IMMOBILIZATION OF CELLULOLYTIC BACTERIUM (A)

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JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI - 110 067, INDIA

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CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full, for any other Degree or Diploma of any University.

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... To those who laboured in their laboratories to make this world a happier place to live in.

CONTENTS

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INTRODUCTION

Depleting fossil fuel resources the world over has forced the mankind to look for alternative energy sources. Coal and petrolium, the conventional energy resources are being exploited by man at such a rate that they may not be available in the near future with the present rate of development. The search for new energy sources has opened up many areas such as nuclear, solar, tidal, wind, oceanic ionic and oceanic thermal gradients etc. In this search for new technology, the biologists and biotechnologists are also trying to tackle these problem by way of converting the municipal and agricultural wastes into usable compounds with the help of microorganisms.

The bioconversion of solid municipal and agricultural wastes has many potential advantages. Besides giving acceptable food and feed stocks, it also promises to provide the Single Cell Protein (S.C.P.), organic fertilizers, biofuels and organic chemicals. Apart from these, it also lends solution to the pollution problem bogging the major metropolis all over the world related to disposal of the solid wastes.

The major component of municipal and agricultural wastes is cellulose. It is probably the most abundant organic compound in nature and is going to be the major renewable energy source in the long run. It is estimated that cellulosic materials are produced at a rate of 10^{11} tons

per year in the world (Pathak and Ghose,1973). So practically there will not be any dearth of this most abundant natural material.

Though seed-bearing plants are the major synthesizers of cellulose, algae, fungi and bacteria can also produce it in small quantity. The natural sources of cellulose in decreasing abundance are : a) the stems of woody angiosperms (hard woods) containing about 40-55% cellulose,b) the stems of woody gymnosperms (soft woods) with about 45-50% cellulose, c) the stems of monocotyledons most of which contain 25-40% cellulose, d) nonlignified parenchyma cells of most leaves, most of which contain 25-40% cellulose and, e) certain nonlignified or partially lignified fibers such as seed hairs of cotton and the bast fibres of flax containing 80-95% cellulose (Cowling and Kirk, 1976). Thus it is evident that in nature most of the times cellulose is always strongly associated with other naturally occuring materials such as lignin and hemicelluloses.

Any bioconversion involves three steps. Firstly, the upstream processing in which the substrate is processed in such a way that it can readily participate in the reaction. Secondly, the conversion itself i.e. the substrate is catalysed into product and finally, the down stream processing which involves the separation of product from the catalyst and substrate. The conventional batch or continuous culture reactors that are prevalent cannot be

used repeatedly for the fact that in down stream processing i.e. the separation step of product from the catalyst (in bioconversion, the enzyme in most of the cases) invariably renders either the loss or inactivation of the catalyst. Thus a novel approach of immobilization was tried towards solving this problem which envisages to overcome this difficulty. This technique was also successfully employed on the industrial scale.

Immobilized cells or enzymes are defined as 'cells or enzymes physically confined or localized in a certain defined region of space with retention of their catalystic activities, and which can be used repeatedly and continuously'. Thus this definition covers three aspects. The first aspect, dealing with 'confinement or localization', is of geometrical nature. The second aspect is the 'retention of enzymatic activity'. The third aspect of the definition is the 'repeated use in a continuous fashion'.

The present study is aimed at immobilizing the cells of Bacillus thermoalkalophilus, a bacterium isolated from termite infested mound soils with cellulolytic activity (Paul et al., 1985), for the production of the enzyme cellulase(s) which can hydrolyse the soluble cellulose. Two different immobilization methods have been used and it has been concluded that the successful application of these methods depends upon their efficiency inter alia and and over that of the batch culture.

REVIEW OF LITERATURE

The faster depletion of fossil fuel resources, petroleum and natural gas has led to a search for renewable energy sources. Plant material being one of nature's largest biomass resource has naturally attracted the attention as an important energy food source. The largest component of plant material is cellulose. If it is utilized properly, it can easily substitute the petroleum and natural gas and allay the fear of anticipated energy crisis. Keeping this point in view a lot of attention is being paid to this area and extensive research work has been initiated in recent past with increasing budget to make this dream true. The present work is also a concerted approach towards tackling this problem.

Cellulose and its occurrence :

There are more carbohydrates in the biosphere than any other organic matter. The carbohydrates are dominated by two polysaccharides, both polymers of D-glucose i.e., starch and cellulose. Bellamy (1974) estimated that 6% of the total CO_2 fixed (22 b tons/year) is converted into cellulose, that is, 24 tons per person per year. Cellulose is the major structural constituent of the comparatively rigid cell wall of plant cells. Cellulose in plant cell walls is associated mainly with hemicellulose and lignin. In the stems of woody angiosperms (hard woods) hemicellulose constitutes 24-40% whereas the lignin content ranges between

18-25%. Similarly in the stems of woody gymnosperms (soft woods) the content of hemicellulose and lignin ranges between 25-35%. Most of the stems of monocotyledons contain 25-50% hemicellulose and 10-30% lignin. On the other hand, the parenchyma cells of most leaves contain 80-85% pectin and hemicellulose, the rest being cellulose. Cellulose constitutes the major proportion (80-95%) with a small amount of hemicellulose (5-20%) in certain nonlignified or only partially lignified fibers such as seed hairs of cotton and the bast fibers of flax (Cowling and Kirk, 1976).

Besides the above, algae, fungi and certain bacteria, e.g. Acetobacter xylinum (Savidge and Colvin, 1985), Velonia venticosa (Marx-figini, 1982) etc. can also synthesize cellulose.

Cellulose also does not occur in pure form in most forest and agricultural residues or in urban trash such as food and paper processing wastes. Most forest and agricultural residues contain about 20-50% of cellulose and 50-80% of hemicellulose and lignin. Sugarcane bagasse and wheat or paddy straw, for example contain 25-40% cellulose, the rest being hemicellulose and lignin. News papers have about the same general composition as the woods from which they are derived. Waste fibers, paper prepared from chemical pulp such as corrugated fiber board and Kraft bag paper usually contain about 60-70% cellulose, 10-20% hemicellulose and 5-10% of lignin. Waste fibers from chemical

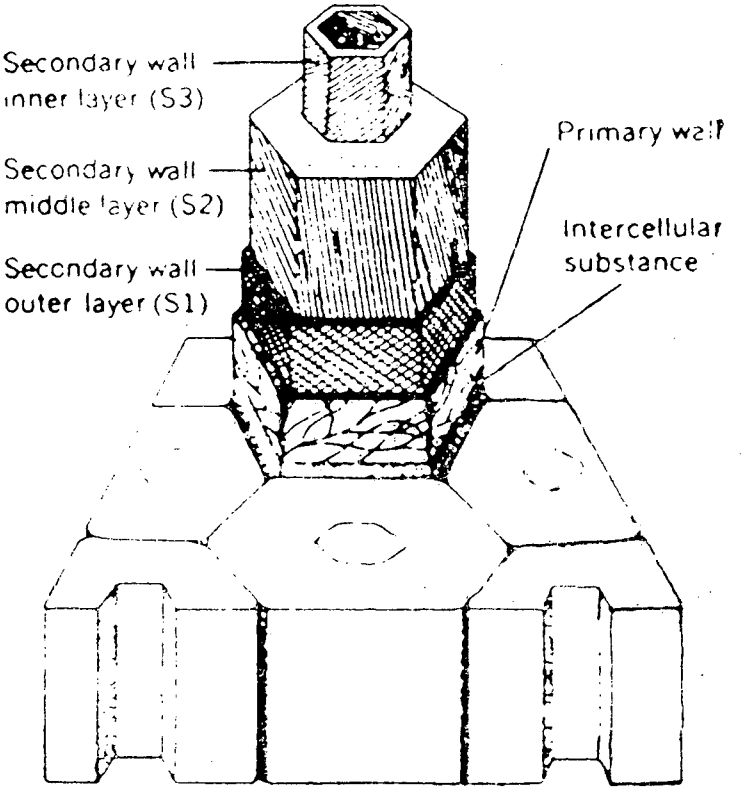


Fig. 1 Diagrammatic sketch of wood cell walls. S_1, S_2 and S_3 are outer, middle and inner layers of secondary wall respectively (from : Properties of cellulose materials by E.B. Cowling and T.K. Kirk).

pulping processes typically contain about 60-80% cellulose, 20-30% hemicellulose and only 2-10% lignin (Cowling and Kirk, 1976).

Keeping in mind the above information, the naturally occuring source should be properly modified to suit the best utilization of its individual components.

Structure of cellulose fibers :

Cellulose in cotton fibers and wood cell walls is very similar in molecular structure despite its varying content (cotton fibers contain 90% cellulose whereas wood contain only about 45%). Cotton fibers contain no intercellular substance and wood fibers on the other hand, form a cohesive three dimensional structure with large amounts of intercellular substances of which lignin is a major constituent.

Both cotton and wood fibers have a thin primary wall surrounded by a thick secondary wall. This secondary wall consists of three layers (S1,S2 and S3). S2 layer is of variable thickness but forms the bulk of cell wall substance. The cellulose molecules in S1 and S3 layers are deposited in a flat helix with respect to the fiber axis whereas those in S2 layer are deposited in a series of concentric zones.

Within each layer of secondary wall, cellulose and inner cell-wall constituents are aggregated into long slender

bundles designated as microfibrils.None or very few cellulose molecule cross over from one microfibril to another. Within each microfibril, the linear molecules of cellulose are bound laterally by hydrogen bonds and are associated in various degrees of parallelism-regions that contain highly oriented molecules called crystalline and those of lesser order are called paracrystalline or amorphous (Cowling and Kirk, 1976).

Regarding the structure of microfibril, three concepts were presented by Mülethaler (1976).According to one concept, the microfibril is about 50 x 100A^{o} in cross section and consists of crystalline core surrounded by amorphous region which contains mainly cellulose molecules in cotton while it contains hemicellulose and lignin molecules too in the wood. On the other hand, another concept proposes that cellulose molecules are less ordered at certain points along the length of the microfibril. And third concept proposed by Manley (1964) suggests that the cellulose molecules exist in a folded chain lattice formed as a ribbon which in turn is wound in a tight helix. Rowland and Roberts (1972) proposed that there are coalesced surfaces of high order, readily accessible slightly disordered surfaces and readily accessible surfaces of strain-distorted tilt and twist regions.

Since microfibrils, and larger aggregates of cellulose molecules are not visible in untreated cotton or wood fibers

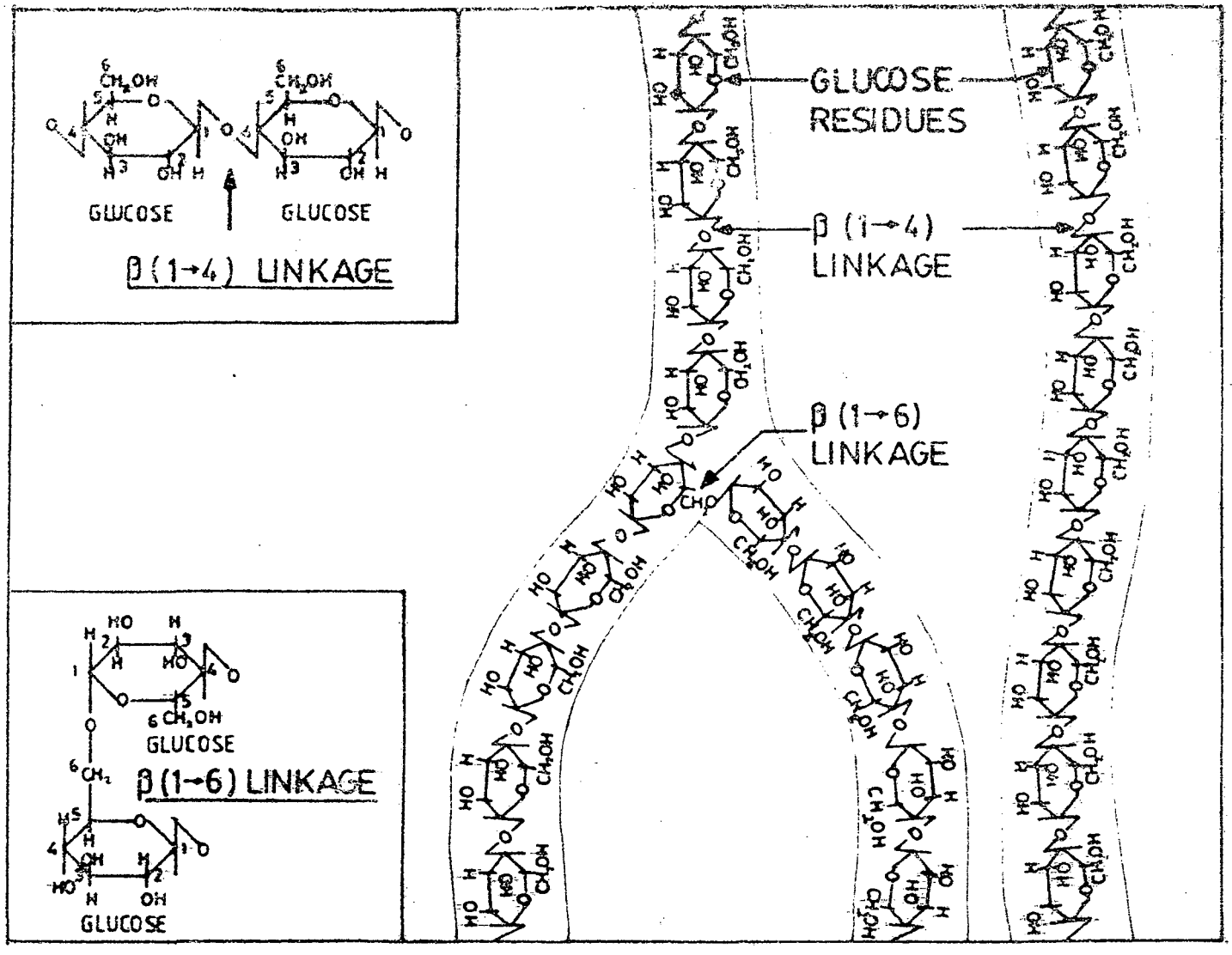


Fig. 2 Structure of cellulose molecule.

by X-ray crystallography, the exact structure of cellulose is still eluding.

Cellulose is a polymer of D-glucose, the glucose sub-units being linked into a chain by ß-(1-4)-glucosidic bonds. But Atalla (1983) regards disaccharide cellobiose as the functional unit of cellulose instead of glucose. These chains are further cross-linked by ß-(1→6) linkages. The degree of polymerization ranges from 15 or less to 10,000 and above. This gives a molecular weight of over one million and a length of about 5 mm per chain.

Hydrolysis of cellulose :

Hydrolysis of crystalline cellulose is a complex process and was well reviewed by Ljungdhal and Eriksson (1985). This requires the participation of at least three enzymes. They are : 1. Endo-(1,4)-ß-D-glucanase (Endo-ß-glucanase) (E.C. 3.2.1.4).

It is sometimes reffered to as endocellulase, carboxymethyl cellulase (CMCase), 'C_x' or as avicelase. Its molecular weight ranges between 18,000 and 60,000. It initiates attack on crystalline cellulose.

2. Exo-(1,4)-ß-D-glucanases (exoglucanases) :

It is also known as C_1. There are atleast two types of exoglucanases. THey are :

i) (1,4)-ß-D-glucan cellobiohydrolase (cellobiohydrolase CBH) (EC.3.2.1.91) : This removes cellobiose units one by one from nonreducing ends of cellulose chain.

ii) (1,4)-ß-glucan glucohydrolase (Glucohydrolase) (EC.3.2.1.74) : This removes glucose molecules one by one from nonreducing ends of the chain.

3. ß-(1,4)-Glucoside glycohydrolase (ß-D-glucosidase) : This is also known as cellobiase. Two types of ß-glucosidases are reported, one which is active towards aryl ß-glucosidase but not on polymeric substrates (aryl-ß-glucosidase), and the other with a broad activity towards different glucosidase. In general, endoglucanases are more thermostable than exoglucanases.

ß-glucosidases and glucohydrolases share common substrates. The ß-glucosidases differ from exoglucanases in four respects :

i) They have greater activity on dimers than on higher cellooligosaccharides and polymeric substrates.

ii) They retain anomeric configuration during hydrolysis.

iii) They are more sensitive to inhibition by glucono- - lactone and

iv) they hydrolyze ß-1,1, ß-1,2, ß-1,3, ß-1,4 and ß-1,6 linkages whereas exoglucanases are highly specific for ß-1,4 linkages. The ß-glucosidases are reported to be specific for hydroxyl group at C_4 (Sadana, 1985).

A two step mechanism for the hydrolysis of glucosides by ß-glucosidase has been proposed. The first step involves splitting of a glycogen moiety with simultaneous formation of an enzyme-glycosyl complex. This intermediate complex

then reacts with water, yielding glucose.

Different hypotheses were put forward to explain enzymatic degradation of cellulose. But the perfect mechanism still remains debatable. These hypotheses are discussed as follows :

a) $C_1 - C_x$ hypothesis :

This was proposed by Reese et al., in 1950. According to this hypothesis native cellulose is attached by free-hydrolytic 'C_1' factor which breaks the hydrogen bonds. As a result native cellulose would be converted into linear anhydroglucose chains which are then acted upon by hydrolytic 'C_x' enzymes producing soluble sugars.

b) Modified Reese hypothesis :

Due to mounting evidences against his hypothesis, Reese proposed a modified hypothesis in 1971. According to this, the first step in hydrolysis of native cellulose involves a special type of an endoglucanase, C_1 which is limited in its action only on crystalline surfaces and is unable to hydrolyse (unlike other endoglucanases) the products of its own action.

c) Hypothesis involving sequential action :

According to this, the endoglucanase action is followed by CBH (Eriksson, 1969). This hypothesis envisages that C_x enzyme initiates the attack on crystalline cellulose, thus generating new chain ends for the 'C_1' to act. This hypothesis enjoyed a high degree of experimental evidence.

d) Further modified Reese hypothesis (1977) :

Reese presented a further modified form of his hypothesis in which he postulated that native cellulose is converted into swollen cellulose by the action of C_1 and which in turn acted upon by endoglucanases, glycohydrolases and CBH to give oligomers, glucose and cellobiose, respectively.

e) Hypothesis based on oxidative attack :

Vaheri (1982) reported that an oxidative reaction catalyzed by a flavin-like hydrophobic compound takes place in the early stages of crystalline cellulose degradation.

f) Hypothesis based on microfibril generating factor :

Proposed by Griffin et al. (1984), this hypothesis envisages that a low molecular weight microfibril generating factor (MGF) interacts with native cellulose to yield microfibrils without significant release of soluble sugars. They interpreted that one major element in cellulose is physical fracturing of superstructure of cellulose fibers into smaller units that are accessible to other cellulase components.

g) Hypothesis involving sequential action :

Sadana and Patil (1985) suggested a new hypothesis. According to them, the action on crystalline cellulose is initiated by CBH and modified cellulose is synergistically acted upon by endoglucanase and CBH.

Klyosov et al., (1986) studied the activity and adsorption of cellulases in their efficiency of degrading and crystalline cellulose. The major factor controlling the enzymatic hydrolysis of crystalline and amorphous cellulose seems to be the adsorption capacity of endoglucanases on cellulose, which in turn may explain the different rates of hydrolysis of cellulose.

Cellulose degraders :

The ability to hydrolyze cellulose is present in wide range of organisms like bacteria, actinomycetes, fungi, higher plants and protozoa, but there is no authentic report of cellulase being produced by other animals. These organisms are predominantly present in the soil. Some cellulolytic bacteria occur in symbiotic relation with some animals and are found in rumen of vertebrates (Hungate, 1955, 1963; Bryant et al., 1958), in low quantity in intestine of rat and in high number in ceca (Montgomery and Macy,1982),in cockroaches (Cruden and Markovetz, 1979) and in gut of millipedes (Anderson and Bignel, 1980; Taylor, 1982; Paul et al., 1985). Some cellulose degrading actinomycetes (Mauzynska and Janota-Bassalic, 1974) and a fungus, Neocallimastrix frontalis (Wood et al., 1986)from rumen of vertebrates and fungi from the gut of millipedes (Anderson and Bignel, 1980; Taylor, 1982) have also been reported. Cellulose degrading microorganisms have also been reported from wood eating small insects (Reyes

and Tiedje, 1976; Ulrich et al., 1981; Martin et al.,1981), earthworm (Parley, 1963) and pigs (Varel et al., 1987). These organisms include mesophiles, thermophiles and alkalophiles as well.

Cellulases have also been reported from higher plants e.g. pea (Byrne et al., 1975). In pea it was found that with the treatment of growth hormone (Indole Acetic Acid, IAA) there was enhancement of cellulase production. IAA plays major role in cell elongation. So they proposed that the cellulases losen the rigid cell wall, thereby facilitating fresh deposition of cellulose. The mechanism of induction of cellulase production by IAA is not clear (Byrne et al., 1975; Verma et al., 1975; Maclachlan and Wong, 1979).

The most active cellulases till date are produced by Trichoderma reesei, a fungus isolated at U.S.Army Natick Development Center. Two mutants of Trichoderma reesie (QM 9123 and QM 9414) are very active on cellulose and in fact, much of our knowledge on cellulose saccharification is a result of the work carried out on these strains. These strains are also known as Natick strains. Besides Trichoderma reesie a number of other fungi also produce considerable amount of cellulases. Prominent among them are Trichoderma koningii, Penicillium funiculosum, P.notatum, Aspergillus fumigatus, A.wentii, A.aureus, A.terreus (Doelle, 1984) and Eupenicillium javanicum (Tanaka et al., 1980). Thermophilic, cellulolytic fungi so far isolated include Sporotrichum

thermophile, Chaetomium thermophile var. dissitum and Humicola sp. (Dolle, 1984).

Among bacteria the major cellulose producers are ; Cellulomonas uda, Cellvibrio fulvus, Clostridium sps. (Lee and Blackburn, 1975). Mutants of Cellulomonas fimi have also been raised (Paul et al., 1987).

Thermophilic bacteria which can degrade cellulose include Clostridium thermocellum (Viljoen et al., 1926) and Sporocytophaga (Bellamy, 1980). A number of other thermophilic cellulolytic bacteria had also been reported (Suchardová, et al., 1986).

Horikoshi and Akiba (1982) isolated a number of alkalophilic, cellulolytic bacteria. Cellulolytic and alkalophilic bacilli, Bacillus sp. No. 1139 (Fukumori et al., 1985) had also been reported. A new Bacillus sp. which is both alkalophilic and thermoalkalophilic besides having cellulolytic activity was isolated by Paul et al. (1985).

Tansey and Brock (1978) proposed that the thermophilic microorganisms are not extensively involved in cellulose decomposition, since thermophilic environments (50^{o}C) are quite limited in distribution. This might be true with alkalophilic, cellulolytic microorganisms as well.

Though a number of microorganisms produce cellulases, the rate of cellulose degradation varies among them (Gong and Tsao, 1979). Degradation is dependent on the composition and amount of cellulases produced, as well as on the nature of cellulosic substrates (Mandels et al., 1976; Manning and

Wood, 1983). Johnson et al. (1982) suggested that cellulases in thermophilic Sporocytophaga and Clostridium are quite active on highly crystalline cellulose but less so on amorphous substrates. One of the major problem with bacterial cellulases has been the low detectable activity in culture filtrates. Cultures of Clostridium thermocellum grow faster than T.reesei on native cellolose, yet the cellulase activity in the bacterial broth is generally about 100 times lesser than that in fungal broth (Gordon et al., 1978).

Trichoderma and Clostridium cellulases were compared for end product inhibition. It was found (Johnson et al., 1982) that fungal enzyme complex was less sensitive to cellobiose inhibition but much more sensitive to glucose inhibition. Cellulase production in fungi was shown to increase by addition of surfactants such as Tween 80 (Reese and Maguire, 1969). But Duong (1981) working with Clostridium thermocellum did not get any increased enzyme production with Tween 40.

The activity profile of cellulase enzyme has also been found to differ in different organisms. Fungal cellulases are generally active in the acidic range. Horikoshi et al. (1984) purified the cellulases from an alkalophilic Bacillus strain. They could differentiate two fractions of cellulase, one active at neutral pH and the other in the alkaline range.

There can be synergism between the action of different cellulases from different organisms. It was indeed the case

as Wood et al.(1980) had shown. It was also shown that synergism in action is present between CBH of Penicillium funiculosum and endo-(1-4)-ß-D-glucanases of Trichoderma koningii and Fusarium solani (Wood and McCrae, 1972 ; Gum and Brown, 1977). Although various forms of CBH have same substrate specificity and mode of action, these may differ in their amino acid and carbohydrate composition and pH (Gum and Brown, 1977; Wood and McCrae, 1972). This may suggest their symbiotic action in nature.

I Induction : Cellulase synthesis has also been reported to be inducible by cellulose and certain disaccharides in several fungi and bacteria. Cellulose being insoluble cannot enter the cell. Thus a soluble product of cellulose was proposed to be the actual inducer of cellulase(s) (Mandels and Reese,1960).According to this hypothesis a small amount of cellulase is produced constitutively and hydrolyses small amount of cellulose.The product was proposed to induce the enzyme system. They proposed that cellobiose was involved in cellulase induction, but later Mandels et al. (1962) showed that sophorose (2-0-ß-D-glucopyranosyl-D-glucose), which may be formed from cellobiose as a transglycosylation product of ß-glucosidase (Okada and Nishizawa, 1975; Marja et al., 1979; Fukumori et al., 1985), was a much more potential inducer. Labelled leucine was not incorporated into CMCase unless sophorose was present. Sternberg and Mandels (1979)

also showed that synthesis of CMCase stopped when the mycelia of T.reesei were separated from induction medium containing sophorose. The experiments of Loewenberg and Champan (1977) and Sternberg and Mandels (1979) suggest that the metabolism of sophorose may be important for CMCase production. Sternberg and Mandels (1980) showed that sophorose was responsible for repression of ß-glucosidase synthesis, which is responsible for the formation of sophorose from cellobiose.

Similarly in other organisms also cellulase synthesis was shown to be inducible and regulated by catabolite repression. For example, Enebo (1954) reported that in Clostridium thermocellaseum, cellulase synthesis is more sensitive to catabolite repression by glucose and cellobiose with cellobiose being more repressive. In Clostridium thermocellum also (Barker, 1983) had shown that the system is inducible.

II Catabolite Repression : Soluble, rapidly-metabolized carbon sources such as glucose and cellobiose dramatically decrease cellulase synthesis in cellulolytic bacteria and fungi. This lowering of cellulase production is probably caused by catabolite repression. Nisizawa et al., (1972) showed that glucose prevented the formation of CMCase and ß-glucosidase in T.reesei. They suggested that inhibition of CMCase production occurred at translational level since the kinetics and degree of inhibition resembled to that by

puromycin but not actinomycin D. Further evidence was provided when glucose was added after transcription and was found to be inhibitory. In Trichoderma, cellulase repression is not related to the levels of cyclic AMP (Montenecourt et al., 1980). Thus catabolite repression appears to be the most common mechanism for the regulation of cellulase synthesis.

Factors affecting cellulose hydrolysis :

The following factors were shown to be affecting the enzymatic hydrolysis.

a) Moisture content of the fibre : Cellulosic materials are protected from deterioration by microorganisms so long as their moisture content is maintained below some critical amount which is characteristic of the material and organisms involved. For wood, this was shown to be slightly above the fiber saturation point (Etheridge, 1957) while for cotton, about 10% moisture was shown to be adequate (Siu, 1951). The fiber saturation point for cotton and most wood species has been noticed between 24 and 32% of the dry weight. This critical moisture content is achieved by adsorption of moisture in equilibrium with 100% relative humidity. It is that moisture content at which all sorption sites within cell walls are fully saturated with water having no free water available at the lumina.

Moisture swells the fiber by hydrating the cellulose molecules, thus opening up the fine structure so that the

substrate is more accessible to cellulolytic enzymes and other reagents. Also, the elements of water are added to the cellulose during hydrolytic cleavage of glucosidic bond.

b) Size and diffusibility of cellulolytic enzymes and other reagents in relation to the capillary structure in cellulose: The accessible surface of cellulose is defined by the size, shape and surface properties of the microscopic and submicroscopic capillaries within the fiber in relation to the size, shape and diffusibility of the enzymes themselves. When the substrate exists as an insoluble polymer in a complex structural matrix as in cellulose, the specific affinity of the enzyme towards substrate drastically reduces the diffusion of the enzyme.

c) Degree of crystallinity :

The influence of the degree of crystallinity on enzymatic hydrolysis has been studied by Norkrans (1950), and Reese et al. (1957). Using X-ray diffraction, Norkrans (1950) showed that cellulolytic enzymes more readily degrade the amorphous regions than crystalline regions. Besides it was also shown that, the accessible portions of cellulose hydrolyzed, the residual portion became more crystalline and thus more resistant to further enzymatic hydrolysis.

d) Unit cell dimension of the crystallinities present :

Cellulose occurs in four reognised crystal structures designated as cellulose I,II,III and IV (Honeyman, 1959).

Cellulose I is the crystal form of native cellulosic material. Cellulose II occurs in regenerated celluloses such as cellophane etc. Cellulose III and IV are formed by treatment with anhydrous ethylamine and certain high temperatures, respectively. These in turn will also influence the hydrolysis rate.

e) Conformation and steric rigidity of the anhydroglucose units :

King (1961) suggested that the greater reistance of crystalline cellulose may not only be due to its physical inaccessibility to enzyme molecules but also due to the conformation and steric rigidity of the anhydroglucose units within the crystalline region in which they occur in chain (C_1) conformation with alternate units oriented in opposite direction. However, it was not yet proved experimentally.

f) Degree of polymerization of the cellulose :

The degree of polymerization of cellulose varies within a fiber and may have an affect on the rate of enzymatic hydrolysis, particularly by those enzymes which cleave the cellulose molecules by endwise mechanism.

g) Nature of the substances with which the cellulose is associated and the nature of their association :

Cellulose is normally associated with lignin and the other elements that are essential for microbial growth. Gascoigne and Gascoigne (1960) have concluded that mercury, silver,copper, chromium and zinc salts are generally

inhibitory to microbial degradation whereas manganese, cobalt, magnesium and calcium inpresence of phosphate are stimulatory. The presence of specific enzyme inhibitors such as phenols (Mandels and Reese, 1965) also will effect the enzymatic hydrolysis.

The nature, concentration and distribution of substituent groups :

The susceptibility of substituted cellulose derivatives to enzymatic hydrolysis increases as they become more water soluble and less crystalline upto the point of complete solubility (Reese, 1957).

Pretreatment of cellulose materials :

The cellulose molecules in their native forms are not freshly accessible to cellulase(s) for the reasons cited in the previous section. So pretreatment of the cellulosic substances became a necessary to make them more accessible to the enzymes and thus increasing the rate of hydrolysis.

Two approaches of pretreatment are in practice. One is the removal of polymers other than cellulose from natural cellulosic materials to get pure cellulose. The other is extraction of cellulose. A number of methods are in practice.

The most common pretreatment is ball milling or very fine grinding (Pew, 1951). He suggested that the overall increase in digestibility due to ball milling was apparently

a result of decreased particle size and increased available surface rather than a result of reduced crystallinity.

Marchessault and St. Pierre (1978) reported a so called explosion process that could be applied to deciduous wood. The initial stage involved a short prehydrolysis for 30 seconds at 220-240^{o}C and 600 psi followed by a rapid decompression whereupon the hemicellulose was rendered almost completely water-soluble. Then 90% of the lignin was solubilised in ethanol : water (9:1) leaving cellulose highly accessible to enzymes. Other pretreatments with SO_2 (Ben-ghedalia and Miron, 1981), electron irradiation, cryogenic grinding (Millet et al., 1976) etc. have also been reported but did not evoke any acceptance.

A number of approaches could contribute to better hydrolysis of cellulose which render it more accessible to the enzyme, isolation of better cellulase producers and raising mutants with improved cellulase production and resistant to product inhibition. A better understanding of genetics and regulation of the enzyme synthesis will be of great help.

Bioconversion :

In bioconversion batch cultures are playing a major role. In batch culture the substrate and the biocatalyst (enzyme) are incubated together and after the reaction is over the product is separated from the enzyme by denaturation

using pH adjustment or heat treatment. The denatured enzyme cannot be used again.

To make the use of enzyme economically viable and to increase the stability of the enzyme, a new approach of its immobilization on solid and liquid supports had been developed. The first report of immobilized enzyme was that of invertase from yeast adsorbed on charcoal (Nelson and Griffin, 1916). Since then a lot of development had taken place and whole range of immobilization techniques are available now. This technique had also been applied in a number of industries.

At the Enzyme Engineering Conference held at Henniker, New Hampshire in 1971, the definition of immobilized enzymes was proposed as 'enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously'.

The first aspect of the definition, localization of enzyme, means that there is a macroscopic catalytically active solid phase dispersed in or in contact with a liquid reactant medium. This compartmentalization, in fact, leads to very high enzyme density thus enabling to reduce the reactor volume considerably over that of batch culture. In the solid phase, transport of reactants and products to and from the reaction site is governed by diffusion only. As

a result of diffusion transport resistance, gradients of concentration or pH may be established in the solid particles. Thus, the environmental situation of immobilized species may become different from that of liquid bulk phase.

The second aspect is the retention of enzymatic activity. This retention need not be complete but should be high enough to be of practical interest.

The third aspect dealing with continuous and repeated use is more important in industries since it brings down the cost of production. Upon immobilization the stability of the enzyme increases and consequently the half-life of enzyme is greatly enhanced. This half life depends upon the method of immobilization. Kokubu et al. (1981) reported a half life period of 20-50 days under operational conditions without applying any stabilization techniques. With the chemical treatment for stabilization after immobilization Chibata (1979) had reported that operational stability can be increased to a year.

Hence, the following advantages can be envisaged with immobilized enzyme systems,

a) Stability of the enzyme is improved,

b) Enzymes can be reused,

c) Continuous operation becomes possible,

d) Reactions require less space,

e) Higher purity and yield of products may be obtained, and

f) Resources can be conserved and pollution minimized.

But the extraction and purification of enzyme itself may be quite costly and tedious process. Particular reactions are catalysed by more than one enzyme as with cellulose hydrolysis. Some enzymes require continuous cycling of cofactors etc. These disadvantages can be overcome by using immobilized cells.

Immobilized cells are essentially defined in a similar way as that of immobilized enzymes except that the term enzyme is replaced by cell and in some cases the retention of the viability of cell is important.

A number of methods are available for immobilizing the cells. These can be broadly classified into binding and entrapping. Binding can be either carrier-binding or cross-linking. Carrier-binding methods can be classified into adsorption method, chelation method and covalent binding. Similarly, entrapment can be either gel entrapment, fiber entrapment or micro-encapsulation. Gel entrapment is again classified into entrapment by polymerization, by ionic net work formation or by precipitation.

Cross-linging of cells utilizes the free amino groups in the bacterial cell walls. The covalent attachment of cells is brought together by bi or multifunctional reagents such as aldehydes (Chibata et al.,1974; Poulsen and Zittan, 1976; Hughes and Thurman, 1970) or amines (Lartigue and Weetall, 1976). The degree of cross-linking depends upon the structure of cell walls. The most commonly used bifunctional aldehyde

for this purpose is glutaraldehyde, though a multifunctional epoxypolyamine (EPA) has also been reported (Hu, 1986). The major disadvantages with these chemicals are their toxicity and low retentivity of enzyme activity (Munton and Russel, 1973).

Adsorption, on the other hand, is mainly based on elecrtostatic interactions-i.e. Vanderwaals forces, and ionic and hydrogen bonds between the cell surface and support material. Zeta potential on both of them plays an important role in this type of interaction. Cells will be attracted to surfaces of opposite zeta potential. Even if the cells and surfaces have the same zeta potential adhesion is still possible provided the electrostatic barrier can be penetrated by small surface projections (Van Oss et al., 1975; Grinnell, 1978). A number of carriers, both organic and inorganic have been used for this process. The organic carriers include wood chips (Moo Young et al., 1980) etc. The inorganic support includes glass (Heinrich and Rehm, 1981; D'Souza et al., 1986), porus brick (Ghose and Bandopadhyay, 1980), stainless steel (Atkinson et al., 1979) etc. But adsorption has a week binding force with a high retention of activity.

Hydrous titanium (IV) and Zirconium (IV) oxides are the most commonly used carriers for immobilization by chelation (Kennedy and Kay, 1976). The actual mechanism is still not clear. But it was envisaged that hydroxyl groups on the surface of metal hydroxide is replaced by the ligands

from enzyme or cell such as side-chain hydroxyl groups of L-serine or L-threonine, carboxymethyl groups, L-glutamic acid or L-aspartic acid and or ϵ-amino group of L-lysine residues. The gel formation by these hydroxy metal oxides is pH dependent, hydrous oxide of titanium being more effective in acidic range and that of zirconium in alkaline range.

Covalent coupling on the other hand, involves direct linkage of cells to an activated support. The components of cell surface which can take part in covalent linkage are amino, carboxyl, thiol, hydroxyl, imidazole or phenol group of proteins. This is the most stable immobilization method but with a very low retention of activity. Weetall (1976) reported silanization of silica and ceramics by trialkoxysilane derivatives with an organic functional group. Coupling of these derivatives to the carrier presumbaly takes place by displacement of the alkoxy residues on the silane by hydroxy groups on the oxidized surface of the inorganic support to form a metal-O-si linkage.

Entrapment method of immobilization is, infact, the most commonly used method of immobilization. This is based on occlusion of cells within a constraining structure tight enough to prevent the leakage of cells but allowing the diffusion of substrate and product.

Dinelli (1972) first reported a method of entraping cells within microcavities of synthetic fibers. These fibers

are resistant to weak acids or alkalis, high ionic strength and some organic solvents. Entrapment is achieved by dissolving a fiber-forming polymer in an organic solvent immiscible with water and emulsifying this solution with the aqueous suspension of cells containing glycerol. The emulsion is extruded through a spinnet into a liquid coagulent (toluene or petroleum ether) which precipitates the polymer in filamentous form. The main draw-back of this system is the use of water-immiscible liquid as polymer solvents and coagulents which in some cases may cause damage to the cells.

Microencapsulation method was first reported by Chang (1964). In this, the cells or enzyme molecules were entrapped within microcapsules. However, these microcapsules were too fragile to be used in reactors and thus were limited to medical and analytical applications.

Entrapment by polymerization was first reported by Mosbach and Mosbach (1966) using polyacrylamide. In this method, the acrylamide solution containing cells were polymerized by cross-linking of the monomers with bifunctional compounds (N,N'-methylene bis acrylamide, BIS) in presence of a catalyst (N,N,N',N'-tetramethylethylenediamine) and an initiator (potassium persulphate). The major disadvantages of this method are the toxicity of the acrylamide monomer, the cross-linking agent (BIS), TEMED and the high temperature produced at the time of reaction which some cells may not be able to tolerate. Another method of radiation-induced

polymerization of a glass-forming monomer, such as 2-hydroxyethyl methacrylate, at low temperatures was reported by Kumakura et al., (1978).

Updike et al., (1969) showed that the cells entrapped in acrylamide retained their viability. Franks (1971, 1972) reported that cells immobilized by this method were stable over 11 days. Kennedy and Cabral (1983) have shown that polyacrylamide immobilized thalli retained their catalytic activity over 3 months.

In entrapment by ionic network formation, only nontoxic compounds are used. The most popular method in this case is entrapment of living bacterial cells in calcium alginate (Kierstan and Bucke, 1977; Cheetam, 1979). Entrapment of plant cells in Ca-alginate was also reported (Brodelius et al., 1979, 1980).This was originally developed by Thiele (1954). The underlying principle in this method is that alginate being a linear block copolymer of D-manuronic and L-guluronic acids, can be cross-linked by multivalent cations such as calcium and aluminium. Care should be taken, in this case , that no calcium chelating agents should be present in the medium.

Entrapment in k-carrageenan (Wada et al., 1979;Chibata, 1980), collagen (Veith and Venkatasubramanian, 1979) and agar (Tada and Shoda, 1975; Margalith and Holeberg, 1981) are the most commonly used entrapment methods by precipitation. Agar was melted and cooled to 40^{o}C followed by mixing of

the cells. This was then extruded into toluene or tetrachloroethylene to form pellets (Toda and Shoda, 1975).

Cheetam et al., (1979) had shown that the distribution coefficients, which in turn is determined by diffusion and partition coefficients, is not dependant upon external diffusional limitations but on the porosity of the matrix. Moreover, since cells are entrapped, larger pore spaces could be tolerated facilitating diffusion of larger molecules. The same authors had also shown by using chloramphenicol in the medium that the increased number of cells in the medium is actually the result of division of leaked cells and not the increased rate of cell leakage.

Regarding the changes in metabolic behaviour, Mattiason and Hahn-Hagerdahl (1982) proposed a model in which decreased water activity results in changed metabolic activity and product formation of the immobilized cells. The changed water activity would be due to the high polymer concentration of the microenvironment; these macromolecules 'organize' water and thereby decrease the amount of water available to the cells. This hypothesis was in agreement with the work of Karube et al., (1980) and Holeberg and Margalith (1981) who showed that the presence of polymer even in low concentration had substantial effects on biochemical reactions which are water dependent.

Best (1985) concluded that the share of cellulase(s) in commercial utilization is less than one percent despite

the large abundance of cellulose materials. For the commercial utilization of cellulase(s), the enzyme has to be supplied at low cost with a consistently high quality.

The present study is aimed at producing the endoglucanase enzyme by immobilizing a unique thermophilic and alkalophilic organism (Bacillus thermoalkalophilus sp. nov.) isolated from termite mound soil in polyacrylamide and agar gel matrics. The efficiency of these methods was also checked inter alia.

MATERIAL AND METHODS

Carboxymethyl cellulose (DS 0.65-0.8), Coomassie Brilliant Blue G, Bovine Serum Albumin, Acrylamide, N,N-Methylene-bis Acrylamide, N,N, N',N',-tetramethyl ethylenediamine (TEMED) were obtained from Sigma Chemical Company, St. Louise, U.S.A. Dinitrosalicylic acid (DNSA) was from Merck, Germany. Yeast extract and agar were from Difco, Michigen, U.S.A. Sucrose was obtained from Hi Media, Bombay, India. All other chemicals were of local manufacture and were of analytical grade.

Organism : The strain of Bacillus thermoalkalophilus sp. nov. isolated from termite gut,was taken from the stock maintained at the Microbiology laboratory, School of Life Sciences, Jawaharlal Nehru University.

Maintenance of the culture : The strain was maintained in liquid medium as well as in solid medium on slants and was stored at $4^{o}C$.

Medium for growth, maintenance and preservation : The culture was grown in prescribed medium (Paul et al., 1985) unless otherwise mentioned. The medium contained (g/l) : $CH_3COO.Na$ 3.0, Na_2SO_4. $7H_2$= 0.4, KCl 0.8, Fe SO_4. $7H_2O$ 0.01,EDTA 0.03, K_2HPO_4 0.3, KH_2PO_4 0.3, Mg SO_4. $7H_2O$ 0.2,yeast extract 2.0. The pH was adjusted to 9.0 using 1N NaOH. Before autoclaving, 1% carboxymethyl cellulose was added. For the growth of bacterium on lactose, cellulose was substituted by 1% lactose. Agar (1.5%) was used for making the solid medium.

Sterilization : The glasswares used were thoroughly washed in detergent water, acid water (1%H_2SO_4), running tap water followed by rinsing in single and double distilled water. The flasks used were tightly plugged with non-absorbant cotton and wrapped with cotton gauge. Further, the cotton pluggs were covered with aluminium foils. All the glass-wares were then kept in an oven at 180°C for 4-5 hours. Liquid media and distilled water were sterilized in an autoclave at 122.4 Kpa for 15min. Laminar air flow chamber was sterilized by ultraviolet irradiation for 15 minutes.

Inoculum : 0.1 percent inoculum was used in all the experiments with 10 hr old late log phase culture.

Culture conditions : The bacterium was grown aerobically at 60°C in a thermostatically controlled waterbath shaker (New Brunswick) at 150 rpm. Petri plates were incubated in an incubator.

Growth measurements : Growth was monitored by measuring the change in absorbance of cells in medium at 550 nm by Shimadzu UV-150-02 with uninoculated medium as a blank. The growth rates were determined from the slope of the growth curve for the culture during logarithmic growth phase. Generation time was calculated (Thayer, 1975) from the slope using the equation :

$$G = \frac{(t_2 - t_1)\ 10g2}{10g\ O.D_2 - 10g\ O.D_1}$$

Where G = generation time

$O.D_1$ = Absorbance at time t_1 in hours.

$O.D_2$ = Absorbance at time t_2 in hours.

Specific growth rate, μ was calculated from generation time using the formula,

$$\mu = \frac{1n\ 2}{G}$$

Change in pH : The change in pH of the growing culture was monitored periodically at an interval of 10 hours over a period of 40 hrs.

Effect of surfactant on release of enzyme from cell surface

To check the effect of surfactant on the release of enzyme into culture medium, 0.015% (V/V) of Tween 80 was added to the culture medium and the supernatant was checked for CMCase activity over a period of time.

Protein estimation :

100 mg Coomassie Brilliant Blue (G-25) was dissolved in 50 ml of 9% ethanol. To this solution 100 ml of 85% phosphoric acid was added. Then the solution was diluted to 1 l and filtered through Whatman No. 1 filter paper.

Procedure : Protein estimation was done according to the method of Bradford (1976) using BSA as standard. 0.1 ml of sample was taken and to that 5 ml of Bradford reagent was added. They were mixed properly, kept at room temperature for 20 minutes and absorbance was read at 595 nm by Shimadzu UV-260. A standard curve was plotted using different

concentrations of Bovine Serum Albumin.

Gram's stain :

Jacob and Gerstein (1965) method followed :

Reagents : i) Crystal violet solution.

Crystal Violet - 2g

Ethanol (95%) - 20ml Sol. (A)

Ammonium Oxalate - 0.8g

Distilled water - 80 ml Sol. (B)

Solution (A) and (B) were mixed and stored at room temperature for 24 hours, filtered through paper into staining bottle.

ii) Gram iodine (mordant) Solution

Iodine - 1g

KI - 2g

Distilled water - 300ml

Iodine and KI were ground slowly in a mortor and water was added slowly and stored.

iii) Acetone alcohol

95% ethanol - 100 ml

Acetone - 100 ml

IV) Counter-stain

Stock solution : Safranine 2.5 g

Ethanol 100ml

Working solution : 10 ml of stock solution +

90 ml of distilled water.

Procedure : On a clean glass slide one or two loopful suspensions of exponentially growing culture were taken and were heat fixed. Crystal violet solution was flooded onto the smears and kept for one minute. Then it was washed in running water and excess water was drained off. Smears were flooded with mordant solution and kept for 1 minute. Then the slides were again washed with tap water. Smears were destained with acetone-ethanol solution until flowing solution was colourless. Slides were washed in tap water. Smears were then counterstained with safranine for 10 seconds, washed, blot dried and the cells were observed under microscope (100x with oil immersion). The size of the bacteria was determined using ocular and stage micrometers.

Cellulase estimation :

Cellulase(s) were estimated by estimating the reducing sugars as a result of enzymatic action.

Reducing sugars were estimated by the method of Miller (1959). The reagent was prepared as described below :
40g dinitrosalicylic acid (DNSA), 8g phenol, 2g sodium sulfite and 800g potassium sodium tartarate (Rochelle salts) were dissolved in 2 l of 2% sodium hydroxide and was then diluted to 4 l.

CMCase (Endo-ß-1,4-glucanase) estimation :

This was done using the method of Mandels et al., (1976). Caboxymethyl cellulose (CMC) reagent was prepared

by stirring 10 g CMC in 1 l of 0.05 M phosphate buffer for 3 hours. To this 0.01% sodium azide was added and kept at $4^{o}C$.

The assay was standardized for optimum incubation temperature, quantity of CMC reagent mixture to be added and pH of the reaction. The enzyme was assayed by incubating 0.5 ml of the enzyme solution with CMC reagent for 30 minutes. To this 3 ml DNSA reagent was added and boiled for 5 minutes and absorbance was noted at 540 nm on the Shimadzu UV-260. The amount of sugars were estimated from a standard curve plotted for glucose.

Filter paper assay for saccharifying cellulase :

Mandels et al., (1976) method was followed for assaying saccharifying cellulase. This is typically similar to that of CMCase assay except that instead of CMC reagent, 6 cm x 1 cm strip of Whatman No. 1 filter paper (50 mg) was added and 1 ml of 0.05 M phosphate buffer was added. The reaction mixture was incubated for 1 hour.

In both cases spectro zero was adjusted with substrate blank and against this absorbance of an enzyme blank having the enzyme containing solution was taken to estimate the reducing sugars present in the enzyme solution. This value was subtracted from the value of reducing sugars to give the amount of reducing sugars produced.

The unit of enzyme was taken as u mole of glucose

produced per ml of the enzyme solution per 30 and 60 minutes for CMCase and saccharifying cellulase respectively.

Experiments on Assay :

In all these experiments supernatant of 48 hour culture was used.

Effect of pH on activity : The effect of pH on activity of CMCase was tested by preparing the CMC reagent in 0.05M K- phosphate buffer in a pH range of 6.0 to 10.5 with 0.5 units interval.

Effect of temperature : To find out the optimal temperature of incubation for CMCase activity, the reaction mixture was incubated at different temperatures of 30, 40, 50, 60, 70 and 80°C.

Effects of substrate concentration : To study the effect of substrate concentration on enzyme activity, different concentrations of CMC ranging from 0.005 to 5% were prepared in 0.05 M phosphate buffer (pH 7.0). 2ml of this reagent was added to the reaction mixture and the enzyme was assayed.

Location of Cellulase :

Cell free Cellulase :

Cultures at different time intervals of 6-60 hours were taken and centrifuged at 10,000 x g for 15 minutes at 4°C in Sorvall centrifuge. The supernatant was assayed for both cell free CMCase and filter paper activities.

Cell bound Cellulase :

The cell pellet from the above mentioned process was resuspended in 3 ml phosphate buffer (0.05 M) of pH 7.0 and the cell walls broken by ultrasonic disintegrator (MSE) for 3 minutes (90 seconds burst followed by 30 seconds gap to avoid heating of samples at the amplitude of 12 KHZ with titanium probe). THe sonicated sample was thencentrifuged at 15,000xg. for 10 minutes and the supernatant was used for assay studies.

Membrane associated cellulase :

This was measured suspending the cell debris in 3 ml of 0.05 mM phosphate buffer of pH 7.0 and assayed for CMCase and for saccharifying cellulase.

Effect of time on sonication :

To find out the optimum time of sonication of the cells, the cells that got pelleted by centrifugation of 30 hour old culture at 10,000 x g for 15 minutes were suspended in 3 ml phosphate buffer of 0.05 M (pH 7.0). They were then sonicated by placing the container having cells in ice in beaker for 1, 1.5, 2, 2.5, 3, 3.5, and 4 minutes with 30 seconds gap at half the time. These sonicated samples was centrifuged at 15,000 x g for 10 min. and the protein in the supernatant was determined.

Effect of surfactant on enzyme production and release :

To check the effect of surfactant, 0.015% (V/V) of

Tween 80 was added to the culture medium and the supernatant was checked for CMCase over a period of time.

Fresh weight of bacteria :

The fresh weight of bacteria in late logarithmic phase was determined by taking the culture when absorbance at 550 nm was 1.0 against the medium without cells. The culture was centrifuged at 10,000 x g for 15 minutes, the supernatant was drained off completely and the weight was determined in a Mettler balance by substracting the weight of empty centrifuge tube.

Immobilization :

Matsunaga et al., (1980) method was followed :

a) Immobilization in agar :

40 mg (fresh weight) cells from late logarithmic phase(O.D at 550 nm 1.0) were taken and centrifuged at 10,000 x g for 15 minutes. These cells were suspended in sterile physiological saline (sodium chloride 0.9%). Different concentrations of agar (140, 150 and 160 mg) were dissolved in 7 ml of physiological saline by heating it to 70°C. This was deaerated and cooled to 40°C and then the cells (in 3ml Physiological saline) were mixed. This mixture was extruded through a sterile syringe into a mixture of sterile water and paraffin to form the spherical beads of about 3 mm diameter. The beads were thoroughly washed on a rotary shaker in physiological saline for five to six times to remove

paraffin oil and cells adsorbed on the surface.

Immobilization in acrylamide gel :

5 gm weight cells were suspended in sterile water. 10 ml of 0.2 M phosphate buffer (pH 7.0) was chilled in ice and to this different concentrations of acrylamide (2.7, 2.85, 3.0, 3.5 g) and bis acrylamide (140, 150 and 160 mg) were added and dissolved with 50 ul of TEMED. Then the cells were mixed with the above solution keeping it in ice bucket and poured into sterile petri plates. 10 mg ammonium persulphate was added to the petri plates and it was allowed to polymerize for one hour. This was then cut with the help of sterile scalpel into squares of about 1.5 cm and washed in sterile physiological saline for three to four times.

Stability of gels :

Both acrylamide gel and agar beads were tested for their stability at different temperatures (30, 40, 50 and 60^{o}C).

Determination of cell leakage :

The beads and acrylamide gel having entrapped cells were put in 250 ml of physiological saline. At periodical intervals of 8 hours over 64 hours, one ml aliquot of the saline was taken and plated to determine the number of cells that leaked out of the gels.

Design of the reactor :

A 30 cm reactor was designed with two jackets - the

inner one containing the immobilized cells and the outer one for circulating water at 60°C. The inlet and outlet of the outer jacket were connected to multitemperature II thermostat to maintain the temperature at 60°C. The inner jacket had an inlet and outlet for the medium connected to a flask containing the medium without sodium acetate so that there is no multiplication of cells. The inner jacket was fixed with a glass tube for bubbling air through an aerator and an outlet for air, fixed with glasswool to prevent any possible microbial contamination. The outlet for medium also contained glasswool at the base so as to prevent beads or gel moving out.

The column was sterilized before loading the immobilized cells. The cells were loaded upto a height of 15 cm so as to maintain a height to diameter ratio of 3.0 to prevent serious diffusion problems.

Electron microscopy :

The specimens were essentially prepared according to the method of Shi et al. (1987). The gel matrices were sliced into thin sections and were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 6% sucrose on weight per volume basis for 3 hours at room temperature. Then they were washed in the same phosphate buffer. It was then transferred to 1% osmium tetraoxide in phosphate buffer of pH 7.2 and kept at room temperature for 2 hours. The specimens were washed and dehydrated in

graded 50-100% (30, 40, 50, 70, 90 and 100%) acetone for 10 minutes each. These samples were dried in critical point dehydrator and were then coated with silver. They were then scanned in Phillips (Model 501 B) electron microscope.

Electron microscopy of free cells :

The free cells were fixed on a glass slide, the glass slide was cut with a diamond knife and was coated with silver and scanned in an electron microscope (Phillips - model 501B).

Oxygen uptake :

30 mg (fresh weight) of cells were immobilized in agar and polyacrylamide gels. The gels were then cut into thin sections. The viability and metabolism of both free and immobilized cells was checked by monitoring the oxygen taken up in a YSI model 53 oxygen monitor at $37^{o}C$.

The cells/sections of gel were suspended in 3 ml of 0.05M phosphate buffer. 3 ml buffer saturated with air was put in the sample chamber and the meter was adjusted to 0.9. umoles of oxygen consumed per minute by gram fresh weight of cells was calculated using the formula,

$$\frac{423 \times \text{number of divisions moved / min.}}{1.8}$$

Enzyme production by immobilized cells :

Samples were collected at periodical intervals and were tested for enzyme production and protein.

RESULTS

I) Growth of organism :

The growth of the organism (Bacillus thermoalkalophilus sp. nov.) with both lactose (1%) and CMC (1%) as carbon source is depicted in Fig. 3. With CMC as carbon source, a very short lag phase of 2 hours was noticed, whereas in lactose containing medium the lag phase extended upto 10 hours. In CMC medium the stationary phase was obtained within 12 hours, whereas lactose medium took 18 hours to reach stationary phase. With lactose, the stationary phase was of very short duration (one hour) followed by the death phase. With CMC as carbon source, the stationary phase was comparitively longer (2 hours). After 14 hours, there was again a logarithmic growth phase in CMC medium, reaching the second stationary phase by 20 hours.

The generation time with lactose as carbon source in the medium was found to be 3.25 hours and the specific growth rate was 0.09/hr. In CMC, the generation time was smaller (3 hours) and the specific growth rate was calculated to be 0.1/hr.

II) Change in pH of the growing culture :

The medium which was adjusted to a pH of 9.0 has gone down to pH of 8.05 after autoclaving (Fig. 4). The pH more or less remained constant for about 10 hours, but increased to 9.2 in 20 hours.30hours old culture had a pH of 9.35 which

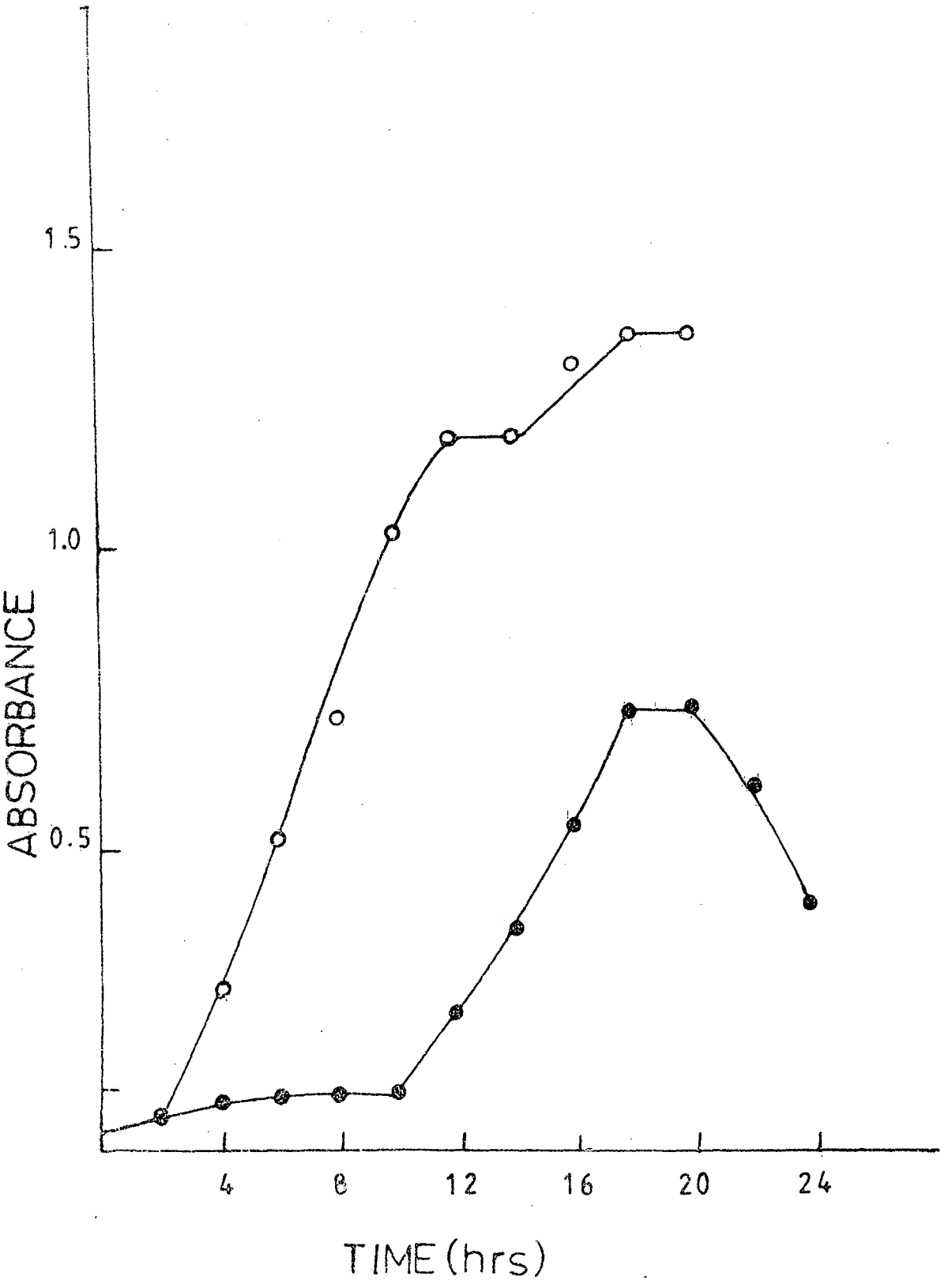


Fig. 3 Growth of Bacillus thermoalkalophilus sp. nov. Growth was measured in terms of absorbance at 550 nm. o——o CMS (1%) as carbon source ●——● lactose (1%) as carbon source.

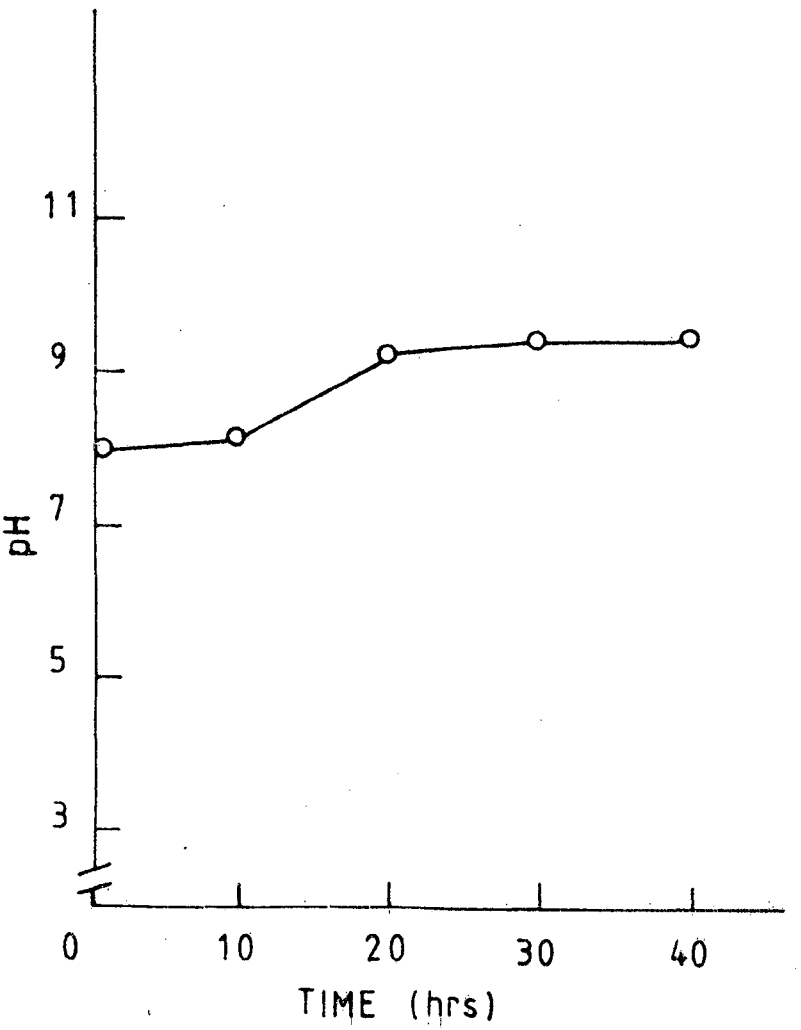


Fig. 4 Changes in pH of culture medium in the growth period. pH of the medium was noted at 10 hours intervals.

remained almost constant thereafter.

III) Bacteriological observations :

The mature colony was observed to be unpigmented, opaque, round regular, dry , flat and non-spreading type (Fig. 5). Cells were gram positive, straight rods, measuring 3-5μ in length with an average of 4μ (mean of 30 cells) and approximately 0.5μ in width.

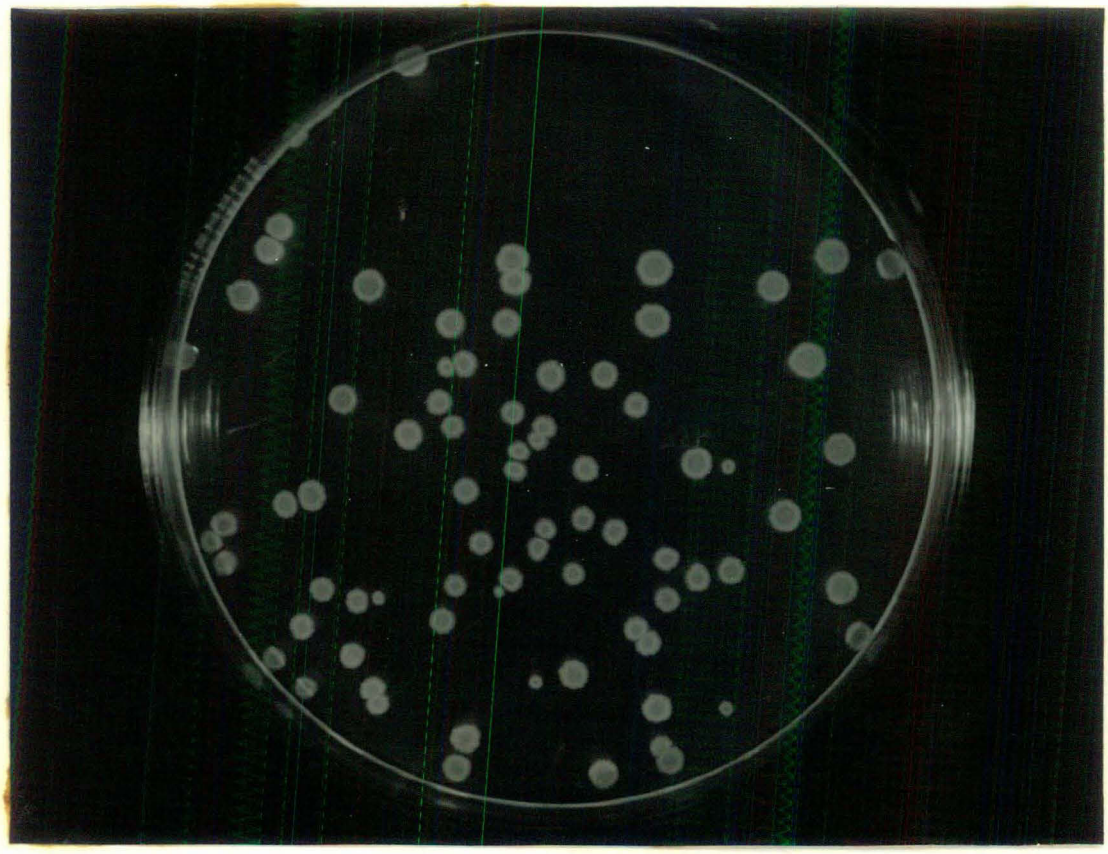
IV) pH activity profile of CMCase :

The standard curve of glucose was depicted in Fig.6. Effect of pH of reaction mixture on the rate of reaction was presented in Fig. 7. Two peaks were clearly observed. A peak of high activity was observed at pH 7.0 which dropped steeply when the pH was increased by 0.5 units. Then with increasing pH the activity also increased again showing a peak at pH 9.5 which later started declining with further increase in pH. The activity at pH 9.5 was smaller than that at pH 7.0.

V) Effect of incubation temperature on CMCase activity :

Fig. 8 indicates the temperature activity profile of the enzyme(s) in the cell free supernatant. Here again two peaks are clearly visible - one at 30°C and another at 60°C with a steep depression between. On other words, peaks of high activities were preceeded and followed by low activity.

Fig. 5 Colony morphology of *B. thermoalkalophilus* sp. nov.



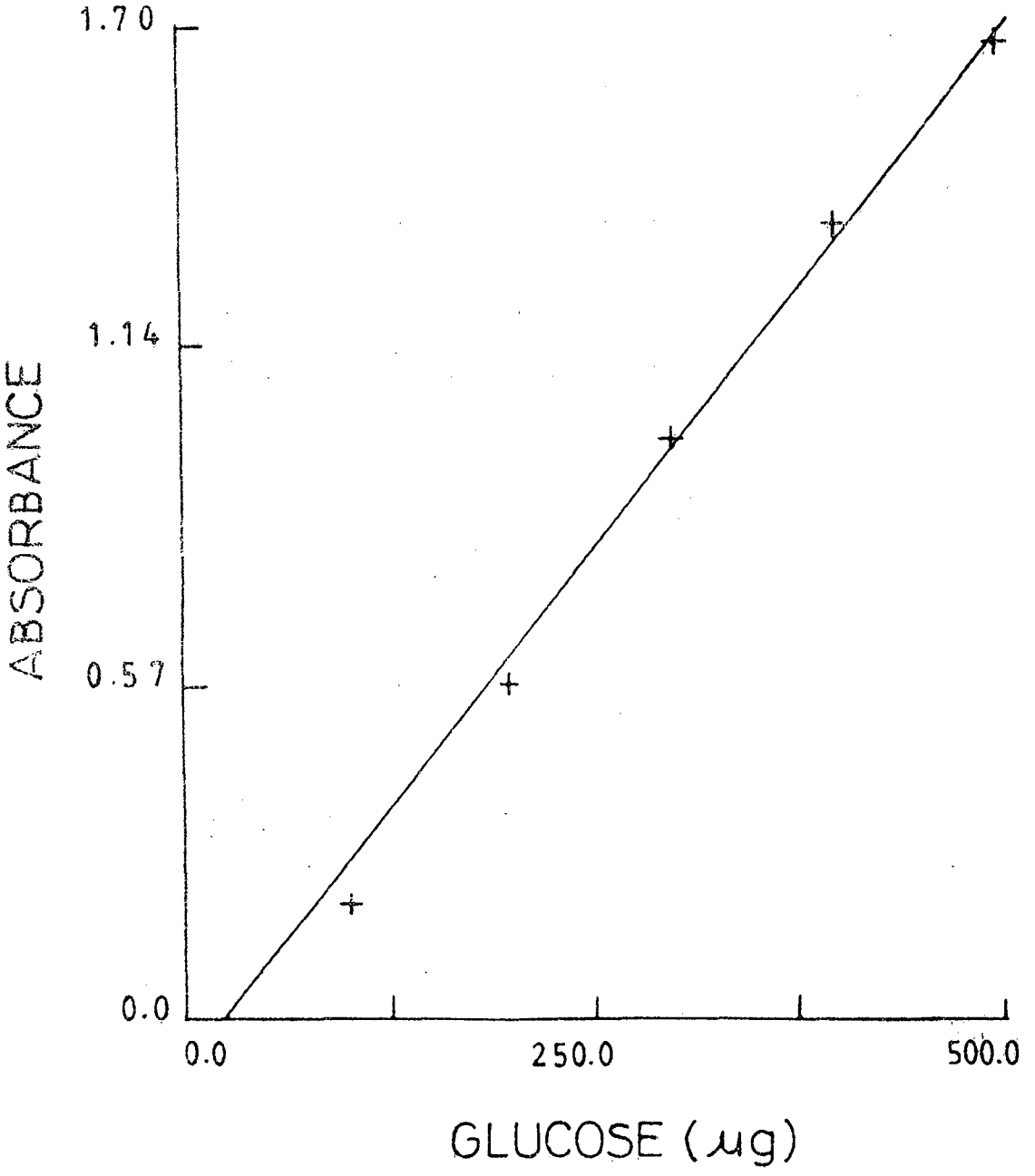


Fig. 6 Standard curve of glucose. 0.5 ml of different concentrations (0 to 500 ug) of glucose was added to 2 ml of distilled water. 3 ml of DNSA was added to it, boiled, cooled and O.D. taken at 540 nm.

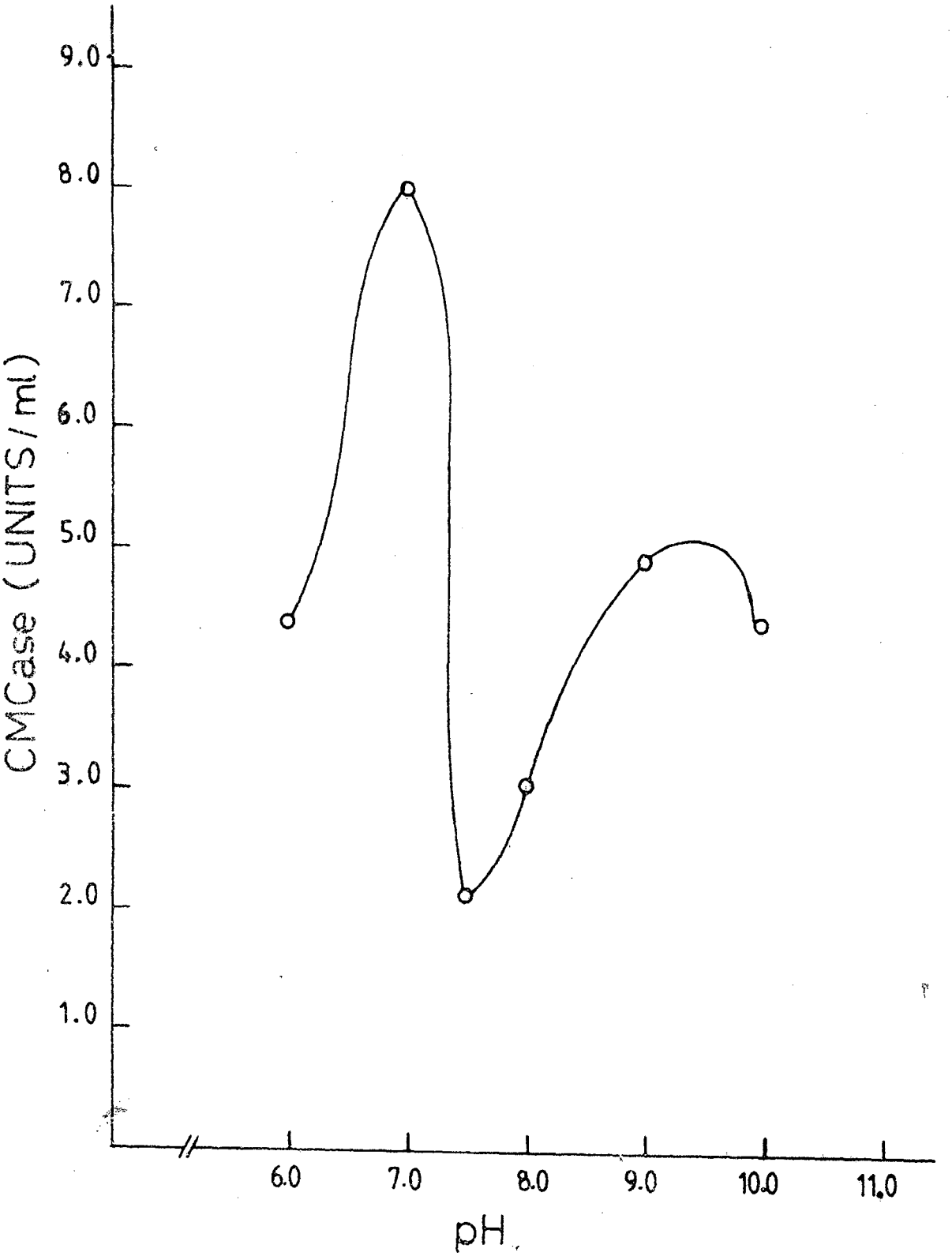


Fig. 7 PH activity profile of CMCase. 0.5 ml enzyme with 2 ml of 1% CMC solution in different buffers (pH ranging from 6.0 to 10.0) was incubated for 30 minutes. Reaction was stopped by adding 3 ml DNSA, samples boiled, cooled and O.D. taken at 540 nm.

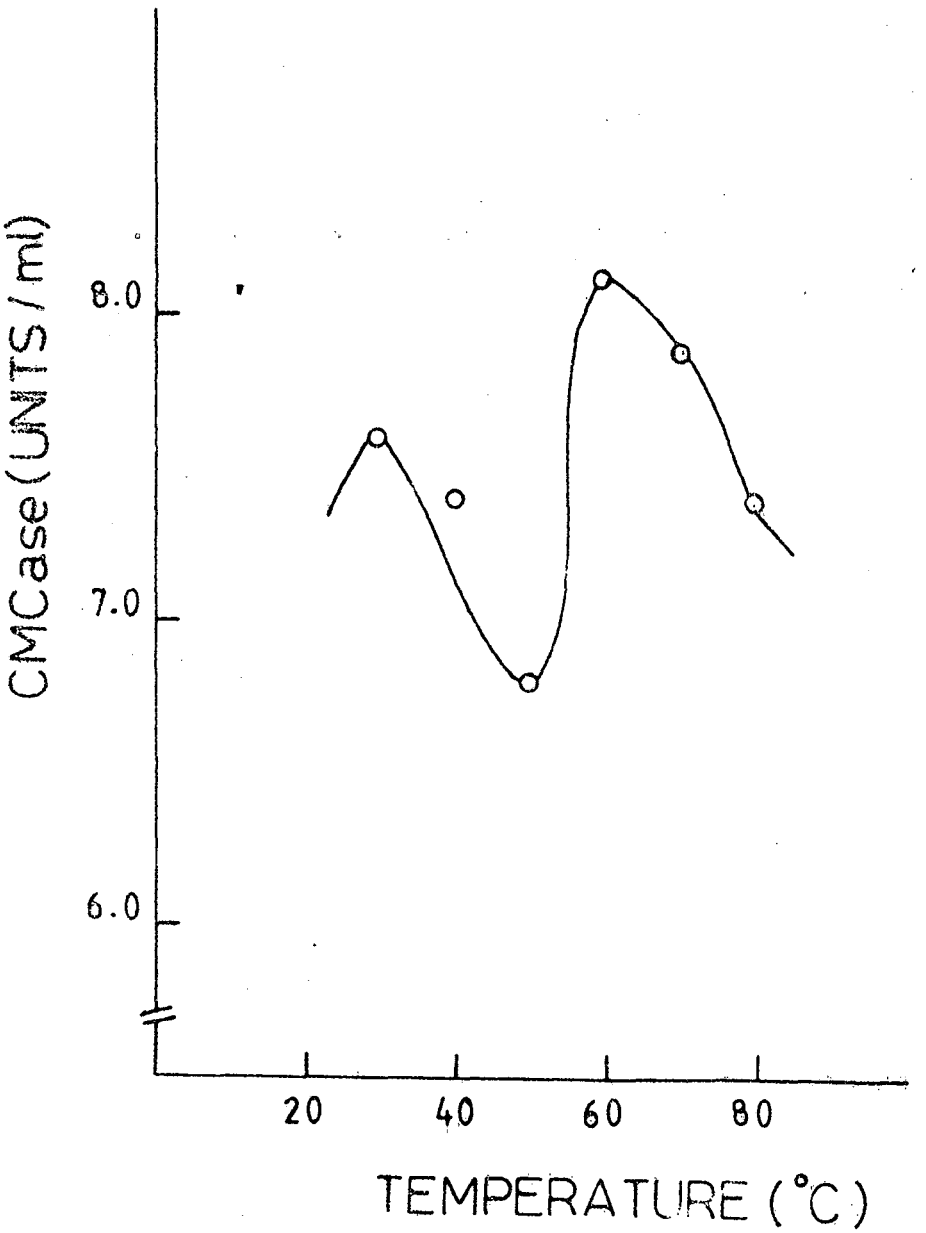


Fig. 8 Temperature activity profile of CMCase. The reaction mixture was incubated at different temperatures (25 to 80°C with 5°C interval).

VI) Effect of substrate concentration on enzyme activity:

The result shown in Fig. 9 shows that with increasing CMC concentration from 0.1% to 1% the activity of the enzyme(s) increased. Thereafter, no any significant increase was found and a plateau was obtained.

VII) Optimum time of sonication :

The standard curve of protein was shown in Fig.10. Fig. 11 shows the effect of duration of sonication on the breakage of cell wall and release of intracellular protein. It is evident from the figure that the optimum time of sonication was 3.0 minutes with 30 seconds gap after 1.5 minutes so as to reduce the effect of temperature being produced. Above that period there was no any significant increase in protein being released from cells.

VIII) Localization of cellulase :

The localization of CMCase and protein were presented in figure 12 and 13 respectively. The cell free cellulase concentration was very low upto a period of 12 hours after inoculating the medium which then increased rapidly by 18 hours and remained more or less same upto a period of 30 hours. It again increased rapidly by 48 hours and was almost constant upto a period of 60 hours. The protein component in the supernatant, on the other hand, increased at a slower rate upto 42 hours and, thereafter, increased with an enhanced rate upto 60 hours.

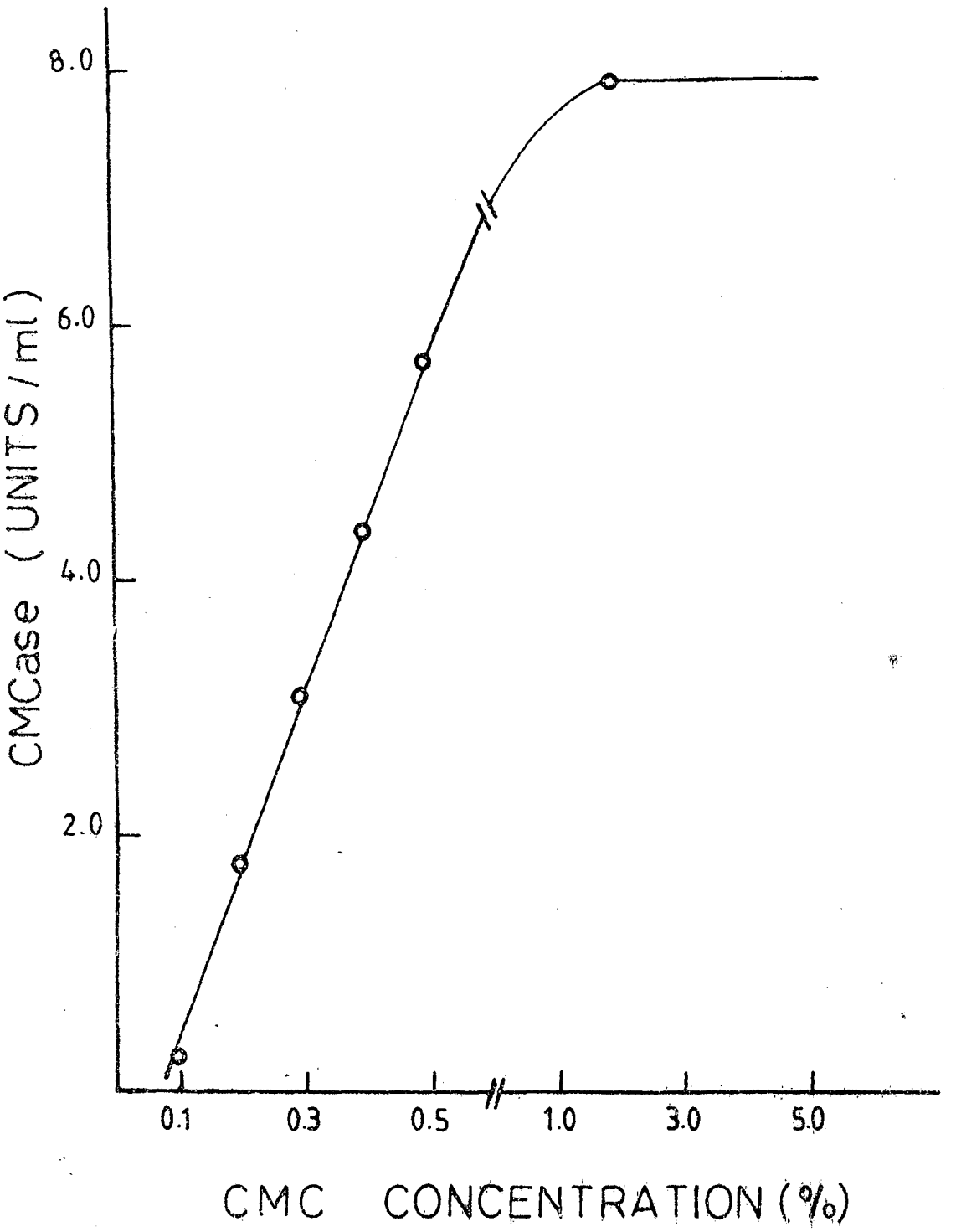


Fig. 9 Effect of CMC concentration in the reaction mixture on the activity of CMCase. The enzyme with 2 ml of different concentrations of CMC solution (0.1 to 5%) was incubated for 30 minutes.

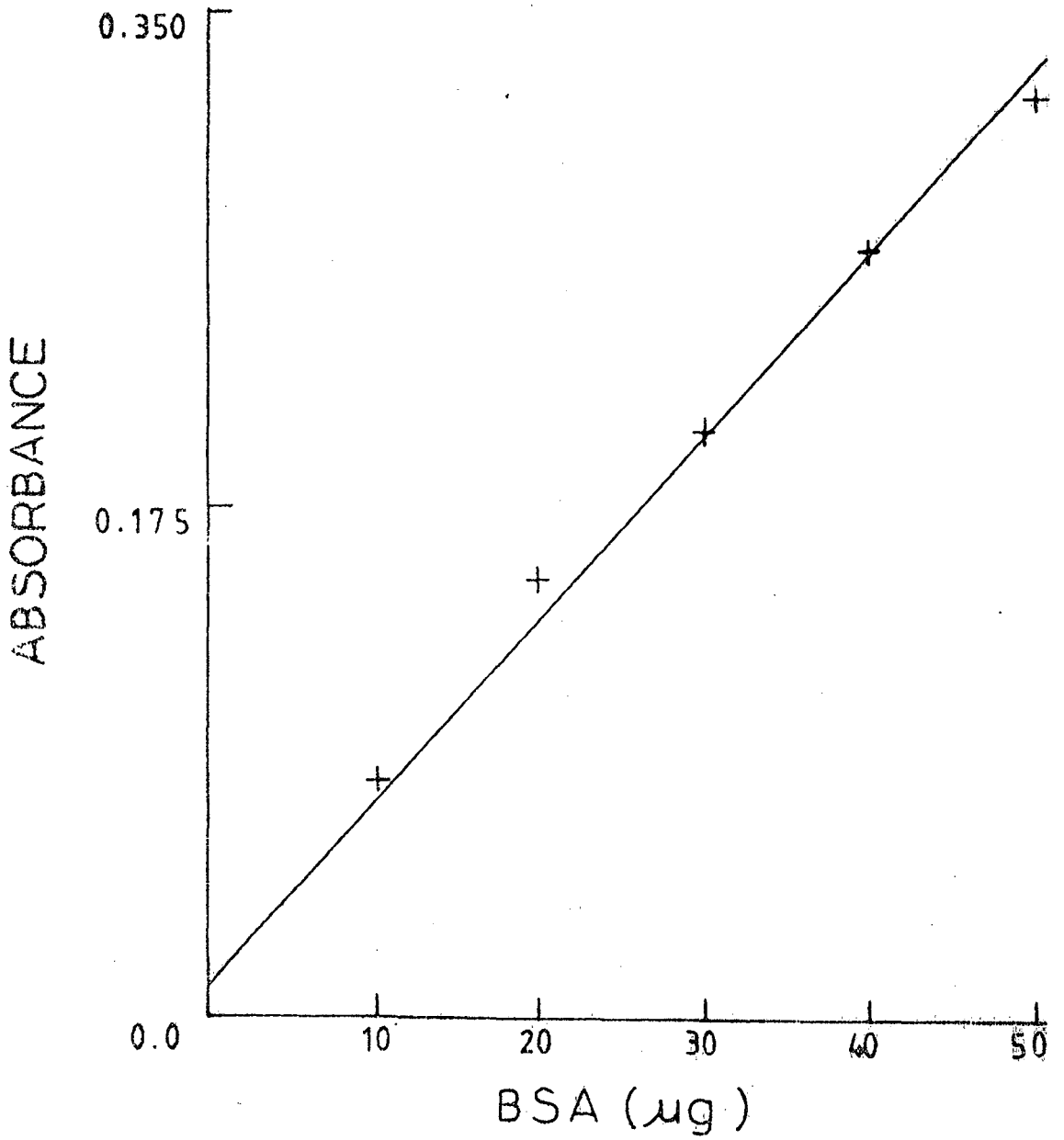


Fig. 10 Standard curve of protein. 5 ml Bradford reagent was added to 0.1 ml of different concentrations of BSA (0-50 ug) and O.D. was taken at 595 nm.

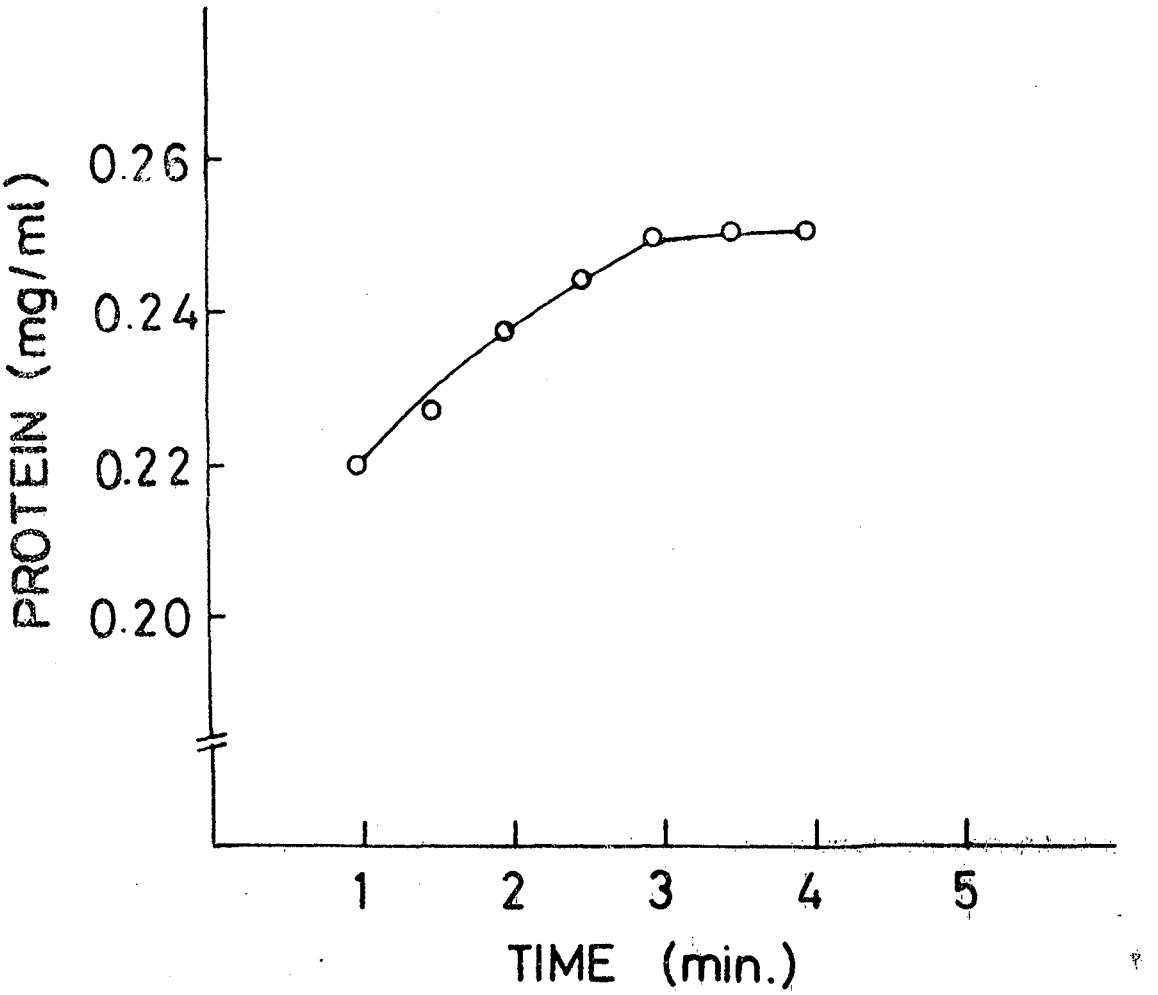


Fig. 11 Effect of duration of sonication on release of intracellular protein.

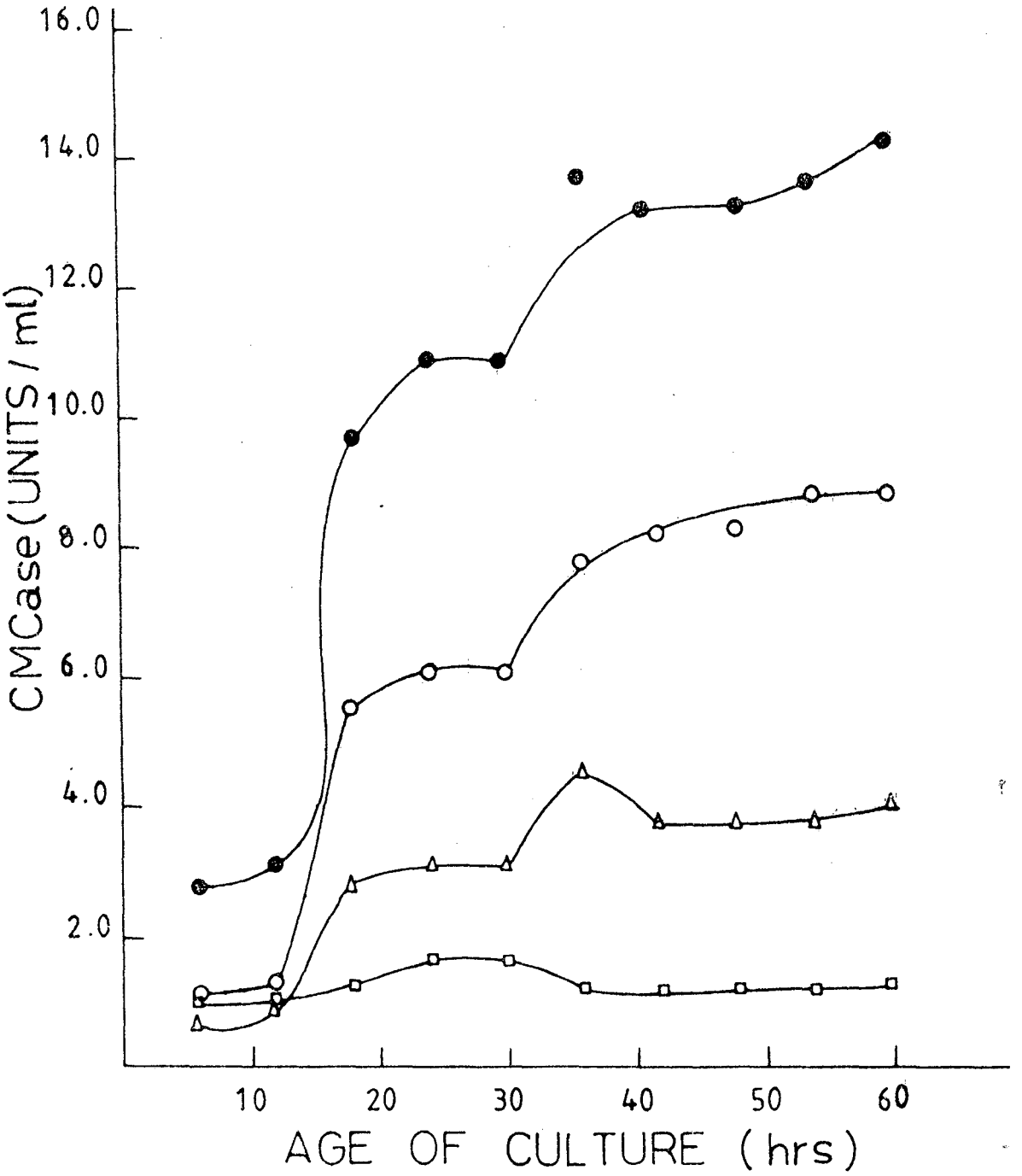


Fig. 12 Localization of CMCase. The enzyme was assayed at 8 hours interval during growth period in CMC medium. ●——● total, ○——○ extracellular △——△ membrane bound and □——□ intracellular /Extracellular (in lactose containing medium) enzymes.

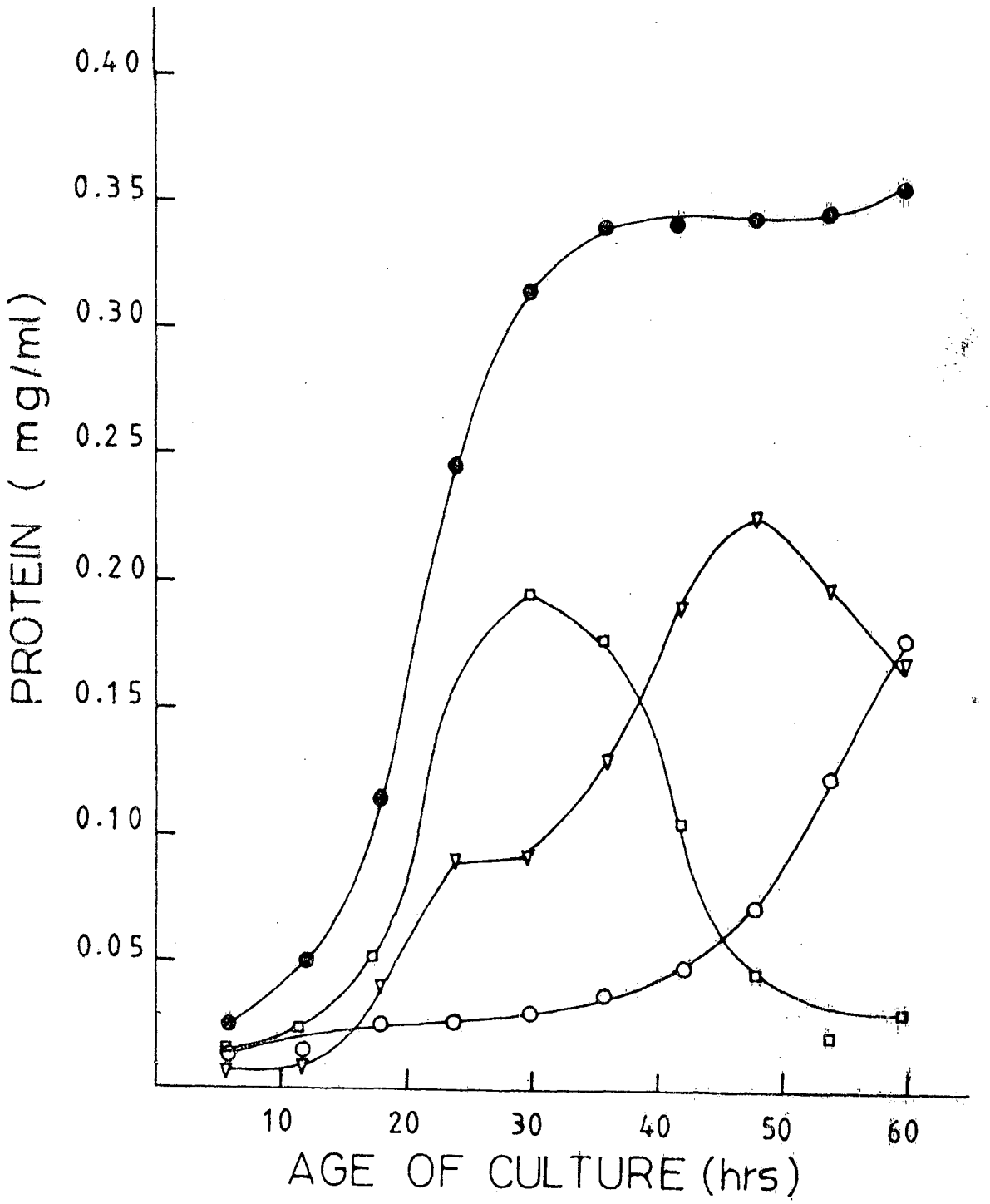


Fig. 13 Protein concentration of different fractions. Protein was assayed from samples at 8 hours interval during growth period. ●——● total, o——o extracellular, △——△ membrane bound and □——□ intracellular protein concentration.

The intracellular CMCase concentration, on the contrary, was fairly constant throughout the experiment except that there was a slight increase after 18 hours which remained constant till 30 hrs. and dropped back to initial level by 36 hours. The intracellular protein showed a rapid increase upto a period of 30 hours which sharply declined afterwards.

CMCase concentration, that was bound to membrane showed an increase upto a period of 18 hours and thereafter remained more or less constant. Protein in this component was shown to have increased rapidly upto a period of 48 hours which declined meagerly afterwards.

It was also evident from figure 12 that the extracellular enzyme in a medium containing lactose as carbon source was almost constant throughout and its level was approximately equal to that of intracellular CMCase levels.

It is clear from figure 12 that CMCase level was high in supernatant though the protein content was quite low (figure 13) in comparision to both membrane bound, intracellular levels of CMCase and protein respectively. The intracellular CMCase was the least in quantity amongst all the three components though its protein component was as high as membrane bound protein. The protein level in the supernatant was noted to increase once the intracellular and membrane bound protein had shown a declining tendency.

The saccharifying cellulase quantity in cell free supernatant shown as filter paper units showed a similar pattern to that of cell free CMCase (Fig. 14). But the enzyme levels were quite low, nearly 1/10 to that of CMCase levels. However, no membrane bound and intracellular saccharifying cellulase could be detected.

The specific activity of CMCase was presented in (figure 15)and of saccharifying cellulase in figure 14. It is obvious from the figures that specific activity of CMcase in supernatant was the highest reaching a maximum in 18 hours and declining thereafter rapidly. The membrane bound CMCase showed a decreasing specific activity at a slower rate. The specific acticvity of intracellular CMCase showed a sharp decline upto 24 hours, then remained constant upto 36 hours and increased with rapid rate afterwards. The specific activity of saccharifying cellulase in supernatant was very low. It increased upto 24 hours and showed a decline thereafter. It was also evident that once the specific activity of CMCase in supernatant started declining, the intracellular one showed an increase.

IX Effect of surfactant on the release of enzyme from intracellular environment :

The effect of surfactant (Tween 80) on the release of CMCase from the cell is presented in Fig. 16. There was no significant effect of Tween 80 on the release of enzyme

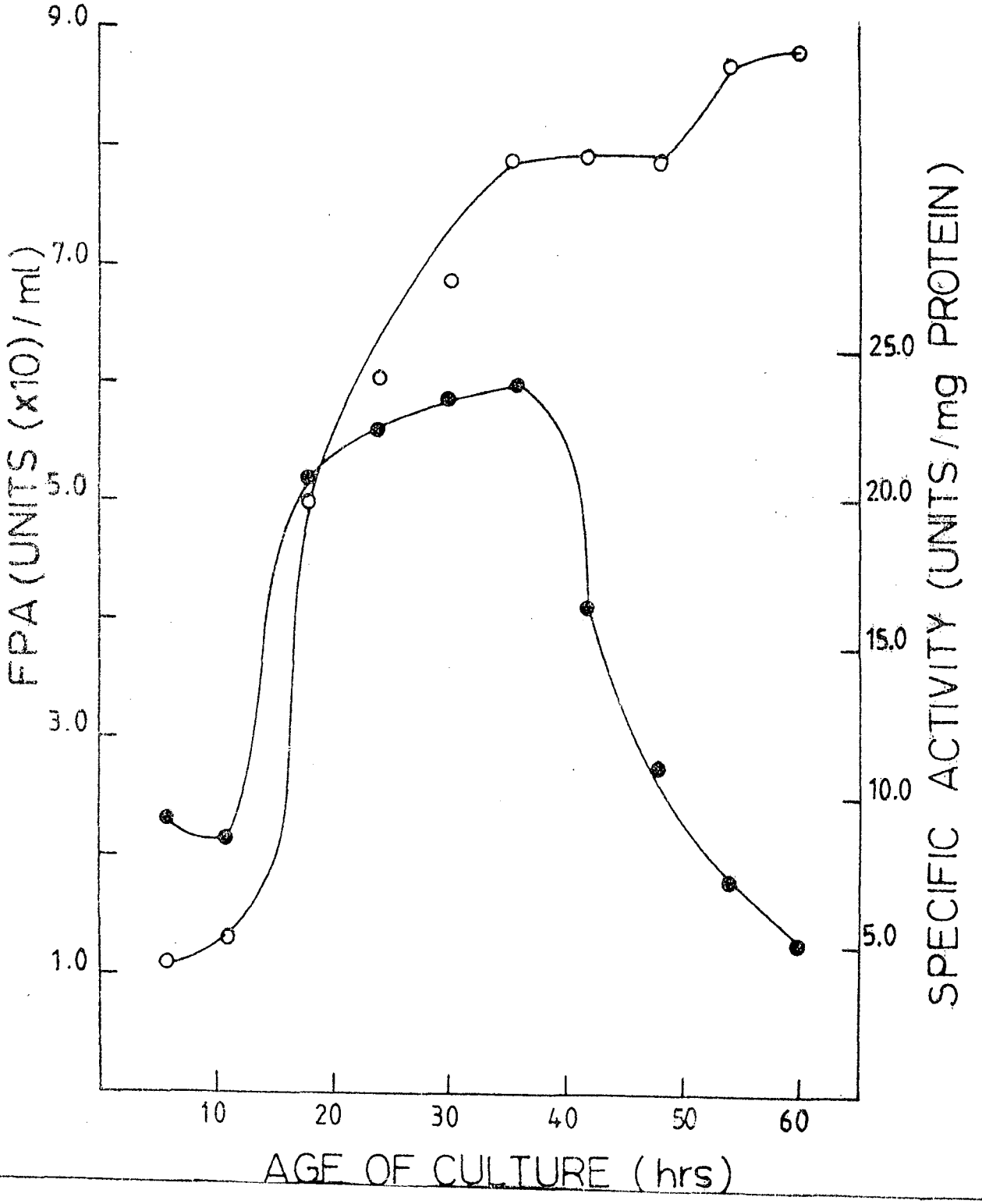


Fig. 14 Rate of saccharifying cellulase production. 0.5 ml of supernatant with 6 c m x 1 cm of whatman No.1 filter paper incubated for 60 minutes. Reaction was stopped by adding 3 ml DNSA. The samples were boiled, cooled and O.D. was taken at 540 nm. ○——○ FP units and ●——● specific activity.

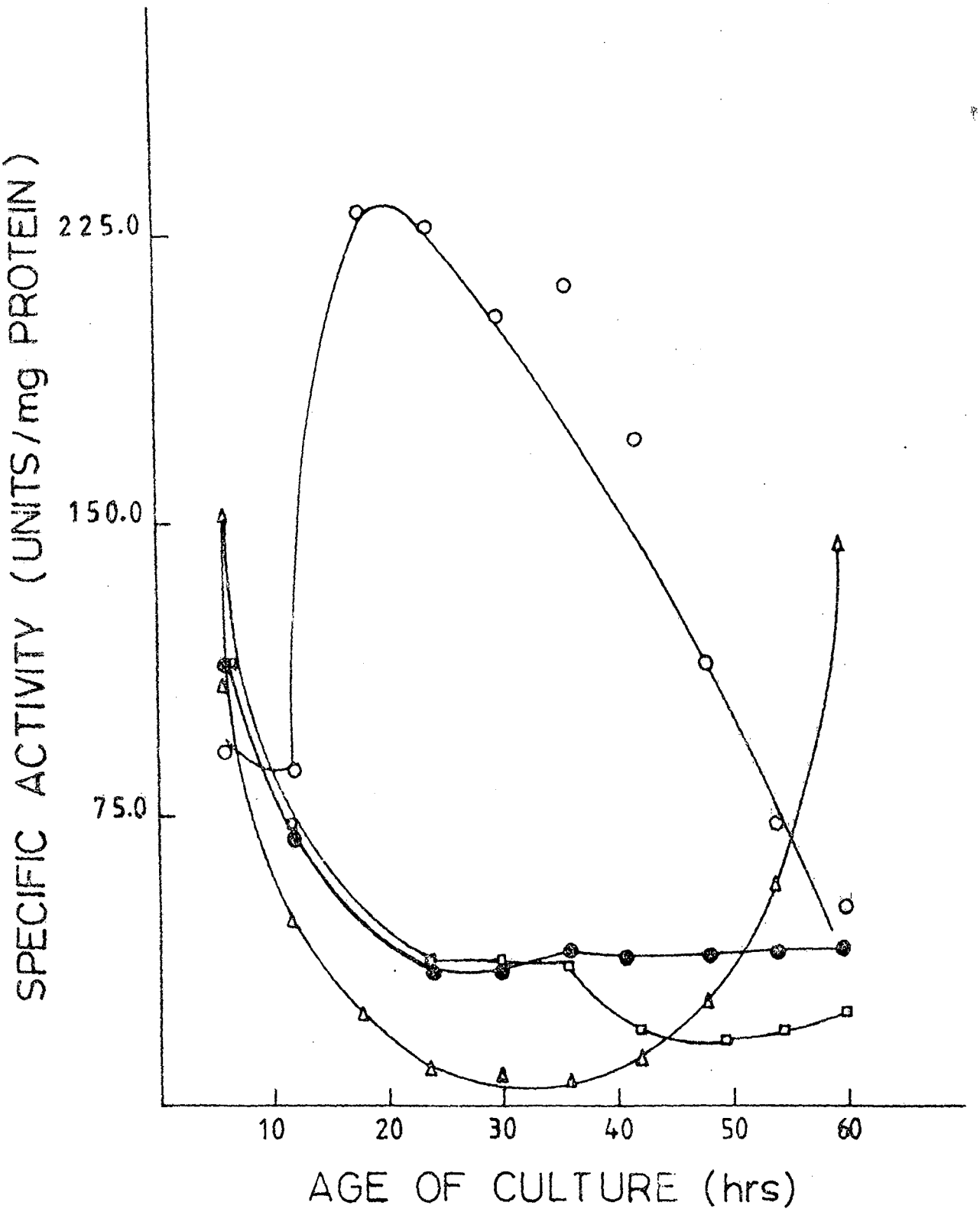


Fig. 15 Specific activity of CMCase from different fractions during growth period. •——• total, o——o extracellular, Δ——Δ membrane bound and □——□ intracellular enzymes.

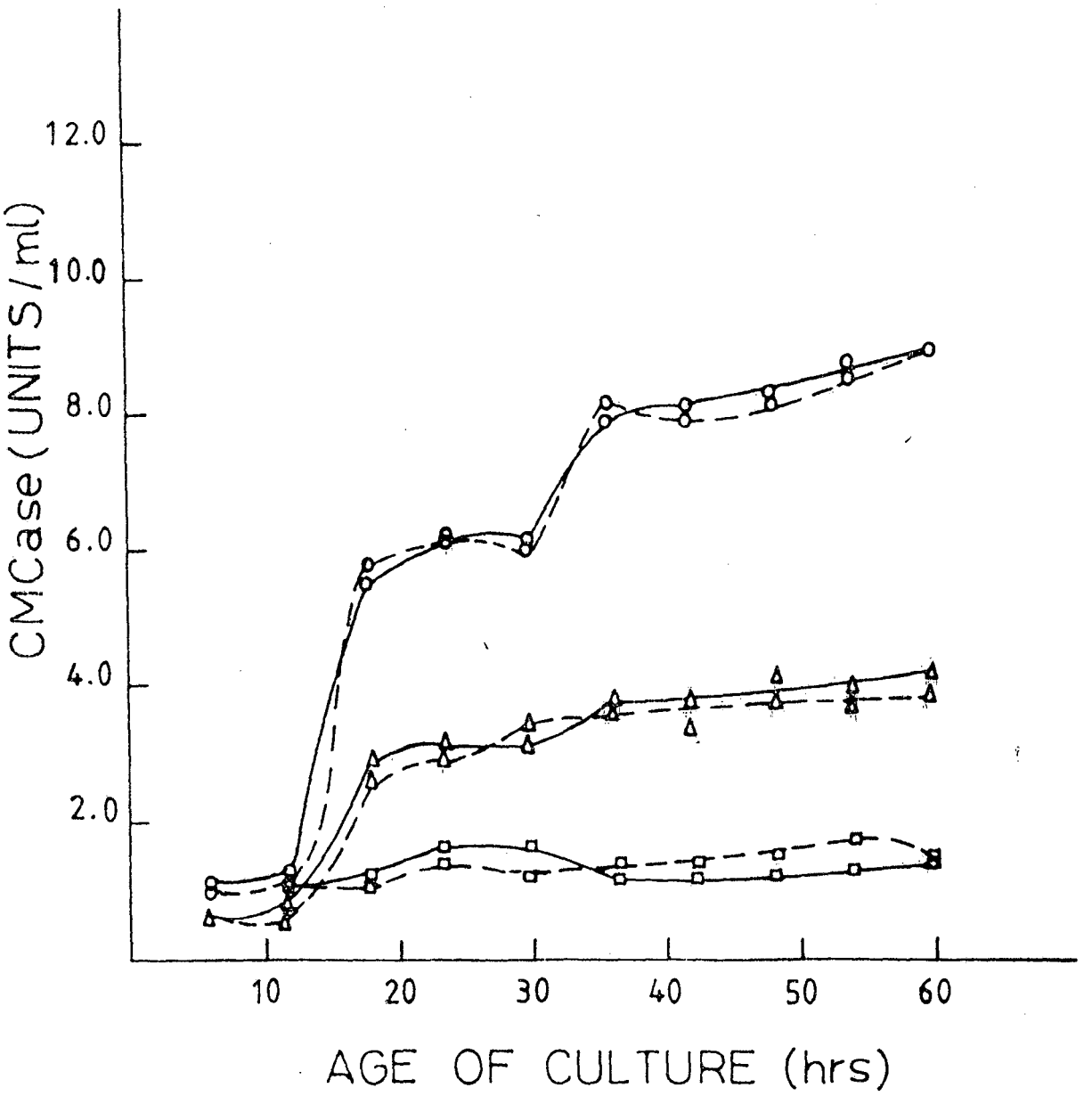


Fig. 16 Effect of Tween 80 on CMCase production. Samples were assayed for the enzyme at 8 hours internal during growth period. o——o extracellular, Δ——Δ membrane bound, □——□ intracellular enzyme ——— without and — — — — with Tween 80.

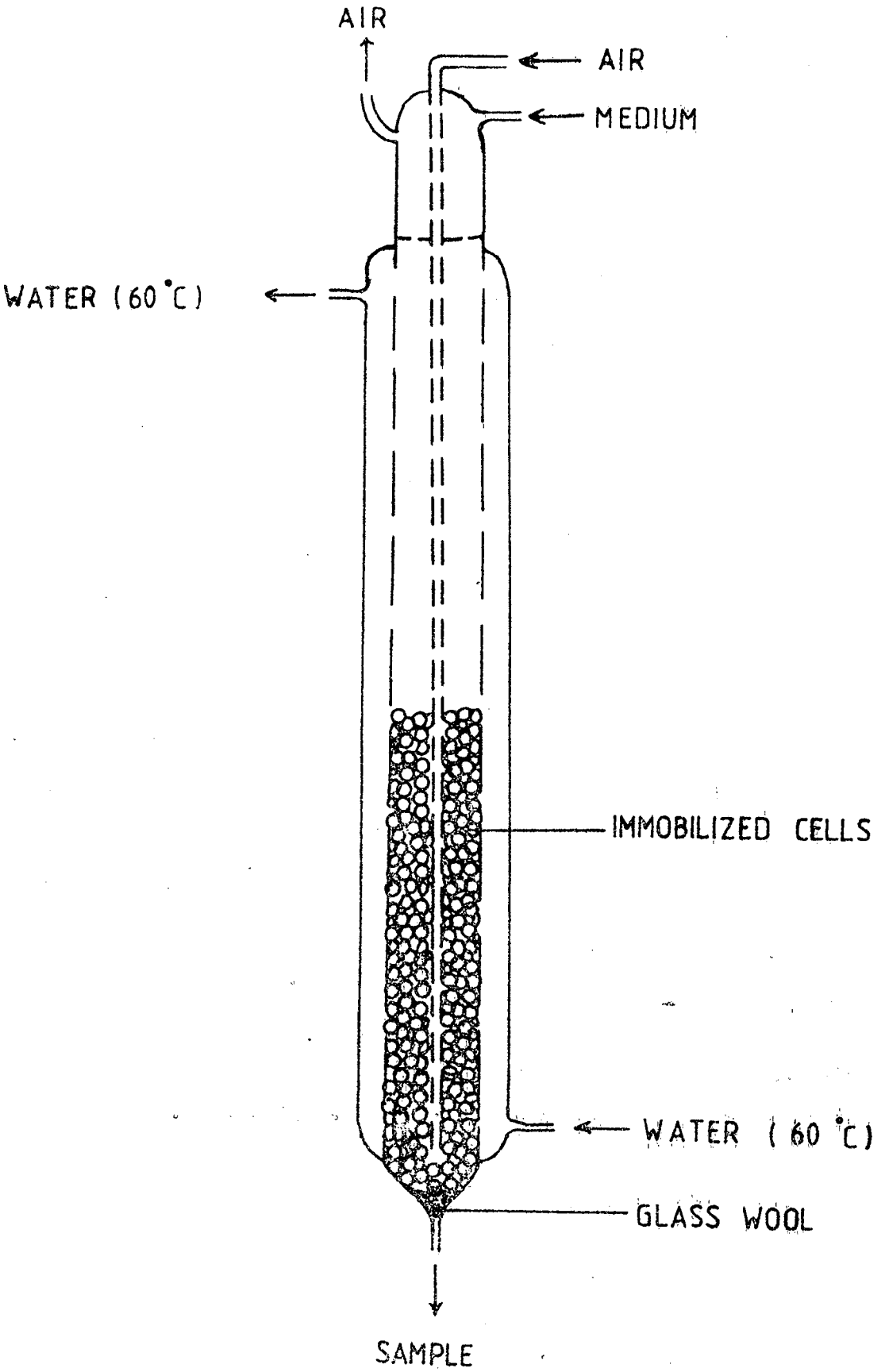


Fig.17 Diagrammatic representation of reactor.(scale 1 : 2.)

as there was no change in the supernatant, membrane bound and intracellular enzyme levels.

X Stability of gels :

The stability of both agar and polycralamide gels from 30 to 60^{0}C was checked. Both the gels showed the stability at 60^{0}C. Agar gel was stable only for a week whereas acrylamide gel was stable more. The agar beads became softer in comparison to when they were prepared. On the other hand they were quite stable upto a temperature of 50^{o}C for about the same period. Polyacrylamide gel did not show any such tendency.

XI Cell leakage :

After immobilization leakage of cells was checked and estimated in refrence to concentration of agar, acrylamide and bisacrylamide. Even after 50 hours of entrapment the leakage was highly insignificant and was in the order of 0.008 to 0.01% of the total immobilized cells.

Morphology of immobilized cells :

The scanning electron micrographs of free cells, cells immobilized in agar and polyacrylamide gels were presented in figure 18, 19 and 20 respectively. The cells were rod shaped and were of 3-5 x 0.5 (approximately) μ in size.

Oxygen uptake :

Oxygen uptake by cells immobilized in agar was shown

Fig. 18 Scannihg electron micrograph of B. thermoalkalophilus sp. nov. (×5000)

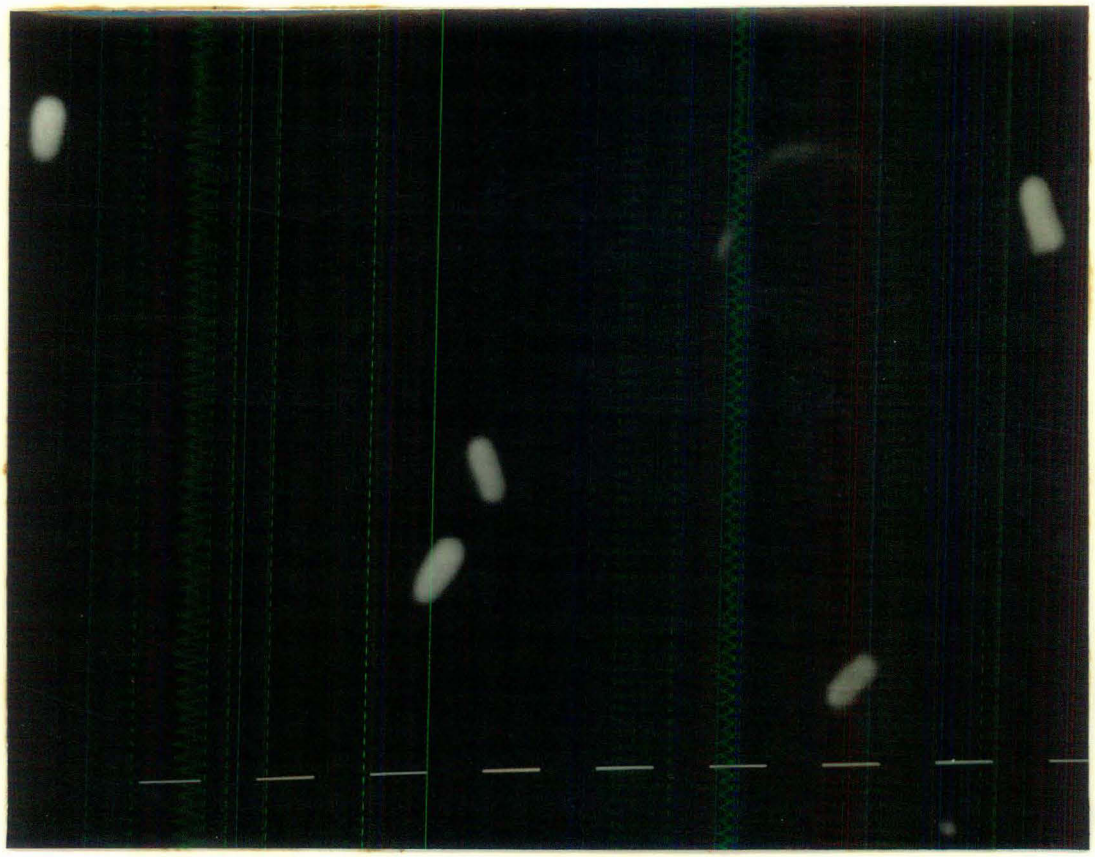
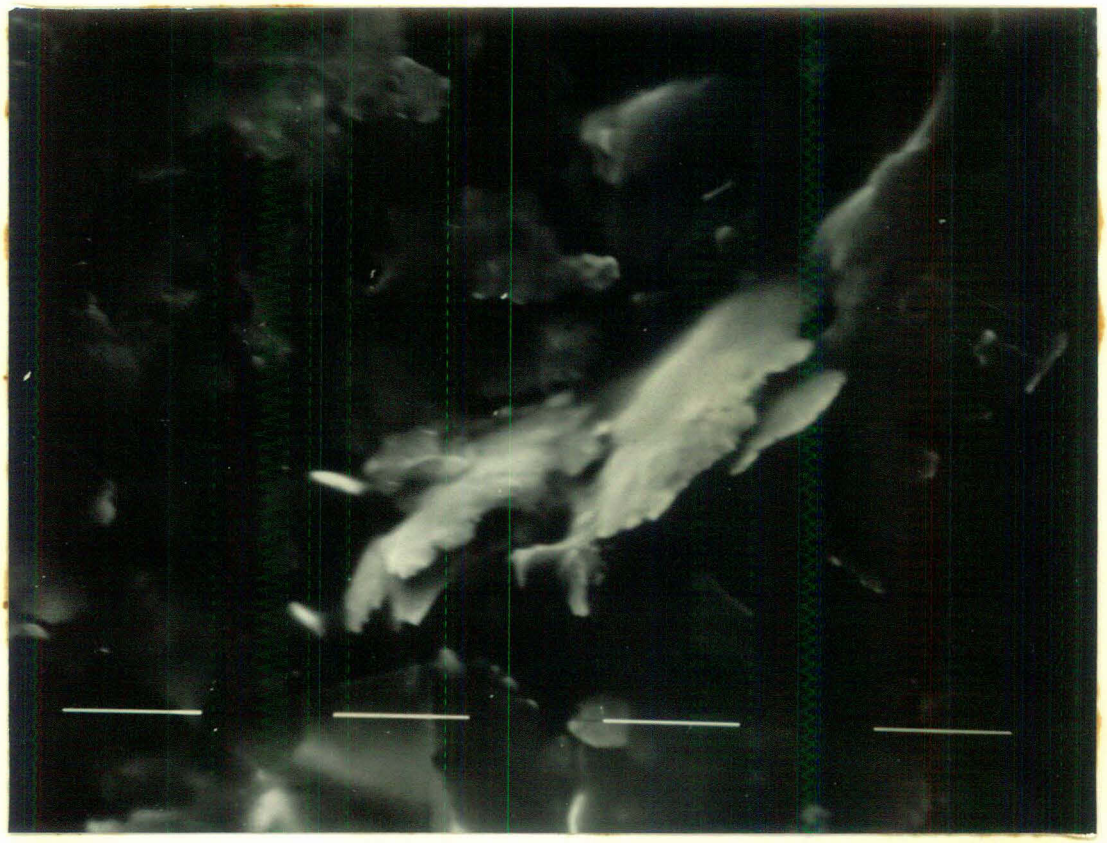
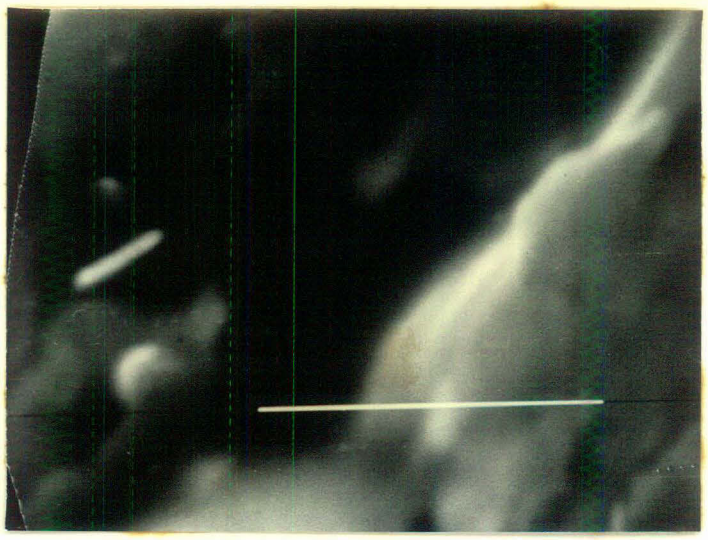


Fig. 19 Scanning electron micrograph of critically dried B. thermoalkalophilus sp. nov. immobilized in agar. a. number of cells (x 1250). b. single cell (x 2500).

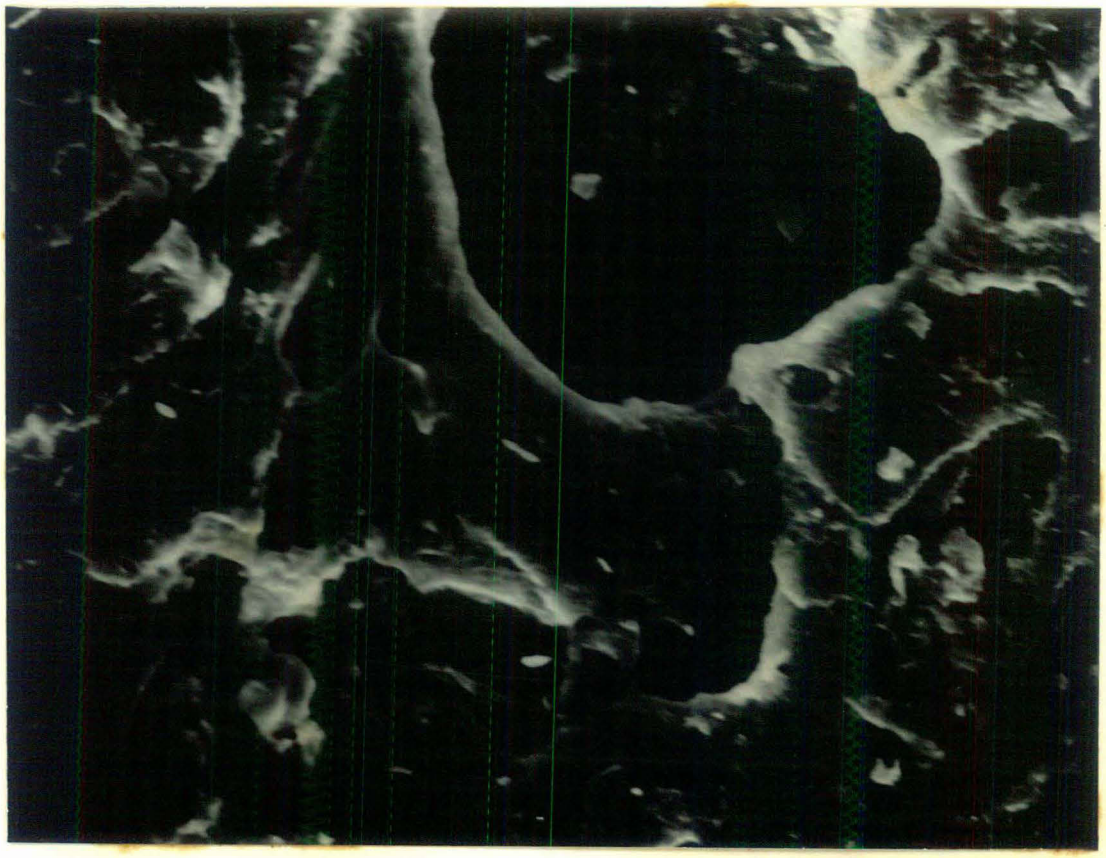


(a)

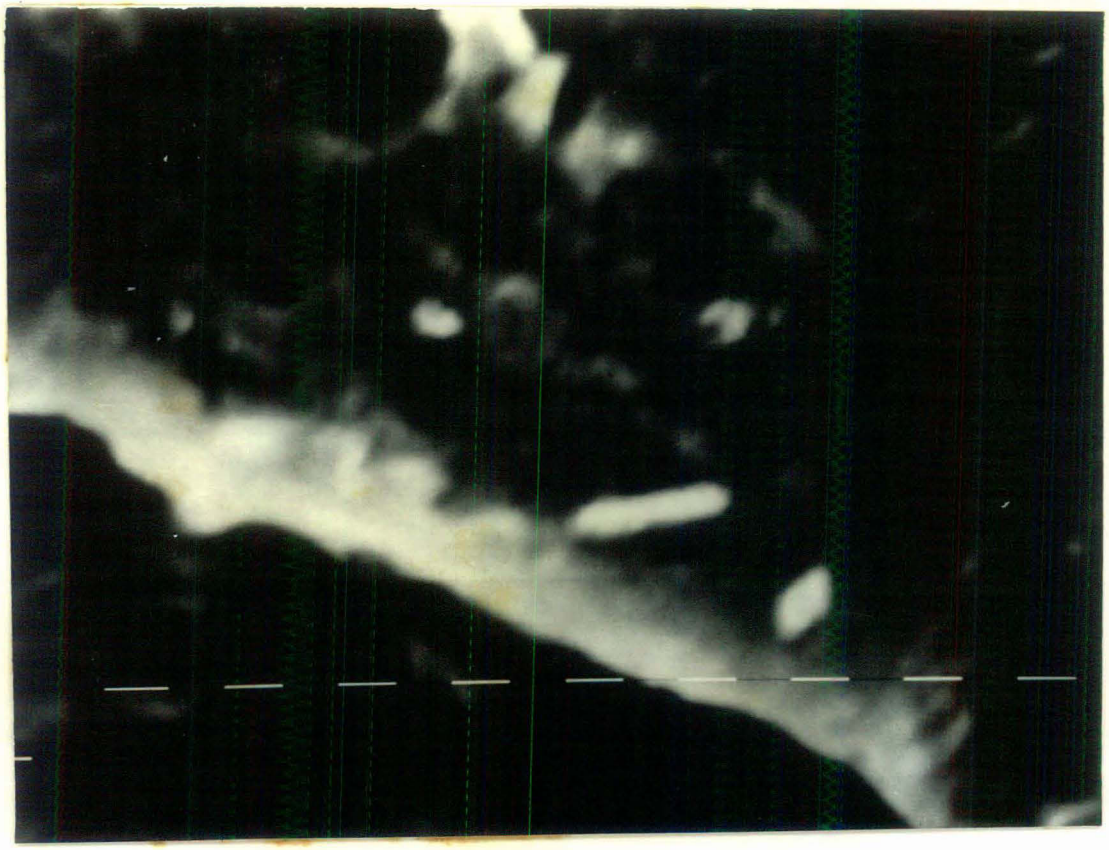


(b)

Fig. 20 Scanning electron micrograph of critically dried B. thermoalkalophilus sp. nov. immobilized in polyacrylamide. a. number of cells (x 1250), b. single cell (x 5000).



(a)



(b)

in figure 21. It clearly shows that upto 2 minutes there was no oxygen consumption. But, thereafter oxygen consumption by cells was at a fairly constant rate. A similar result was also obtained with the cells immobilized in acrylamide.

Table 1 : Oxygen consumption by free and immobilized cells

Matrix of immobilization	O_2 consumed (m moles/g fresh wt. of cells/min)	% O_2 consumption over free cells
Free cells	3.031	-
Agar	1.097	36.19
Polyacrylamide	1.200	39.59

Table 1 shows m moles of oxygen consumed on an avarage per minute per gram fresh weight of cells. It is clear from the table that oxygen consumption by immobilized cells is nearly one third that of free cells. Both the immobilized systems showed almost comparable results.

Table 2 : Enzyme production by gram fresh weight of free and immobilized cells

Matrix of immobilization	Enzyme produced (U)					
	1 day		4 days		7 days	
		% product -ion		% product -ion		% product -ion
Free cells	520	100	830	100	850	100
Agar	47.95	9.2	221.9	26.73	627.8	73.86
Polyacrylamide	30	5.8	150	18.1	490	57.65

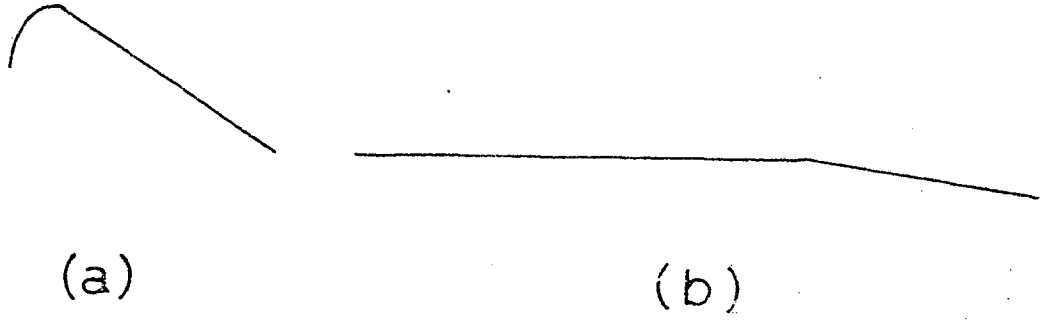


Fig. 21 Oxygen consumption by free cells (a) and cells immobilized in agar (b). Chart speed 3 cm/min.

Enzyme production by immobilized cells :

The enzyme produced by both immobilized and unimmobilized cells is presented in table 2. The enzyme produced per gram fresh weight of cells immobilized both in agar and polyacrylamide gels was smaller (25-45% less) after 7 days of that produced by gram fresh weight of unimmobilized cells. In both the methods of immobilization, the enzyme produced increased with duration of time. In terms of enzyme production, both the methods of immobilization showed a small variation upto a period of 4 days, but thereafter the cells immobilized in acrylamide showed greater potential. Free cells did not show any significant new synthesis of the enzyme.

Figure 22 represents the concentration of protein produced in both immobilized and unimmobilized forms. The concentration of proteins produced by free cells increased upto a period of 4 days and remained more or less stable. It is higher than that of the immobilized cells in both the matrices. The protein concentrationproduced by cells in both the matrices increased upto a period of 4 days and then decreased. The protein that was produced by cells entrapped in agar was greater than that produced by cells entrapped in acrylamide.

The specific activity as depicted in figure 23 showed that specific activity of the enzyme produced by free cells was greater than that produced by cells in both the matrices.

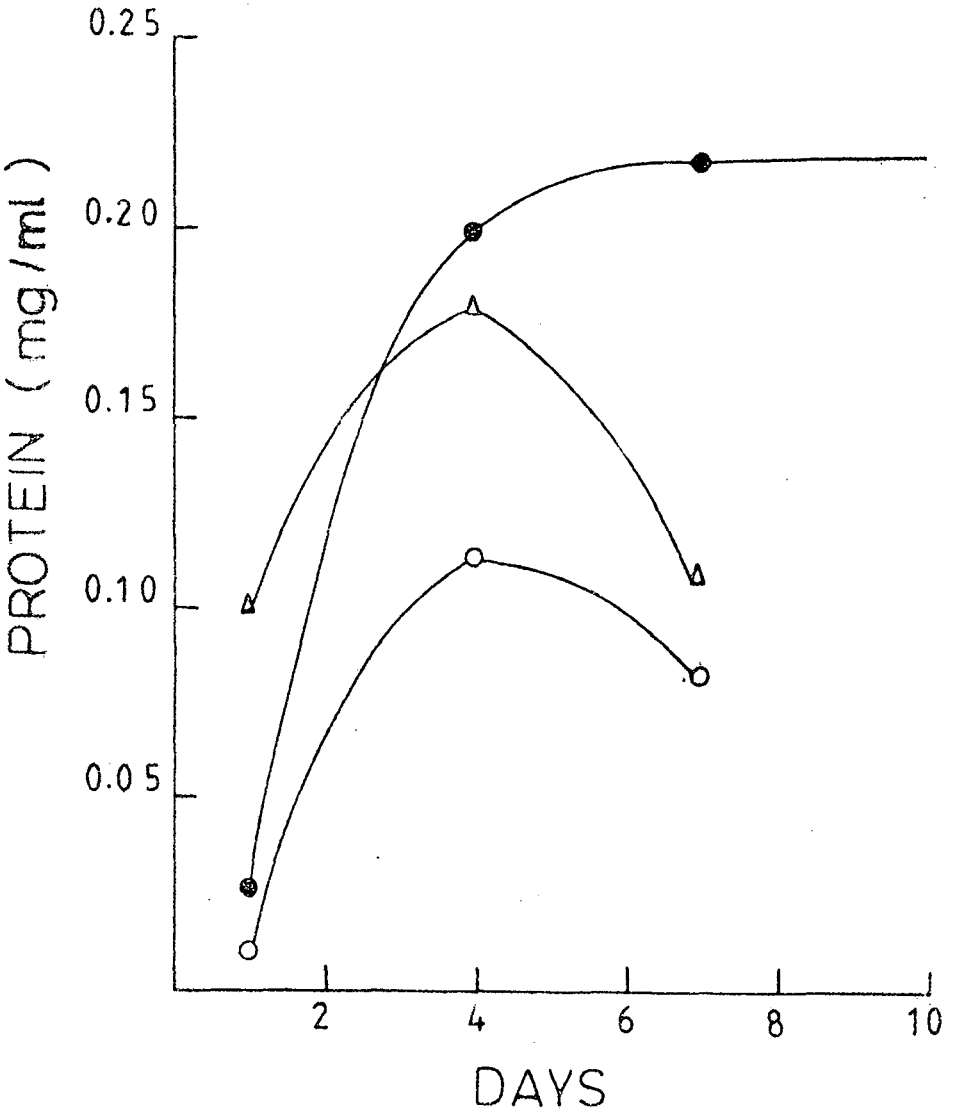


Fig. 22 Protein concentration in immobilized and free cell systems. ●——● free cells, ◿——◿ cells immobilized in agar, o——o cells immobilized in polyacrylamide.

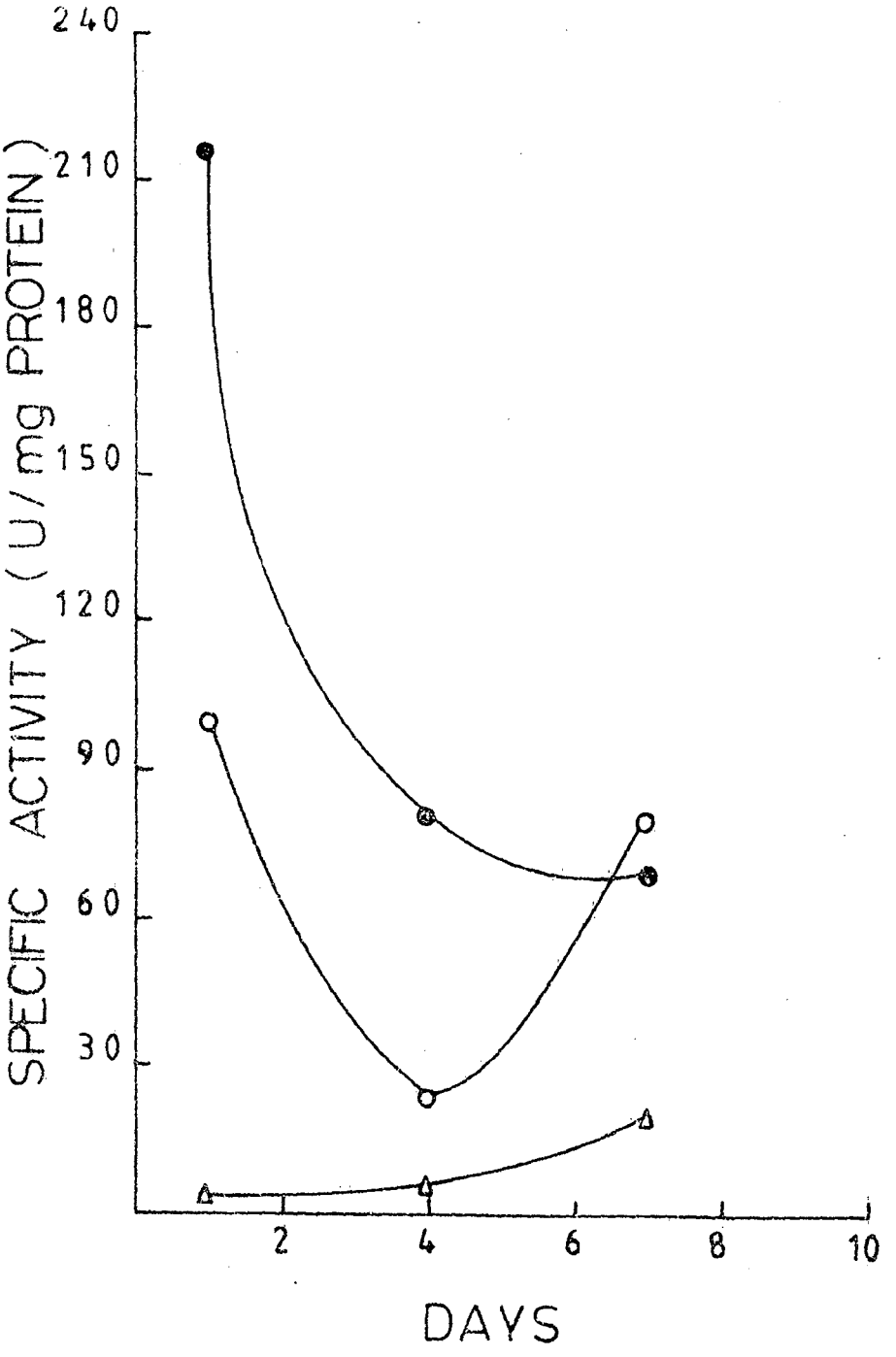


Fig. 23 Specific activity of CMCase of free and immobilized cells. ●——● free cells, △——△ cells immobilized in agar, o——o cells immobilized in polyacrylamide.

Specific activity of the enzyme produced by cells in agar matrix showed a steady increase whereas that of the enzyme that was produced by cells in polyacrylamide matrix, showed a steep fall upto 4 days and then a rapid increase thereafter. The specific activity of the enzyme produced by cells entrapped in acrylamide matrix was greater than that synthesized by cells in agar matrix.

DISCUSSION

Cellulose is the most abundant carbon containing organic substance found in nature comprising about one third on the total vegetation of this planet and its estimated production is about 10 tons per year (Bisen et al., 1982). This is about 40% of the biomass produced by photosynthetic carbon di-oxide fixation (Ghose and Ghosh, 1978). Despite its large abundance, little attention was paid to it till recently. The degradedproducts can be converted into a number of useful products such as food and feed stocks, important organic chemicals and biofuel. The degradation is brought about by a complex enzyme known as cellulase(s) which involves atleast three types of enzymes (Ljungdahl and Eriksson, 1985). Cellulase(s) are present in many organisms, but the rate of cellulose degradation varies among them (Gong and Tsao, 1979). A number of thermophilic (Viljoen et al., 1926; McBee, 1948; Bellamy, 1980) and alkalophilic (Horikoshi and Akiba, 1982; Horikoshi et al., 1984; Fukumori et al., 1985) organisms which can degrade cellulose have been reported. A thermophilic as well as alkalophilic organism which can degrade cellulose was reported for the first time from this laboratory (Paul et al., 1985).

For the commercial utilization of the enzyme system, the enzyme has to be supplied at low cost with a consistently high quality. Batch cultures have a draw-back in this regard that they can not be used repeatedly and continuously.

This draw-back can be overcome by the immobilized cell system. For utilizing the immobilized cell system, deep understanding of the organisms and the localization of the enzymes have to be known.

Physiological Considerations :

The growth curve of the organism in lactose and carboxymethyl cellulose as carbon source (Fig.3) shows that the organism prefers carboxymethyl cellulose (CMC) as carbon source than lactose. The generation time in lactose containing medium was longer (3.25 hours) than in CMC containing medium (3 hours). As a result the specific growth rate in CMC medium was higher (0.1/hr.) than in lactose medium (0.09/hr). Probably the organism is not an efficient producer of ß-galactosidase(s), the lactose degrading enzyme, as the cellulase(s). In the medium containing CMC the organism showed multiple stationary phases. This is probably because of the fact that initially the organism utilizes the most readily available nutrients and afterwards it starts utilizing CMC.

The medium in which the bacteria were growing showed an increase in pH. In the initial stages upto 10hours, the pH did not alter much. But as soon as the organism starts utilizing CMC the pH increased. This could be either because of low amounts of CMC or the increased production of some alkalis into the medium or a combination of both. This could

also be due to protone uptake because of which hydroxyl ion concentration of the culture medium might have increased, thereby increasing the pH of the medium.

Enzyme assay studies :

The two peaks in Fig. 7 (pH activity profile) might be because of the presence of two enzymes, one active at neutral pH and another in the alkaline pH (9.5). Horikoshi et al.,(1984) also got a similar result. They purified the enzyme and with one fraction they got activity at neutral pH and with another in the alkaline range.

The enzyme that is active in alkaline range showed lower activity than that at neutral pH.

This result can also be interpreted by assuming that the enzyme has two substrate binding sites, one which can bind to the substrate at neutral pH and the other in alkaline range. The binding sites might be highly pH dependent.For this to be confirmed the enzyme has to be purified.

The temperature activity profile (fig.8) also showed two peaks of activity of the enzyme - one in mesophilic range and the other in thermophilic range. If there are two enzymes of CMCase it may be that one enzyme is active in mesolphilic range whereas the other in thermophilic range. Again, for this to be confirmed the enzyme has to be purified . This result can be assumed to be because of two binding sites of one or the other enzyme.

The effect of CMC concentration on the avtivity of the enzyme (Fig.9) shows that the optimum concentration of CMC is 1%. At lower concentrations, it is limiting the activity of the enzyme where as at higher concentration enzyme get saturated.

Localization of cellulase :

Figure 12 shows that most of the CMCase is extracellular with a little amount bound to the membrane and a small amount inside the cell. Extracellular CMCase was also reported in some other bacteria like Sporocytophaga myxococcoides (Vance et al., 1980 , Cellvibrio fulves (Berg et al., 1972) and Pseudomonas fluorescens (Suzuki et al., 1969; Horikoshi et al., 1984; Fukumori et al., 1985).

Extracellular CMCase concentration was low at the initial stage of incubation (upto 12 hours). But the enzyme concentration increased thereafter. From figure 3 it was concluded that CMC utilization starts after 12 hours. So CMC degradation might be having a role in the induction of the enzyme. When the organism was grown on lactose medium very low amount of enzyme (CMCase) was observed. This could be the constitutive level of the enzyme and the enzyme is being induced in presence of CMC. But CMC is a big molecule and cannot enter the cell. So it was proposed that in cellulolytic organisms there is always a small amount of constitutive enzyme which can degrade the substrate. These

degradation products then enter the cell and induce the enzyme system (Mandels and Reese, 1959). Mandels et al.(1962) showed that sophorose (2-O-ß-D-glucopyronosyl-D-glucose), produced from cellobiose (one of the products of cellulose degradation) by the transglucosylation action of ß-glucosidase was in actuality the inducer of the enzyme.

The constant levels of extracellular CMCase after 18 hours of inoculation is probably due to the regulation of enzyme synthesis. This view was supported by the findings of Nisizawa et al.,(1972) (formation of CMCase and ß-glucosidase being prevented by glucose in Trichoderma reesei) They further suggested that inhibition of CMCase synthesis occurred at translational level. Glucose was still inhibitory when added after transcription has ceased. Motenecourt et al. (1980) have shown in Trichoderma that cellulase repression is not related to cyclic AMP levels. Thus it was proposed that catabolite repression regulates cellulase synthesis.

There is a slight increase in the extracellular CMCase concentration even after 30 hours of culture (fig.12) whereas the protein in the supernatant (extracellular) showed a rapid increase at the same time (fig. 13), thus explaining the sharp fall of the enzyme specific activity (fig.15) during this period due to the secretion other proteins other than the enzyme (CMCase). The declining tendency of specific activity of CMCase between 18 and 30 hours of culture is

is because of fairly constant level of enzyme (fig.12) and slight increase in concentration of other proteins. Between 12 and 18 hours of growth, the CMCase concentration increased rapidly whereas the protein concentration is more or less same. Hence the increased specific activity of the enzyme during this period.

The intracellular CMCase levels, on the otherhand, are fairly constant indicating that the enzyme is transported across the membrane as it is formed leaving some amount inside. This is further supported by the simultaneous increase of extracellular enzyme concentration with the declining levels of intracellular and cell bound proteins. In contrary, the increase in intracellular protein content (fig.13) suggested that a large number of other proteins are being synthesized. This is further supported by decreasing specific activity of the intracellular CMCase (fig.15). The saccharifying cellulase level also showed a similar pattern to that of CMCase in the supernatant (fig. 14). However, the concentration of this enzyme is quite low (about a tenth) in comparison to CMCase. This indicates that more number of enzymes are required for degradation of filter paper than that for CMC (Ljungdahl and Eriksson, 1985). The reaction time proposed and followed for filter paper is also higher (60 minutes) than that of CMCase (30minutes) (Mandels et al.,1976). No intracellular

or membrane bound filter paper activity could be detected indicating that the enzymes other than endoglucanases are not bound to the membrane or present inside the cell but are released to the external cell environment.

The specific activity as represented in figure 15 denotes that the extracellular enzyme had the highest value indicating thereby that very few other proteins in quantity are released into the external environment. Even in extracellular environment the production of proteins other than CMCase was more after 18 hours (fig. 15). Specific activity of intracellular CMCase, on the otherhand, decreased upto 24 hr with the increase in concentration of other proteins, and increased after 36 hours with the decrease in amount of other proteins. The membrane bound CMCase had a decreasing specific activity because of increased accumulation of proteins other than CMCase (fig.15). The increase in specific activity of saccharifying cellulase upto 24 hours and decrease thereafter (fig.14) is also attributed to the above factor but not to the decrease in the enzyme level which was shown to be fairly constant (figure14).

Effect of sonication :

The optimum time for sonication was found to be 3 minutes with a gap of 30 seconds after 1.5 minutes.Sonication ruptures cell wall by cavitation. The application of ultrasonics in liquid creates areas of compression and

rarefaction. Cavities formed in the area of rarefaction rapidly collapse as this area changes to one of compression. The bubbles produced in the cavities are thus compressed to several thousand atmospheres. Subsequent to their collapse shock waves are formed and these shock waves cause cell disruption. (Hugo, 1954).

Effect of surfactant :

Tween 80, a surfactant was shown to increase the enzyme concentration in the supernatant in Trichoderma reesei (Reese and Maguire, 1969, 1970). But with Bacillus thermoalkalophilus, there is no change in CMCase levels in the supernatant (fig. 13). A similar result was also obtained by Duong (1981) working with Clostridium thermocellum. The role of surfactant in enhancement of enzyme is not clear, but Sternberg (1976) proposed that it may be related to increased permeability of the cell membrane, allowing for more rapid secretion of the enzymes which in turn may lead to greater enzyme synthesis. Duong (1981) proposed that in bacterial system in contrast to fungal system the lack of effect of Tween 80 on enhanced enzyme level may be because of the properties of cell surfaces of both bacteria and fungus. It may be that Tween 80 is effective on fungal cell surfaces but not so effective on bacterial cell surface.

Studies with immobilized cells :

The gels of both polyacrylamide and agar are stable

at 60°C. But with the longer treatment of beads at this temperature they became softer. It may be because of the fact that agar melts at 80-100°C (Salle, 1974). Agar beads were stable only for a week.

Morphology of immobilized cells :

Immobilization did not had any drastic effect on the morphological state of the cells as is evident from figure 18, 19 and 20.

Oxygen consumption :

The metabolic state of the immobilized and free cells in terms of oxygen consumption (fig. 21 and table 1) clearly showed that upon immobilization it was drastically affected, but the cells remained viable. Oxygen not being utilized by the immobilized cells for the first two minutes could be because of diffusional resistance to it into the gel matrix. The low oxygen consumption thereafter by immobilized cells in comparison to free cells might have resulted from the alerted microenvironment of the cells or as Mattiasson and Hahn-Hagerdal (1982) suggested could have resulted from the low availability of water molecules of their orientation in presence of the polymer. Both agar and polyacrylamide gels behaved similarly as there was not much difference in oxygen consumption between the cells immobilized in these matrices.

Enzyme production by immobilized cells :

Low amount of enzyme produced by immobilized cells (table 2) could be either because of the diffusional resistance to the inducer or a possible modified microenvironment with low water availability as hypothesized by Mattiasson and Hahn-Hagerdal (1982). Since the medium did not contain sodiumacetate, it might have also affected enzyme production. The better performance of polyacrylamide immobilized cells might be the direct result of pore space or the instability of the agar beads. This might also be because the microenvironment of the cells would have been greatly altered in agar beads than in polycrylamide gel. The new environment of the cells would have resulted in low rate of enzyme production in the beginning.

The concentration of protein produced by agar immobilized cells was greater than that by those immobilized in polycrylamide (fig. 22). These proteins could be either CMCase itself or other proteins as well. The specific activity of the enzyme as shown in figure 23 indicates that the proteins synthesized by cells in agar matrix contain higher quantity of proteins other than CMCase than those synthesized by polyacrylamide entrapped cells. The decrease in specific activity of the enzyme synthesized by cells in polycrylamide gel upto 4 days was mainly because that during that period proteins other than CMCase were produced. The lower specific activity of the enzyme produced by cells

in agar matrix indicates the higher concentration of proteins produced were not majorly CMCase.Though a lower concentration of protein was produced by cells in polyacrylamide matrix, greater part of it was CMCase (in comparison to that produced by cells in agar matrix), as the specific activity clearly showed. This again could be an indication that the microenvironment and diffusional properties in polycrylamide gel were conduc ive than those of the agar gel for CMCase production by the cells.

SUMMARY

Bioenergy might one day successfully substitute the fast depleting conventional energy resources. Cellulose being the most abundant naturally occurring substrate could, in future, be successfully utilized for this purpose as there is no dearth of this material. The complex enzyme system of cellulase can then only be exploited properly if better cellulase producers are screened or raised by genetic manipulation. The easy availability of the enzyme can be of great help in this bioconversion. The present study aimed at producing the enzyme CMCase,(endo-(1,4)-ß-D-glucanase), continuously using immobilization technique.

Bacillus thermoalkalophilus, a bacterium isolated from termite mound soil was selected for the present study for its thermophilic and alkalophilic properties which are very rare to be found in single organism. Even if the pH of the medium was low (between 7 and 9), the organism increased it to above 9.0. It is predominantly active on soluble cellulose but not on native cellulose, probably suggesting that in nature it is present in symbiotic association with other cellulolytic organisms acting on native cellulose. CMCase in this organism might be a mixture of two or more enzymes or a single enzyme with different catalytic sites active at different temperature and pH (60 and 30^{o}C; pH 7.0 and 9.5). The enzyme is predominantly extracellular. Surfactants had little effect on the enzyme production contrary to that in fungal system as with Trichoderma reesei.

Immobilization of cells carried out in agar and polyacrylamide gels showed that there is no effect of immobilization on the morphological state. But metabolically they were drastically affected. They were metabolically less active both in terms of oxygen consumption and enzyme production probably because of microenvironment and low water availability due to the effect of polymer matrix. There is about 25-45% reduction in enzyme production. Production of proteins other than CMCase was also probably affected in immobilized state. Polycrylamide gel entrapped cells can be continuously and repeatedly used for enzyme production. On the otherhand, agar entrapped cells could not be that successfully used in comparison to polycrylamide entrapped cells because of the instability of beads over a long period of time.

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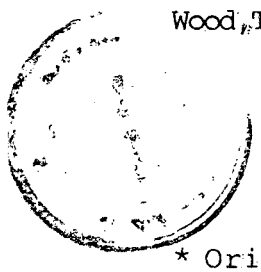

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