STUDIES ON THE BIODEGRADATION OF CELLULOSE BY CELLULASES

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CERTIFICATE

This is to certify that the thesis entitled 'Studies on the Biodegradation of Cellulose by Cellulases', submitted by Shri Arunan, M.C., ID No. 72S87004, for award of Ph.D. degree of the Birla Institute of Technology and Science, Pilani, embodies original work done by him under my supervision.

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ABSTRACT

The availability of high activity cellulase is the major factor in determining the efficiency of biodegradation of cellulose. The slow rate of production, a long lag period for induction, catabolite repression and the lack of a proper method of recovery has been the stumbling blocks in this respect. A method of enzyme production, by starving the mycelia before induction is found, not only to be rapid but also, to cut down the lag period considerably. This method is used further to study the induction-repression mechanism of cellulase enzyme. The inhibitory effect of glucose is found to be in between that of transcriptional inhibitors and translational inhibitors.

The mechanism by which surface active agents like Tween-80 and Triton-X increase the production of enzyme has been studied. It is found that the presence of non-ionic surfactants is more important when the induction medium contains Ca^{++} than when it is absent. The presence of surfactants during growth of the mycelia also has a favourable effect in its cellulase producing capability.

Adsorption-Desorption characteristics of cellulose-cellulase system is studied with a view to understand the possibility of recovery of cellulase. It is found that optimum temperature and pH for enzyme activity are also the optimum conditions for adsorption of the enzyme to cellulose particles and, an abrupt change in temperature caused

maximum desorption of the enzyme thus facilitating its recovery. Endoglucanase (CM case) activity was studied after immobilizing with bentonite and activated charcoal.

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C O N T E N T S

GENERAL INTRODUCTION

CHAPTER-I

GENERAL INTRODUCTION

The main structural substance in the primary and secondary cell walls of plants is cellulose and as such it is the most abundant organic material present in nature. Chemically cellulose is a homopolymer composed of D-Glucose units linked together by β-1,4 glucosidic bonds. Native cellulose consists of bundles of cellulose chains called microfibrils which are bound laterally by hydrogen bonds and vander waals forces into a linear structure. The molecular arrangement within each microfibril is usually regular (Cowling, 1963; Cowling, et al 1969). In nature it exists together with other substances like hemicellulose, lignin and pectin which tend to inhibit its biological degradation. Cellulose degradation is consequently a slow process both in vivo and in vitro and only relatively a few groups of bacteria and fungi are able to degrade cellulose (Norkans, 1962).

Cellulose is one of the major components of municipal waste, the disposal of which has been drawing considerable attention for many years. The composition of the municipal refuse in an industrialised society is given in Table 1.1. Further, agricultural wastes also have a considerable amount of cellulose. The incineration process by which the solid wastes are degraded, in

effect, only shifts the form of pollution from land to atmosphere. Chemical degradation processes also instead of reducing, invariably, complicates pollution problems. As cellulose in nature is found associated with many different substances, acid or alkali hydrolysis results in many abnoxious byproducts.

Cellulose can be effectively degraded by micro-organisms. Reese, et al (1972) has discussed the advantages of enzyme hydrolysis over acid and alkali hydrolysis. Enzymes generally known as cellulases hydrolyse the β-1,4-glycosidic linkages of cellulose resulting in unit molecules. Glucose and other soluble cellooligosaccharides are effectively converted into protein, chemicals and energy (Mandles, 1975, Mitra and Wilke 1975; Ghose, et al, 1975). In biogas production, cellulose degradation has been identified as a rate limiting step (Bryant, 1972). An effective method to remove this bottleneck may be by means of enzyme pre-treatment of the substrate by cellulases.

There are many problems that confront the process of effective biodegradation of cellulose wastes.

1. The nature of waste materials: Cellulose found in nature is interwoven with substances like lignin, pectin etc., which form a protective layer.

2. Cellulolytic Organisms: Though many cellulolytic organisms are available, their ability to produce

Table 1.1

The composition of average municipal refuse (Bell, 1964)

Component	Weight percentage
Moisture	20.73
Cellulose, Sugar, Starch	46.63
Lipids (fats, oils, waxes)	4.50
Protein	2.06
Other organic (Plastics)	1.15
Ashes Metal, Glass etc.	24.93

adequate amount of enzyme is less. Mutations could increase the production rate of enzymes only by two to four folds (Manels, 1975).

3. Enzyme Purification and Recovery: Almost half the cost of enzymatic hydrolysis process is an enzyme production itself (Mitra, et al, 1975).

4. Product inhibition: The rate of enzyme activity slows down as the product accumulates in the medium.

Cellulolytic Organisms:

The availability of high activity cellulase is the basis for a successful process for enzymatic conversion of cellulose. This depends on selection and improvement of suitable strains for enzyme production and development of fermentation methods for producing cellulase in quantity. Many fungi and bacteria degrade cellulose, producing microbial cells, carbon dioxide and methane. Only a few fungi such as Trichoderma viride (Berghem et al,1973; Mandels et al, 1969 and 1974; Selby et al, 1967; Toyama et al, 1972). T. Lignorum (Eriksson, et al, 1971), T. Koningii (Wood, T.M. 1972a, 1972b and Halliwell, 1971 and 1975), Spirotrichum pulverulentum Syn. Chrysosporium lignorum (Streamer, et al 1972), Aspergillus wenti (Eriksson, 1975) and Fusarium solani (Wood T.M. 1972a) are known for their ability to produce high activity cellulases capable of degrading insoluble cellulose into soluble sugar, in vitro. In addition, there are many

organisms that produce cellulase preparations which degrade only soluble derivatives of cellulose such as carboxymethyl cellulose, while a few organisms produce very little residual cellulase of any type, despite their active growth on insoluble cellulose. (Mandel et al, 1969). This is due to the fact that cellulase is a complex of enzymes and enzyme-like factors and that not all members of the complex are necessarily found in the culture fluid after growth of the organism. The practical saccharification of waste cellulose requires a stable cell-free enzyme preparation with adequate levels of all essential components of the cellulase complex. So production of high levels of enzymes is no adequate criterion for selecting organisms to be used as a source of cellulase (Mandels et al, 1975).

Cellulose degrading forms also occur among the gliding bacteria, among Gram-negative and Gram-positive bacteria and among actinomycetes (Siu, R.G.H., 1951; Goksoyr, et al, 1975).

Cellulolytic activity is also found among obligate aerobes (gliding bacteria, pseudomonas), facultative anaerobes (Bacillus, Cellulomonas) and obligate anaerobes (Clostridium) (Goksoyr, et al, 1975). Recently, thermophilic organisms have also been studied for their ability to produce cellulases. The ascomycetes, Chaetonomium thermophile var. dissitum, a typical thermophilic fungus is able to produce a cellulolytic system decomposing native

cellulose. Interest in thermophilic organisms has been stimulated by the search for thermostable cellulases. However, cellulases from thermophiles are not necessarily heat stable than cellulases from mesophiles (Mandels, 1975).

Although much work has been done with other organisms, T. viride and T. koningii still remain to be the best source of extracellular cellulases.

One possibility of attaining high overall cellulolytic activity lies in mixing the enzymes produced by different organisms. The advantages of using mixed enzyme preparations for hydrolysis of complex cellulosic substances have recently been shown by Ghose et al (1975). However, the mechanisms of cellulose decomposition may be different in different microbes (Goksoyr, 1975).

Cellulase Biosynthesis:

The production of cellulase depends on selection and improvement of suitable microbial strains and on the development of fermentation methods. Most of the research concerning production methods have been done using T. viride in spite of its long cultivation time. Mutants of T. viride have brought about only three to four fold increase in cellulase yields which is low in comparison with the hyperproduction of some other fungal enzymes (amylases, proteases etc.) The former may be a result of a different induction mechanism of cellulases (Enari et al, 1971). Catabolite repression is another regulatory mechanism by which concentration of cellulase is controlled (Nisizawa et al, 1971 and 1

Trichoderma Species produces various carbohydrases like xylanase, mannase etc, besides cellulase. Therefore, for waste cellulolytic materials, it may be desirable to use the same waste materials as a carbon source to induce the proper mixture of enzymes. The basic media for growth and cellulase production by T. viride contain pepton or urea as nitrogen source, different cellulose preparations as carbon source and necessary minerals. It has been well established that surface active agents like Tween-80 stimulate the production of extracellular enzymes (Reese et al, 1969, Ghose et al, 1975).

Cellulase is an inducible enzyme complex in T.viride (Nisizawa, et al, 1971a and 1971b), but has been shown to be constitutive in Pseudomonas flurescens (Suzuki, 1975). Cellulase is produced when T. viride is grown on cellulose, lactose, glucose and cellobiose (Mandels et al, 1957). Glucose does not appear to be an inducer since a high initial concentration is required and synthesis of cellulase begins only after glucose is totally exhausted from the medium. The slight inducing effect of glucose is presumably caused by sophorose found during acid hydrolysis of starch (Nisizawa, et al, 1971a and 1971b; Brown et al, 1975; Mandels et al, 1960). Though sophorose has been found by Mandels et al, (1962) to be a potent inducer, it has not been proved that it would be involved in the synthesis of cellulase in natural conditions.

Cellulose is converted mainly into cellobiose and glucose during hydrolysis with cellulase enzymes. Cellobiose in turn, markedly stimulates the production of cellulase if the fungal growth is restricted by culturing under suboptimal conditions (Mandels et al, 1960). Although cellobiose at low concentration stimulates the production of cellulase (Ghose et al 1975) its precise role in the induction mechanism has not yet been shown. In growing fungal cultures, cellulases are also inhibited by products of their action (Mandels et al, 1960 and 1975; Ghose et al, 1975). Crystalline cellulose can be used as the sole carbon source for cultures (Mandels et al, 1957, 1960 and Tomita et al, 1968). In washed cells neither cellulose nor cello-oligo saccharides induce the formation of cellulase (Nisizawa et al, 1971).

Sophorose is not a degradation product of cellulolysis and therefore if it is a natural inducer, it must be synthesized during the induction phase. Nisizawa and coworkers (1971b) demonstrated that sophorose addition considerably enhanced the formation of xylanase as well as that of β-glucosidase whereas cellulase was synthesized only in the presence of sophorose, showing that it also causes de novo cellulase synthesis. It has been shown that most of the increase in glucose producing enzyme activity by sophorose is due to the enhancement of exoglucanase activity (Nisizawa et al, 1971b).

Cellulolytic Enzyme Systems:

The degradation of crystalline cellulose is a complex process requiring the participation of many enzymes. Reese et al, (1950) first suggested the conversion of native cellulose to soluble sugars based on a two step process. The so-called C_1 component of the enzyme was postulated to cause some disorganisation of the cellulose chain as a preliminary to the hydrolysis by C_X enzymes. According to these workers, micro-organisms which grow only on soluble cellulose synthesize only C_X components, whereas micro-organisms capable of growing on highly ordered forms of cellulose produce both C_1 and C_X. The latter group of microorganisms include Trichoderma viride, T. koningii, Fusarium solani, Penicillium funiculosum, Spirotrichum pulverulentum etc. However, it was on the basis of observations made with actively growing fungi that C_1 - C_X hypothesis was first formulated and it was many years later until a cell-free filtrate from T.viride was isolated which was sufficiently active on highly ordered cellulose to conclude that a C_1 component was present (Mandels et al, 1964). Native cellulose on exposure to cellulase from this group of organisms shows extensive change in its physical properties like increased capacity of alkali and moisture absorption, transverse cracking, loss of tensile strength etc., before producing any measurable amount of reducing sugars (Halliwell, 1965, Marsh, C.A., 1966 and Rantela et al, 1968).

There is substantial evidence for the existence of a C_1 component in multi-component enzyme systems of true cellulolytic microorganisms. However, controversy still exists about the specificity of various C_1 components and about the original two-step sequential C_1 - C_X mechanism. There are contradictory claims on the matter. Some of the important observations are in the following paragraphs.

Selby and Maitland (1963) isolated C_1 component, a C_X component and a cellobiase fraction from cultures of T. viride using sephadex and ion-exchange chromatography. The C_1 fraction by itself did not support any cellulose degradation but it was active in presence of C_X fraction. The important findings of these observations were the striking synergestic action of C_1 and C_X. Two possible hypotheses were proposed. C_1 makes cotton fibre more susceptible for C_X action or that C_1 is inhibited by the products of its own reaction. The second possibility finds support from the fact that additional synergestic action of cellobiase fraction was observed. Furthermore, C_1 activity was found to increase by 50 percent when simultaneous dialysis against water was carried out (Halliwell et al 1972 and 1973). Recent advances however, elucidated the mechanism of C_1 action and suggest it to be a cellobiohydrolase acting as an exoglucanase against native and derived forms of cellulose (Halliwell et al 1972; Berghem et al, 1973).

Many reports contrary to the proposed hypothesis that C_1 initiates attack on crystalline cellulose have

appeared in the literature. Wood (1962) and Wood and McCrae (1972) purified C_1 component from cellulase complex of T. koningii and F. solani using gel filtration, ion exchange chromatography and electrofocussing methods. The purified C_1 component was found to possess a limited ability to produce reducing sugars from a solution of CM-Cellulose, but was not able to attack the highly ordered cellulose by itself. It attacked oligo-saccharides (Cellotetrose and Cellohexose) and cellobiose was virtually the sole product of the reaction. Similar results were also reported by Petterson et al (1972) with purified C_1 component from T. viride. Nisizawa et al (1972) reported that purified CM-Cellulase fraction degraded cotton at a much faster rate than purified C_1 fraction. Similar results were also reported by these workers from culture filtrates of Irpex Lacteus. Streamer et al(1968) observed that if dewaxed cotton was pretreated with endo-1, 4-β-glucanase, the exo-1,4-β-glucanase enzyme released much more degradation products than from an untreated cotton. All these observations strongly supported the alternative theory that the C_X (endoglucanases) acting randomly over the cellulose chain go in first and open up chain ends where C_1 (exoglucanases) can act.

At present, the most generally accepted view is that C_1 enzyme is an exo-β-1,4-glucanase which has been shown to be a cellobiohydrolase in case of T.viride and T. koningii (Halliwell, et al, 1975). According to the present view complimentary action of endo and exo-glucanases

hydrolyse crystaline cellulose to soluble cello-oligosaccharides, mainly cellobiose (Wood, 1975, Pettersson 1975, Wood et al, 1975 and Bisaria 1977). The current trend is to consider the cellulase enzyme system to be composed as follows (Reese 1975).

1. Endo-β-1,4-glucanases are present with several components varying in degree of randomness. One of these may be the enzyme that acts first on 'Crystalline' Cellulose.
2. Exo-β-1,4-glucanases are present in varieties. β-1,4-glucohydrolase, removing single glucose units from the non reducing end of the chain. The cellobiohydrolase is being equated with the old C_1 enzyme by many investigators (Halliwell, et al 1973, Pettarsson, 1975, Wood et al, 1975 and Tomita et al, 1974).
3. β-glucosidase (cellobiase) is also present. The relationship of this enzyme to the glucohydrolase has not yet been clarified.

For complete activity on crystalline cellulose, it is necessary that several of preceding components be present in the reaction mixture.

Purification and Properties of Cellulases:

One of the main difficulties with the purification of cellulolytic enzymes is the choice of substrates. Pure cellulose is composed of very long chains of β-1, 4-linked glucose units, nevertheless, there is controversy on how

these chains are arranged in the microfibrils in wood and cotton. The question whether regions of less well-ordered cellulose chains exist, in the microfibril is of fundamental importance and is yet to be solved. Native cellulose fibres such as cotton are poorly defined as enzyme substrates and are unsuitable for enzyme assay, since their rate of hydrolysis by enzymes is very low. Moreover, some celluloses do not have a readily determinable effect on crystalline cellulose such as cotton. Commercial cellulose preparations such as Avicel are more rapidly hydrolysed but cannot be used as an universal substrate, since it is not attacked by all of the enzymes present in cellulolytic culture fluids. A cello-oligosaccharide, such as cellotetrose, is attacked by all known types of cellulolytic enzymes but is rather difficult to prepare. It may be concluded that if one wished to discover all the different cellulolytic enzymes present in culture fluid several different substrates are required (Selby, 1968). Another factor tending to render the purification difficult is that the cellulase enzymes are nearly always present as isoenzymes, often differ only slightly in isoelectric pH and are therefore difficult to separate. It is also not certain whether the components resolved, for instance, by isoelectric focussing actually represent species of the same type of enzymes or if they differ in specificity. Wood (1972 and 1975) separated the cellulase complex produced by T. koningii into six pure components using gel filtration and isoelectric focussing

techniques. These components were a single exoglucanase (C_1), three endoglucanases (C_X) and two β-glucosidases. The complex thus consists of many isoenzymes. The exoglucanase was found to split off cellobiose from non-reducing end of the cellulose chain (Wood et al, 1972) and thus systematically designated as β-1, 4-glucan cellobiohydrolase (E.C. 3.2.1.-). The endoglucanase hydrolysed β-1, 4-glucans in a random fashion and were systematically called β-1, 4-glucan-glucanohydrolases (E.C.3.2.1.4.). The endoglucanases can be differentiated by the randomness of their attack on carboxy methyl cellulose and by the rate of solubilization of phosphoric acid-swollen cellulose (Wood, 1972). Working with the cellulase system of the rot fungus S. pulverulentum Eriksson and coworker (1975a and 1975b) isolated five endo-β-1, 4-glucanase. Pettersson et al (1975), fractionated the cellulase complex of T. viride into four components. Two of the components were endoglucanases. The exoglucanases were shown to be cellobiohydrolases. Some of the properties of cellulase components isolated from T. viride are summarized in Table 1.2 (Petersson, et al, 1975).

Thermostability is one of the most important technical property of cellulases since the hydrolysis of cellulose proceeds faster at higher temperatures. Endoglucanases are quite stable upto 4 hours at 60°C and pH 5.0. β-glucosidase and exoglucanase of T. koningii resemble one another in their heat stability at 60°C; they lose about 80 percent of their original activity at 60°C and pH 5.0 in less than 4

Table 1.2

Some properties of cellulolytic enzymes, isolated from Tricholerma viride. (Pettersson, et al, 1975).

Types of enzymes	Mole wt.	Isoelect. point	Carbohydrate percentage.	Activity towards different substrates			
				CMC	Microcryst. cellulose	Repreci-pitated cellulose	Cellotetrose
Exo-β-1, 4-Glucanase	42,000	3.79	9	-	+	+	+
Enco-β-1, 4-Glucanase I	12,500	4.60	21	+	-	+	+
Enco-β-1, 4-Glucanase II	50,000	3.39	12	+	-	+	+
β-Glucosidase	47,000	5.74	0	-	-	-	+

hours. (Wood, 1975). In the presence of cotton, the cellulases of T. koningii and F. solani are remarkably stable showing no loss of activity when incubated for four weeks at 37°C and pH 5.0 (Pettersson, et al, 1975).

The initiation mechanisms of the degradation of cellulose has not yet been completely clarified. Eriksson and coworkers strongly feel that at least in S. pulverulentum an oxidizing enzyme is involved in the initiation of cellulose degradation. These authors suggested that the role of oxidizing enzyme was to oxidise cellulose by inserting uronic acid meeties and thus breaking hydrogen bonds between two cellulose chains. S. pulverulentum further has been shown to produce the enzyme cellobiase: quinone oxido-reductase which participated in the degradation of cellulose in wood (Wood, et al 1975, Westermark et al, 1974a and 1974b). The discovery of this enzyme indicated that at least when wood was the substrate the mechanism of fungal cellulose degradation was not simply a synergestic action between endo and exoglucanases. The mechanisms of action of the cellulase complex may be very different in different organisms (Wood, 1975).

Objective of the Present Work:

It has been reported (Mitra and Wilke, 1975) that the cost of enzyme make up during hydrolysis is nearly 50 percent of the cost of cellulose hydrolysis. So the availability of a high activity cellulase and the development of an enzyme recovery process can considerably increase the efficiency of

the process. Even the most effective mutation technique could yield only four fold improvement in cellulase production. This low hyperproduction of cellulases compared to the hyperproduction of some other fungal enzymes (amylases, proteases etc.) is suspected to be the result of the possible difference in the mechanism of production of cellulases. This is all the more pertinent as it has been found that the induction-repression processes of extracellular enzymes may be different in eukaryotic organisms. Further the characteristic insoluble nature of the initial substrate also is significant with reference to the induction of cellulases.

Cellulase release is enhanced by non-ionic surface active-agents. But the exact mechanism by which the production of extracellular enzymes increase is not yet found out. The knowledge of the mechanism of increased enzyme production/ release with the help of surface active agents would go a long way to optimise the process of production of enzyme with high activity.

The study of the cellulose-cellulase adsorption characteristics is very important not only in enzyme fermentor process design but also in the process of recovery of used enzyme components. Proper techniques may be developed to purify the enzyme by utilizing the knowledge of specific adsorption-desorption characteristics of cellulose-cellulase system.

The slow rate of enzyme production in cellulase

producing organism has been a matter of utmost concern for long. However, Nisizawa et al (1971) could obtain a faster production of enzyme by washing the mycelia, before transferring to an induction medium. A better method of rapid production of enzyme not only would have significance in the field of application of cellulose degradation process, but also would aid tremendously in elucidating the basic mechanism of production and release of cellulase.

Therefore, the present work is mainly concentrated towards:

1. The biological aspect of cellulase production namely:
   (a) - a rapid method production of cellulase
   (b) - the induction repression mechanism of extracellular cellulases
   (c) - the mechanism of action of surface active agents in the enhancement of cellulase production;

and,

2. The biochemical properties of cellulase enzyme namely
   (a) - the adsorption-desorption characteristics of cellulose-cellulase system and
   (b) - the immobilization of the enzyme and the possible change in reaction kinetics, with a view to reuse active cellulases in order that the process of utilization of cellulose becomes more efficient.

MATERIALS AND METHODS

CHAPTER-II

MATERIALS AND METHODS

ORGANISM AND CELLULASE SAMPLES:

Trichoderma viride QM 9414 obtained from U.S. Natic Army Laboratories, Natic, Mass. USA, was used as a source of exo- and endoglucanases-rich cellulase enzyme for preparation of enzyme samples. The culture was maintained on potato-dextroxeagar medium. The composition of the medium used for cellulase production was as follows:

	gm/litre		mg/litre
$(NH_4)_2SO_4$	1.4	$FeSO_4 7H_2O$	5.0
KH_2PO_4	2.0	$MnSO_4.H_2O$	1.6
Urea	0.3	$ZnSO_4.7H_2O$	1.4
$CaCl_2$	0.3	$CoCl_2$	2.0
$MgSO_4.7H_2O$	0.3		

Cellulose	0.75 percent
Tween 80	0.20 percent
Initial pH	5.0

The pH of the medium was adjusted with 1N citric acid or with 1M KH_2PO_4. Temperature of the culture was maintained at 29°C, in an incubator water bath (SEW).

The culture was centrifuged or filtered to remove the solid mycelia and the culture filtrate was used as the crude enzyme preparation.

DETERMINATION OF CELLULASE ACTIVITY:

Cellulase activity was determined following Filter paper assay method. (Mandels et al, 1976). This method as developed at Natic has been used for a simple, easily reproducible measurement of Saccharifying cellulase to predict the action of the enzyme in extensive conversion of concentrated cellulose. Filter paper activity is the mg. of glucose produced when 0.5 ml. of enzyme solution acts on 50 mg. of Whatman No. 1 filter paper at pH 4.8 and 50°C for one hour. Filter paper units are calculated as the micromoles of glucose produced per minute in the above assay based on the enzyme dilution to give 2.0 mg. of glucose. The cutoff value of 2.0 mg. was chosen because the hydrolysis curve is fairly linear to above this value and it represents 4 percent hydrolysis of the filter paper.

FILTER PAPER ASSAY PROCEDURE:

1. Reagents: Whatman No. 1 filter paper cut into 1 x 6 cm. strips (50 mg.). Buffer 0.05 M sodium citrate pH 4.8 Glucose standards in buffer. Dinitro salicylic acid (DNS) reagent for reducing sugar.

2. Filtered or centrifuged culture sample to remove solids.

3. Placed 0.5 ml. of enzyme solution and 1.0 ml. buffer in 18 mm. test tube. Added a filter paper strip and mixed on

a Vortex mixer to coil the filter paper in solution. Incubated for one hour at 50°C. Added 3 ml. of DNS reagent to stop reaction. Placed the tubes in boiling water for 5 minutes and determined the reducing sugar as glucose. Included a blank tube (without filter paper) to correct for any reducing sugar present in the enzyme preparation. The milligram of glucose produced in this test is the filter paper activity.

4. The DNS reagent measures reducing sugar nonspecifically. When glucose was used as standard, values for cellobiose were 15 percent low and values for xylose were about 15 percent high on a weight basis.

ANALYTICAL PROCEDURES:

Estimation of reducing sugars: Dinitrosalicylic acid (DNS) method (Summer and Somers, 1954).

DNS Reagent: To 300 ml of 4.5 percent NaOH were added 800 ml. of 10 percent dinitrosalicylic acid and 255 gm of Rochelle salt. To 10 gm. of crystalline phenol was added 22 ml. of 10 percent NaOH, diluted to 100 ml. and mixed. 6.9 gm. of sodium bisulfite was added to the DNS solution. The resulting solution was mixed well until all of the Rochelle salt was dissolved and it was kept in coloured, tighly stoppered bottle. 3 ml. of the reagent contained, by titration with phenolphthalein as indicated, the equivalent of 5 to 6 ml. of the tenth-normal NaOH.

To 1 ml. of sample containing 0.2 to 2.0 mg. of reducing sugars 1 ml. of citrate buffer (0.5M, pH 4.8) and 3 ml. of DNS reagent were added. The mixture was heated for five minutes in boiling water bath and diluted to 10 ml. after cooling. The absorbance of the developed colour was measured by Spectronic-20 at 600 nm. Glucose standard was prepared for each set of determination.

DETERMINATION OF SOLUBLE PROTEIN BY FOLIN-CIOCALTEAU METHOD (FOLIN AND CIOCALTEAU, 1927).

Reagent A: 20 gm. Na_2CO_3 + 4 gm NaOH dissolved in water and made upto 1 litre.

Reagent B: 2.5 gm. $CuSO_4 . 5 H_2O$ + 5 gm. sodium citrate dissolved in water and made upto 500 ml.

Reagent I: 50 ml. of A + 1 ml. of B.

Reagent II: Folin-Ciocalteau's reagent, (Biochemicals Unit, V.P. Chest Institute), diluted 100 percent (V/V) with distilled water. Reagent I and II were prepared immediately before use. 5 ml. of reagent I were added to 1 ml. of sample containing 20-200 µg of protein, thoroughly mixed and allowed to stand at 30°C for 10 minutes. 0.5 ml. of reagent II was then added rapidly and mixed on a Vortex mixer. The tubes were allowed to stand at 30°C for 30 minutes and the absorbance of the colour was read at 680 nm. Bovine Serum albumin was used as standard protein.

ASSAY OF CELLULASE ACTIVITIES:

The enzyme activities of cellulase are expressed in terms of international units, i.e. 1 μ mole of reducing sugars formed in 1 minute. The specific activities are expressed in units per mg. of soluble protein.

RAPID METHODS OF PRODUCTION OF CELLULASES

CHAPTER-III

RAPID METHOD OF PRODUCTION OF CELLULASES

3.1.0 INTRODUCTION:

Crystalline cellulose can be used as the sole source of carbon for cultures of cellulolytic organisms. (Mandels, et al, 1957, 1960 and Tomita et al, 1968). However, where the mycelia was in the process of production of cellulases, induced by sophorose, addition of cellulose on cellobiose inhibits the process (Ghose et al 1975). In washed c... cells also cellulose or cello-oligo saccharides did not induce the formation of cellulase whereas sophorose did. (Nisizawa et al, 1971). It should be understood that these observations were made with an enzyme production system of very slow rate. Further, whereas Mandels, et al (1957) took many days to observe an experimental result Nisizawa et al (1971) could obtain the enzyme production with a lag period of 4 hours after induction. However, the findings by the latter workers showed an advancement in the sense that the washed mycelia when induced, produced the enzyme more rapidly than the unwashed mycelia. The lag phase was also reduced remarkably. But no attempt was made to explain the reason for a faster induction. That the enzyme production is repressed directly or indirectly by catabolites has been established by many workers including Nisizawa, et al, (1971 and 1972). It is therefore reasonable to assume that

the inhibition of enzyme production observed when cellulose or cellobiose were introduced is due to catabolite repression, considering that in all cases the time taken for induction process is long and the rate of production is slow. This is corroborated partly by the finding that cellobiose can serve as an inducer when the mycelia are grown under suboptimal conditions (Mandels, et al, 1960). Further, one of the observations by Nisizawa et al (1971) that the same glucose grown mycelia samples, one at 15th hour and another at 30th hour, when induced, had shown a lag period of 15 hours and 2 hours respectively before the production of cellulase, also gives credence that either glucose or some of its metabolites are possibly acting as agents of repression. That the washed mycelia induced faster production of enzyme led to the logical extension of the work to allow the washed mycelia to be kept for more time in the washing solution or in effect, to starve the mycelia and to study its effect on enzyme production.

It is observed that the mechanism of enzyme induction-repression may not be the same as put forward by Jacob and Monod (1961) for E. Coli (Nisizawa, 1971a). Further, the induction-repression mechanism of extracellular enzyme may be different from that of cell-bound enzymes. Earlier studies on cellulases of T. viride were less than convincing due to the longer induction period and the slow rate of enzyme production. Hence with the development of a method

for rapid production of cellulases, which is described in this chapter the studies on induction-repression mechanism is made more reliable. This is more significant because of the starvation the mycelia is put to prior to induction, that it would be proved whether cellulase production is the result of de novo synthesis.

3.2.0 MATERIALS AND METHODS:

Glucose as well as cellulose grown mycelia of Trichoderma viride QM 9414 of different age groups were used for the work. The culture conditions were given in Chapter-II.

The mycelia were harvested and washed with phosphate buffer (pH 4.8) and kept suspended in the buffer and submerged in a starvation medium for specific time. The starvation medium contained all other constituents of the culture medium except the carbon source. At definite intervals the starved mycelia were transferred to the medium equivalent to the starvation medium with inducer. The FPA was estimated at definite intervals and compared with that of the unstarved mycelia put under induction. Before transferring to the induction medium the mycelia were taken out of the starvation medium, pressed in between two charred Whatman No.1 filter papers to dryness and weighed. Equal amount by weight of mycelia was then transferred to the induction medium.

Table 3.1

Effect of inducers in the production of cellulase after the mycelia put under starvation for 15 hours.

Time minutes	FPA						
	Control non-starved.	Cellulose	Cellubiose	Sophorose	Starch	Glucose	Glycerol
0	0	0	0	0	0	0	0
30	0.32	1.2	1.5	1.78	0.27	0.25	0.72
60	0.327	2.1	2.7	2.75	0.268	0.252	1.25
90	0.48	2.75	3.54	3.84	0.27	0.27	1.35
120	0.69	3.45	3.87	3.96	0.35	0.29	1.52
150	0.87	3.85	3.94	4.2	0.27	0.25	1.60
180	1.25	4.4	4.25	4.48	0.32	0.268	1.8
210	1.48	4.87	4.57	4.72	0.27	0.279	2.5

Table 3.2

Effect of starvation time on induction.

Starvation time (hours)	Extracellular cellulase FPA/120 minutes
0	2.96
1	4.46
3	5.33
8	5.83
12	6.58
15	7.87
16	7.85
22	5.78

glucoside are also poor inducers. Cellulose, cellobiose and Carboxy Methyl Cellulose acted as inducers.

3.3.4 Performance of Glucose Grown Mycelia as Against Cellulose Grown Mycelia in Rapid Production of Cellulases:

Equal amounts by weight of 25 hour old glucose grown and cellulose grown mycelia were starved for 15 hours and induced with 1 x 10^{-1} M Sophorose. The enzyme activity was tested after 12,24, 48 and 87 hours. Glucose grown mycelia in each case, showed more enzyme production than the cellulose grown mycelia. (Table 3.4 and figure 3.1).

3.3.5 Effect of Ageing on the Rapid Production of Cellulases:

Glucose grown mycelia of different age representing stationary phase (57 hours), late log phase (39 hours), middle log phase (25 hours) and early log phase (16 hours) were transferred to an induction medium with 0.5 percent Sophorose, after putting the mycelia under starvation for 12 hours. The enzyme activity in FPA was estimated 60 minutes after induction. The results are shown in Table 3.5 and Figure 3.2.

Within 60 minutes of induction the more aged mycelia were found capable of producing more enzyme with the exception of the stationary phase mycelia.

Figure 3.3 shows the effect of age on the rapid production of mycelia in more detail. The amount of enzyme, the late log (39 hours) phase mycelia produced in 30 minutes,

Table 3.4

Effect of carbon source during growth on the induction of enzyme by starved mycelia.

Induction time (hours)	F P A	
	Cellulose grown	Glucose grown
12	6.54	7.852
24	8.45	9.45
48	12.78	14.475
87	9.83	12.44

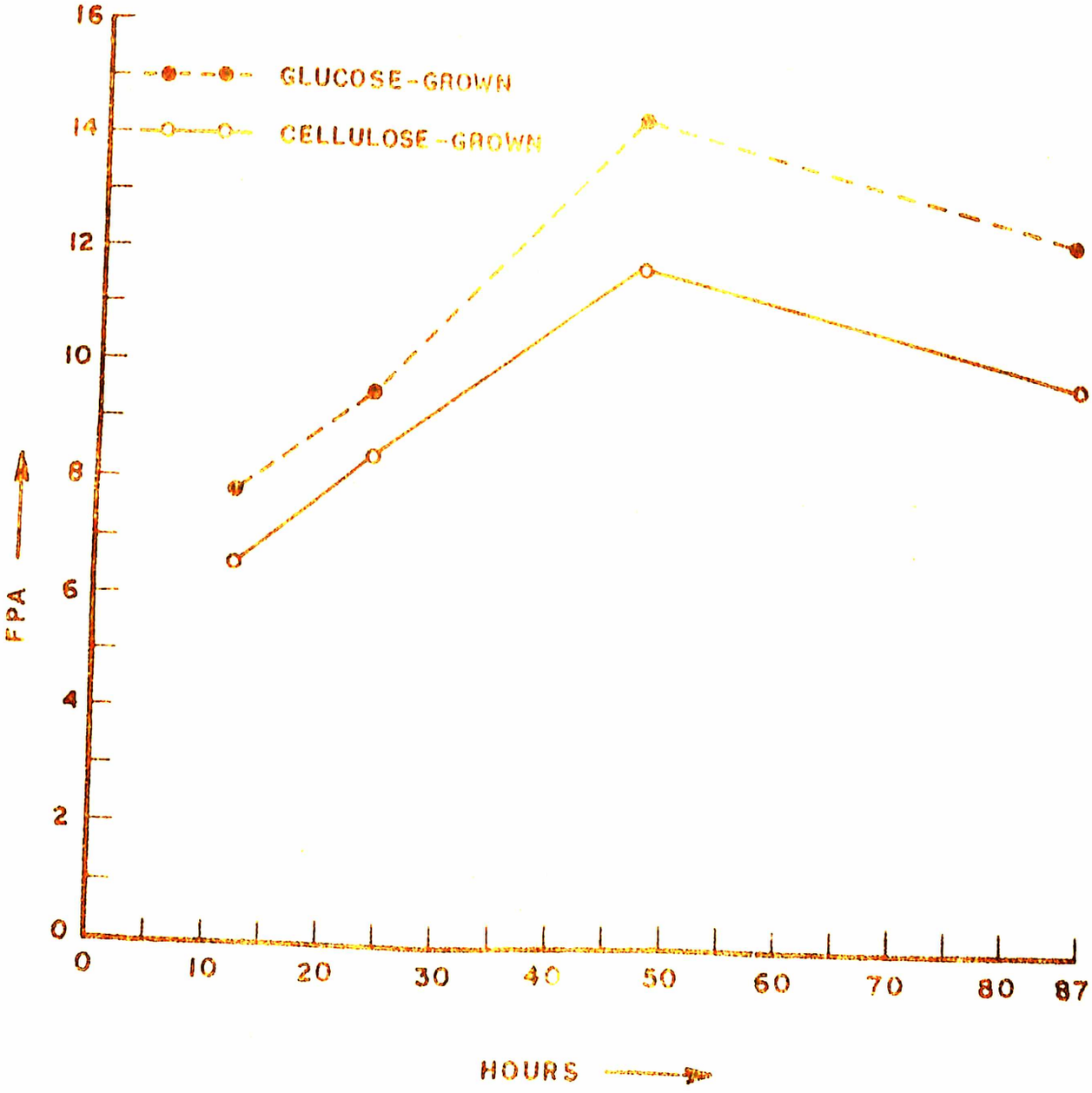

FIG. 3.1 GLUCOSE - GROWN AND CELLULOSE - GROWN MYCELIA ON INDUCTION.

Table 3.5

Effect on ageing of the mycelia on rapid induction of cellulase

Age (hours)	FPA After 60 minutes of induction
57	1.349
39	1.48
25	1.12
16	0.99
unstarved 25 hours	0.87

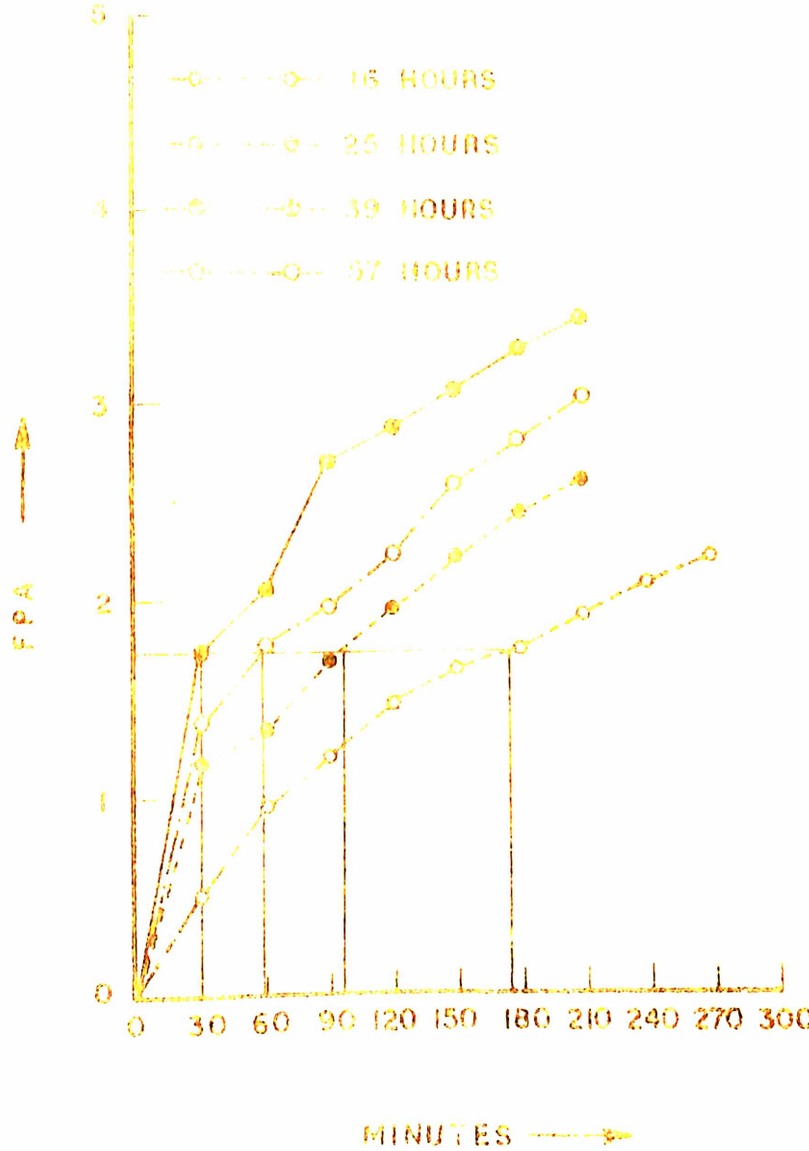

FIG. 3.3 EFFECT OF AGE ON RAPID INDUCTION OF CELLULASES.

the early log phase (16 hours) mycelia produced only within 174 minutes, whereas middle log phase (25 hours) mycelia and stationary phase (57 hours) mycelia produced that much enzyme within 96 minutes and 54 minutes respectively. The capacity of older mycelia to produce more enzyme within a short time was observed. However, after 5 days of induction the mycelia of different ages reached a stabilizing peak and the FPA did not show any significant change from each other.

3.3.6 Effect of Induction Medium in Rapid Production Cellulase:

25 hour old glucose grown mycelia after 15 hour starvation were transferred to a medium containing culture medium free of carbon source. Adequate amount of Sophorose (1×10^{-1} M) was added as an inducer. FPA was determined and compared with those in which phosphate buffer (pH 4.8) and distilled water replaces the culture medium and induced by Sophorose (1×10^{-1} M). The results are shown in Table 3.7.

Within 75 minutes the induction medium did not influence the production of enzyme significantly though the culture medium sample showed slight increase in activity compared to other samples. This was amplified after 48 hours, as there was significant difference in the total enzyme activity (Table 3.7).

3.3.7 Effect of Cyclic AMP on Nonstarved Mycelia cultured in Glucose on Production of Cellulase:

25 hour old glucose grown mycelia were washed with phosphate buffer and induced in presence and absence of

Table 3.6

Effect of ageing on enzyme production

Age (hours)	F.P.A.	
	60 mts.	150 mts.
16	0.87	2.75
25	1.57	3.58
39	2.78	3.79
57	2.49	4.28

Table 3.7

Effect of medium in rapid induction

Time	F P A		
	Culture medium	Buffer	Distilled water
75 mts.	1.78	1.47	1.43
2 days	5.79	3.47	2.88

different amounts of cyclic AMP. 25 hours old glucose grown mycelia were starved for 15 hours and were treated with and without cyclic AMP that served as controls. (Table 3.8).

Nonstarved mycelia produced more enzyme in presence of cyclic AMP, though the amount of cyclic AMP had no significance within the range. Cyclic AMP has no perceptible effect on starved mycelia.

3.3.8 Effect of Removal of Inducer on Cellulase Production:

Since cellulose was found to be a successful inducer upto 150 minutes without causing inhibition, the effect of the presence and absence of an inducer once induction was started, was studied by removing the solid cellulose particles (rayon pulp pieces) from the induction medium. Diffused particles of inducers may still be available for some more time, but sufficient time was given for the system to degrade whatever particulates remained.

25 hours old glucose grown mycelia were starved for 15 hours and transferred to the induction medium where rayon pulp pieces were used as the inducer. After 15 and 48 minutes of induction the pieces were picked up with the help of a stainless steel forceps from some of the flasks and other flasks were treated as controls with inducers all along the experimental period. Results are shown in Table 3.9.

Table 3.8

Effect of cyclic AMP on non-starved and starved mycelia

Starved mycelia	F P A		
	+Cyclic AMP		-Cyclic AMP
	.005/ml	.25/ml	
15 minutes	0.75	0.84	0.87
75 minutes	2.15	2.78	2.75
Unstarved mycelia			
15 minutes	0.57	0.54	0.25
75 minutes	0.99	0.97	0.25

Table 3.9

Effect of removal of inducer during induction

Time (minutes)	FPA/mg x 10^2		
	Cellulose present (control)	Cellulose removed	
		at 15th mts.	at 48th mts.
15	0.242	0.25	0.242
30	0.39	0.375	0.395
45	0.485	0.435	0.495
65	0.547	0.52	0.69
75	0.675	0.57	0.72
150	1.25	0.75	0.99

Removal of inducer had remarkable effect on further enzyme production as in both cases the enzyme production was repressed when the inducer was removed.

In another experiment where cellobiose and sophorose were used as the inducer the mycelia were washed thoroughly by suspending and agitating in phosphate buffer and were then transferred to a non-inducer medium at the 15th minute. A control was maintained as one group of samples was transferred to an induction medium after washing with phosphate buffer. Results are shown in Table 3.10.

The presence of inducer is found to be essential throughout the induction period.

3.3.9 Effect of Rifampicin, Glucose, Cyclohexamide, Chloramphenicol, Penicillin-G, Actinomycin-D and Streptomycin during Induction of Cellulases:

Drugs like Rifampicin, Cyclohexamide, Chloramphenicol, Penicillin-G, Actinomycin-D and Streptomycin were administered to the induction medium after induction started. The effect of cyclic AMP and glucose was also studied.

Starved, glucose-grown mycelia were induced in a cellobiose (1×10^{-1}M) inducing medium and cyclic AMP (0.05 μ gm/ml) Rifampicin (500 μ gm/ml), Glucose (1×10^{-1} M) Cyclohexamide (500 μ gm/ml), Chloramphenicol (500 μ gm/ml), Penicillin-G (500 μ gm/ml), and Streptomycine (500 μ gm/ml) were introduced to samples in duplicates at the 15th minute, the results are shown in Table 3.11.

Table 3.10

Effect of removal of inducer during induction

Time minutes	FPA/mg x 10^2			
	Cellobiose		Sophorose	
	Experimental	Control	Experimental	Control
15	0.024	0.22	0.0022	0.42
30	0.022	0.45	0.00242	0.57
45	0.022	0.45	0.00242	0.574
75	0.0249	0.75	0.00224	0.78
150	0.0052	0.998	0.00242	0.97

Table 3.11

Effect on drugs on production of cellulases

Time minutes	F P A per mg x 10^2								
	Control	Cyclic AMP	Rifam-picin	Actino-mycin-D	Penici-llin-G	Strep-tomycin	Glucose	Chloramph-enicol	Cyclohex-amide
15	0.4422	0.4357	0.4427	0.4422	0.4378	0.4445	0.4354	0.4444	0.4428
30	0.5784	0.5727	0.5475	0.5478	0.5797	0.5825	0.5427	0.4487	0.4475
45	0.7543	0.7527	0.7242	0.7278	0.7884	0.7591	0.5744	0.4488	0.4487
65	0.8875	0.8764	0.7984	0.7444	0.8975	0.8845	0.5754	0.4489	0.4485
75	0.8945	0.8872	0.7287	0.7248	0.9675	0.8954	0.5754	0.4478	0.4487

The effect of drugs on enzyme activity was studied by administering appropriate amount of drugs in control culture filtrates and by studying their effects on cellulase activity. It has been found that none of the drugs including cyclic AMP and glucose showed any significant effect at the administered quantity on enzyme activity.

Figure 3.4 shows that the effect of glucose is in between that of transcriptional level inhibitors like Rifampicin and Actinomycin-D and the protein synthesizing inhibitors like Chloramphenicol and Cyclohexamide. Streptomycin, Penicillin-G and Cyclic AMP did not show any effect during induction at the administered level.

In another experiment the effects of cyclic AMP during the inhibition of cellulase induction by Cyclohexamide, Rifampicin and Glucose were studied.

The inducer used was 1×10^{-1} M Sophorose. At appropriate intervals cyclic AMP (0.05 μ gm/ml) was added. Samples in duplicates were taken and the results shown in Table 3.12 is the average of two identical experiments.

The result showed that in Glucose inhibited mycelia cyclic AMP reverses the repression (Fig. 3.5) whereas Rifampicin treated and Cyclohexamide treated as well as in control cyclic AMP has no significant effect at the quantity administered.

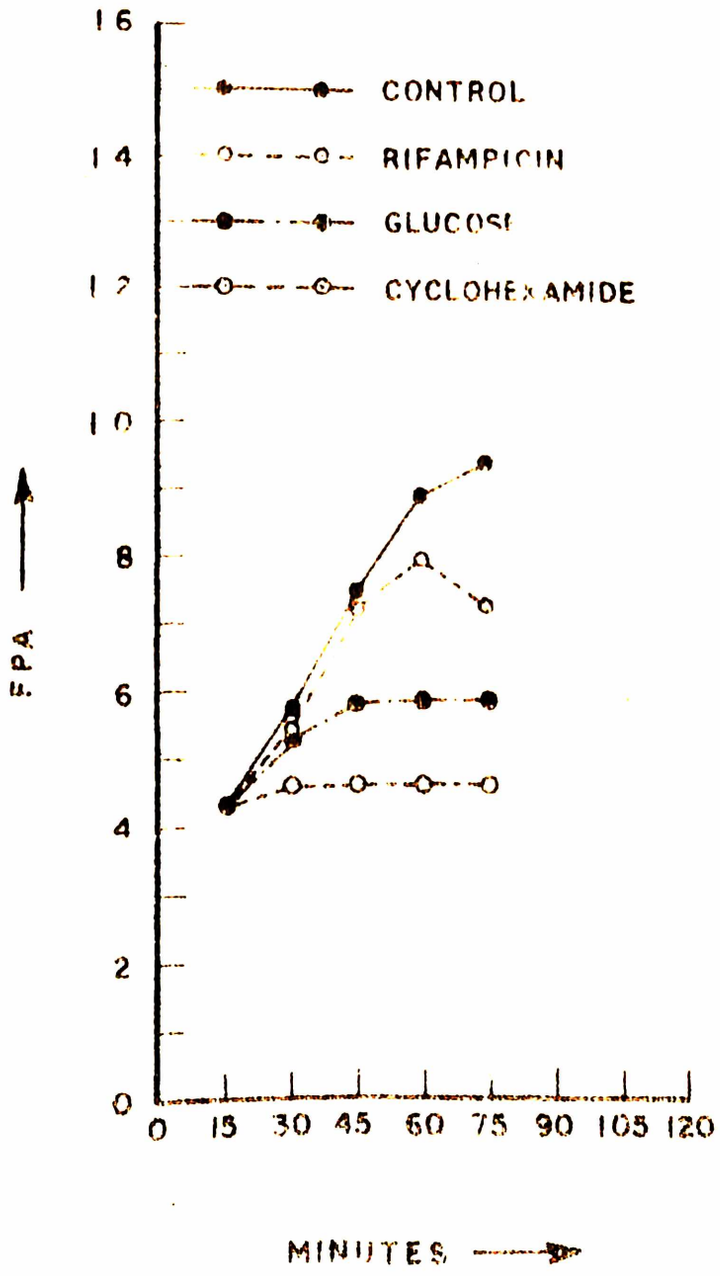

FIG. 3.4 EFFECT OF CHEMICALS ON INDUCTION OF CELLULASES.

Table 3.12

Effect of drugs on the production of cellulases

Time minutes	FPA/mg x 10^2							
	Control		Rifampicin		Glucose		Cyclohexamide	
	-	+	-	+	-	+	-	+
5	0.242	-	0.242	-	0.242	-	0.242	-
Rifampicin, Glu. Cyclohex-amide added	-	-	-	-	-	-	-	-
16	0.447	-	0.447	-	0.448	-	0.342	-
Cyclic AMP added	-	-	-	-	-	-	-	-
18	0.457	0.459	0.457	0.457	0.452	0.457	0.358	0.357
35	0.7925	0.7894	0.7845	0.7844	0.484	0.574	0.3587	0.359
Cyclic AMP at 44th minute	-	-	-	-	-	-	-	-
45	0.9922	0.9875	0.7898	0.7875	0.5272	0.598	0.3588	0.3584
75	1.228	1.228	0.7975	0.7972	0.528	0.597	0.358	0.358

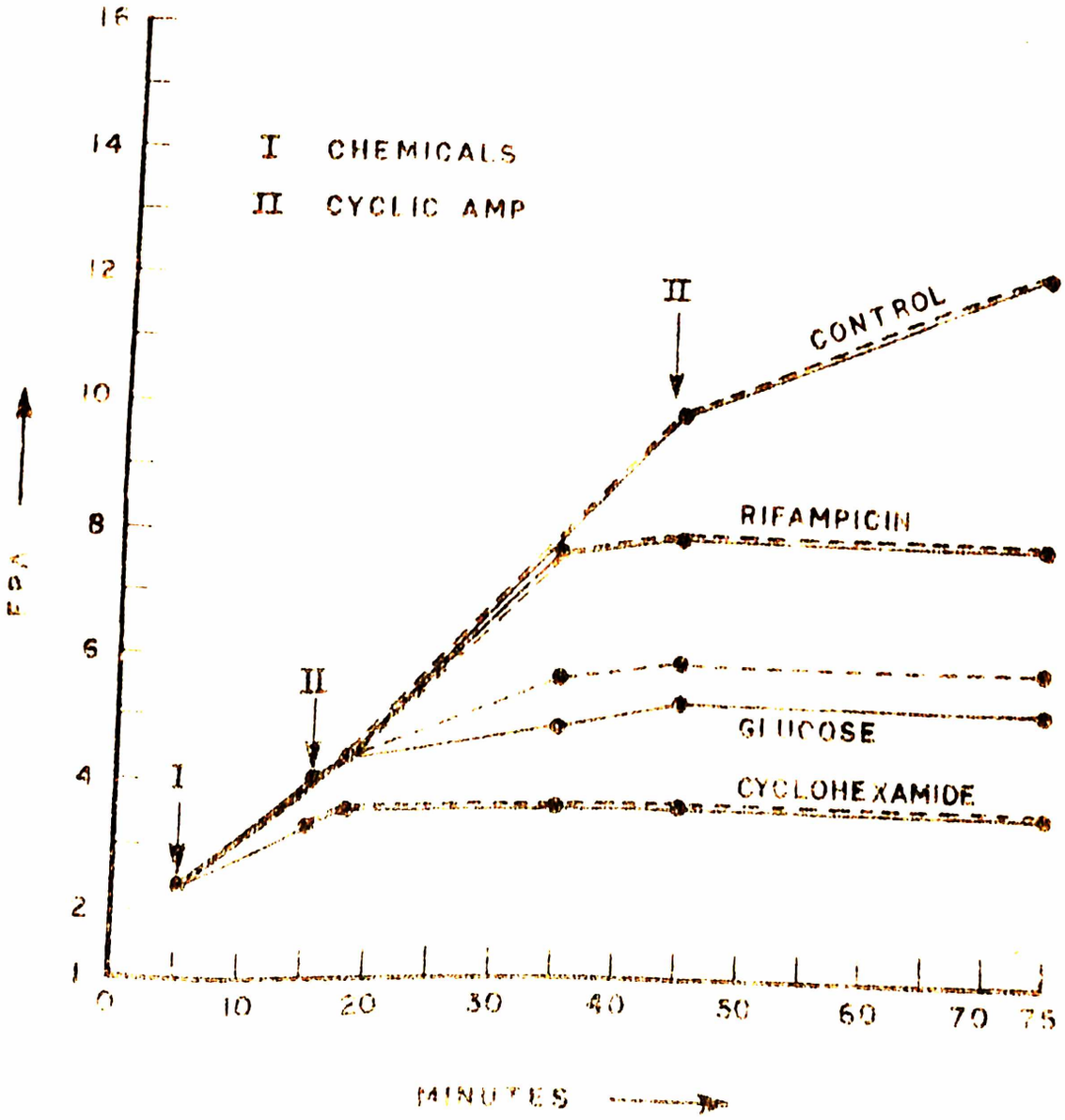

FIG. 3 EFFECT OF CHEMICALS ON THE INDUCTION OF CELLULASE

Table 3.13

Effect of Glucose on Rifampicin treated and nontreated mycelia during rapid induction of cellulases

Time minutes	FPA/mg x 10^2			
	Rifampicin treated		Rifampicin non-treated	
	Control	Glucose	Control	Glucose
0	0.422	0.424	0.422	0.422
15	0.78	0.729	0.976	0.769
30	0.8875	0.754	0.985	0.844
45	0.8844	0.7542	1.22	0.887
60	0.8842	0.7542	1.257	0.889
75	0.8852	0.7524	1.358	0.969

inducers as sophorose. This may be made possible because within such a short interval the products are not excessively formed which could inhibit enzyme activity. Also since the mycelia were starved the whole amount of glucose formed as a result of spurt in enzyme activity might have been, sooner, consumed by the glucose-starved system. This should explain the sustained enzyme production for further more time.

Around 15 hours of starvation was found to be optimum for the rapid production of cellulase (Table 3.2).

Glucose grown mycelia were capable of producing more cellulase activity than cellulose grown mycelia (Table 3.4). Though an explanation is warranted, with these limited observations any categorical assertion might only prove immature.

A similar observation calling for explanation was made when the effect of ageing of mycelia in their capacity to produce cellulase rapidly was studied. (Table 3.5 and 3.6 and Figure 3.2). Stationary phase mycelia (57 hours) produced much less enzyme compared to 39 hours old mycelia. However, in actively growing mycelia as the age increases the capacity for production of cellulase increases.

The importance of induction medium is ascertained in the experiment where the inducer was added to a buffer or distilled water (Table 3.7). The salt medium was found

to sustain the rate of enzyme production whereas distilled water almost inhibited the production after sometime (presumably caused by the leaking of salts from inside the mycelia). The presence of certain ions are probably a prerequisite for sustained production and activity of cellulases.

That unstarved mycelia on addition of cyclic AMP behaved like starved mycelia in the rapid production of cellulases may point towards a possible involvement of a cyclic AMP mediated process in regulating the enzyme production.

Nisizawa et al, (1971) in their studies with washed mycelia did not attempt explaining the reason for a faster induction of cellulase, though some of their observations, mentioned earlier, leads to conclude that the presence of glucose or some intermediary metabolites are possibly the agents of repression. The starvation experiments and especially the effect of the duration of starvation shows that a catabolite repression mechanism may be causing the delay and resulting in the slow rate of production of enzyme. That cellulose grown mycelia are also affected by starvation leads to think that glucose and more so some of its catabolites are regulating the synthesis of cellulase. Cyclic AMP effect only confirms this point and implies a possible involvement of a cyclic AMP system in the production control of cellulases.

When sophorose was used as an inducer and when glucose added to the induction medium at the 15th minute after induction started, cellulase production was inhibited within 45 minutes. Therefore the inability of cellulose and cellobiose as inducers noticed by earlier workers (Mandels, et al, 1960, 1961 and 1962 and Nisizawa et al, 1971 and 1972) and further the inhibition effect of cellulose and cellobiose during cellulase induction (Sternberg, 1976) in a growing culture of T. viride may be due to the absence of a rapid method of induction of cellulase. The desirability of studying the whole induction-repression process based on rapid enzyme induction process need not be over-emphasized.

The necessity of the presence of inducer throughout the induction period is established when the induction was found to terminate at the removal of the inducer from the medium (Table 3.8 and 3.9). Cyclic AMP, Streptomycin and Penicillin-G did not have any effect during induction whereas Rifampicin, Glucose, Actinomycin-D, Chloramphenicol and Cyclohexamide inhibited the production of Cellulases. Rifampicin and Actinomycin-D acted by inhibiting the transcription where as Chloramphenicol and Cyclohexamide inhibited the enzyme production at the site of protein synthesis as the drugs are known for the respective activities. (Burger 1972). Glucose unlike in earlier studies, instead of behaving like Rifampicin was inhibiting to the extent that the effect is intermediate and in between that of mRNA

inhibitors and protein synthetic inhibitors. The peculiar glucose effect was more pronounced in another experiment where the effect of cyclic AMP during the action of inhibitors of induction was studied (Table 3.13). It was found that cyclic AMP reverses the glucose effect at least for certain time just after its administration whereas with other inhibitors of induction cyclic AMP has no effect. This again suggests that the inhibitory effect of glucose may be totally in a different fashion to that of other inhibitors tested. Further, it is worth suggesting that a cyclic AMP mediated mechanism may be acting in the induction-repression process of cellulases, at least where glucose is involved. Another observation made earlier that cyclic AMP induces a starvation effect in non-starved mycelia further corroborates it.

EFFECT OF SURFACE - ACTIVE AGENTS

CHAPTER-IV

EFFECT OF SURFACE-ACTIVE AGENTS IN THE PRODUCTION OF EXTRACELLULAR CELLULASES

4.1.0. Introduction:

That surfactants have a role in enhancing the production of cellulase is known. But the explanation how surfactants act to increase enzyme yields is largely conjectural. It is known that the agents used are not generally toxic to micro-organisms and also that the release of enzymes occurs sometime (days) after the complete consumption of substrate, i.e., probably from old cells, but not from cells undergoing autolysis, since there is no accumulation of autolysis products (Reese and Maguire, 1969).

The enzyme cellulase was induced when cellobiose octaacetate served as the carbon source for the fungi which also produced the necessary esterases. By this means, yield of cellulase were very much higher (4 to 100 times) than those obtained on cellobiose (Mandels and Reese, 1960). Sometime later these workers found that other fungi growing on sucrose monopalmitate give yields of sucrase (10-16 times those of obtained on sucrose)(Reese and Mandels, 1962). Sucrose plus palmitate could not substitute for sucrose palmitate. As for cellulase, it appeared that supplying the inducer at a low level, through hydrolysis of ester was the basis

for increased yield (Reese and Maguire, 1969).

Tween-80 and related surfactants have been used for sometime in bacterial cultures to assist in growth. They have also been found to promote entrance of compounds into cells. Thus mycobacterial cells grown with Tween-80 have an increased permeability to triphenyl tetrazolium (Paumeson, et al, 1964). Pseudomonas aeruginosa cells grown in a medium containing Tween-80 are much more susceptible to inhibition by benzalkonium chloride and polymyxin-B (Dastuge, et al, 1959 and Brown and Richards, 1974.) and are much more reponsive to anaesthetics (Cocaine, Xylocaine etc.) in the presence of surfactants. These indicate that surfactants promote both entrance and exit of compounds from cells. Surfactants have effect on fungi other than those reported above. Takahashi et al, (1960), reported that there is a tendency for growth in shaken flasks to change from pellet form to mycelium of a more dispersed type. Reese and Maguire (1969) confirmed these findings in their report. Krezminski, et al (1969) reported an increased production of carotene in Neurospora. In this instance the product remained inside the cells. Apparently surfactants do not work in the same way.

Actions of Tween-80, Triton-X and other detergents like Sodium Dodecyl Sulphate (SDS) and deoxycholate and Ethylene Diamine Tetraacetic acid are studied. The role of non-ionic surfactants are studied further with a

view to establish at which stage the surfactants act to enhance the production of enzyme. The study is also aimed at finding out other factors that influence the increase in production of extracellular cellulases.

4.2.0. Materials and methods:

The rapid method of production of cellulase (Chapter-III) is followed. Tween-80, Triton-X (BDH), SDS, Deoxycholate and Ethylene diamine tetraacetic acid (BDH) were used. Ca^{++} ionophore (2244) was kindly supplied by Base Chemicals, New York.

Mycelia were grown in media with and without Tween-80. Phosphate buffer (pH 4.8) was used. Basal medium is the culture medium without a carbon source.

4.3.0. Results:

4.3.1. Effect of Surfactants on cellulase production:

Tween-80, Triton-X, SDS, EDTA and deoxycholate were used to study the effect on cellulase production. 0.05 percent of these chemicals were added to the culture medium in place of 0.1 percent Tween-80 in the original culture medium (Chapter-II). The starvation and induction media were also supplied with 0.05 percent of these chemicals instead of the original 0.1 percent Tween-80. Equal weight of mycelia were used to induce cellulase production. Growth of mycelia in EDTA was very less and so the concentration was reduced to 0.005 percent in the culture medium for EDTA sample. A control

was used without any surfactants. The results are shown in Table 4.1.

Tween-80 and Triton-X enhanced the production of cellulase significantly. The starvation medium and the induction medium showed the presence of ultravoilet absorbing compound when Deoxycholate and Ethylene diamine tetraacetic acid were used. SDS did not cause any leakage in the cells as is shown by the absence of ultraviolet absorbing compounds and also did not enhance the cellulase production.

4.3.2 Effect of concentration of surfactants on cellulase production:

Tween-80 and Triton-X at different concentrations were used to find out the maximum concentration for optimum production of cellulases. The results are shown in Table 4.2.

As the concentration increases the production also increases. But when the concentration goes beyond 0.15 percent the growth seems to be affected and a slight colour difference in the mycelia is also observed. However, ultraviolet absorbing substances were not found even at this concentration. Tween-80 at a concentration of 0.15 percent did not affect enzyme activity.

4.3.3. Effect of Tween-80 grown mycelia in cellulase production:

25 hours old Tween-80 grown and non-Tween-80

Table 4.1

Effect of surfactants on cellulose production

Time mts.	F.P.A./mg. x 10^2					
	Tween-80	Triton-X	Deoxycho-late	EDTA	SDS	Control
45	0.844	0.8725	0.0528	0.0744	0.22	0.048
75	1.242	1.279	0.212	0.0884	0.78	0.087

Table 4.2

Effect of concentration of surfactants on cellulase production

Concentration percent	F.P.A./mg. x 10^2	
	Triton-X	Tween-80
0.01	0.8422	0.7825
0.05	0.99	1.088
0.10	1.44	1.528
0.20	1.87	1.96
0.75	2.79	2.2482

grown mycelia were starved for 15 hours in a Tween 80 medium and transferred to a Tween-80 induction medium. Another sample was induced in a medium containing no Tween-80. The results are shown in Table 4.3.

The presence of Tween-80 during mycelial growth has got effect in determining the production of cellulase, as shown by the difference in activity between C and D (Table 4.3).

The effect of the presence of Tween-80 in induction medium is also shown (The difference between A and B in Table 4.3).

4.3.4 Effect of Tween80 during starvation:

The starvation medium was added with and without Tween-80 (1 percent). The results are shown in Table 4.4.

For Tween-80 mycelia starvation medium did not have significant effect whereas for non Tween-80 mycelia the presence of Tween-80 in starvation media is important. This is more pronounced in the case when the induction medium does not contain Tween-80.

4.35: Effect of salts on cellulase production in presence of Tween-80:

To study whether other factors influence the enhanced cellulase production by means of Tween-80, induction was carried out in a medium containing all the salts of the culture medium and compared with that of the induction

Table 4.3

Effect of Tween-80 during growth in cellulase production

		A	B
		Tween-80 induction FPA/mg x 10^2	Non-Tween-80 induction FPA/mg. x 10^2
Tween-80 mycelia	C	2.75	1.7875
Non-Tween 80 mycelia	D	1.8744	0.5745

Table 4.4

Effect of Tween-80 during starvation

	Tween-80 induction FPA/mg. x 10^2	Non-Tween 80 induction FPA/mg. x 10^2
Tween-80 mycelia starved in Tween-80 medium	2.75	1.7875
Tween-80 mycelia starved in non-Tween 80 medium	2.5784	1.8745
Non-Tween 80 mycelia starved in Tween-80 medium	1.8744	1.5745
Non-Tween 80 mycelia starved in non-Tween 80 medium	1.7597	0.4544

in a medium comprised of buffer. Tween-80 mycelia were used in both cases. The starvation medium was without Tween-80. The results are shown in Table 4.5.

Tween-80 and non-Tween-80 mycelia in presence of Tween-80 in induction medium favoured basal media to buffer. But when Tween-80 was not present in the induction media, basal media or buffer did not make any difference for both Tween-80 and non-Tween-80 mycelia. When Triton-X was used in an identical experiment the result was not different.

4.3.6 Effect of specific cations during induction:

When the basal medium used for induction was free of Tracemix (Chapter-II) the result did not differ significantly (Table 4.6).

Basal medium in presence of Tween-80 showed no difference in influencing the production of cellulase whether with or without Tracemix. The possibility of cations like Fe^{++}, Zn^{++}, Mn^{++} and Co^{++} influencing the cellulase production is ruled out.

The other possible cations are Ca^{++} and Mg^{++}. In an experiment $CaCl_2$ was replaced by $MgCl_2$ in both starvation and induction media. The results are shown in Table 4.7.

Basal medium in presence of Tween-80 showed more cellulase production when Ca^{++} was not substituted by Mg^{++}. When Tween-80 was absent the substitution did not

Table 4.5

Effect of salts on cellulase production in presence of Tween-80

		A	B
		Tween-80 induction FPA/mg. x 10^2	Non-Tween 80 induction FPA/mg. x 10^2
Tween-80 mycelia	C	(Basal) 2.84 (Buffer) 2.24	(Basal) 2.23 (Buffer) 2.23
Non-Tween-80 mycelia	D	(Basal) 1.87 (Buffer) 1.44	(Basal) 0.99 (Buffer) 0.98

Table 4.6

Effect of specific cations during induction

	Basal medium				Buffer	
	With tracemix		Without tracemix			
	-Tween-80	+Tween-80	-Tween-80	+Tween-80	-Tween-80	+Tween-80
Tween-80 mycelia	1.78	2.79	1.744	2.88	1.729	1.753
Non-Tween-80 mycelia	0.48	1.96	0.57	1.87	0.78	0.87

Table 4.7

Effect of Calcium ions during induction

	Basal medium				Buffer	
	- Tween-80		+ Tween-80		- Tween-80	+ Tween-80
	$- Ca^{++}$	$+ Ca^{++}$	$- Ca^{++}$	$+ Ca^{++}$		
Tween-80 mycelia	1.745	1.87	1.57	2.574	1.22	0.98
Non-Tween-80 mycelia	0.54	0.57	1.242	1.984	0.88	0.87

evoke as significant response as when it was present. This was more pronounced in the case of non-Tween-80 mycelia.

In another experiment the induction medium was buffer. $CaCl_2$ (1.5 percent) was added to it before the mycelia were transferred. A control was maintained without $CaCl_2$. Enzyme production was estimated in presence and absence of Tween-80. The results are shown in Table 4.8.

The presence of Ca^{++} enhanced the Tween-80 effect in cellulase production. The enzyme production is enhanced considerably only when both Ca^{++} and Tween 80 were present.

Tween-80 mycelia starved in a medium without Ca^{++} was used in another experiment where Ca^{++} ionophore (2244) was used. Tween-80 replaced ionophore in another set. The basic induction buffer medium did not contain Tween-80. The results are shown in Table 4.9.

The influx of Ca^{++} is presumably enhanced by ionophore (2244) and the parallel effect when Tween-80 was used goes to show that apart from other activities Tween-80 might somehow enhance the calcium influx resulting in more release of the enzyme.

4.4.0. Discussion and conclusion:

Tween-80 and Triton-X enhanced the cellulase production while SDS did not show any promoting effect in

Table 4.8

Effect of Calcium ions during induction

Time minutes	Tween-80 Buffer		Non-Tween-80 Buffer	
	- Ca^{++}	+ Ca^{++}	- Ca^{++}	+ Ca^{++}
15	0.5784	0.84	0.4878	0.4544
30	0.7844	0.958	0.5722	0.547
60	0.9675	1.5978	0.8478	0.7825
75	0.9724	1.8757	0.8488	0.844

Table 4.9

Effect of Ca^{++} and Tween 80 during induction

Ca^{++} concentration mM	With 0.01 µM ionophore	Without ionophore	With 1.0 percent Tween-80	Without Tween-80
0.05	0.7848	0.4457	0.5748	0.4588
0.1	1.2587	0.4878	0.7287	0.4457
0.5	1.4544	0.5784	0.9648	0.4875
0.75	1.7844	0.5475	1.2272	0.5788

the production of cellulase. Deoxycholate (0.05 percent) and EDTA (0.005 percent) caused leakage of cell content as shown by the ultraviolet absorbing materials in both starvation and induction media. SDS however, did not seem to cause leakage in the cell membrane or cell wall.

As the concentration of Tween-80 and Triton-X was increased the cellulase production also was increased.

In order to find out at what stage among the growth, starvation or induction of the mycelia. the presence of surfactants is critical for an enhanced enzyme production, the experiments conducted showed (Table 4.3 and 4.4) that the presence of surfactants during growth has significant effect, but it is also evident that its presence in the induction medium enhances the production of cellulase irrespective of its presence during growth. At least for Tween-80 grown mycelia the presence of Tween-80 in starvation medium was not very significant though in the case of non Tween-80 grown mycelia the presence of Tween-80 in the starvation medium caused the increase in cellulase production when induced in non-Tween-80 medium (Table 4.4). Therefore, it is imperative that surfactants act not only on the structural constitution (cell wall or cell membrane), but its presence is needed for a sustained enzyme production at higher level. It has been found recently that the fatty acid composition of the cell wall changes when surfactants are used in the culture medium, as has been established when the

Tween-80 treated mycelial cell walls contained more unsaturated fatty acids than in the nontreated ones. (Umesaki et al, 1977). It is presumed that as the unsaturated fatty acids increase, the permeability is likely to increase. But, from the above observation it is found reasonable to assume that surfactants may have more than the role of changing the cell wall fatty acid constitution causing permeability difference.

With a view to understand the possible role of other factors influencing the production and release of cellulase, the part played by salts in the induction medium was analysed. It must be noted that in an earlier experiment on induction the salt content of the induction medium was found to influence the amount of extra cellular enzyme (Chapter-III).

It is found (Table 4.5) that the ionic concentration in the induction medium is decisive to a great extent on the amount of cellulase released. At the same time it is established that this role of salts is effective only when the induction medium has Tween-80 (Table 4.5 group B). Triton-X also showed indentical results in a separate experiment.

It is interesting to note that, as stated earlier, the ionic concentration influences the production of extra cellular cellulase, when Tween-80 is present in the medium than when Tween-80 is absent. The cations are

isolated and studied in order to establish if any specific cations are necessary in bringing about the effect. The trace mix containing Zn^{++}, Mn^{++}, Fe^{++} and Co^{++} did not show any effect (Table 4.6). But the basal medium without trace mix (Table 4.6) showed the sustained increase in production of cellulase. Of the cations in the rest of the solution and among the two bivalent cations, Ca^{++} is known to cause an increase in secretory activities. Therefore, when Ca^{++} was replaced by Mg^{++} in an induction medium, the effect, enhancing the production of cellulase, was curtailed when Tween-80 was present than when Tween-80 was absent. This establishes that Ca^{++} is needed for the maximum production of cellulase in presence of Tween-80. This was further confirmed in a study where buffer induction medium replaced basal induction medium when 1.5 percent (weight/volume) calcium chloride was added (Table 4.8).

Since it is known that an extraordinary calcium influx caused more secretory activity it is suspected that the surfactants cause this extraordinary influx of calcium to the mycelia activating the secretory vescicles. In an experiment where Ca^{++} influx was artificially increased by ionophore, the production of the enzyme increased according to the concentration of Ca^{++} present in the medium (Table 4.9). Tween-80 also caused a similar increase in an identical experiment. This is reasonable therefore, to suggest that surfactants cause an influx of Ca^{++} to increase the enzyme secretion.

ADSORPTION OF CELLULASE ON CELLULOSIC MATERIALS

CHAPTER-V

ADSORPTION OF CELLULASE ON CELLULOSIC MATERIALS

5.1.0. Introduction:

There are only a few documented information desdribing the mechanism of adsorption of cellulases on cellulosic materials. Since adsorption of cellulases on cellulosic substances is a pre-requisite step for their breakdown, an indepth study of the adsorption characteristics of cellulases can provide a better insight into the process of enzymatic hydrolysis. The studies conducted on adsorption of cellulase have been based on two reasons: (a) for assessment of the adsorbed enzyme in the cellulose hydrolysis (Mandels, et al, 1971), and (b) recovery of the cellulase from the adsorbed and the homogeneous phases after hydrolysis (Wilke and Yang, 1975). On milled Solka Floc SW 40A, a kind of wood (spruce) pulp, the cellulase was strongly adsorbed and the quantity of the enzyme and protein taken up was proportional to the cellulose concentration. More than 90 percent of the enzyme and protein was adsorbed by 10 percent cellulose at 25°C. At 50°C the maximum adsorption was reported at about 8 hours and by 24 hours the enzyme started going back into the solution as about 50 percent of the cellulose had been hydrolysed. The extent of adsorption obviously depends on the type of cellulosic materials used. Wilke and

Yang (1975) obtained about 70 percent adsorption of cellulose on 10 percent ball-milled news print. There are contradictory reports on the possibility of recovering the enzyme from the adsorbed cellulosic phase. Some workers (Toyama 1969) adsorbed exoglucanase on gauze or Avicel and then eluted it with distilled water. Mandels and coworkers (1971) were however, not able to desorb the cellulase from cellulose except by digestion. Apparently the cellulase used by Mandels et al (1971) was held much more strongly by the cellulose. The addition of a number of additives to the crude undialized enzyme at pH 4.8 like; NaCl, $CaCl_2$, $MgCl_2$, Ethylene diamine Tetraacetic acid, Tween-80 and Bovine Serum albumin had no marked effect on the adsorption of cellulase on cellulose. Wilke and Yang (1975) on the other hand, obtained a distribution coefficient of 0.04, (FP units/ml)/(FP units/gm of solid), based on their washing experiments for the recovery of cellulase from the adsorbed phase at 30°C. In a more recent finding, Peitersen et al (1977), described their data by a hyperbolic function similar to the Langmuir isotherm.

$$P_{ads} = \frac{k_p P_{ads,m}}{1 + k_p P}$$

where P = Protein concentration in supernatant, mg/ml.

P_{ads} = Protein concentration adsorbed, mg/mg of cellulose

$P_{ads,m}$ = Maximum protein adsorbed mg/mg cellulose

k_p = Constant, $(mg/ml)^{-1}$

The same expression was used for adsorption of the cellulase activity in which P was replaced by the cellulase activity E. The adsorption of protein and enzyme was found to be largely independent of pH, but depended strongly on temperature and type of cellulose used. According to these authors an increase in temperature generally resulted in decrease in the values of $P_{ads,m}$ and $E_{ads,m}$; the difference was however not much marked.

Amidst these controversial notes still many parameters are left untried in the study of adsorption-desorption characteristics that might prove to be of much significance for the recovery of enzyme and in the study of the mechanism of enzymatic hydrolysis of cellulose.

5.2.0 Materials and Methods:

Cellulose powder is obtained from E. Merck and the average size of cellulose particles used for adsorption and hydrolysis studies was 97 microns (150/170 mesh) unless otherwise stated.

Cellulose pulp sheets from Gwalior Rayons, Nagda, ball-milled into appropriate size, were also used.

To 1 ml. of properly diluted enzyme solution in citrate buffer (0.5M, pH 4.8) 1 ml. of 1% CMC (degree of substitution 0.5) was added. The mixture was incubated at 50°C for 30 minutes and the resulting sugars were measured by DNS method (Chapter-II). Total cellulase is

measured in terms of FP activity (Chapter-II).

1 gm. of CMC (DS-0.5) was added slowly to 100 ml of boiling citrate buffer (0.5M pH 4.8) and heated for 30 minutes at this temperature. After cooling the solution was made upto 100 ml and centrifuged. The clear supernatant was used for endoglucanase activity determination.

Adsorption experiments were done in batch samples. Desired quantities of cellulose were contacted with enzyme solution in 0.5 M citrate buffer at a particular pH and temperature. The amount of original activity remained in the solution was measured after removal of solids by filtration under centrifugation.

The adsorbed enzyme-cellulose complex was suspended in 0. 5M citrate buffer of desired pH at a particular temperature. The amount of desorbed enzyme was similarly determined in the solution after removal of solids, The suspended solids were mixed in a Vortex mixer.

5.3.0 Results:

Standardisation experiments conducted by means of E. Merck cellulose as the adsorbant showed that within 5 minutes at 45°C and pH 4.8 the solubilization is not significant as per the DNS reagent test. However, 50°C has shown very little solubilization. The amount of original cellulase activity in terms of FP units was the minimum in the remaining solution at a contact time

of 5 minutes plus 2.5 minutes (centrifugation time) (Table 5.1). However, the specific adsorption was not the maximum at that contact time. A contact time of 25 minutes showed only very little adsorption and the specific adsorption at that time was considerably less. The period also caused solubilization of cellulose. The specific adsorption in general showed a decreasing trend with the increase in contact time. Though the specific adsorption was more at the first and second minute, the actual adsorption was considerably less at that time.

5.3.1 Effect of temperature on adsorption:

Temperature from 5°C to 50°C during adsorption showed marked change in the effect of adsorption (Table 5.2).

The specific adsorption was also the maximum at 45°C. At temperature higher than 50°C, the loss of activity was significant.

5.3.2 Effect of pH on adsorption:

The pH (3.5 - 7.5) effects showed that the adsorption of enzyme components increased when pH was raised or lowered from pH 5 (Table 5.3) whereas the specific adsorption values were maximum at 4.8 and decreased on either side of this pH.(Fig. 5.1).

5.3.3. Effect of temperature on desorption:

The solid was resuspended in 0.5 M citrate buffer

Table 5.1

Effect of contact time on adsorption

Time of contact	Percentage adsorption	Specific adsorption
1	26.25	0.522
2	31.22	0.435
3	37.84	0.438
4	41.85	0.348
5	58.22	0.358
6	55.43	0.322
7	57.92	0.324
8	47.46	0.314
25	31.12	0.258

Table 5.3

Effect of pH on adsorption

pH	Percentage adsorption	Specific adsorption
3.5	32.76	0.484
4.0	42.56	0.475
4.5	51.52	0.587
5.0	55.44	0.750
5.5	55.48	0.727
6.0	51.78	0.522
6.5	37.52	0.425
7.0	36.56	0.457
7.5	41.43	0.252

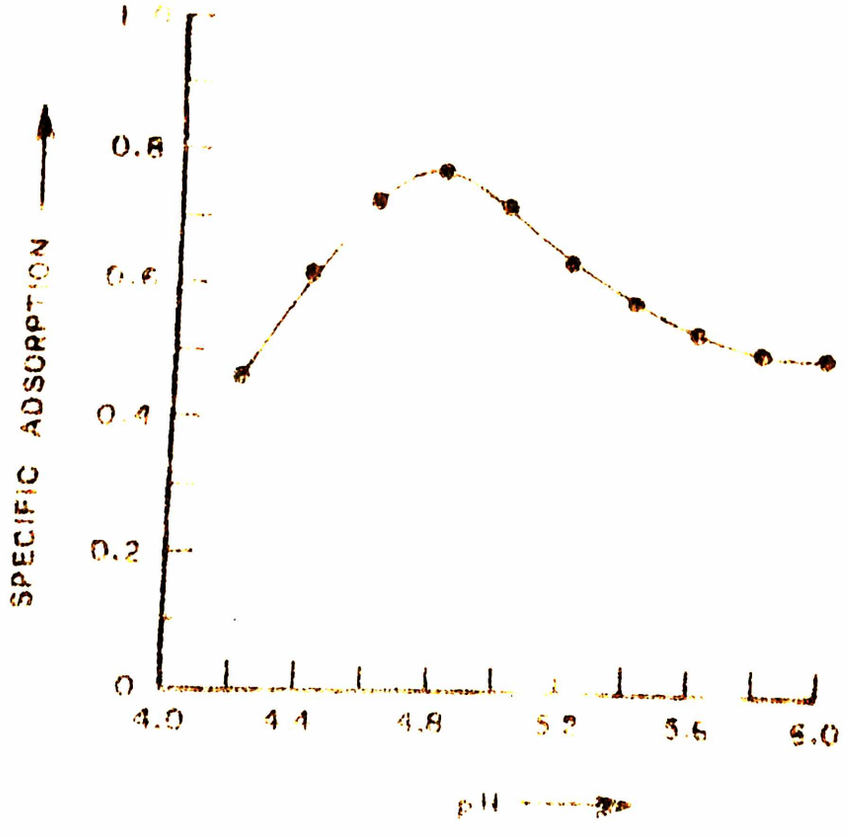

FIG 5.1 EFFECT OF pH ON SPECIFIC ADSORPTION.

at pH 4.8 and agitated and mixed over a Vortex mixer for 5 minutes at appropriate temperatures. Desorption data showed that percentage desorption increases with decrease in temperature. Further an abrupt change in temperature caused the desorption of more active enzyme than the gradual change in temperature. (Table 5.4 and 5.5a and 5.5b).

In the case of the desorption due to gradual change in temperature, the buffer was of the same temperature to that of the solid and slowly brought to the required temperature where it was kept for 5 minutes before centrifuging again.

5.3.4. Effect of reducing sugars in adsorption:

Glucose was added to a suspension of cellulose pulp in enzyme solution (0.5 M citrate buffer 1 pH 4.8) in order to make a 2 percent, 5 percent, 10 percent and 15 percent glucose concentration and the extent of adsorption was measured after contact time of 5 minutes under agitation. To avoid the effect of cellulose hydrolysis on adsorption, the experiments were done at 25°C ± 2°C at which the solubilization within 5 minutes was not significant. The results are shown in Table 5.6.

5.3.5. Effect of Protein in Specific adsorption:

When more protein was added in the same way as glucose in the experiment described above in the form of bovine serum albumin, specific activity in the

Table 5.4

Effect of temperature on desorption

Temperature ^{o}C	Percentage desorption	Specific desorption
5	88.44	0.525
10	72.75	0.534
15	75.44	0.529
20	45.75	0.578
25	53.88	0.481
30	42.57	0.425
35	41.52	0.274
40	42.22	0.256
45	46.48	0.154
50	15.84	0.128

Table 5.5a

The comparative effects of abrupt change and gradual change in temperature during desorption

Temperature	Percent Desorption	
	Abrupt	Gradual
$25^{o}C \pm 2^{o}C$	27.00	27.00
$15^{o}C \pm 2^{o}C$	48.78	35.00
$5^{o}C \pm 2^{o}C$	75.44	42.84

Table 5.5b

Effect of abrupt change in temperature in enzyme activity

Temperature drop ^{o}C	Enzyme activity drop % specific activity	After 2 hours activity percentage of loss
45 - (5 ± 2)	24.87	48.005
25 - (5 ± 2)	12.75	72.44
45 - (25 ± 2)	5.40	78.80
25 - (15 ± 2)	16.27	-
28 - 28	- 2.50	-

Table 5.6

Effect of reducing sugars on adsorption of cellulase by cellulose pulp

Concentration percentage	Percentage adsorption	Specific adsorption
Control	55.73	0.570
2	54.13	0.540
5	54.52	0.548
10	46.52	0.482
15	42.56	0.534

remaining solution changed remarkably with concentration (Table 5.7). However, 5 percent protein addition with varying temperature showed a maximum specific adsorption at 45°C, at pH 4.8 and a contact time of 5 minutes plus 2.5 minutes (Table 5.8).

5.3.6. Endoglucanase adsorption-desorption characteristics:

Total cellulase activity in terms of filter paper units and endoglucanase activity in terms of CMC-ase activity were studied during adsorption and desorption (Table 5.9). While 44.77 percent of the cellulase activity is recovered only 23 percent of endoglucanase component could be recovered.

5.4.0. Discussion and conclusion:

Maximum adsorption of cellulase on both E. Merck cellulose and ball milled cellulose pulp was at 45°C and at a pH around 4.8 when the contact time was 5 minutes plus 2.5 minutes (centrifugation at 8000 G). Afterwards conspicuous solubilization took place and this resulted in the release of enzyme to the solution. Therefore the contact time is of maximum importance in the study of adsorption characteristics.

That higher temperatures caused more adsorption is contradictory to the earlier findings (Bisaria, 1977), and specific adsorption, like Bisaria's, in this case also was higher at higher temperatures upto 45°C. As for the effect of pH there was no perceptible change

Table 5.7

Effect of protein on adsorption of cellulase by cellulose

Concentration percentage	Specific activity in the remaining solution	
	Before	After
Control	0.468	0.475
1.5	0.247	0.155
5.0	0.057	0.005

Table 5.8

Effect of temperature on adsorption when 5 percent protein is added

Temperature °C	Percentage adsorption	Specific adsorption
5	6.43	0.224
15	12.75	0.187
25	30.56	0.284
35	42.20	0.322
45	42.60	0.484

from earlier observations. It only confirmed that adsorption of enzyme is the maximum at optimum temperature and pH. This finding is probably made possible because of the specific time of contact allowed with a view to study maximum adsorption characteristics. As for desorption, abrupt temperature change caused more active enzyme to be released (75.44 percent) compared to gradual temperature change (42.84 percent) at 4°C from 25°C. (Table 5.5). Specific desprption was however, not affected for a wide range of temperature (Table 5.4), the abrupt change in temperature did not affect the original enzyme activity considerably, once it is again warmed upto the optimum temperature. With regard to the effect of protein, the specific adsorption was controlled by temperature (Table 5.7).

The adsorption-desorption characteristics of components may be different from that of the total cellulases. An experiment to find out the quantity recovered by adsorption-desorption technique by means of an abrupt change in temperature after adsorption at 45°C showed that while 44.77 percent of cellulase activity was recovered only 23 percent of the original endoglucanase activity could be retained after recovery. This points towards a problem of changing the proportion of constituents due to recovery. Further it may be suggested that a strict proportion of constituents is not critical for total cellulase activity. Probably endoglucanases

are more sensitive to change in temperature.

In addition to confirming the possibility of enzyme recovery from a mixture of enzymes and products or of purifying the enzymes from a crude extract the present study stresses on two aspects in the study of adsorption-desorption characteristics,

(a) the contact time of 5 minutes plus 2.5 minutes does not affect the distribution coefficient (FP/ml/FP/mg) and that within this contact time the optimum temperature and optimum pH for enzyme activity are the optimum conditions for specific adsorption also;

(b) an abrupt temperature difference releases most adsorbed enzyme than when the temperature change is gradual.

IMMOBILIZATION STUDIES

CHAPTER-VI

IMMOBILIZATION STUDIES

6.1.0 Introduction:

During the past several years a number of methods have been developed for retaining an enzyme within a limited region of space. Such methods fall under the general term of 'enzyme immobilization', although a number of other terms such as matrix - entrapped, insolubilized, encapsulated and support - coupled enzymes have been used. Several reasons are apparent for developing methods to immobilize enzymes. Through immobilization, enzyme recovery can be made easier and the resulting economics for *in vitro* applications, at least in theory, may become highly favourable. A second reason for immobilization of enzymes is to provide an additional tool for investigating the mechanisms of enzyme activity and the factors that affect stability. And, finally since most intracellular enzymes are attached to membranes or appear to be associated with interfaces, the study of immobilized enzyme may be a tool to improve understanding of the mode of action of intracellular membrane-bound enzymes as well as a guide for developing novel *in vitro* devices for enzyme controlled processing (Wingrad Jr., 1972).

Goldman, Goldstein and Katchalski (1971) have reviewed the methods and literature on the immobilization of enzymes through most of 1970. They list 16 enzymes immobilized by entrapment in polyacrylamide or starch

gel (32 reported cases), 17 immobilized by adsorption on various cellulosic and synthetic polymers and to porous glass (143 reported cases) and 7 enzymes immobilized by intermolecular crosslinking by glutaraldehyde and other disfunctional reagents (14 reported cases). Both the adsorption and gel entrapment, in general, provide only temporary immobilization owing to reversible desorption and leaking of enzyme from the gel matrix. Covalent bonding methods lead to permanent attachment of the enzyme to a support; however, many enzymes undergo loss of activity during the coupling process. In another recent review, Melrose (1971) has given a fairly complete coverage of the literature on the immobilization of enzymes. He provides details of 178 reported cases of enzyme immobilization involving 54 different enzymes. Another review is by Goldstein (1969).

In addition to gel entrapment, adsorption and covalent bonding (including cross-linkage) enzymes have been immobilized through encapsulation by semipermeable membranes. A few additional examples of enzyme immobilization, reported after earlier review papers were described by Wingrad Jr. (1972). A group of persons active in enzyme immobilization studies (Sundaram and Pye, 1972) have suggested that the method of immobilization be included when describing an immobilized enzyme.

The stability of an enzyme to temperature, pH, ionic

strength, substrate and enzyme concentrations, trace materials and other factors is of prime interest for any process or device utilizing enzyme catalysis. It appears from the available data that a moderate number of immobilized enzymes are more stable under certain conditions than are the same enzymes free in solution. In many cases the stability, kinetic activity and other parameters are modified for immobilized versus free enzymes. A second generality regarding many immobilized enzyme systems is the extensive diffusional resistance for transport of substrates and product to and from the active site.

Recent reports on the stability and activity of immobilized enzymes are noted. Neurath and Weetall (1970) reported increased DNA - ase coupled to porous glass, supposedly from increases in the contact of partially degraded DNA with enzyme. Gable et al (1970) showed rather dramatically that in some cases markedly increased stability to materials such as urea, that normally causes chain unfolding, could be obtained by immobilization; these authors obtained retention of 60 percent activity of trypsin in 8 M urea over 7 days by coupling the enzyme to sephadex G-200. Unbound trypsin showed no enzymatic activity in 8M urea. Weetall (1970) has evaluated the storage stability of papain, trypsin and glucose oxidase bound to a variety of carriers. For all the enzymes there was at least one enzyme support

combination that exhibited 100 percent retention of activity after storage for upto 68 days at 4°C to 5°C either dry or in distilled water. He concluded that enzymes covalently bound to inorganic supports showed greater stability than those bound to organic supports. Although there are numerous reports citing increased stability of enzymes on immobilization, there are other cases where decreased stability has been observed. It seems logical to assume that certain changes in micro-environment around the enzyme may increase the stability of the enzyme for long term usage. However, in general it has not been possible to explain why the stability increases or decreases upon immobilization on a particular support or better still to predict correctly the effect of specific support on the stability of an enzyme without the need to resort to experimental testing. It seems premature to make a general statement, that immobilization of enzymes leads to improved stability. It is still a trial-and-error task, based largely on experimental testing as guided by some qualitative concepts, to obtain immobilized enzymes having improved stability.

Cellulases especially pose many problems the most formidable of which probably would be the insoluble nature of most of the substrates. But the soluble substrates like short chain cellooligosaccharides or carboxymethyl cellulase could be effectively used as substrates for cellulases immobilized to a solid support •

(Ghose et al 1977). The stability of the enzyme (endoglucanase) adsorbed to certain solid supports as well as some of the parameters of enzyme kinetics have been studied with a view to understanding the possibility of cellulases being immobilized for reuse in enzyme fermentors.

Goldman, Goldstein and Katchalsky (1971) cited a number of specific examples of changes in kinetic parameters due to enzyme immobilization. However, a number of cases of unchanged kinetic parameters also have been demonstrated for immobilized enzymes (Wingrad Jr. 1972). In general, the effect on kinetic parameters of immobilization of a specific enzyme by a certain technique can not be predicted at present.

6.20 Materials and Methods:

Cellulase with a specific activity 22 from a culture of Trichoderma viride QM9414 was used to study the immobilization of endoglucanases. Bentonite (Clay and Minerals, Ahmedabad) Glass beads (Corning, Jaipur) and activated charcoal (Bengal Chemicals) were used as solid support adsorbants. Endoglucanase activity was measured following the method described in chapter-V.

6.3. . Results:

6.3.1. Adsorption Technique to Immobilize Endoglucanase by Bentonite:

Bentonite was equilibrated with phosphate buffer pH 4.8 and cellulase with specific activity 22 (5 ml)

was added to it while stirring. It was kept for incubation at 29°C for 75 minutes and then washed thoroughly with 15 ml of phosphate buffer (pH 4.8.). After every washing the washed solution was tested for enzyme activity. The results are shown in Table 6.1.

After 8 washings the bentonite was dried by incubating at 29°C. The dried enzyme-immobilized-bentonite was added to 2 ml of carboxymethyl cellulose preparation or 2 ml of cellobiose and incubated at 50°C after adding 2 ml of phosphate buffer (pH 4.8). FPA was estimated. Another portion of immobilized enzyme on bentonite was added to 2 ml of phosphate buffer and mixed by shaking. The supernatant after centrifuging is measured for cellulase activity using carboxymethyl cellulose as the substrate. Protein was estimated by Folin-Ciocalteau method.

There was no cellulase activity seen in the supernatant though 0.0048 μ gm/ml protein was found in the supernatant.

Endoglucanase specific activity was estimated to be 15.75. The incubated immobilized enzyme even after 15 days retained a specific activity of 12.

The process was repeated by using activated charcoal and glass beads. The results are shown in Table 6.2a. Activated charcoal immobilized enzyme

Table 6.1

Effect of washing on immobilization

Number of washing	Activity µg/ml of reducing sugar
1	5.2
2	3.5
3	2.7
4	0.78
5	0.044

Table 6.2a

Effect of immobilization on different solid supports

Samples	Endoglucanase soluble	Endoglucanase immobilized
Bentonite	22	15.75
Activated charcoal	22	18.48
Glass beads	22	4.48

showed more activity though after every use the activity decreased tremendously.

6.3.2. Effect of temperature on bentonite-immobilized-endoglucanase.

The effect of temperature on the activity of bentonite-immobilized-endoglucanase was studied by estimating the reducing sugar after 75 minutes. Table 6.2b, Figure 6.1 shows that the temperature profile of immobilized enzyme does not have marked effect as against the soluble enzyme though slight shift towards lower temperature optima is observed.

6.3.3. Effect of pH on immobilized endoglucanase activity:

The effect of pH on immobilized endoglucanase activity was studied by treating the substrate with bentonite-immobilized-endoglucanase at different pH. The results are shown in Table 6.3 and Figure 6.2. For the immobilized enzyme the pH optima shifted slightly to the alkaline side.

6.3.4. Effect of reuse on immobilized endoglucanase:

The immobilized endoglucanase activity was tested during reuse. After every use the immobilized enzyme fraction was washed in 0.05 M phosphate buffer (pH 4.8) and incubated at 27°C to dryness. This was again used for testing the endoglucanase activity. The results are shown in Table 6.4. Bentonite retained more enzyme in contrast to activated charcoal after every reuse.

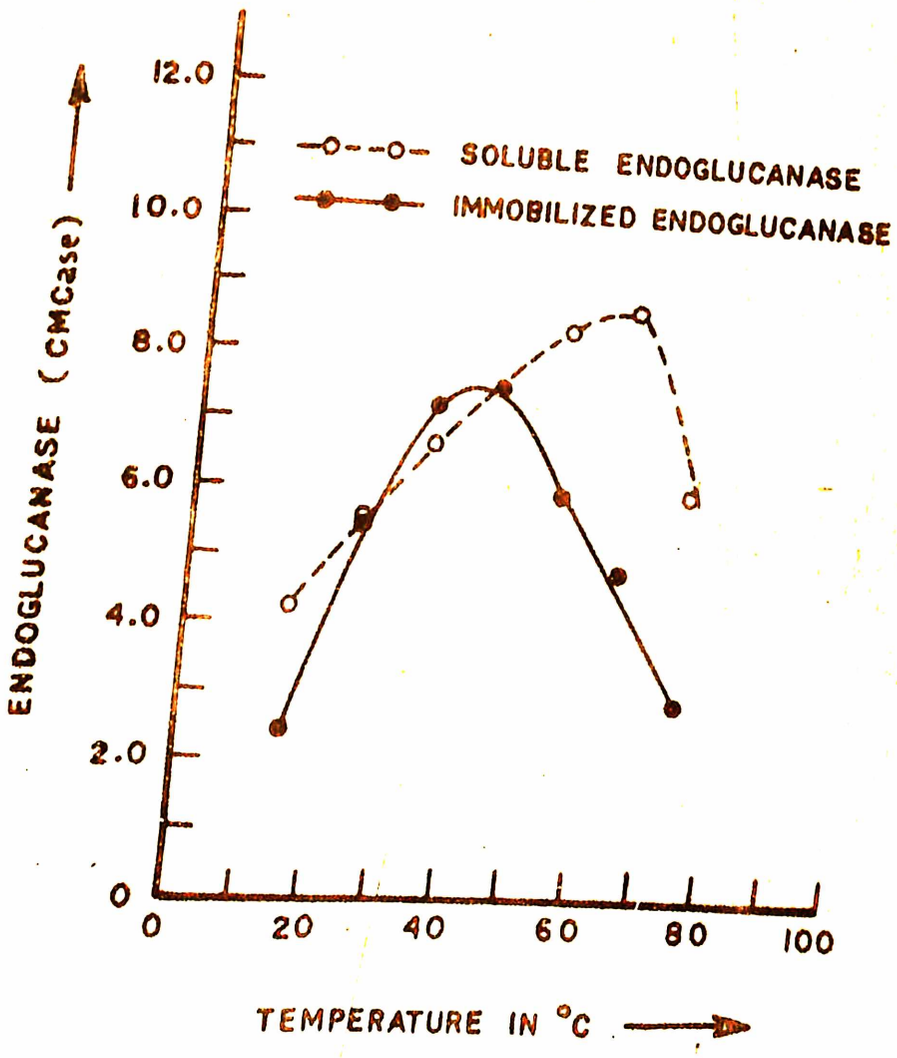

FIG. 6.1 EFFECT OF IMMOBILIZATION ON TEMPERATURE PROFILE.

Table 6.2b

Effect of temperature on bentonite - immobilized endoglucanase

Temperature ^{o}C	Immobilized endoglucanase activity	Soluble endoglucanase activity
5	0.048	0.57
15	2.448	4.228
25	5.48	5.528
35	7.225	6.69
45	7.45	7.54
55	5.88	8.422
65	4.75	8.79
75	2.84	5.99

Table 6.3

Effect of pH on immobilized endoglucanase activity

pH	Immobilized glucanase activity	Soluble endoglucanase activity
2.5	0.479	4.750
3.5	2.484	7.242
4.5	5.754	8.970
5.5	5.840	8.422
6.5	5.970	5.540
7.5	4.540	2.880
8.5	2.790	0.750
9.5	2.574	0.780

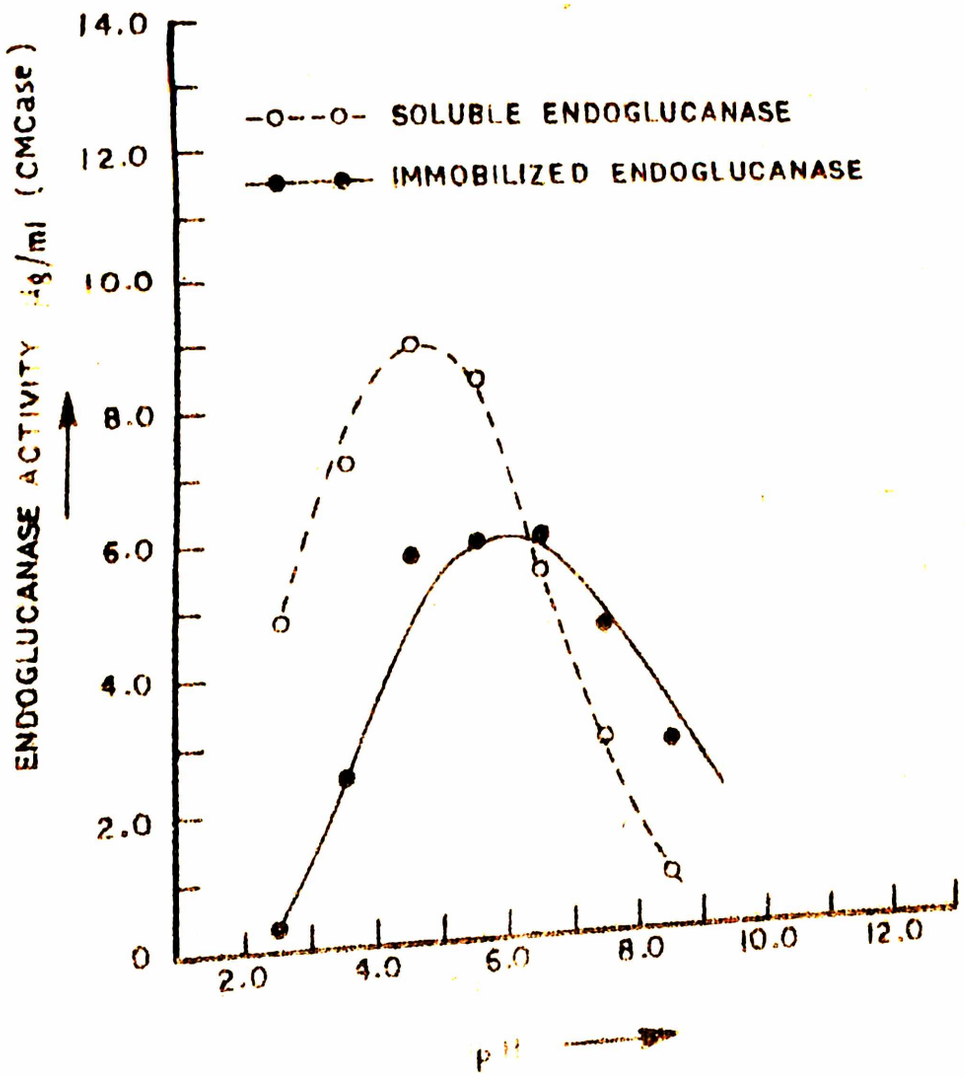

FIG. 6.2 EFFECT OF IMMOBILIZATION ON pH PROFILE OF ENDOGLUCANASE

Table 6.4

Effect of reuse on immobilized endoglucanase on bentonite

Number of time used	Endoglucanase activity	
	Bentonite	Activated charcoal
0	5.457	8.440
1	4.870	4.790
2	4.422	2.484
3	3.750	0.870
4	3.480	0.450
5	2.880	0.084

Though activated charcoal absorbs more enzyme, immobilized enzyme on bentonite exhibited significant amount of activity even at the 5th reuse.

6.4. Discussion and conclusion:

Endoglucanase was adsorbed to bentonite, activated charcoal and glass beads and its kinetic parameters like temperature and pH were studied. Though both bentonite and activated charcoal immobilized significant amount of endoglucanase, activated charcoal could not retain the enzyme to the extent bentonite could do. Glass beads were found to be an inadequate support for enzyme immobilization by adsorption. Enzyme immobilized bentonite performed satisfactorily even at the fifth reuse. As for the kinetic parameters of immobilized endoglucanase, temperature optima was found to be at a lower temperature for the immobilized enzyme while the optimum pH showed a shift to higher pH for the immobilized enzyme. This should be as a result of the change in microenvironment of the active site. For the immobilized enzyme, lower pH from 3.5 downwards was found to cause considerable loss of enzyme activity than for the soluble enzyme. Higher pH however, showed much less effect for immobilized enzyme than for the soluble enzyme.

SUMMARY AND GENERAL CONCLUSION

CHAPTER-VII

SUMMARY AND GENERAL CONCLUSION

The biodegradation of cellulose by cellulases as an economically viable process for the production of energy, protein and chemicals is in experimental stages only. The major problems in this process are enumerated elsewhere (Chapter-I). The present work was concentrating partly on the biological aspect of cellulase production and partly on the biochemical properties of cellulase enzyme, with a view to make available more active cellulases in order that the process of utilization of cellulose becomes more efficient.

On the biological front a rapid method of production of cellulase has been developed that formed definitely, an advanced technique to that is present todate in cellulase production. The starved mycelia with a definite starvation period when transferred to an induction medium evoked an immediate response that resulted in a spurt in enzyme production without a perceptible lag period. This when compared to earlier studies (Nissizawa et al, 1971) is remarkable in the sense that the latter could obtain comparable amount of enzyme only with a lag period of 2-4 hours and at a slow rate. The implication is obvious. For large quantities of enzyme production for industrial level application the starved mycelia put under induction will be more useful than all other

methods available for the production of cellulase enzyme. Though as to the exact quantity of enzyme thus can be produced in large scale and how long the mycelia can be used for the production by such a process are problems to be tackled during pilot plant level studies and also during studies on scaling up further, the process looks feasible.

Apart from this, the rapid method of production of cellulase has opened new possibilities for exploring the fundamental aspects of cellulase production especially, the induction - repression processes. That cellulose and cellobiose were successfully used as inducers for the production of significant amount of cellulase, following this techmique, is also important. That cellulose grown mycelia are also affected by starvation, leads to think that glucose and its intermediary metabolites are regulating directly or indirectly the synthesis of cellulase. Cyclic AMP effect on non-starved mycelia converting it to function in the form of a starved mycelia implies a possible involvment of a cyclic AMP system in the production control of cellulases. The duration in which the mycelia were starved was found to be important and 15 hours starvation was found to be the optimum. Though both glucose grown and cellulose grown mycelia were effective, glucose grown mycelia were found more suitable, as the enzyme activity per mg. of mycelia in that case was more than that of cellulose grown mycelia.

The mycelia of different ages were studied to estimate their capacity to produce cellulase and it was found that among the actively growing mycelia, as the age increased, their capacity to produce enzyme in terms of FPA/mg. of mycelia also increased. The non-starved mycelia when treated with cyclic AMP prior to induction behaved like starved mycelia. The induction medium had pronounced influence over induction as the basal medium containing many bivalent cations caused more cellulase production than buffer or distilled water.

The presence of inducer was found to be necessary during the whole course of induction as the removal of the inducer during the course of induction virtually stopped the enzyme production. Rifampicin, Actinomycin-D, Chloramphenicol, Cyclohexamide and glucose inhibited cellulase production, though unlike in earlier experiments (Nissizawa, et al, 1971) glucose did not follow the course of translational inhibitors and its effect was in between that of rifampicin and cyclohexamide. However, glucose effect on inhibition was partially reversed by the administration of cyclic AMP suggesting the possible mediation of a cyclic AMP system during the catabolite inhibition. There was no effect of cyclic AMP on the inhibition caused by rifampicin and cyclohexamide.

Tween-80 and Triton-X was found to enhance the production of cellulase. But SDS, deoxycholate and

Ethylene diamine tetraacetic acid did not cause any increase in cellulase production. The presence of Tween-80 and Triton-X was not only important during the growth of the mycelia but also during starvation (for non-Tween-80 mycelia) and induction. During induction Tween-80 is found to have a major role when used in basal medium. It was further noted that the presence of bivalent cations and especially Ca^{++} plays a very important role during induction and this is more pronounced when Tween-80 is present in the medium. That the creation of an artificial influx of Ca^{++} using ionophores resulted in an increase in production of cellulase in close parallel with the effect of Tween-80 suggests a possible role of these surfactants in the calcium transport. However, other well established role of surfactants during the increased production of enzymes like the bringing about of changes in fatty acid composition of cell wall and cell membrane causing increased permeability is not contested.

The maximum adsorption of cellulase on cellulose was at 45°C and at a pH around 4.8, when the contact time was 5 minutes plus 2.5 minutes. The contact time is found to be of maximum importance in the study of adsorption characteristics.

Higher temperatures upto 45°C caused more adsorption. The effect of pH only confirmed the earlier observation (Bisaria, 1977) that adsorption of enzyme was maximum at

optimum temperature and pH of enzyme activity. As for desorption abrupt temperature change caused more active enzyme release. Reducing sugars, did not affect the adsorptivity of cellulase to cellulose. The adsorption - desorption characteristics of cellulase components may be different from total cellulases. While 44.77 percent of cellulase activity was recovered in an adsorption-desorption experiment only 23 percent of the original endoglucanase activity could be recovered.

Bentonite, activated charcoal and glass beads were used to immobilize the soluble endoglucanase. Bentonite was found to be a better base for cellulase immobilization than activated charcoal or glass beads, as the former retained more enzyme activity after reuse.

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