml at 250 - 600 mg N/litre broth. The enzyme activity decreased from 0.03 to 0.02 units/ml, when the concentration was increased beyond 450 mg N/litre broth.

The PPase activity increased from 0.025 to 0.037 units/ml up to 500 mg N of sodium nitrate and then declined to 0.03 units/ml, when the concentration was increased up to 600 mg N/litre broth. The PPase activity was highest with the addition of corn steep liquor as a nitrogen source for the growth of \( \text{A. terreus} \), and was in the range of 0.07 - 0.09 units/ml at 250 - 600 mg N/litre broth.

4.6.0. FERMENTATION OF UNTRATED AND ALKALI-TREATED BAGASSE SUBSTRATE BY \( \text{A. terreus} \) AT DIFFERENT TIME INTERVALS

The effect of fermentation of 1.0% untreated or treated bagasse substrate by \( \text{A. terreus} \) in flask trial with 600 mg N of corn steep liquor/litre broth, under optimum cultural conditions and at different time intervals, on cellulase enzyme activity, utilisation of sugarcane bagasse cellulose, protein production and water soluble carbohydrate content is presented in Figs. 15 - 26.
FIG. 12: EFFECT OF DIFFERENT NITROGEN SOURCES ON FILTER PAPER ENZYME PRODUCTION BY A. terreus GN1 WITH ALKALI-TREATED BAGASSE SUBSTRATE.
4.6.1.1. *Cellulase (CMCase and PMase) Enzyme Production*

The effect of untreated bagasse substrate on the growth and enzyme activity of *A. terreus* GA, during seven days fermentation is shown in Fig. 15. The CMCase activity in culture filtrate was in the range of 0.26 - 0.55 units/ml. The cellulase activity was zero during first 24 hr and increased after second day. The PMase activity ranged from 0.009 to 0.027 units/ml and corresponded with CMCase activity. The maximum CMCase activity of 0.55 units/ml and PMase activity of 0.027 units/ml were obtained on the 7th day of fermentation.

4.6.1.2. *Cellulose Utilization and Crude Protein Production*

The extent of bagasse cellulose utilization and biomass crude protein content is shown in Fig. 14. During seven days fermentation, *A. terreus* GA utilized 8.5 to 23.5% of bagasse cellulose. During first two days, cellulose was not utilized by the mold. Maximum utilization of bagasse cellulose (23.5%) was noted on the 7th day of fermentation. The crude protein content of the fungal biomass ranged from 9.6 to 14.9%, as compared to 2.01% crude protein in untreated bagasse control. With 23.5% utilization of bagasse cellulose, the biomass crude protein was 14.9% on the 7th day, whereas, with 17.0% cellulose utilization also, the biomass crude protein was 14.4 and 14.9% on the 5th day and 6th day, respectively.
FIG. 13. EFFECT OF GROWTH OF _A. terreus_ GN1 WITH UNTREATED BAGASSE ON CELLULASE ENZYME ACTIVITY.
4.6.1.3. **Protein Recovery and Water Soluble Carbohydrate**

The variation in water soluble carbohydrate content and protein recovery at different fermentation time intervals is shown in Fig. 15. The initial water soluble carbohydrate content of untreated bagasse substrate was 11.24, which decreased to 7.12% after 24 hr fermentation. The utilization of cellulose from bagasse substrate after 6th day of fermentation, corresponded with concomitant increase in water soluble carbohydrate and no increase in crude protein per cent. Maximum protein recovery of 16.0 g/100g bagasse was obtained on the 5th day of fermentation.

4.6.1.4. **Hydrogen Ion Concentration**

The change in pH of the fermenting slurry due to the growth of *A. niger* is shown in Fig. 16. There was a continuous increase in the pH (4.0 to 7.2) during seven days fermentation.

4.6.2.0. **With Alkali-Treated Bagasse Substrate**

4.6.2.1. **Cellulase (CMCase and PFase) Enzyme Production**

The effect of different fermentation times on cellulase activity of *A. niger* is shown in Fig. 17. The CMCase activity in culture filtrate was in the range of 0.10 - 1.02 units/ml. Maximum CMCase activity of 1.02 units/ml was noted on the 7th day of fermentation. The PFase activity ranged from 0.03 to 0.09 units/ml and corresponded with the CMCase activity. The maximum PFase activity of 0.09 units/ml was obtained on the 7th day of fermentation. The cellulase activity was less during initial 24 hr and increased from
FIG. 14. EFFECT OF GROWTH OF *A. terreus* GNI WITH UNTREATED BAGASSE ON CRUDE PROTEIN AND CELLULOSE UTILIZATION
FIG. 15. EFFECT OF GROWTH OF *A. torreyus* GN1 WITH UNTREATED BAGASSE ON CRUDE PROTEIN RECOVERY AND WATER SOLUBLE CARBOHYDRATE
FIG. 16. EFFECT OF GROWTH OF *A. terreus* GN₁ WITH UNTREATED BAGASSE ON pH OF FERMENTATION BROTH
FIG. 17. EFFECT OF GROWTH OF *A. terreus* GN1 WITH ALKALI-TREATED BAGASSE ON CELLULASE ACTIVITY

- CARBOXY METHYL CELLULASE ACTIVITY ($Y_1$)
- FILTER PAPER ENZYME ACTIVITY ($Y_2$)
second day onwards. The comparison of CMCase and FPase activities on untreated and alkali-treated bagasse substrate by A. terreus C101 is depicted in Figs. 18 and 19.

4.6.2.2. Cellulose Utilization and Crude Protein Production

The extent of bagasse cellulose utilization and biomass crude protein content is shown in Fig. 20. A. terreus C101 utilized 6.9 - 82.7% bagasse cellulose during 7 days fermentation. However, 69.8% of the bagasse cellulose was utilized during 24 to 72 hr of fermentation. Maximum utilization of bagasse cellulose (82.7%) was noted on the 7th day of fermentation. The crude protein content of the fungal biomass ranged from 9.6 to 32.8%. With 69.8% bagasse cellulose utilization on the 3rd day, the biomass crude protein content was 21.6%. The comparison of biomass crude protein content and cellulose utilization during fermentation of untreated and treated bagasse substrates by A. terreus C101 is shown in Figs. 21 and 22.

4.6.2.3. Protein Recovery and Water Soluble Carbohydrate

The variation in water soluble carbohydrate content and protein recovery at different fermentation times is shown in Fig. 23. The initial water soluble carbohydrate content of treated bagasse substrate was 6.56%, which decreased to 3.6% after 24 hr fermentation. After one day initial fermentation, the water soluble carbohydrate content increased to 6.2% in the later phase. The results indicate that there was a positive correlation among the bagasse cellulose utilization, protein recovery, crude protein content, CMCase and FPase
FIG.18: EFFECT OF GROWTH OF \textit{A. terreus} GN4 ON CELLULASE ENZYME ACTIVITY (CMCase) WITH SUGARCANE BAGASSE SUBSTRATE
FIG. 19: EFFECT OF GROWTH OF *A. terreus* GN1 ON CELLULASE ENZYME ACTIVITY (FPase) WITH SUGARCANE BAGASSE SUBSTRATE
FIG 2: EFFECT OF GROWTH OF *A. terreus* GN 1 WITH ALKALI-TREATED BAGASSE ON CRUDE PROTEIN AND CELLULOSE UTILIZATION.
Fig. 21. Effect of growth of *A. terreus* GN1 on crude protein content with sugarcane bagasse substrate.
Fig. 22. Effect of growth of A. terreus GN₁ on water soluble carbohydrate with sugarcane bagasse substrate.
FIG23 EFFECT OF GROWTH OF \textit{A. terreus} GN1 WITH ALKALI-TREATED BAGASSE SUBSTRATE ON CRUDE PROTEIN RECOVERY AND WATER SOLUBLE CARBOHYDRATES.
activities, which were maximum on the 7th day of fermentation (Figs. 17, 20 and 23). The protein recovery of the biomass ranged from 9.6 to 20.1 g, as compared to the control value of 3.04 g/100g treated bagasse. The maximum protein recovery of 20.1 g/100g bagasse was obtained on the 7th day of fermentation. The protein recovered in biomass and variation in water soluble carbohydrate during fermentation of untreated and treated bagasse by A. asperculus GN1 are compared in Figs. 24 and 25.

4.6.2.4. Hydrogen Ion Concentration:

During the fermentation of treated bagasse substrate by A. asperculus GN1, the initial pH 4.0 increased gradually to 6.05 in the fermenting slurry on the 7th day of incubation (Fig. 26).

4.6.3.0. Pilot Scale Fermentor Trial with Treated Bagasse Substrates

4.6.3.1. Cellulase (CMCase and FPase) Enzyme Production:

The fermentation of 1.0% treated bagasse substrate by A. asperculus GN1 for cellulase production during four days fermentation in a pilot scale (10-litre) fermentor is shown in Fig. 27. The CMCase activity was zero during initial 24 hr of fermentation and then slightly increased up to 48 hr. Then, there was a significant increase in CMCase activity up to 96 hr. The CMCase activity was in the range of 0.02 - 0.6 units/ml during 94 hr fermentation. The maximum CMCase activity was observed at 84 hr fermentation and decreased further. The FPase activity ranged from 0.012 to 0.032 units/ml during 96 hr fermentation. Even though the CMCase activity was nil during initial 24 hr fermentation, the culture
FIG 25 EFFECT OF GROWTH OF _A. terreus_ G1 ON CELLULOSE UTILIZATION WITH SUGARCANE BAGASSE SUBSTRATE
FIG. 26. EFFECT OF GROWTH OF A. terreus G\textsubscript{4} WITH ALKALI-
TREATED BAGASSE ON pH OF FERMENTATION BROTH
filtrate showed very little Fase activity. The maximum Fase activity of 0.032 units/ml was obtained at 96 hr of incubation, as compared to CMase activity, which was maximum at 84 hr fermentation.

4.6.3.2 Cellulose Utilization and Crude Protein Production

The sugarcane bagasse cellulose utilization and the biomass crude protein content with A. niger G14 is shown in Fig. 26. The mold utilized bagasse cellulose in the range of 23.6 - 72.5% during 96 hr fermentation. There was no initial utilization of cellulose in the treated bagasse substrate during initial 24 hr. The cellulose utilization increased from zero to 25.6% at 36 hr fermentation. Maximum bagasse cellulose was utilized at 84 hr incubation time, which remained constant up to 96 hr. The biomass crude protein content ranged from 10.5 to 29.8%, as compared to 3.04% crude protein in control treated bagasse sample. The crude protein content of 25.0% was obtained with 72.5% utilization of bagasse cellulose at 84 hr incubation period. However, 39.8% crude protein was present in the biomass harvested at 96 hr incubation.

4.6.3.3 Water Soluble Carbohydrate and pII

The variation in water soluble carbohydrate content and pII during fermentation of 1.0% treated bagasse substrate by A. niger G14 for 96 hr is shown in Fig. 27. The results indicate a decrease in water soluble carbohydrate from the initial value of 6.56% to 3.68% during 24 hr fermentation. There was an alternate decrease and increase in water soluble carbohydrate during the fermentation process.
Fig. 27: Effect of growth of *A. terreus* GN4 with alkali-treated bagasse on cellulase enzyme activity in 10 litre fermentor.
**FIG. 28. EFFECT OF GROWTH OF A. terrae GN2 WITH ALKALI-TREATED BAGASSE ON CRUDE PROTEIN AND CELLULOSE UTILIZATION IN 10 LITRE FERMENTOR**
Fig. 29. Effect of growth of *A. terreus* GN1 with alkali-treated bagasse on water soluble carbohydrate and pH in 10 litre fermentor.
The initial pH of 4.0 increased gradually to 6.7 during 72 hr fermentation followed by slight decrease to 6.42 at 96 hr incubation.

4.7.0. EVALUATION OF SIMPLE CELL PROTEIN QUALITY

4.7.1. In Vitro Rumen Digestibility

The quality of microbial protein as evaluated by its in vitro rumen digestibility is presented in Table 13. The pure fungal mycelia of A. terreus GN had an in vitro digestibility coefficient of 71.2%. It was noted that the initial in vitro rumen digestibility of treated bagasse was 84.4%, which decreased to 81.0, 78.0, 76.0, 74.0, 70.0, 69.0 and 68.8% in the biomass during the daily fermentation up to 7 days with A. terreus GN.

4.7.2. Amino Acid Analysis

The amino acid content of the biomass protein obtained by growing A. terreus GN on treated bagasse substrate is presented in Table 14. Almost all the essential and non-essential amino acids were present in the microbial protein. The level of all essential amino acids was higher in single cell protein obtained from A. terreus GN, but only phenylalanine was less. The glutamic acid and proline contents were very high, i.e., 22.75 and 14.29 g/100g protein, whereas, threonine, serine and tyrosine contents ranged from 7.92 to 9.56 g/100g protein and other amino acids were less than 5.81 g/100g protein.

4.8.0. PARTIAL PURIFICATION OF CELLULASE ENZYME

The recovery of partially purified enzyme from A. terreus GN,
Table 13

Effect of Incubation Time on Biomass Production by A. terreus GH₁ and In vitro rumen Digestibility

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Incubation time (days)</th>
<th>Biomass yield (mycelium + unfermented bagasse) (mg/100 ml broth)</th>
<th>In vitro rumen digestibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>1000</td>
<td>84.4</td>
</tr>
<tr>
<td>2.</td>
<td>One</td>
<td>980</td>
<td>81.0</td>
</tr>
<tr>
<td>3.</td>
<td>Two</td>
<td>944</td>
<td>79.0</td>
</tr>
<tr>
<td>4.</td>
<td>Three</td>
<td>820</td>
<td>76.0</td>
</tr>
<tr>
<td>5.</td>
<td>Four</td>
<td>689</td>
<td>71.0</td>
</tr>
<tr>
<td>6.</td>
<td>Five</td>
<td>629</td>
<td>70.0</td>
</tr>
<tr>
<td>7.</td>
<td>Six</td>
<td>623</td>
<td>69.0</td>
</tr>
<tr>
<td>8.</td>
<td>Seven</td>
<td>613</td>
<td>68.0</td>
</tr>
</tbody>
</table>
Table 14

Amino Acid Composition of Biomass Protein
Produced by \( \Delta \text{Luxus} \) strain

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Particularity of amino acids</th>
<th>Concentration of amino acids (g/100 g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aspartic acid</td>
<td>5.15</td>
</tr>
<tr>
<td>2.</td>
<td>Threonine</td>
<td>9.56</td>
</tr>
<tr>
<td>3.</td>
<td>Serine</td>
<td>8.18</td>
</tr>
<tr>
<td>4.</td>
<td>Glutamic acid</td>
<td>22.74</td>
</tr>
<tr>
<td>5.</td>
<td>Proline</td>
<td>14.28</td>
</tr>
<tr>
<td>6.</td>
<td>Glycine</td>
<td>4.78</td>
</tr>
<tr>
<td>7.</td>
<td>Alanine</td>
<td>1.09</td>
</tr>
<tr>
<td>8.</td>
<td>Valine</td>
<td>5.03</td>
</tr>
<tr>
<td>9.</td>
<td>Methionine</td>
<td>2.44</td>
</tr>
<tr>
<td>10.</td>
<td>Isoleucine</td>
<td>5.21</td>
</tr>
<tr>
<td>11.</td>
<td>Leucine</td>
<td>4.97</td>
</tr>
<tr>
<td>12.</td>
<td>Tyrosine</td>
<td>7.92</td>
</tr>
<tr>
<td>13.</td>
<td>Phenyldalanine</td>
<td>1.33</td>
</tr>
<tr>
<td>14.</td>
<td>Histidine</td>
<td>5.81</td>
</tr>
<tr>
<td>15.</td>
<td>Arginine</td>
<td>0.51</td>
</tr>
</tbody>
</table>
is presented in Table 15. The protein precipitated at the range of 35-75% ammonium sulphate concentration contained maximum cellulase units. After passing (desalting) through Sephadex G-25, this resulted in a 5.4 and 5.8 fold purification with 80 and 83.7% recoveries of CMCase and Fase activities, respectively.

4.9.0. PROPERTIES OF CELLULASE ENZYME

4.9.1. Effect of Incubation Time

The CMCase and Fase activities were positively correlated with respect to time of incubation up to 120 min for the enzymes obtained from the partially purified filtrate of 7 days old fermenting slurry of A. niger var. G1 (Fig. 30).

4.9.2. Effect of Enzyme Concentration

The effect of varying concentrations of enzyme on CMCase and Fase activities is shown in Fig. 31. A linear relationship between CMCase, Fase activities and enzyme concentration was observed up to 4.8 and 9.6 mg protein (0.05 and 0.1 ml enzyme solution).

4.9.3. Effect of Temperature of Incubation

The effect of temperature of incubation of cellulase enzyme is shown in Fig. 32. The enzyme exhibited an optimum temperature of incubation at 60°C for CMCase and 55°C for Fase activities.

4.9.4. Effect of Heat Treatment

The effect of heat treatment on CMCase and Fase activities, when the enzyme solution alone was heated at different temperatures
Table 15

Partial Purification of Cellulase Enzyme from *Anarcirrillus terrimus GN-1*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Purification step</th>
<th>Total enzyme (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CMCase</td>
<td>FFase</td>
<td>CMCase</td>
<td>FFase</td>
<td>CMCase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Grade enzyme (150 ml)</td>
<td>416.7</td>
<td>12.0</td>
<td>97.5</td>
<td>4.3</td>
<td>0.12</td>
</tr>
<tr>
<td>2.</td>
<td>Sephadex G-25 (after ammonium sulfate fractionation) (35-75%)</td>
<td>333.9</td>
<td>10.0</td>
<td>14.4</td>
<td>23.2</td>
<td>0.70</td>
</tr>
</tbody>
</table>
FIG. 30. EFFECT OF INCUBATION TIME ON CELLULASE ACTIVITY
FIG. 31. EFFECT OF ENZYME CONCENTRATION ON CELLULASE ACTIVITY.

CARBOXYMETHYL CELLULASE ACTIVITY ($Y_1$)
FILTER PAPER ENZYME ACTIVITY ($Y_2$)
FIG. 32. EFFECT OF INCUBATION TEMPERATURE ON CELLULASE ACTIVITY.
is shown in Fig. 33. The cellulase enzyme was stable up to 55°C. However, further increase in temperature during heat treatment, resulted in a rapid loss of both CMCase and FPase activities at 70°C and showed zero activity at 75°C.

4.9.5. Effect of pH

Effect of hydrogen ion concentration on CMCase and FPase activities is shown in Fig. 34. Maximum CMCase and FPase activities were observed at pH 4.5 and 5.5, respectively.

4.9.6. Effect of Substrate Concentration

Lineweaver-Burk plots of cellulase enzyme with carboxymethyl cellulose and filter paper substrates are shown in Figs. 35 and 36. $K_m$ and $V_{max}$ values of the enzyme with CM-cellulose as the substrate were 11.1 mg/ml and 38.9 μM/mg protein/min, whereas, with filter paper substrate, the $K_m$ and $V_{max}$ were 80 mg/ml and 2.7 μM/mg protein/min, respectively.

4.9.7. Effect of Metal Ions

The effect of various concentrations of metal ions on CMCase and FPase activities is presented in Table 16. It was observed that Mn$^{2+}$ ($10^{-2}$M) stimulated the CMCase activity, whereas, Hg$^{2+}$, Cu$^{2+}$, (10$^{-3}$ and 10$^{-2}$M), Ag$^+$ and Fe$^{3+}$ ($10^{-2}$M) significantly inhibited the CMCase activity. On the other hand, FPase activity was stimulated by lower concentration (10$^{-4}$M) of Ni$^{2+}$ and Cu$^{2+}$, but Mn$^{2+}$ and Ag$^+$ (10$^{-2}$M) significantly inhibited FPase activity. However, almost all concentrations of Co$^{2+}$, Mg$^{2+}$, Ni$^{2+}$ and Zn$^{2+}$ did not have any significant effect on both
FIG. 33 EFFECT OF HEAT TREATMENT ON CELLULASE ENZYME.
FIG. 34. EFFECT OF pH ON CELLULASE ACTIVITY

- CARBOXYMETHYL CELLULASE ACTIVITY ($Y_1$)
- FILTER PAPER ENZYME ACTIVITY ($Y_2$)
FIG. 35 EFFECT OF SUBSTRATE CONCENTRATION ON CARBOXYMETHYL CELLULASE (CMCase) ACTIVITY.
FIG. 36. EFFECT OF SUBSTRATE CONCENTRATION ON FILTER PAPER ENZYME (F Pase) ACTIVITY.
<table>
<thead>
<tr>
<th>Metal Ions</th>
<th>Relative carboxymethyl cellulase activity (4)</th>
<th>Relative filter paper enzyme activity (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-2}</td>
<td>10^{-3}</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>77</td>
<td>96</td>
</tr>
<tr>
<td>Hg^{2+}</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>90</td>
<td>87</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>16</td>
<td>50</td>
</tr>
<tr>
<td>Na^{2+}</td>
<td>130</td>
<td>110</td>
</tr>
<tr>
<td>Fe^{3+}</td>
<td>62</td>
<td>75</td>
</tr>
<tr>
<td>Hg^{2+}</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>Ag^{+}</td>
<td>0</td>
<td>77</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>87</td>
<td>87</td>
</tr>
</tbody>
</table>
CMCase and FFase activities.

The lower concentration of all metal ions (10^{-4} M) did not show any inhibitory or stimulatory effect on both CMCase and FFase activities except Ni^{2+} and Cu^{2+}, which stimulated FFase activity at this concentration.

4.9.8. Effect of Effectors/Modulators:

The effect of some activators and inhibitors on cellulase enzyme activity is shown in Table 17. Ethylene diamine tetraacetic acid (EDTA), iodosacetamide, thiourea, glutathione, N-ethyl maleimide, 2-mercaptoethanol, aluminium potassium sulphate and cysteine hydrochloride did not have any significant effect on both CMCase and FFase activities except 10^{-3} and 10^{-4} M concentrations of cysteine hydrochloride, which had stimulatory effect on FFase activity only. The α-α- dipyridyl, β-hydroxy quinoline and sodium thiosulphate at the concentration of 10^{-2} M inhibited only CMCase activity and not FFase activity. However, diethyl dithio carbamate at the concentration of 10^{-3} and 10^{-2} M inhibited both CMCase and FFase activities. Sodium tungstate (Na_{2}WO_{4}) did not have any significant effect on CMCase activity, but stimulated the FFase activity at all the concentrations tried.

Ascorbic acid (vit-C) was found to be the best positive modulator for cellulase activity, which stimulated the CMCase activity approximately by six fold and FFase activity by nine fold at 10^{-2} M concentration of ascorbic acid.
Table 17

Effect of Modulators on CMCase and Fase Activities of Cellulase from *S. terrae* G*ᵣᵣ*

<table>
<thead>
<tr>
<th>Activators/Inhibitors</th>
<th>Relative CMCase activity (¢)</th>
<th>Concentration of Activators/Inhibitors</th>
<th>Relative Fase activity (¢)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻²</td>
<td>10⁻³</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>Aluminium potassium sulphate</td>
<td>80</td>
<td>96</td>
<td>104</td>
</tr>
<tr>
<td>Sodium tungstate</td>
<td>93</td>
<td>96</td>
<td>86</td>
</tr>
<tr>
<td>p-chloro mercuribenzoate</td>
<td>0</td>
<td>6</td>
<td>85</td>
</tr>
<tr>
<td>Ethylene diamine</td>
<td>76</td>
<td>77</td>
<td>84</td>
</tr>
<tr>
<td>tetra acetic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium thiosulphate</td>
<td>63</td>
<td>89</td>
<td>86</td>
</tr>
<tr>
<td>Thiourea</td>
<td>89</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Sodium diethyl dithio carbonate</td>
<td>56</td>
<td>74</td>
<td>82</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>82</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>Glutathione</td>
<td>91</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>81</td>
<td>100</td>
<td>103</td>
</tr>
<tr>
<td>β-Hydroxy quinoline</td>
<td>49</td>
<td>76</td>
<td>90</td>
</tr>
<tr>
<td>N-Ethyl maleimide</td>
<td>85</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>α,α'-dipyridyl</td>
<td>59</td>
<td>84</td>
<td>93</td>
</tr>
<tr>
<td>Ascorbic acid (vit-C)</td>
<td>566</td>
<td>181</td>
<td>123</td>
</tr>
<tr>
<td>2-Nerceptoethanol</td>
<td>104</td>
<td>108</td>
<td>102</td>
</tr>
</tbody>
</table>
Table 13

Economic Evaluation of SCP Process with 8-Litre Working Volume of Flask and Fermentor Trial

<table>
<thead>
<tr>
<th></th>
<th>Flask (€)</th>
<th>Fermentor (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Expenditure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw material</td>
<td>11.10</td>
<td>11.10</td>
</tr>
<tr>
<td>Water and Electricity charges</td>
<td>19.20</td>
<td>24.00</td>
</tr>
<tr>
<td>Labour cost</td>
<td>56.00</td>
<td>70.00</td>
</tr>
<tr>
<td>Depreciation of Equipment and Building</td>
<td>19.20</td>
<td>40.20</td>
</tr>
<tr>
<td>Breakage</td>
<td>28.40</td>
<td>-</td>
</tr>
<tr>
<td>Interest on Investment</td>
<td>66.00</td>
<td>145.00</td>
</tr>
<tr>
<td><strong>Total cost</strong></td>
<td>199.90</td>
<td>290.30</td>
</tr>
<tr>
<td><strong>B. Product value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCP (56.0 - 65.6 g)</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Cellulase(Sigma)</td>
<td>32,400</td>
<td>1,458.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11,700</td>
</tr>
<tr>
<td></td>
<td></td>
<td>526.50</td>
</tr>
<tr>
<td><strong>C. Margin of Profit</strong></td>
<td>1237.25</td>
<td>236.35</td>
</tr>
</tbody>
</table>
4.10.0. ECONOMIC EVALUATION ON SCP PROCESS

The cost of production of SCP and cellulase in the flask and fermentor trials using 1.0% alkali-treated bagasse in 8-litres modified Czapek’s broth with corn steep liquor at 600 mg N/litre was evaluated as per the methods described earlier and is presented in Table 18. The total cost of production of SCP and cellulase in flask and fermentor trials were approximately Rs. 200.00 and Rs. 290.00, respectively. The products recovered in the SCP process were 65.6 g biomass (43.7% of the original bagasse substrate) with 20.57% crude protein content and 32,400 Sigma units of cellulase in flask trials and 56.0 g biomass (37.5% of the original bagasse substrate) with 24.5% crude protein content and 11,700 Sigma units of cellulase in fermentor trial.

The cost of production of cellulase enzyme per 1000 units (Sigma) was Rs. 6.11 in flask trial and was Rs. 24.80 in fermentor trial.

The SCP cost was taken as almost negligible, as the market cost of this SCP produced being hardly 0.15 paisa. The market cost of 1000 units of cellulase (Sigma) was Rs. 45.00. It could be seen that the SCP could be produced at no additional cost, when the method developed is used for commercial production of cellulase enzyme.
DISCUSSION

The shortage in production and supply of protein in food and feed in the developing countries have led to malnutrition of human beings and livestock. Every effort is being made to supplement the land and animal based protein production by unconventional methods. Use of microorganisms for single cell protein (SCP) production on different substrates, has been attracting much attention (Matales and Tannenbaum, 1969; Kihlberg, 1972; Tannenbaum and Wing, 1975).

Protein derived from microorganisms have been increasingly becoming popular because of ease of production in view of the high speed of multiplication of the microbes, the suitability of different substrates for their growth and the absence of need for land. Single cell protein can be cultivated on a wide range of substrates including wastes.

Agricultural cellulose wastes have been considered as a potential source of raw material for different product recovery viz., SCP, cellulase etc., thus decreasing the immense waste disposal problems. The sugarcane bagasse, a by-product of sugar industry, in India is being burnt away as fuel in factory itself and is also considered as a feeding material for ruminants as well. The present investigation, aims to utilise the delignified sugarcane bagasse as substrate for fermentation by cellulolytic molds to increase its nutritional value in relation to its protein and recovery of cellulase enzyme as a by-product.
The original dried sugarcane bagasse had 2.01% crude protein, 30.83% cellulose and 11.18% water soluble carbohydrate. During hot alkali treatment and subsequent washing, the bagasse lost 45% of its dry weight including 7.85% cellulose and 67.71% water soluble carbohydrate content. There was also delignification and losses of carbon components. In the treated bagasse, the crude protein content was 3.04% and cellulose was 51.67% and it was more biodegradable and could yield more total biomass protein and cellulase enzyme than the untreated bagasse. Different physical and chemical treatments of bagasse were reported to break the ligno-cellulose complex. Treatment with hot alkali like 4.0% sodium hydroxide, 20% ethylene diamine and endoxen made the treated bagasse cellulose more accessible for enzymic degradation (Han and Anderson, 1974; Badisch et al., 1978).

Among the microorganisms, the cellulosic substrates are utilized by some bacteria viz., Cellulomonas, Bacillus, Pseudomonas and a number of molds, which convert them with greater efficiency into microbial biomass. In the present study, a number of mold isolates have been screened for utilization of cellulosic substrates (Ampelomyces flavus, A. sydowi GN, A. flavus GN, A. aureus sp. GN, Aspergillus niger, Trichoderma viride 64GN and YU, Hypocystium verrucaria, Fusarium moniliforme GN-1224 and Alternaria tenius).

The maximum biomass dry matter of 74 mg/g carboxymethyl cellulose was obtained with Alternaria tenuis followed by Fusarium sp., and Hypocystium verrucaria. The biomass dry matter yield
ranged from 34 to 53 mg/g substrate in case of *Aspergillus* spp. and *Trichoderma* spp. The biomass crude protein content of *A. terreus* 1 and 3, was maximum ranging from 40.02 to 41.60, whereas, that of other molds was 11.62 to 26.99% only. Hence, the total protein recovery was maximum (2.08%) with *A. terreus* 3, as compared to the other molds having total protein recovery of 0.977 to 1.59% CMC (Table 2).

On the basis of the growth of different molds with 1.0% untreated or alkali-treated bagasse substrates as sole carbon source, *A. terreus* 3, gave the maximum total crude protein recovery of 4.65 g/100g untreated bagasse and 11.03 g/100g treated bagasse as compared to the other molds yielding total crude protein recovery of 2.01 - 3.86 g/100g untreated bagasse and 3.04 - 9.73 g/100g treated bagasse (Tables 4 and 5). However, it was noted that extent of degradation of both untreated and treated bagasse substrates was comparatively low with *Alternaria tenuis* i.e. 142.48 mg/g untreated bagasse and 383.65 mg/g treated bagasse, but its efficiency of fungal biomass conversion was maximum i.e. 29.53 and 47.77% for untreated and treated bagasse substrates. Hence, there was low protein recovery by *Alternaria tenuis* on bagasse substrate. The degradation of both untreated and treated bagasse was high with *A. terreus* 1, i.e. 314.22 mg/g untreated bagasse and 683.19 mg/g treated bagasse and the efficiency of fungal biomass conversion was only 20.40 and 35.47% for untreated and treated bagasse (Tables I, II, Appendix). The higher total protein recovery with *A. terreus* 3, on untreated and treated bagasse substrates was closely followed by *A. terreus* 1,
Anamum sp. 2, 3 and A. niger with treated bagasse and by
A. terreus 1, 2, viride 6, 9 and H. verrucaria with untreated
bagasse substrate. In cases of higher total protein recovery by
some molds, the molds had higher biomass crude protein content,
degraded more of the substrate and had comparatively high biomass
conversion efficiency also, as compared to molds of lower total
protein recovery. Hence, A. terreus GN, was found to be the best
protein producer and higher bagasse cellulose degrader in the case
of both untreated and treated bagasse substrates.

All the cellulolytic molds produced more crude protein and
protein recovery with the treated bagasse substrate as compared to
the untreated ones. The pretreatment of bagasse considerably increased
the fungal growth rate and also increased the biodegradability of
bagasse by its delignification, decrystallization and depolymerisation.
The utilization of substrate was improved by the fact that some
regions on the cellulose fibers were made accessible for enzymic
hydrolysis. A highly crystalline and lignin containing cellulosic
waste was more difficult to utilize and yielded a biomass with a
lower crude protein content (Cowling and Brown, 1969; Eriksson and
Marsson, 1973; Chahal and Jang, 1978; Hsu et al., 1979; Hoo-Young
et al., 1979). It was noted that the biomass dry matter yield was
less with higher crude protein content and conversely, with higher
dry matter yield, the crude protein content was low. For the
production of more crude protein, more energy was required for the
breakdown of cellulosic substrate. The substrate mass balance for
protein production by molds further indicated that most of the nitrogen
was retained in microbial biomass and nearly half of the carbohydrate substrate was lost as carbon dioxide for the synthesis of highly ordered biological macromolecules (Irvin and Righelato, 1976). However, Rogers et al. (1972) and Petersson (1975 a,b) recovered protein in the range of 13.0 - 21.26 % by using various cultures and substrates. When Chaetomium globosum was grown on bagasse substrate, 12.0 % crude protein was obtained, which could be increased up to 17.5 - 23.75 % by various treatments (Sharma et al., 1973). Thus, it was observed that by growing A. terreus OR1 on untreated and treated bagasse substrates, the biomass crude protein content was increased from an initial 2.01 to 5.40 % and 3.04 to 19.98 %, respectively (Tables 4 and 5).

Mixed inoculation with combination of cellulolytic molds viz., A. terreus J2, Myrothecium verrucaria, Fusarium sp. J2, and Alternaria tenuis did not show synergistic effect on protein production (Table 3). However, the mixed cultures of Cellulomonas sp. and Alcaligenes faecalis, Phanerochaete chrysosporium, and Candida utilis, T. viride and Candida utilis grown on cellulosic substrates yielded an overall increase in protein and cellulase production. In the mixed culture studies, the first organism being cellulolytic and second non-cellulolytic, the former degraded the cellulose and the latter grew at the expense of degradation product and increased cell mass and protein production (Han et al., 1971; Ek and Eriksson, 1975; Petersson, 1975 a,b; Yu et al., 1976).

The fermentation of substrate by microorganisms requires rigorous control of cultural conditions like, hydrogen ion concentration
temperature, extent of aeration and shaking, size of inoculum etc., for their excellent growth, cellulase and protein production.

The cellulase activity of the fermenting slurry was assayed for both CMCase and βCase. Complete saccharification of cellulose requires the action of complex enzyme system containing endo- and exo-β-glucanase (cellulase) and β-glucosidase (cellobiase). Assay procedures based upon hydrolysis of a complex substrate, though realistic, would be time consuming; assay procedures based upon a very simple substrate would be unrealistic. A simple substrate such as cellotetraose proposed by Petterson (1975) and a complex substrate such as cotton proposed by Talliswell (1975) did not find acceptability as substrates for assaying total cellulase activity. Enzyme preparations lacking exo-β-glucanase would scarcely hydrolyze insoluble cellulose but would readily breakdown derivatives such as carboxymethyl cellulose. For such enzyme preparations, carboxymethyl cellulose would be the substrate of choice. Mandels and Weber (1969) recommended filter paper as the substrate, because this substrate was insoluble and was neither too susceptible nor too resistant. It was, therefore, decided to use both carboxymethyl cellulose as well as filter paper as the substrates for assaying the activity of cellulase enzyme.

The optimum pH for maximum crude protein production by <i>Aspergillus terreus</i> CM was found to be 4.0. However, the per cent crude protein in biomass obtained at pH 4.5 to 5.5 was only slightly less than that obtained at pH 4.0. Higher protein recovery was also noted in the pH range of 4.0 to 5.5 (Table 6).
Even though, the crude protein recovery was maximum (3.4%) and fungal biomass conversion efficiency was 27.53% at pH 5.5, the overall efficiency in terms of weight of bagasse degraded (760.43 mg/g bagasse), crude protein recovery (9.2%), cellulase production - C1ase (54 units/g bagasse) and C2ase (6.3 units/g bagasse) were maximum at pH 4.0 (Table III, Appendix). However, Chhabl and Chhabl (1973) reported maximum crude protein production at pH 4.0, when *Fusarium moniliforme* was grown on wood pulp substrate. Andreotti et al. (1977) reported maximum production of PM and cellulase by *T. viride* pH-4.414 at pH 3.5. Biterz et al. (1978) found the pH range of 3.5-6.0 for the maximum protein production by *Aspergillus niger*, *Penicillium curvatum* and *Mucor miehei* sp. Dhillon and Chahal (1978) reported pH 5.0 to be most suitable for *Aspergillus oryzae*, *Mucor miehei*, *Cochliobolus* sp. and *Rhizoctonia* sp.; pH 5.0 for *Aspergillus terreus*, *Penicillium sp.* and *Aspergillus niger* and pH 4.0 for *Geotrichum* sp. Dhillon et al. (1991) observed an optimum pH of 5.0 for SCP production by *Chrysomium cellulolyticum*.

The pH of the culture broth affects both the biomass composition and the nature of the mold metabolism, but the molecular basis of these effects is not understood. Possibly the proton motive force generated across the plasma membrane in chemiosmosis is affected by the medium pH value. Hence, under optimum pH ranges, the relative metabolic efficiency of the organism is high (Pirt, 1975).
The optimum temperature of incubation was 30°C for maximum crude protein production (17.8%) by \textit{A. niger} CH, which was comparable with the crude protein obtained at 25°C (17.5%). Hence, optimum incubation temperature for the cultivation of \textit{A. niger} CH for cellulase and protein production was 25–30°C (Table 7). Though the temperature of incubation at 25 and 30°C was found to be optimum range for more growth and cellulolytic activity of \textit{A. niger} CH, 30°C incubation yielded more degradation of bagasse (73.81 mg/g bagasse); more enzyme activity (units/g bagasse) - CMCase, 54 units and \textit{P}ase, 45 units and crude protein recovery of 9.24% (Table III, Appendix). However, Romanelli \textit{et al.} (1975) reported the optimum temperature range of 46-51°C for \textit{T. thermovacuum} and 36-43°C for \textit{Sporotrichum thermophile}. Chahal and Hawksworth (1976) and Dhillion \textit{et al.} (1931) noted 57°C as optimum for the maximum growth of \textit{Chaetomium cellulolyticum}, while 40°C was found to be inhibitory. Andreotti \textit{et al.} (1977) observed an optimum temperature of 27°C for maximum SCP and cellulase production by \textit{T. viride} CH-9414.

The effect of different temperatures on growth rate could be predicted in terms of the activation energy required for growth. Above the optimum temperature, cell degradation probably becomes dominant over the growth process and with sub-optimal temperature, the regulation of metabolism may fail (Pirt, 1975). Hence, under optimum temperature, the mold could utilize the carbon substrate better, with other optimum cultural and nutritional conditions.
The maximum crude protein and cellulase units were obtained in continuously shaken flask cultures as compared to intermittently shaken and still cultures. But, the biomass protein recovery was comparable both in continuously shaken and intermittently shaken flask cultures (Table 8). Thus, continuous shaking was more suitable for efficient growth and activity of \textit{A. terreus MN} than intermittent shaking. The optimum culture broth for fermentation was 1/5 (v/v), which yielded higher cellulolytic activity (U/Case, 63 units, U/Bse, 4.5 units) and crude protein content (Table III, Appendix). Mezlo (1968) and Rawi (1970) observed that the conversion of cellulose to SCP under aerobic conditions was much more efficient than anaerobic conditions.

The extent of aeration affects the growth of microorganisms, which in turn, influences the protein content and recovery of biomass. During continuous shaking of fermenting mold slurry (1/5 v/v), the mold received optimum aeration under submerged culture so that it could grow more efficiently.

The optimum dose of 5.0\% (7 days old) inoculum of \textit{A. terreus MN} resulted in maximum fungal biomass conversion efficiency of 35.4\%, protein recovery of 11.2\% and degraded 691.1 mg/g beechwood substrate. However, Sharma \textit{et al.} (1978) reported the maximum protein production with 20\% inoculum of \textit{Cheskucum globosum}.

Since, the mold inoculum was of the pellet or atmomatic form during continuous shaking, the fungal mycelium grew exponentially until the substrate concentration became limiting or when the growth of pellet of mycelium became diffusion limited.
Under the optimum cultural conditions, \textit{A. terreus} \textsuperscript{23}, yielded proportionally higher protein and cellulase activity of both CMCase and \textit{FPase}. The extent of cellulose degradation depended on the synergistic action of different cellulase enzyme components. The induction of the components of cellulase enzyme system was different in different fungi. In \textit{A. terreus}, the CMCase and \textit{FPase} enzyme components of cellulase might remain associated with one another. Hence, positive correlation was obtained between the highest CMCase and \textit{FPase} activities and highest crude protein per cent in the biomass produced. However, Chahal and Jhaliwal (1973), Vogt and Staffeldt (1977) and Chahal \textit{et al.} (1977) reported that there was no clear relationship between the individual enzyme activities and biomass and/or crude protein production, when \textit{Rhizoctonia solani}, \textit{Trichoderma lignorum}, \textit{Chactomium globosum}, \textit{Pyrrothium} sp. and \textit{Chactomium cellulolyticum} were grown on various cellulolytic substrates.

The effect of substrate charging rates revealed that 1.04 (w/v) treated bagasse concentration was optimum for maximum substrate degradation of 677.34 mg/g, crude protein recovery of 11.5%, fungal biomass conversion efficiency of 37.4% and also maximum cellulase production (Table III, Appendix). At the optimum bagasse substrate concentration, the mold utilized the substrate efficiently as both carbon and energy sources by the enzymatic saccharification of cellulose into simple sugars for further assimilatory processes. However, by increasing the substrate concentration from 2.0 to 4.0%, the total protein recovery decreased from 8.5 to 5.6% (Table 11). This might
be attributed to either too much packing of the substrate, severely limiting the oxygen availability, solution flow rate, depletion of other nutrient supply or due to change in hydrogen ion concentration, which might be inhibitory to the growth of the mold and induction of enzyme synthesis. The results are in agreement with Sitaran et al. (1973), who obtained maximum protein by Aspergillus niger, Penicillium chrysogenum and Mucor sp., at 1.0% (w/v) level of cellulolytic substrate.

The nitrogen from inorganic and organic sources are metabolized by the mold for the synthesis of protein, nucleic acid and cell wall polymers. The organic nitrogen sources viz., corn steep liquor, urea and cattle urine and inorganic non-ammonium nitrogen source like sodium nitrate were preferentially utilized for greater protein recovery and cellulase production than other inorganic nitrogen sources. Among the organic nitrogen sources, corn steep liquor at 600 mg N/litre broth yielded the maximum protein recovery of 22.78%, degradation of 847.93 mg/g bagasse substrate, maximum cellulase activity (CMCase, 94.5 units and BMCase 8.10 units) and biomass crude protein content of 32.90% with corn steep liquor, maximum growth and protein recovery by Aspergillus terreus 8%, were obtained, which might be due to the additional supply of carbon (lactic acid, complex polysaccharides resembling gums and variable amounts of reducing sugars) and amino acids (alanine, asparagine, glutamic acid, leucine, proline, isoleucine, threonine, valine, phenylalanine, methionine and cystine), calcium, phosphorous, potassium and growth factors (riboflavin, niacin, pantothenic acid, biotin and pyridoxine) (Rhodes and Fletcher, 1977). The cattle urine was inferior to corn steep liquor.
However, cattle urine provided additional nutrients, like potassium, sodium, calcium, magnesium and trace elements (Hutton et al., 1965). With cattle urine at 600 mg N/litre broth, a maximum protein recovery of 11.3%, 607.12 mg/g bagasse degradation and cellulase activity of 43.65 units CMCase and 2.25 units F1ase were obtained (Table III, Appendix). The other nitrogen sources like sodium nitrate and urea, were comparable to cattle urine, but were inferior to corn steep liquor with reference to bagasse degradation, biomass protein recovery and cellulase activity during fermentation of treated bagasse by A. terreus 36.

Among the other inorganic nitrogen sources used for fermentation of bagasse by A. terreus 36, the biomass crude protein recovery and crude protein content with 500 mg N of sodium nitrate were 17.27% and 20.83%, which were next lower to corn steep liquor at 600 mg N/litre broth, yielding 22.08 and 32.97%, respectively. The protein recovery and protein content of the biomass obtained with 600 mg N as corn steep liquor was 1.726 and 1.579 times more than that obtained by using 500 mg N sodium nitrate. Since corn steep liquor supplied additional carbon sources for mold proliferation, carbon utilization pattern of A. terreus 36 fermentation with both sodium nitrate and corn steep liquor sources at 500 mg N was compared.

It was noted that in the case of sodium nitrate (500 mg N and 1.0 g of treated bagasse substrate, containing 567 mg carbon), the biomass recovery was 614 mg/g substrate, the bagasse degradation was 669.27 mg/g substrate and the weight of fungal biomass alone was 283.27 mg, which gave a fungal biomass conversion efficiency
of 42.33%. On the other hand, with cornsteep liquor (at 500 mg N from 1.41 g cornsteep liquor, containing 274 mg additional carbon), the biomass recovery was 594 mg/g substrate, the bagasse degradation was 864.13 mg/g substrate and the weight of fungal biomass alone was 46.13 mg, which gave a fungal biomass conversion efficiency of 52.78% (Table III, Appendix). The higher rate of bagasse degradation of 864.13 mg with 500 mg N cornsteep liquor as compared to 669.27 mg/g bagasse substrate with NaNO₃ at 500 mg N might be due to the synergistic effect of the other nutrient component present in cornsteep liquor, which might have increased the cellolytic activity of both CMCase and EPase, resulting in greater biodegradation. The fungal biomass conversion efficiency with cornsteep liquor was 1.25 times more than NaNO₃ source. The higher protein recovery, protein content and biomass yield was attributed to 1.51 fold higher carbon present plus other growth factors, amino acids and micro and macro nutrients while fermenting 1.0 g bagasse plus 1.41 g cornsteep liquor than fermenting 1.0 g bagasse with sodium nitrate at 500 mg N.

With regard to different nitrogen sources, the activities of the two components of the cellulase enzyme produced by A. niger GN, viz., CMCase and EPase were also maximum with 600 mg N cornsteep liquor, i.e. 94.5 and 8.10 units, respectively as compared to 49.05 and 3.33 units of CMCase and EPase activities with sodium nitrate at 500 mg N. Nearly two fold increase in CMCase and two and half fold increase in EPase activities might also be due to the accelerated growth of mold, which contributed 521.93 mg fungal biomass with
corn steep liquor at 600 mg % as compared to 293 mg fungal biomass/g bagasse substrate with sodium nitrate at 500 mg % level (Table III, Appendix). However, urea nitrogen produced a higher crude protein content, protein recovery and cellulase activity with an optimum dose of 350 mg %. There was a decrease in all above constituents, when urea nitrogen was increased beyond the optimal level. Chahal and Grey (1969, 1970) and Chahal and Dahiwal (1973) reported urea to be the best nitrogen source for microbial protein production by Rhizoctonia solani, Trichoderma lignorum, Pythium vexans and Chaetomium cellulolyticum grown on different cellulosic substrates. They reported that higher concentration of urea resulted in toxicity for mold growth. The inhibitory effect of high urea concentration could be attributed to the accumulation of high levels of ammonia released due to degradation of urea by the enzyme urease. The course of decline in CMCase and β-glucosidase activities with increase in urea concentration might be due to increase in pH by ammonia accumulation and that the maximum cellulase production occurred at acidic pH (Pratt, 1924; Neal et al., 1932, 1933; Neal and Emerson, 1936; Keit and Palmiter, 1937; Saksena and Bhargava, 1943; Sin, 1951; Nachlas, 1953; Dutta et al., 1961; Spalding, 1963; Ueno, 1966; Kawi, 1970).

Chahal and Hawksworth (1976) reported sodium nitrate as the best nitrogen source for maximum growth rate of Chaetomium cellulolyticum, while Sharma et al. (1978), Bhillon and Chahal (1978) and Bhillon et al. (1981) reported potassium nitrate to be the best nitrogen source for the growth of Chaetomium cellulolyticum.
**Passilomyces** sp. and **Cochilobolus** sp.

From the results presented in Table III, it is clear that corn steep liquor (at 600 mg N) was the best nitrogen source for supplementation of treated bagasse for the production of maximum crude protein and cellulase enzyme by **A. terreus** strain. It also provided other nutrients.

Untreated and treated bagasse substrates at 1.0% concentration were fermented with corn steep liquor at 600 mg N/litre broth for 7 days under optimum cultural conditions and the extent of biomass dry matter recovery, enzyme production, cellulose utilization, protein content and recovery were correlated at 24 hr intervals. From the results, the following points were noted:

(i) The metabolic bioconversion of both untreated and treated bagasse substrates with reference to cellulose utilization, protein recovery, protein content and cellulase enzyme activity reached logarithmic phase between 2nd to 4th day of fermentation.

(ii) The above parameters were significantly higher for treated bagasse than untreated bagasses. The cellulose utilization was 69.76% and 82.66% for treated bagasse and 1.5% for 25.5% untreated bagasse on the 3rd and 7th day of fermentation; the total CMCase and FPase activities were 74.7, 6.73 and 91.8, 7.47 units for treated bagasse and 40.5, 1.71 and 49.5, 2.43 for untreated bagasse on the 3rd and 7th day of fermentation; the protein content and recovery were 20.57, 16.87% and 32.79, 20.08% for treated bagasse and 14.0, 14.26% and 14.9, 16.36% for untreated bagasse on the 3rd and 7th day of fermentation, respectively (Table IV, Appendix).
The values of protein recovery, crude protein content of biomass, cellulose utilization, CMCase and FFace production were more significantly different (1.0% level) and were of low efficiency for untreated bagasse as compared to treated bagasse. The 't' values for above parameters were 5.46, 3.55, 4.66, 4.51 and 5.33, respectively.

It was noted earlier that the extent of degradation of untreated bagasse substrate by \textit{A. niger} GM\textsubscript{3}, with sodium nitrate (400 mg N) was 314.22 mg/g bagasse substrate with a protein recovery of 4.05% and fungal biomass yield of 64.22 mg (Table I, Appendix). With cornsteep liquor (600 mg N) and 1.0% untreated bagasse, the biomass crude protein content was 14.9% and protein recovery was 16.36% (Figs. 14 and 15). The higher metabolic rate of biodegradation of untreated bagasse by \textit{A. niger} GM\textsubscript{3}, with cornsteep liquor nitrogen source, which resulted in 3 to 4 fold increase in protein content and recovery over sodium nitrate nitrogen source, was at the expense of the synergistic effect of the additional nutritional components supplied by the cornsteep liquor. However, it was noted that the total CMCase and FFace activities with 1.0% untreated bagasse substrate and cornsteep liquor at 600 mg N were 49.5 and 2.47 units on the 7th day of fermentation, which were very low in comparison to treated bagasse substrate.

As mentioned earlier (Fig. 20), with 1.0% treated bagasse substrate, the extent of cellulose utilization was 69.76% and 92.65% on the 3rd and 7th day of fermentation by \textit{A. niger} GM\textsubscript{3}, which corresponded with both higher fungal biomass yield and extent of degraded bagasse. The fungal biomass yield was 772.79 and 472.92 mg
and degradation of bagasse was 552.79 and 859.94 mg/g treated bagasse substrate on the 3rd and 7th day of fermentation. The efficiency of fungal biomass conversion was 67.44 and 54.99% on the 3rd and 7th day of fermentation, respectively (Table IV, Appendix).

On the basis of extent of bagasse degradation and utilization of carbon with sodium nitrate nitrogen source, only 171 mg out of 544 mg carbon in 1.0 g untreated bagasse was utilized as compared to 396 mg out of 567 mg carbon in 1.0 g treated bagasse. The crude protein content and protein recovery was 3.69 fold and 2.72 fold higher with treated than untreated bagasse during 7 days fermentation. However, with cornsteep liquor at 600 mg N, the crude protein content and protein recovery were only 2.2 fold and 1.23 fold with treated than untreated bagasse due to equal bioconversion of the 319 mg carbon supplied by 1.41 g cornsteep liquor per g bagasse substrates. However, the degradation of treated bagasse was 3.32 fold more than untreated bagasse substrate with cornsteep liquor nitrogen source.

The correlation of the parameters studied during fermentation of untreated bagasse substrate with cornsteep liquor at 600 mg N/litre was highly significant at 1.0% level, i.e. between crude protein, protein recovery, CMCase and FPase. However, the correlation between cellulose utilization and cellulase activity was significant only at 5.0% level. Cellulose utilization was not significantly correlated with crude protein and protein recovery. The effect of time of incubation was highly significant (1.0% level) with enzyme activity and cellulose utilization and significant (5.0% level) with crude protein and protein recovery.
The correlation of all the parameters studied during fermentation of 1.0 treated bagasse substrate was highly significant (1.0% level) except that between time of incubation and CMCase activity, which was only significant at 5.0% level (Table V, Appendix).

Even though, the CMCase activity was nil during initial 24 hr, the filtrate gave a little FPase activity, suggesting thereby that CMCase and FPase activities exist in two different components of multicomponent enzyme complex, which are triggered independently during the initial period of induction. During the initial fermentation for two days, cellulose of untreated bagasse substrate was not degraded by the fungus, probably due to the initial utilization of the water soluble carbohydrate, which was present in a high proportion in untreated bagasse and cornsteep liquor, and thus delayed the induction of cellulase enzyme production. The decrease in water soluble carbohydrate in the untreated bagasse substrate from initial 11.2% to 7.1% after 24 hr microbial fermentation might be due to the growth of \textit{A. terreus} 0%, at the expense of free sugars in bagasse substrate in addition to that present in cornsteep liquor.

It could also be explained that there was a lag period required for the biosynthesis of cellulase enzyme, which was inducible in nature (Sternberg, 1976; Ryu and Mandels, 1980). The alternate decrease and increase in water soluble carbohydrate could be attributed to the induction and repression of cellulase enzyme at a particular concentration of free sugars. The variation in free sugars in the biomass might also be due to simultaneous utilization of initial available sugars and subsequent hydrolysis.
of bagasse cellulose (Fig. 13-15).

The sugarcane bagasse substrate is composed chiefly of lignin, cellulose and hemicellulose. The cellulose and hemicellulose were rather easily attacked by cellulolytic microorganisms (Tobin et al., 1965). The lower cellulase activity observed in untreated bagasse substrate might be due to the lignocellulose complex of the substrate, which retard the cellulase production (Feist et al., 1970; Kirk, 1971; Deshpande et al., 1973; Han, 1978; Sidhu and Sandhu, 1980). Our findings on SCP and cellulase production with treated bagasse substrate are in agreement with the work of Araujo and D'Souza (1978), who reported that \emph{A. terreus} Thom Pale strain brought about 69-85% utilization of treated rice straw cellulose with 44.4% of protein in residual biomass during one week fermentation.

Since, 1.0% treated bagasse substrate with corn steep liquor at 600 mg N/litre broth yielded exponential growth of \emph{A. terreus} G31, higher cellulase activity and protein recovery during initial 3 to 4 days fermentation, a 10 litre fermentor trial (3 litre working volume) under optimum cultural and nutritional conditions was conducted and the various parameters were correlated at 12 hr interval up to 96 hr. In the fermentor trial, continuous aeration at the rate of 2 litres/min and shaking at 6 hr interval at a speed of 120 rpm was given as compared to continuous shaking and 1/5 (v/v) broth culture in flask trial. It was observed that both the biomass protein content and the cellulase production was low up to 48 hr, which increased to logarithmic level only at 60 to 72 hr (Figs. 27 and 28). It was noted in the flask trial, that both the protein
content and recovery as well as the enzyme production with treated bagasse was at an exponential increase during the initial 2 days fermentation itself (Figs. 17, 20 and 23). So, possibly in the fermentor trial, the extent of aeration and intermittent shaking might not have been optimum for biomass protein and cellulase production due to changes in the fermentation conditions initially for 2 days. Hence, for obtaining maximum rate of biomass protein content (24.5%) and cellulase production (CMCase 45.6 and FMase 2.43 units) per g treated bagasse substrate, the time of incubation should be 72 hr only. The correlation of the different parameters during fermentation of 1.0% treated bagasse with A. torreyi 281 was highly significant (Table VI, Appendix).

The efficiency of treated bagasse cellulose utilization and its correlation with the crude protein content of biomass revealed that there was no initial utilization of cellulose substrate in treated bagasse during first 24 hr. This might be due to the preferential utilization of the initial water soluble carbohydrate present in the substrate and the repression of cellulase enzyme synthesis (catabolite repression) due to the threshold concentration of water soluble carbohydrate (Wiszawa et al., 1972; Peitersson, 1977; Gallo et al., 1979; Herr, 1980). When the sugar level went below the critical repression level after 24 hr, the bagasse cellulose induced the synthesis of cellulase enzyme and concomitantly increased the cellulose utilization from zero to 25.6% at 36 hr fermentation. Maximum bagasse cellulose was utilized at 84 hr incubation time, which remained constant afterwards. Thus, there was a positive correlation between cellulase induction and
efficiency of sugarcane bagasse cellulose utilization. The crude protein content of fungal biomass ranged from 10.5 to 29.9% as compared to 3.04% crude protein in control treated bagasse. The crude protein content of 29.4% was obtained with 72.5% utilization of bagasse cellulose at 84 hr incubation period. However, 29.8% crude protein was present in the biomass harvested after 96 hr fermentation, but the bagasse cellulose utilization did not increase from 72.5%. It indicated that after 84 hr, the mold grew at the expense of available water soluble carbohydrate, which decreased from 9.7 to 7.89% during 84 to 96 hr fermentation (Figs. 27 and 28).

The level of water soluble carbohydrate content during fermentation of treated bagasse by A. terreus G1, for 96 hr in the fermentor, indicated an initial decrease from 6.56 to 1.68% during 24 hr fermentation (Fig. 29). The alternate variation in water soluble carbohydrate content, in a regular fashion, may be explained on the grounds of induction and repression of cellulase enzyme at a particular concentration of free sugars. This variation in water soluble carbohydrate content can also be explained on the basis of end product (feed back) inhibition and repression of cellulase enzyme system (Sternberg, 1976; Ryu and Mandels, 1980).

The single cell protein can be a good supplementary diet for ruminant feeding. Hence, the evaluation of protein quality in terms of dry matter in vitro rumen digestibility and amino acid composition would indicate the suitability of the microbial protein as a protein food and feed supplement. The quality of microbial protein evaluated by dry matter in vitro rumen digestibility and quantitative estimation of its
amino acid content are presented in Tables 13 and 14. In vitro rumen digestibility (IVRD) trial revealed that fungal biomass alone had 71.2% digestibility coefficient. It was also observed that the initial digestibility of treated bagasse decreased from 84.4 to 76.0% during 3 days fermentation, which suggested that digestible energy was obtained from the treated bagasse substrate during the SCP process.

The study of amino acid evaluation of microbial protein obtained after 3 days fermentation, revealed that almost all essential and non-essential amino acids are present in microbial protein produced from treated bagasse with A. terreus 38, and were well compared with the FAO reference proteins. The level of all essential amino acids was higher in the SCP obtained from A. terreus 38, but only phenylalanine was less. The other essential amino acid, tryptophan could not be evaluated, as it might have been decomposed due to acid hydrolysis.

From the results of SCP evaluation, it could be deduced that the treated bagasse would form a good substrate for microbial protein production by A. terreus 38, to supplement the feed component of ruminants and for microbial cellulase production.

The fermentation slurry obtained after 7 days incubation on a rotary shaker with 1.0% treated bagasse substrate and 600 mg N cornsteep liquor/litre broth by A. terreus 38, was centrifuged and the supernatant containing the cellulase enzymes was further purified. The crude enzyme in the supernatant was partially purified by ammonium sulphate fractionation. The protein obtained at the range of 35-75% ammonium sulphate concentration, contained maximum cellulase enzyme units after
passing (desalting) through Sephadex G-25, which had 5.4 fold CMCase and 5.8 fold Fase purification with 80% CMCase and 93.7% Fase recovery of activity (Table 15).

The partially purified enzyme was further characterized for its activity in the reaction mixture. The CMCase and Fase activities were linear and positively correlated with respect to incubation time up to 120 min (Fig. 30).

A linear relationship between CMCase activity and enzyme concentration was observed up to 4.8 μg protein concentration. The relationship between Fase activity and enzyme concentration was however, linear up to 9.6 μg protein concentration for cellulase enzyme from A. terreus 3H1 (Fig. 31). Mandels et al. (1976) observed a relationship between Trichoderma viride cellulase and enzyme concentration ranging from 0.01 to 0.1 ml of the culture filtrates and CMCase activity. In case of filter paper, however, they observed a decrease in Fase activity with increase in enzyme concentration. In the present study, the decreased rate of enzyme activity with further increase in protein concentration might be due to limiting substrate concentration.

The optimum temperatures of incubation mixture at 60°C for CMCase and 55°C for Fase were recorded for A. terreus 3H1 cellulase (Fig. 32). However, Ikeda et al. (1967) recorded the optimum temperature of incubation at 40°C for both fractions of A. niger cellulase. Hurst et al. (1977) observed maximum CMCase activity of the A. niger cellulase at 40°C. Misukoshi et al. (1977) observed the optimum temperature for filter paper degrading activity of cellulase produced by Pelllicularia filamentosa at 45°C. Mandels et al. (1976) observed increase in both
CMCase and Pase activities with an increase in temperature of incubation from 40 to 60°C.

The cellulase enzyme was stable up to 55°C. However, further increase in temperature of heat treatment resulted in a rapid loss of both CMCase and Pase activities at 70°C and showed zero activity at 75°C, when enzyme alone was heated for 10 min (Fig. 33). Heat stability of enzyme recorded in the present study is in accordance with similar observations made by Hurst et al. (1977), in case of \textit{A. niger} cellulase and by Selby and Weitland (1967), in case of \textit{E. viride} cellulase. Variation in optimum temperature of incubation as well as heat stability were not only reported in cellulases from different sources but differences in these parameters were also found for different components of the enzyme from a single source.

In the present study, \textit{A. terreus} 3% cellulase had an optimum pH of 4.5 with CM-cellulose as substrate, whereas, with filter paper as the substrate, the optimum pH was 5.5 (Fig. 34). Hurst et al. (1977) reported an optimum pH of 3.8 to 4.0 for \textit{A. niger} cellulase with CM-cellulose as the substrate. A higher pH optima of 4.5 to 6.0 was recorded, when cellulose dextrin sulphate was used as the substrate (Clarke and Stone, 1965). The pH optima of 4.5 to 6.0 was recorded for "Cellulase III" (Okada, 1976) and endo-glucanases II, III and IV (Shoemaker and Brown, 1978) from \textit{E. viride}. The optimum pH for the filter paper degrading activity of \textit{Pellimularia} filamentosa was 5.0 (Mimukoshi et al., 1977). The cellulases from different sources exhibited a wide range of pH optima. Different pH optima were reported with different substrates for the enzyme from the same source.
Km and Vmax values of the enzyme with CM-cellulose as the substrate were 13.1 mg/ml and 31.9 μM/mg protein/min, whereas, the values were 90 mg/ml and 2.7 μM/mg protein/min, respectively, with filter paper substrate (Fig. 35 and 36). However, Clarke and Stone (1965) reported a Km value of 0.25 M for A. niger cellulase with cellulose dextrin sulphate substrate. Hurst et al. (1977) reported for the A. niger cellulase, a Km value of 5.3 - 9.2 CM-cellulose/0.1 mg protein and Vmax of 46.2 - 60.9 units/mg protein/min. Shoemaker and Brown (1976) determined the Km values for three endo-glucanases of T. viride cellulase as 0.25 - 10 mM for cellobiose, and cellobextrin, and 1-5 mM for cellotetraose, respectively. Thus, it is apparent that Km and Vmax values not only varied with the substrate but also with the source of cellulase enzyme.

The enzyme (CMCase) was stimulated to a lesser extent by Mn2+ (10^-2M). However, both CMCase and ββase activities of the enzyme were stimulated to a greater extent by ascorbic acid at 10^-4 to 10^-2M concentrations. The CMCase activity was enhanced approximately by six fold and ββase activity by nine fold by 10^-2M concentration of ascorbic acid. The enzyme was inhibited by Cu2+, Ag2+, As3+, Fe3+, PCMB and sodium diethyl dithio carbonate (Tables 16 and 17). Reddy (1979) reported that ascorbic acid and cysteine hydrochloride slightly stimulated both CMCase and ββase activities of A. niger and T. viride cellulases. Cellulases from A. niger and T. viride have also been reported to be inhibited by Ag2+ phenylmercuricntrate, halogens, heavy metals, detergents, Mn2+, Zn2+, sodium borate, Na2SO4, NaF, \( \text{NH}_2\text{OH}, \text{HCl}, \text{CaCl}_2, \text{As}_3, \text{Hg}^{2+}, \text{Ca}^{2+}, \text{As}_3, \text{Cu}^{2+} \) (Clarke and Stone, 1965;

The economic viability of any proposed industrial fermentation process has to be taken into account the cost of substrates, processing cost and value of the products recovered. The evaluation of production of SCP and cellulase enzyme by fermenting alkali-treated bagasse substrate for 3 days in both flask and fermentor trials, indicates that the SCP could be produced at no additional cost when the by-product, cellulase recovery was taken into consideration. The SCP product recovered has 20.57 to 24.50% crude protein containing most of the essential amino acids with 76% dry matter in vitro rumen digestibility and compares well with other concentrate feed mixtures. The production of SCP on large scale will supplement the conventional protein sources in feeding livestock in India. Besides, the recovery of cellulase as a by-product in the SCP process, opens up new vistas of the important enzyme, which can be commercially used for sugar syrup recovery from delignified cellulose wastes with greater potentials for utilization in ethanol, SCP and pharmaceuticals productions (Anderson et al., 1975; Ladosch et al., 1978; Wilke et al., 1981).

The bagasse substrate is available in enormous quantities as a by-product from sugar industry in India, which could be profitably utilized for SCP and cellulase production. In the flask trial, the cost of producing 1000 Sigma units of cellulase works out to be ₹6.11, whereas, in the fermentor trial, the cost works out to be
No. 24.80 under laboratory conditions. However, when large scale production is undertaken, these costs would be much lower. The possibility of semi-commercial and commercial production of SCP and cellulase has to be explored further. As the SCP availability is very much in short supply and cellulase is mostly imported, the commercial production of both these products will have a tremendous impact on the economy of the country.
1. Twelve cellulytic mold cultures were screened for the production of single cell protein by cultivating on 1.0% carboxymethyl cellulose, sugarcane bagasse and hot alkali-treated bagasse substrates. *Asperillus terreus* GN1 was found to be the most efficient isolate yielding a crude protein content of 19.88% in its biomass and a protein recovery of 11.03 g/100 g treated bagasse. The other mold cultures arranged in the order of decreasing yield of crude protein were: *A. niger* sp. GN2, *Trichoderma viride* TL and 6ACW, A. niger, A. oryzae GN2 and *Fusarium verticillioides*.

2. Inoculation of mixed cultures of cellulytic molds neither increased the protein content nor its recovery in the cell biomass.

3. *A. terreus* GN1 showed a maximum crude protein content of 20.1% in its biomass and a protein recovery of 11.5 g/100 g of substrate, as well as a maximum activity of 0.7 units CMCase/ml and 0.07 units Fase/ml, when cultivated on 1.0% treated sugarcane bagasse substrate supplemented with 0.25% sodium nitrate at the following optimum cultural conditions: pH of medium adjusted to 4.0; incubation at 25 to 35°C for a period of 7 days on a shaker with a continuous agitation; 1/5 (v/v) broth culturing in the flasks and use of 5.0% (v/v) inoculum of a week old mold culture.
4. Among the various organic and inorganic nitrogen sources tried as supplements to 1.0% treated bagasse @ 250 - 600 mg N/litre broth, cornsteep liquor at 600 mg N yielded the maximum biomass crude protein recovery, crude protein content and showed maximum cellulolytic activity during 7 days fermentation.

5. Cultivation of \textit{A. \textit{maximus}} GM\textregistered under optimum cultural conditions on untreated bagasse substrate, supplemented with cornsteep liquor (600 mg N/litre broth) resulted in a maximum protein recovery of 16.36 g/100g substrate with 14.9% biomass crude protein content, as compared to the crude protein content of 2.01% in the control. The organism also exhibited maximum cellulolytic activity of 0.55 CMCase units/ml and 0.027 FCase units/ml with 23.53% cellulose utilization. However, under similar cultural and nutritional conditions, treated bagasse substrate yielded a maximum protein recovery of 20.08 g/100g bagasse with 32.9% crude protein content in the biomass. The organism also exhibited a higher cellulolytic activity of 1.02 CMCase units/ml and 0.033 FCase units/ml with 82.7% cellulose utilization during 7 days fermentation.

The rate of protein and cellulase enzyme production, however, was maximum in the logarithmic growth phase up to 3 to 4 days of fermentation indicating thereby, that the optimum time of incubation for fermenting 1.0% treated bagasse was 3 to 4 days. The rate of protein and cellulase production declined on further incubation.
6. A pilot scale fermentor trial conducted under optimum cultural and nutritional conditions of fermentation indicated a yield of 21.0, 24.5 and 29.8% crude protein content, 0.43, 0.54 and 0.52 CMCase units/ml and 0.027, 0.027 and 0.032 Pase units/ml with 40.0, 55.0 and 72.5% cellulose utilization at 60, 72 and 96 hr incubation, respectively.

7. The biomass obtained on the 3rd day of fermentation of 1.0% treated bagasse by \( \Delta \) \( \text{thermo} \) \( \text{OH} \) had a dry matter \textit{in vivo} rumen digestibility coefficient of 76.0%. The biomass contained almost all essential and non-essential amino acids and was comparable with FAO reference protein.

8. The cellulase produced by \( \Delta \) \( \text{thermo} \) \( \text{OH} \) from treated bagasse substrate, on partial purification through ammonium sulphate fractionation and Sephadex G-25 gel filtration resulted in 5.4 fold increase in specific activity with 80% recovery.

9. A linear relationship of the partially purified CMCase and Pase activities with enzyme concentration was noticed up to 4.8 and 9.6 mg protein, respectively. The CMCase and Pase activities were maximum at pH 4.5 and 5.5 and incubation temperature of 60 and 50°C, respectively. The enzyme was stable up to a temperature of 55°C. \( K_m \) and \( V_{max} \) values of CMCase were 13.1 mg and 10.9 mM/mg protein/min and for Pase, the values were 80 mg and 2.8 mM/mg protein/min, respectively.

Metal ions viz., \( \text{Ca}^{2+} \), \( \text{Hg}^{2+} \), \( \text{Ag}^+ \) and \( \text{Fe}^{3+} \) inhibited both CMCases and Pase activities at the concentrations of \( 10^{-4} \) to
$10^{-2}$, the enzyme activities were inhibited by PCMB and sodium diethyl dithiocarbamate and stimulated to a lesser extent by $Na^{2+}$ and to a greater extent by ascorbic acid (vit-C).

10. The economic evaluation of the fermentation of treated bagasse by *A. niger* G1, indicated that in addition to the SCP recovery, the by-product recovery of true cellulase enzyme would enhance the viability of the process.

**CONCLUSION**

*Aspergillus niger* G1 has the highest mycelial biomass protein content of 41.6% as compared to other molds, when grown on Caspek's broth with 1.0% carboxymethyl cellulose substrate. It utilized the alkali-treated sugarcane bagasse better than untreated substrate at 1.0% concentration.

*A. niger* G1 degraded the treated bagasse cellulose to the extent of 82.65% as compared to only 23.53% of untreated bagasse cellulose during 7 days fermentation under optimum cultural and nutritional conditions. Besides, *A. niger* G1 produced more cellulase enzyme with treated bagasse. The extent of biomass protein recovery was also higher with treated than untreated bagasse. The 10-litre fermentor trial indicated that, 1.0% treated bagasse fermentation by *A. niger* G1 for 72 hr would be ideal for exponential rate of bioconversion into protein and cellulase under optimum cultural and nutritional conditions. The SCP recovered on 72 hr fermentation had a crude protein content of 24.5% and the slurry filtrate
had the cellulase activity of 0.54 CMCase units/ml and 0.027 FPase units/ml. The cellulase was found to be stable up to 55°C. The SCP had a dry matter IVMD coefficient of 76% and comprised of most of the essential amino acids, which compared well with FAO reference proteins. The economic evaluation of the SCP process with A. niger CH4 using 1.0% treated bagasse substrate indicated its viability with the recovery of SCP as main product at negligible cost, which could be utilised to supplement the conventional livestock feed and the cellulase enzyme as by-product, which has commercial application for sugar syrup recovery from delignified cellulosic wastes.
CHAPTER VII

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* Original articles not seen.
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### Table III: Efficiency of Protein Recovery, Cellulase Production and Bagasse Utilization by A. niger 39 on 1.0% Alkali-Treated Bagasse Substrate at Optimum Cultural and Nutritional Conditions of Fermentation

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<th>Crude protein (%)</th>
<th>Fungal biomass (mg)</th>
<th>Undegraded bagasse (mg)</th>
<th>Degraded bagasse (mg)</th>
<th>Efficiency of fungal biomass conversion (%)</th>
<th>Total CMCase (units)</th>
<th>Total β-glucosidase (units)</th>
<th>Efficiency of CMCase (%)</th>
<th>Total Protease (units)</th>
<th>Efficiency of Protease (%)</th>
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<td>1.5 645 13.1 169.68</td>
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| 7. Sodium nitrate 450 596 18.2 234.01 361.99 638.01 36.68 10.8 46.1 3.2 | 8. Urea 450 652 17.0 237.23 414.77 595.23 40.54 10.0 35.7 1.9 | 10. Cornsteep liquor 450 610 32.7 469.36 414.64 859.36 54.62 20.0 85.5 6.8 |
| 500 614 20.8 283.27 330.73 669.27 42.33 12.8 40.1 3.3 | 600 600 17.4 222.66 377.54 622.66 35.76 10.8 43.7 2.3 | 500 640 16.8 223.05 414.95 598.05 39.78 10.8 43.7 2.3 |
| 600 636 17.8 243.12 392.88 607.12 40.04 11.3 43.7 2.3 | 600 674 32.9 521.93 152.07 847.93 61.55 22.1 94.5 8.1 | 600 592 32.8 456.13 135.87 864.13 52.78 19.0 85.5 6.8 |
| 9. Cattle urine 450 616 16.3 311.19 404.81 595.19 52.28 10.0 35.7 1.9 | 10. Cornsteep liquor 450 610 32.7 469.36 414.64 859.36 54.62 20.0 85.5 6.8 |
| 500 640 16.8 223.05 414.95 598.05 39.78 10.8 43.7 2.3 | 600 674 32.9 521.93 152.07 847.93 61.55 22.1 94.5 8.1 | 600 592 32.8 456.13 135.87 864.13 52.78 19.0 85.5 6.8 |
Table IV: Efficiency of Protein Recovery and Cellulase Production by <i>A. niger</i> on Untreated and Alkali-Treated Bagasse Substrates (1.0 g) During 7 Days Fermentation with 600 mg Corn steep Liquor Nitrogen / Litre Broth

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<th>Fungal biomass (mg)</th>
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<th>Degraded bagasse (mg)</th>
<th>Protein recovery (%)</th>
<th>Total CMCase (units)</th>
<th>Total Pase (units)</th>
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Table V: Correlation Coefficient (r) of Various Components During 7 Days Fermentation of Untreated and Alkali-Treated Bagasse Substrates by *L. mesenteroides* CN1 (Flask Trial)

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<th>$x_4$</th>
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* Significant at 5.0% level.

** Significant at 1.0% level.
Table VI: Correlation Coefficient (r) of Various Components During 7 Days Fermentation of 1.0% Alkali-Treated Bagasse Substrate by A. NIGRIPUS CN1 (Fermentor Trial)

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<th>$x_2$</th>
<th>$x_3$</th>
<th>$x_4$</th>
<th>$x_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein ($x_1$)</td>
<td>1</td>
<td></td>
<td>0.9342**</td>
<td>0.9577**</td>
<td>0.9874**</td>
</tr>
<tr>
<td>CMCase ($x_2$)</td>
<td>-</td>
<td>1</td>
<td>0.9342**</td>
<td>0.9796**</td>
<td>0.9122**</td>
</tr>
<tr>
<td>FYase ($x_3$)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.9027**</td>
<td>0.9432**</td>
</tr>
<tr>
<td>Cellulose utilization ($x_4$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.9825**</td>
</tr>
<tr>
<td>Time ($x_5$)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

* Significant at 5.0% level.

** Significant at 1.0% level.
LIST OF PUBLICATIONS

A. PUBLISHED

1. Production of fungal protein on cellulosic substrate.

2. Utilization of cellulosic substrates for fungal biomass production.

3. Effect of cultural factors on cellulase and protein production by Aspergillus niger GII.

B. ACCEPTED

1. Studies on the cellulase enzyme complex from Aspergillus niger GII.

2. Effect of nutritional factors on protein and cellulase enzyme production by Aspergillus niger GII and its evaluation.


C. COMMUNICATED

1. Single cell protein and cellulase enzyme production by A. niger GII with untreated sugarcane bagasse substrates,
VITA

The author, Satyendra Kumar Garg was born on Fifth of April, Nineteen Hundred and Fifty Five at Bapawar Kalan, District Kota, Rajasthan. He was schooled at Bapawar for his Pre-Higher Secondary Studies and completed his Higher Secondary Examination from Government Multipurpose Higher Secondary School, Baran, District Kota, Rajasthan in the year 1972 securing First Rank (71.0%) with distinction in Biology and Chemistry. He graduated from Government College, Baran, University of Rajasthan, Jaipur in July 1975 securing First Rank (69.0%) with distinction in Chemistry and was honoured with Silver Medal. He joined for the Master's programme in the College of Basic Science and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar, Nainital in November, 1975 and completed his degree with major Microbiology and minor Biochemistry in January, 1978 securing O.P.A. 4,800/5,000 (87.0%). During his post graduation, considering his abilities and dedication, he was offered Graduate Research Assistantship in the Department of Microbiology to assist the work on "Pulse Microbiology" project.

He registered for the Doctor of Philosophy programme in Dairy Microbiology at National Dairy Research Institute, Karnal, Kurukshetra University, Kurukshetra (Haryana) in September, 1978 and submitted his thesis in December, 1981. He secured distinction in all courses prescribed by the University. During the tenure of investigation, he was awarded Senior N.D.R.I. merit scholarship in the Department of Microbiology.