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REVIEW

BACTERIAL ADHERENCE TO CELLULOSE: CAN IT BE CAPITALISED?

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ABSTRACT

The tendency of bacteria to adhere to substrates play an important role in various biochemical processes associated with biofilms and flocs. Adhesion is considered to be a prerequisite for hydrolysis of cellulose and therefore is essential for decomposition of crop residues and other lignocellulosic wastes. The cellulose-cellulase system is heterogenous and the hydrolysis reaction involves multi-enzyme system. On adherence by bacteria, the cellulase complex on the bacterial surface bind to the substrate in optimal configuration. In anaerobic cellulolytic bacteria, cellulosome, a complex consisting of both cellulolytic enzymes and specific adhesion proteins play a vital role in the hydrolysis of cellulose. This phenomenon positively help for fibre degradation in biogas reactors and cattle rumen. The role of bacterial adhesion in cellulolysis and its application possibilities is discussed.

Key words : Cellulolytic bacteria, adhesion, cellulolysis, cellulase

Introduction

The adherence of bacteria to insoluble substrates bears physiological and ecological significance. It is a prerequisite for the utilization of substrates like cellulose, chitin etc. Adsorbed bacteria releases cellulase and who's adsorption is reported to parallel the rate of hydrolysis of cellulose. Upon adsorption of the cellulase enzyme on the surface of the cellulose fibers, the enzyme-substrate complex is formed. In few cellulolysers, the membrane bound complex of enzymes located on the bacterial surface consisting of both enzymes and specific adhesion proteins is known as 'cellulosome'. Such adhesion proteins or adhesins could impart a degree of specificity. At times the enzymic polypeptide is glycosylated and posses the binding sites in it. Aggregation of cellulase enzymes has been observed in both fungal and bacterial cellulases (Wood, Wilson and Stewart, 1982). Particular interest is the role of adhesion proteins in the mechanism of cellulolysis. An affinity factor of high molecular weight and a hydrolytic factor of low molecular weight were postulated to be essential features of bacterial cellulolysis (King and Vessal, 1969) and initiated the search for the separate adsorption factor in cellulolytic bacteria especially in anaerobes. However, the details are elucidated. Application of this adsorption phenomenon towards productive uses are on the way.

Why adherence ?

In flowing water bodies the bacteria needs an anchoring. In this regard they settle on inert surfaces or on organic biopolymer. Once settled they gain nutrition by depolymerization of the polymers. Bacteria have a marked tendency to interact with surfaces. Some of these bacteria adhere to surfaces, initially in a reversible association and transforms to an irreversible adhesion (Videla and Characklis, 1992). A bacterial cell initiates the process of irreversible adhesion by binding to the surface using exopolysaccharides of outer cell membrane (Costerton, Geesey and Cheng, 1978) or through special outgrowths like glycocalyx (Costerton et al., 1987). Such adhesion initiated by bacteria is a prerequisite for depolymerisation of polymeric substrate as in cellulolysis (Lamed, Setter and Bayer, 1983)or favours the biofilm development as in waste water treatmen. system (Blenkinsopp and Costerton, 1991). Catalytic potential of the biofilm was the first to be capitalised in trickling filters and biofilters. After initial attachment of bacterial cell through cell wall outgrowths or flagella (Fig. 1), cell division then produces the sister cells that are

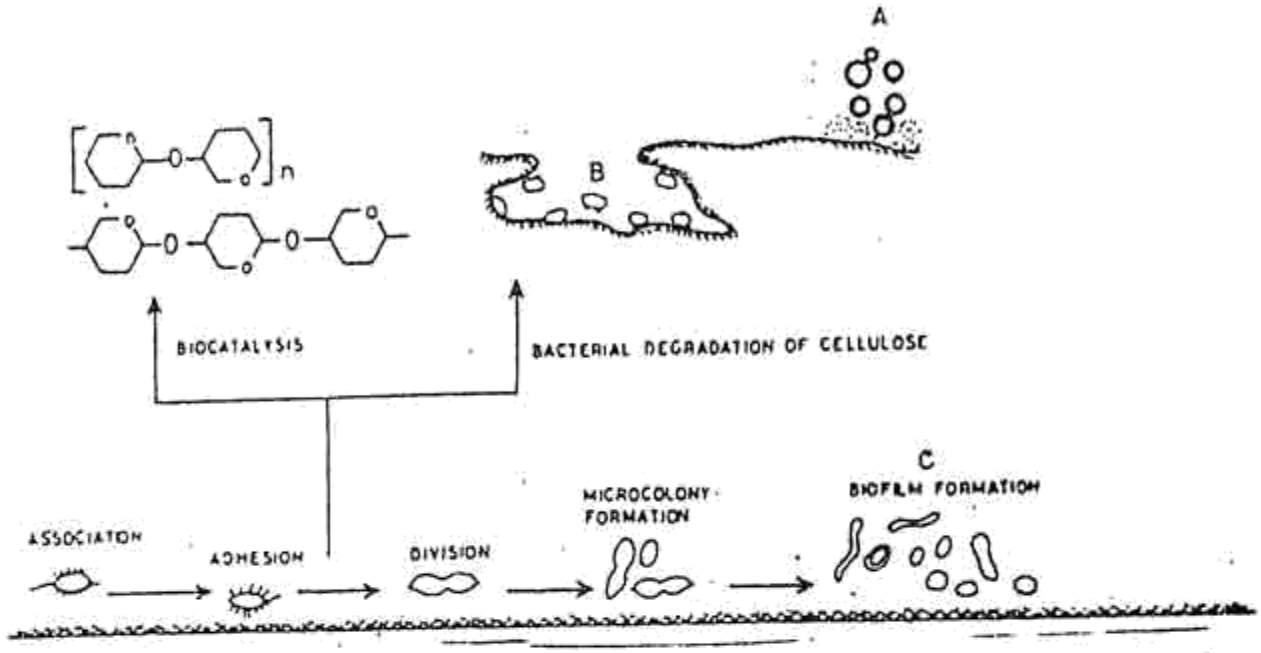


Fig. 1 **Microbial adhesion, biofilm formation and cellulolysis**

a. *Ruminococcus* cells remain at some distance from the substrate surface while their enzymes initiate digestion

b. *Bacteroides* adhere intimately to substrate and detach enzymes that cause a pitting attack

c. Biofilms on biofilters and waste water treatment systems (Costerton *et al.*, 1987)

bound within the glycocalyx matrix, initiating the development of adherent microcolonies. Production of a continuous biofilm in the colonized surface is a function of cell division within microcolonies and new recruitment of bacteria from the planktonic phase. Consequently, the biofilm consists of single cells and microcolonies of sister cells embedded in a highly hydrated predominantly anionic matrix of bacterial exopolymers and trapped extraneous macromolecules. As the bacterial biofilm gradually occludes the colonized surface, newly recruited bacteria adhere to the biofilm itself (Costerton *et al.*, 1987). Two-step and three-step adhesion mechanisms have been proposed for microbial attachment. Bacterial cells initiate the last step, irreversible adhesion, by binding to the surface using exopolysaccharide polymers (Blenkinsopp and Costerton, 1991). Microorganisms in nature are mostly associated with solid surfaces. In nutrient poor environments, they tend to exist as a monolayer of attached cells. Whereas in nutrient rich environments, more extensive biofilms build up. The reason for this phenomenon is that, in most ecosystems like river, soil or mouth, the streaming conditions have plug flow characteristics, or have a high dilution rate. To survive in such environments, microorganisms have to attach to solid surfaces or immobilise themselves in the form of biofilm.

Microbial adherence in various environments

An organism might adhere but unable to grow in a particular environment. When the environment becomes more favourable, the organism may proliferate and constitute a major portion of the biofilm. Net biofilm accumulation results from the combination of (a) transport of cells to the substratum (b) adsorption of cells by the substratum (c) growth and other metabolic processes within the biofilm, and (d) detachment of portions of the biofilm (Videla and Characklis, 1992).

There is a significant role for microbial surface active compounds in adhesion to and deadhesion for microbial surface active compounds (Neu, 1996). Surface active compounds produced by microorganisms mediate the interaction - adhesion and deadhesion between microorganisms and interfaces. Kelley *et al.* (1997) studied the surface colonization characteristics of a predatory prokaryote, *Bdellovibrio bacteriovorus*. The results revealed that *Bdellovibrio* prefer to associate with surfaces and the association provides the predator a rich source of prey bacteria in surface biofilms and protection in the gel-like matrix of the biofilm.

Streptococcal adherence to teeth

Considering the case of tooth surface microbial population, Streptococci, the principal early colonizers bind to acidic proline-rich protein and other receptors like alpha-amylase and sealic acid in the pellicle. Streptococci also participate in intrageneric coaggregation which offers an extra advantage in allowing them to bind to the nascent monolayer of already bound streptococci. In addition, actinomycetes which are other primary colonizers, bind to the acquired pellicle and to the streptococci. During the transition from colonization predominantly by streptococci and actinomycetes in the first few hours to later colonizing genera, a vast array of surface molecules are present in the environment. As each new cell type adheres, its cell body becomes a nascent surface. In this way, dental plaque quickly presents numerous possible receptors and adhesins available for specific recognition among different strains (Fig. 2). The adhesins are presented at this surface by two distinct mechanisms (Whittaker, Klier and Kolenbrander, 1996)

a. Peptidoglycan linkage and surface exposure of N- terminal region of the adhesin, and

b. Cytoplasmic membrane anchoring and surface exposure of the C-terminal region of the adhesin

Adherence of bacteria to cellulose

When biofilms form on the surfaces of insoluble substrates like cellulose, the initial events of adhesion favour specific bacteria that can digest that substrate (e.g. cellulolytic bacteria). The primary colonizers in such a system produce cell-associated digestive enzymes that attack the insoluble substrate and produce soluble nutrients that stimulate the growth of adjacent heterotrophic organisms until a digestive consortium is formed. Biodeterioration of materials including the digestion of insoluble nutrients by bacterial populations in the digestive tract of higher animals usually involves a focused enzymatic attack on particular loci at the material surface. Physical attachment is necessary for active cellulose digestion and the enzymes involved remain in particularly close association with bacterial cells growing in biofilms on the surface of cellulose fibers. The colonization of cellulose fibers exposed to normal rumen flora is very rapid. The specific adhesion of bacteria to cellulose is the essential first step in ruminant digestion. When the cellulose fibers are completely digested, all of the bacterial components of this digestive biofilm become perforce, planktonic organisms that await the provision of similar nutrient substrates that will be specifically colonised and rapidly digested by a reconstituted digestion biofilm.

The overall facility with which a particular enzyme system acts on cellulose depends on many factors:

a. The recognition and binding of the soluble, particulate or cell bound enzyme on to cellulose

b. The diffusion into or movement of the cellulases in the solid cellulosic matrix

c. The hydrolytic reactions necessary to form soluble sugars

d. The relative efficiency of hydrolysis of crystalline regions or the capacity to convert crystalline regions to amorphous regions, and

e. Product inhibition characteristics of the various cellulose regions

Hsing and Canale-Parola (1992) observed that *Clostridium* sp. strain C7, when grown on cellulose, the bacterial cells multiplied in association with the sedimented cellulose and the

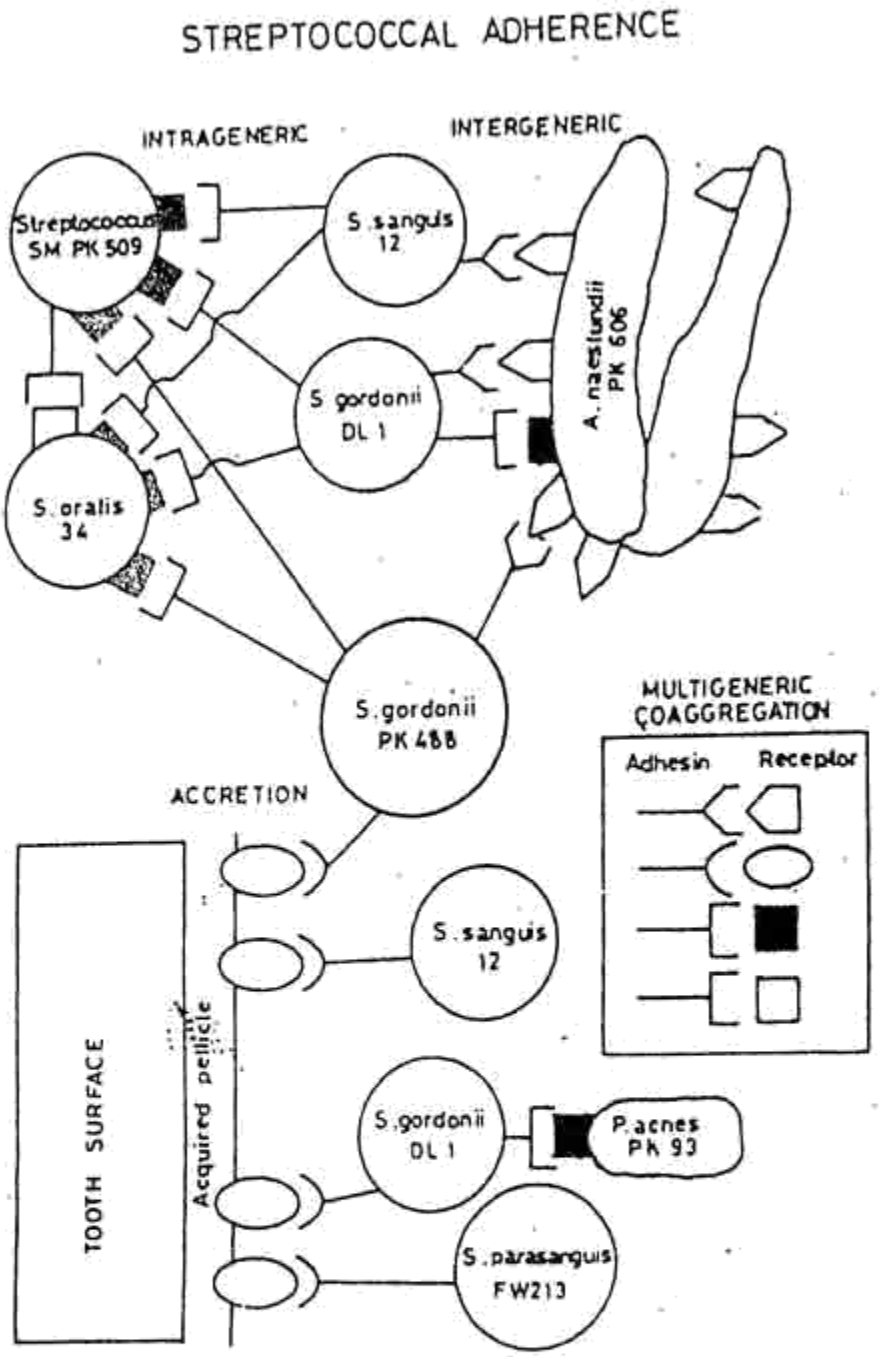


Fig. 2 Streptococcal adherence to tooth surface
Diagrammatic representation of sterptococcal adherence to other genera of oral bacteria and to the acquired pellicle coating on teeth. Streptococci participate in intrageneric coaggregation, thus binding to the nascent monolayer of already bound streptococci. Primary colonizers like Actinomycetes bind to the acquired pellicle and to the streptococci (Whittaker *et al.*, 1996).

supernatant fluid of broth culture remained clear. They attributed the accumulation of cellulolytic bacteria near the cellulose fibers to some type of chemotactic response. However, as cellulose is insoluble in water and cannot elicit a direct chemotactic response, it appeared probable that the response could be attributed to a soluble cellulose hydrolysis product formed by the activity of an extracellular cellulase bound to the cellulose fibers. Chemotactic responses toward products of cellulose hydrolysis may play an important role in the overall process of cellulose degradation in natural environments. Furthermore, it seemed possible that, in nature, hydrolysis products of polymers present together with cellulose in plant cell walls could serve to attract motile cellulolytic bacteria towards cellulose-containing plant material. Hsing and Canale-Parola (1992) opined

that chemotaxis of bacteria towards cellobiose occurs in natural environments. They further stated that concentration gradients of cellobiose is created upon hydrolysis of cellulose and the motile cellulolytic bacteria are attracted towards cellulose, utilizing their constitutively synthesized cellobiose chemoreceptors. As a result, the cellulolytic bacteria either attach to cellulose fibers or remain in their vicinity resulting in higher cellulose degradation.

Biofilms in waste water recycling

Biofilms in waste water treatment plants and ponds have been used to remove heavy metals from solution because the exopolysaccharide components of their glycocalyx matrices have a high affinity for metallic cations. It is also possible to use biofilms in the selective removal of targeted metals from mining and refining effluents. Their organic-nutrient trapping capabilities are successfully used in waste water treatment plants to reduce organic content before release. The oldest forms of biofilm reactors are trickling filters and biorotors. In both these types of reactors, the biomass is attached to an inert support and are retained for biocatalysis in the reactor. Advantage of bioreactors or trickling filters is their simplicity and low maintenance cost (Van Loosdrecht and Heijnen, 1993). But in fluidized bed or air-lift reactors biofilms grow on small suspended particles and thereby increase the biofilm surface area which improved the efficiency of these type of reactors.

Methods to assess adherence

Progress in the research of adherence has been hampered by lack of reliable quantitative techniques for assessing the level of adherence. Attempts have been made to measure bacterial adherence to cellulose that is suitable for quantitative research.

Radioactive assay

Morris and Cole (1987) developed radioactive assay method to study the adherence of *Ruminococcus albus* to cellulose. They labelled the cells by growing in ^{14}C sodium acetate. The cells were harvested during the stationary phase and suspended in mineral buffer. The suspension was then added to 100 mg cellulose (previously washed with distilled water and mineral buffer) in scintillation vials. The suspension was agitated for 30 min at room temperature. After that it was diluted with 20 ml buffer and the supernatant was removed by aspiration after the cellulose was allowed to settle. The radioactivity was measured in Liquid scintillation counter after adding scintillation fluid to the washed cellulose. Control vials without cellulose were maintained to assess and deduct the adherence to the vials.

Rasmussen, White and Hespell (1989) developed an alternate method which employed adhesion of cells to acid swollen cellulosic disks instead of cellulose particles as the chain forming bacteria such as *R. flavefaciens* sedimented with cellulose particles giving differential results. They explained that these microbes were entrapped in the cellulosic suspension and in some strains, the cells were sedimented at low speed centrifugation even when no cellulose was present. Such entrapment could not be distinguished from true adherence.

Turbidometric method

Bhat, Wallace and Orpin (1990) estimated the adherence of *R. flavefaciens* and *Fibrobacter succinogens* to straw by turbidity assay. The cell suspension was added to straw in Hungate's tube inverted several times and incubated at 39°C under CO_2 for 30 min. The mixture was then filtered under vacuum through a sintered glass filter and the optical density was measured at 650 nm. The adherence was calculated by the following formula

Adherence = 1-(D-B)/(C-A), where

A= O.D. of filtrates of uninoculated medium.

B= O.D. of uninoculated medium to which straw is added,

C= O.D. of bacterial suspension and

D= O.D. of bacterial suspension to which straw is added

Morag, Bayer and Lamed (1990) studied the adherence of *Cl. thermocellum* by using avicel instead of straw. The cell suspension (A_{650} = 2 with phosphate buffer saline) was added to 10 mg

avicel, vortexed for 20 seconds and rocked gently at room temperature. After 20 minutes the mixture was centrifuged at 1500 rpm for 4 minutes and the turbidity was measured at A_{400} and compared with control. Bayer, Kenig and Lamed (1983) followed a similar method but used all the cellulosic substrates at the rate of 20 per cent except cotton (@ 1 per cent).

Filter paper method

Gehin et al. (1995) studied the adherence of *Cl. cellulolyticum* to filter paper. The cells were suspended in Tris HCl buffer and added to Whatman No.1 filter paper in a glass tube. After incubation with gentle agitation for 30 min., the optical density of the supernatant was determined (OD_{600}). The cells were harvested and dried to constant weight at 70°C. The adherence was calculated from the dry weights of pellet both before and after adherence.

Adherence in relation to cellulolysis

Since native cellulosic materials are water-insoluble solid substrates, the cellulose - cellulase system is heterogenous and the hydrolysis reaction involves several steps. Among these, the adsorption of enzyme molecules on susceptible sites of the cellulosic surface is a prerequisite step for subsequent catalytic reaction. Direct physical contact between cellulolytic enzyme and its substrate was required for catalytic reaction by cellulase (Ramasamy, 1980). The binding of enzyme on a specific binding site of substrate provides the desired specific forces for adsorption of cellulase enzyme on the cellulose. Cellulose hydrolysis by adsorbed cellulase was found to be controlled by effective binding (Lee, Shin and Ryu, 1982). Enzyme binding to cellulose through hydrogen bonding would disrupt the hydrogen bonding networks in the crystal lattice and thereby the ordered structure of the substrate is broken. Adherent interactions of microorganisms with insoluble polymeric surfaces is an important phenomenon implicated in a variety of biochemical response at both cellular and molecular level. It has been suggested that the binding of cellulolytic bacteria to plant cell wall and cellulose matrix facilitate cellulose fibre degradation (Imaim and Kuru, 1991).

The cellulolytic enzymes produced by *Cellulomonas* species appear to bind tightly to the cellulose substrate (Beguin, Eisen and Roupas, 1977). They observed that most of the cellulolytic activity during the growth phase was cell bound, and the activity appeared in the culture fluid only during the stationary phase. The cellulose-bound activity remained on the cellulose even after extensive washing with buffers of different concentrations and pH (Ljungdahl *et al.*, 1983). About 70 - 80 per cent of the extracellular endoglucanase activity consistently adsorbed to cellulose and was eventually determined to be part of the cellulosome.

Morris (1988) observed that the optimum parameters for adherence may not always be the same as those for enzyme activity. The rumen bacteria that are capable of degrading the more-highly ordered forms of cellulose tend to adhere closely to the substrate. Wood *et al.* (1982) postulated that the polyanionic coat of *R. albus* served as a medium for adhesion of the bacteria to their substrate and the diminution of the coat decreased the ability of the cells to degrade the more-highly ordered substrates (Wood *et al.*, 1982). Possible competition between bacteria for adhesion sites was investigated by using enzyme markers for each species. This method was developed as an alternative to radiolabelling. The enzymes were cell associated and the activity was measured by using cells that were suspended from the filtrate after centrifugation (Bhat *et al.*, 1990). Cell-free enzyme extracts are not as effective as the whole cells in degrading cellulose, demonstrating that some essential factor present on the bacterium is missing in the cell- free extract. In contrast, Kauri and Kushmer (1985) found that for a variety of bacteria, degradation of cellulose did not depend on cell-fibre contact. Similarly, Morris and Cole (1987) also found that adhesion is a prerequisite for effective cellulose degradation, but was not necessarily followed by cellulolysis. They found that there was no correlation between the extent of attachment to cellulose and the ability to degrade it. Adherent bacteria played a decisive role in the

biogas production from cowdung. By selective fractionation and enzyme assay (Sivakumaran and Ramasamy, 1986) it was observed that the cellulose binding bacteria had a direct relation to hydrolysis of solids in the slurry. Further studies (Sivakumaran, Nagamani and Ramasamy, 1992) showed that these adherent bacteria were *Acetivibrio* sp., *Bacteroides* sp. and *Clostridium* sp.

Factors affecting adherence and cellulolysis

Factors affecting the adsorption of cellulases to cellulose include the nature of the substrate, its purity, pretreatment and the extent of which it is crystalline or amorphous, as well as the enzyme/substrate ratio, the affinity of the multicomponent enzyme system for the substrate used, the fact that the topography of the substrate changes as digestion proceeds, together with factors such as inactivation of the bound or free enzyme and the effects of products of action or other substrates that might promote or inhibit adsorption.

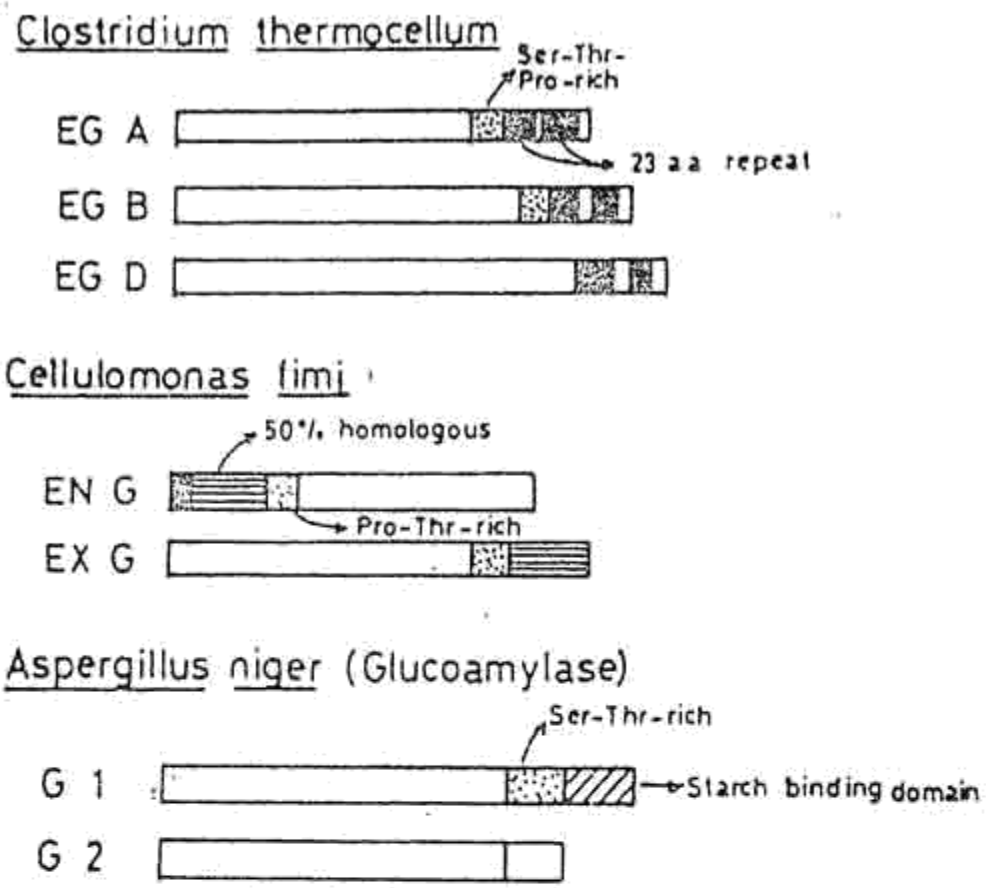
Substrate

Cellulolytic enzymes were found to bind with different degrees of tenacity to cellulosic substrates (Coughlan, 1985). When relatively pure celluloses were used, the cellulases were rapidly adsorbed. The initial phase was followed by a gradual release of enzyme to the solutions as hydrolysis proceeded.

Cellulose is the best substrate for enzyme production by microorganisms, but some substrates are rapidly degraded and so yield only low levels of enzyme, while others, such as cotton are not broken down readily enough to support adequate growth. However, Tokatlidis, Dhurjati and Beguin (1993) reported that the thermostable cellulase system produced by *Cl. thermocellum* has a very high specific activity towards cotton, a form of cellulose that is most recalcitrant to enzymatic hydrolysis due to its high degree of crystallinity. The synthesis of cellulases was induced by the presence of cellulose and repressed by the presence of dextrose or other readily metabolized sugars in the growth medium. Evidence supporting this conclusion has been obtained for bacteria e.g. *Acetivibrio cellulolyticus* and *Cellulomonas uda* (Stoppok, Rapp and Wagner, 1982). The structural properties of cellulosic materials have a profound influence on the adsorption and the overall hydrolysis reaction. The adsorption of cellulase on spent bagasse and newspaper resulted in a decreased hydrolysis rate and the extent of adsorption was found to be mainly due to the change of cellulose structure by the hydrolysis reaction (Lee *et al.*, 1982).

Morris (1988) reported a marked inhibition of cellulolysis by extracellular cellulases of *R.albus* in the presence of cellobiose and to a lower extent with glucose. Sternberg and Mandels (1980) found that sophorose had two regulatory roles in *T.reesei*, on the one hand it induced the synthesis of endo and exocellulases and on the other, it repressed the production of ß-glucosidase.

The nature of substrate greatly influence adsorption. Lee *et al.* (1982) compared the adsorption/desorption behaviour of cellulases on a number of substrates. They concluded that enzymes were continuously adsorbed when the initial adsorption was hindered by the inaccessibility of the substrate or by the presence of non-hydrolysable materials. By contrast, enzyme proteins that adsorbed maximally at the initial stages of hydrolysis were gradually released with reaction time as the crystalline and inaccessible fractions of substrate increased. The extent of cellulase adsorption to a given cellulose increased as the substrate particle size decreased i.e. the surface area increased. Adsorption of soluble protein at initial reaction time was related to the specific surface area of cellulose (Imam and Kuru, 1991). In contrast, Goel and Ramachandran (1983) found that particle size and crystallinity had only a limited effect on the adsorption of the cellulases in *T. reesei*. However, Lee *et al.* (1982) concluded that the adsorption affinity as well as structural properties have a significant influence on adsorption. Also, Lee and Fan (1983) found that the marked decline in the hydrolysis rate during the early period of reaction was due to product inhibition and conversion of the substrate to a less digestible form with an increased crystallinity index and a decreased specific surface area. They claimed that product inhibition was caused by deactivation of the adsorbed protein by the



Structural organization of the cellulase genes from *Clostridium thermocellum*, *Cellulomonas fimi* and glucoamylase genes from *Aspergillus niger*.
The terminal domains and the putative hinge regions rich in hydroxyl aminoacids and proline are indicated. The catalytic domains are represented by open boxes and the intron positions are shown by solid bars (Singh and Hayashi, 1995)

products. Deactivation was said to be linearly related to dextrose concentration, but related to cellobiose concentration in a hyperbolic fashion (Coughlan, 1985).

Adhesion of cellulolytic bacteria can be inhibited and even reversed, by substrate analogs such as methyl cellulose (MC) and carboxymethyl cellulose (CMC). MC also inhibits cellulolytic enzyme activity. Morris and Cole (1987) found that the adherence of *R. albus* was inhibited by various soluble cellulosic substrates. Lignified regions of the plant cell wall was found to prevent attachment of rumen microorganisms. It was reported by Imam and Kuru (1991) that the adsorption of cellulase on spent bagasse and newspaper resulted in a decreased hydrolysis rate and the extent of adsorption was found mainly due to the change of cellulose structure by the hydrolysis reaction.

Temperature

Adsorption in the initial rapid phase is greatest under the conditions of temperature that is optimal for hydrolysis. However, desorption in the succeeding slow phase is also greatest under these conditions. Thus, for example, at 4°C, the extent of adsorption in the initial phase was low, but such adsorption continued until much more enzyme is bound to the substrates than at higher temperatures. Presumably, the greater rate of hydrolysis of substrate at the higher temperatures effected a more rapid rate of decrease on the number of sites to which the enzymes bind, thereby it gave rise to a greater rate of desorption (Coughlan, 1985). Ooshima, Sakata and Harano (1983) found the ratio of adsorbed components to be temperature dependent, the endoglucanases being preferentially bound at low temperatures (5°C), whereas the exocellobiohydrolase was more tightly adsorbed at

50°C, the temperature at which activity was maximal. The optimum temperature for adhesion was found to be around 30°C.

Microbial adhesion of *R. albus* was measured at temperatures ranging from 4°C to 50°C and at two different pH, It was observed that the optimum temperature was around 30°C. *R.albus* did not adhere at all to cellulose at 4°C but showed greater adherence at 38°C. The decrease was more marked at high temperature (50°C), suggesting the involvement of an enzyme or protein which could be inactivated at high temperature (Morris, 1988). Imam and Kuru (1991) reported that maximum binding of *Lactobacillus amylovorans* to starch granules occurred at 24°C.

Time

Most of the bacterial adhesion occurred within five minutes of addition of a cell suspension to cellulose. However, 30 minutes was taken as a standard incubation period (Morris and Cole, 1987). The stage of growth also influenced adhesion. Peak adhesion occurred during mid to late exponential phase. Adhesion did not decline to less than one-half of the maximum level at other phases of the growth cycle (Bhat *et al.*, 1990). Imam and Kuru (1991) reported that the number of *L. amylovorans* cells bound to corn starch granules increased with time, reaching a maximum of 60 to 75 per cent in 30 minutes, after that decreased to about 50 per cent and remained stable for upto three hours.

pH

Adherence was reduced markedly at pH 4.5-5.0. A slight peak around pH 7.0 represented the optimum for specific adherence to cellulose (Morris, 1988). A second adherence maximum was seen around pH 5.5. This peak may represent non-specific adherence to cellulose and inter-bacterial aggregation. The broad pH optimum for adhesion of *R. albus* covered the physiological pH range likely to be found in the rumen even under the acid conditions associated with rapid starch fermentation. It was suggested that the decrease in cellulolysis associated with high concentrate diets is due to the effect of pH on cellulolytic activity and adhesion (Morris, 1988). Bhat *et al.* (1990) reported a substantial adhesion at both pH 4.0 and 8.0. Bound *L. amylovorans* cells can be removed from the starch granules either by high ionic strength or low pH (Imam and Kuru, 1991).

Addition of chemicals

Addition of NaCl at 2.5 M concentration enhanced adherence (Morris, 1988). Further they observed that a lack of inhibition by potassium thiocyanate confirmed that hydrophobic interactions are not involved in attachment of the cell to the substrate. Imam and Kuru (1991) reported about 69 - 91 per cent removal of the starch granules bound *L. amylovorans* by 2M $MgCl_2$. Adherence of cellulase was unaffected by the presence of salts and mild detergents. Numerous monosaccharides and polysaccharide including various cellulose derivatives and degradation products, failed to inhibit cellular adherence to cellulose. Various other natural and synthetic polymers were non-inhibitory to adherence even at very high concentrations. Only polyethyleneimine was found to interfere significantly with adherence.

Yellow affinity substance in adherence

The binding of the cellulolytic enzyme system to cellulose *in vivo* seems to be facilitated by a low molecular weight yellow affinity substance (YAS) that is produced during the early stages of growth (Coughlan *et al.*, 1985). YAS binds tightly to cellulose to form YAS-cellulose. The YAS produced slightly ahead of the enzyme system, adheres to the cellulose fibres and facilitates binding of the enzyme system to the insoluble substrate. SDS- polyacrylamide gel electrophoresis showed that each complex was composed of 15-20 different types of polypeptides ranging from M_r 45,000 to M_r 2,10,000 and that many copies of some of these polypeptides were present. This substance is characteristically formed by *Cl. thermocellum* during cellulose fermentation. An additional low molecular weight component was associated with binding of the cellulase system to cellulose. The YAS increased the adsorption of the cellular complex to cellulose.

A multi component complex binds to YAS cellulose in the presence of salt solution. It was termed the free bindable (FB) complex. The other free fraction does not bind to YAS-cellulose and was termed the free-non bindable (FNB) complex. Hon-nami *et al.* (1987) suggested that the YAS secreted by the bacteria coated the substrate. This induced the synthesis of the cellulase system which is later assembled as a complex on the surface of the bacteria. The enzyme complex acts as a bridge between the bacteria and the substrate.

Catalytic mechanism of cellulase

During cellulose hydrolysis, the exact catalytic mechanism of cellulases on the substrate is still unresolved. This is due to the complexity of the cellulase mixture required for efficient hydrolysis and the fact that cellulose hydrolysis is a two-phase reaction (Yu and Saddler, 1995). A cellulase mixture must contain three general types of enzyme activities *viz.*, cellobiohydrolase (CBH), endoglucanase and ß- glucosidase for effective hydrolysis of cellulose. Since multiple cellulase components of similar type of activity exist in cellulase mixtures, the exact number of cellulase components required for efficient hydrolysis is still unknown (Lee and Fan, 1980).

Endo-ß-1,4-glucanase : This contains several components with varying degrees of randomness. The enzyme acts randomly mainly on carboxymethyl cellulose, phosphoric acid swollen cellulose and cellodextrin. This component does not act on cellobiose. The main products are cellobiose and cellotriose (Lee and Fan, 1980).

Exo-ß-1,4-glucanase : This is present in several forms. ß1,4-glucan glucanohydrolase removes a single glucose unit from the non-reducing end of the chain (Lee and Fan, 1980). ß-1,4-glucan cellobiohydrolase (CBH) removes a cellobiose unit from the non-reducing ends of the chain. This component has the greatest affinity for cellulose; it cannot attack carboxymethyl cellulose and can act very slowly on phosphoric acid swollen cellulose. Although it is unable to attack crystalline cellulose to any significant extent, it can degrade cellulosic substrates by removing cellobiose residues successively from the chain ends. When CBH is combined with endoglucanase and ß-glucosidase, it plays a major role in hydrolysis of cotton or avicel like crystalline cellulose.

ß-glucosidase : This hydrolyses cellobiose and short chain oligosaccharides to glucose, but has no effect on cellulose. While it rapidly hydrolyses cellobiose and cellotriose, the hydrolysis rate decreases markedly with an increasing degree of polymerization (Lee and Fan, 1980). Many of the ß-glucosidases are glycoproteins, the carbohydrate content varying from 0 to 90 per cent by weight. Molecular weight values range from 35 kDa to 440 kDa. ß-glucosidases differ from the ß-glucanase components of the cellulase system by their cellular localization.

Reese and his associates (1950) postulated that the microbial conversion of native cellulose to soluble sugars involved two step process: C_1 activated or disaggregated the cellulose chains and the enzyme classified as C_x then hydrolysed the substrate. After cellulase purification and characterization, they found that the C_1 factor from fungal cellulase systems was cellobiohydrolase. But contradictory to Reese's C_1-C_x concept, Streamer, Eriksen and Petterson (1975) found a strong synergistic effect of exoglucanase and endoglucanases on the hydrolysis of crystalline cellulose. They proved that endoglucanase initiated the attack on crystalline cellulose. So, Reese (1976) modified the original C_1 - C_x concept. He proposed that C_1, or the first enzyme to act on cellulose is an endoglucanase which possessed special properties such as activity on crystalline cellulose, ability to split hydrogen bonds, lack of activity on carboxymethyl cellulose and inability to act on its own reaction products. Confirmation of Reese's hypothesis was difficult because of the complexity of cellulase systems. Later Din *et al.* (1994) tried to confirm it. They found the endoglucanase from the bacterium *Cellulomonas fimi* to be composed of a catalytic domain and a nonhydrolytic cellulose binding domain that can function independently. Further they speculated that the catalytic domain corresponds to the hydrolytic C_x system and the cellulose binding domain corresponded to the nonhydrolytic C_1 system of Reese. Din *et al.* (1991) explained that the non hydrolytic

component of the cellulase system termed C_1, destabilizes the cellulose structure rendering the substrate accessible to hydrolytic enzymes. The swelling, defibrillation and production of 'short fibers' or 'small particles' from cotton by particular cellulolytic components was cited as evidence of C_1 and the activity was enhanced when the organism was grown on cellulose. Such binding ability might constitute the 'C_1' activity (Morris and Cole, 1987).

Interaction of cellulase components

Cellulose hydrolysis is a heterogenous reaction. The soluble cellulases have to be adsorbed into the insoluble cellulose substrate in order to carry out hydrolysis. This implied that the adsorption of cellulase components on to substrate has to be included in the study of possible mechanisms of cellulose hydrolysis. Since different cellulase components act synergistically in cellulose hydrolysis, a good understanding of the adsorption-desorption phenomena of cellulase components may provide information on the reaction mechanisms and their synergistic action. Synergism between the cellulase components appeared to be a general mechanism by which crystalline cellulose is degraded (Wu, Johnson and Demain, 1988).

Ryu, Kim and Mandels (1984) postulated that the endoglucanases and cellobiohydrolases adsorb at distinctly different sites on cellulose, corresponding to the sites of hydrolysis. Moreover, the addition of cellobiohydrolase to substrate to which endoglucanase is bound effects the latter to speed up its action and bring about desorption. Conversely, endoglucanases, on binding to cellulose increased the rate of scission by cellobiohydrolase. They also found that the endoglucanase fraction consisted of adsorable and non- adsorable components. Yu, Lee and Saddler (1995) reported that the proportion of cellulase components bound to the substrates did not change during cellulose hydrolysis.

Cellulosome

The cellulosome is a discrete multienzyme protein complex responsible for the efficient degradation of cellulosic substrates. The cellulosome is found both in the culture medium and at the surface of the bacteria, where it mediates adhesion of the cells to the substrate. Cellulosomes are also found to be bound to cellulose. It is a multi subunit complex with a molecular weight of 2.1 million daltons, responsible for adherence of the cell to the cellulosic substrate and contains both endoglucanase and exoglucanase systems (Coughlan, 1985). They can aggregate into macromolecular complexes termed polycellulosomes which range in size from $5X10^7$ to $8X10^7$ Da. Cellulosome of different strains are reported to contain 14 to 50 proteins and their molecular mass ranged from 20 kDa to 250 kDa (Romaniec *et al.*, 1992).

Subunits of cellulosome

When the cellulosome was dissociated into its subunits, two general types of subunits were found. There was a large, nonenzymatic subunit and several enzymatic subunits. The large, nonenzymatic subunit was essential for degradation of the crystalline cellulosome and for the binding of the endoglucanase subunits. The enzymatic subunits that dissociated from the scaffolding protein were capable of digesting only the soluble or amorphous forms of cellulose. Thus, the complex between the scaffolding protein and the endoglucanases and exoglucanases were necessary for the digestion of crystalline forms of cellulose (Doi *et al.*, 1994). The subunits of cellulosome are packed into polycellulosomal protuberance-like organelles known as protubozymes. The cellulosomes mediate cellular adhesion to cellulose and, upon binding, the protubozymes undergo a dramatic conformational change, forming protracted contact corridors between the cell and the substrate. The process is further facilitated by noncellulosomal cellulases and as the cell matures, cellulosomes are also released into the extracellular matrix, where they continue their cellulolytic activity (Bayer, Morag and Lamed, 1994).

Cellulose binding domains

Many glycoside hydrolases have substrate binding domains that function independent of the catalytic domain. Cellulose-binding domains are

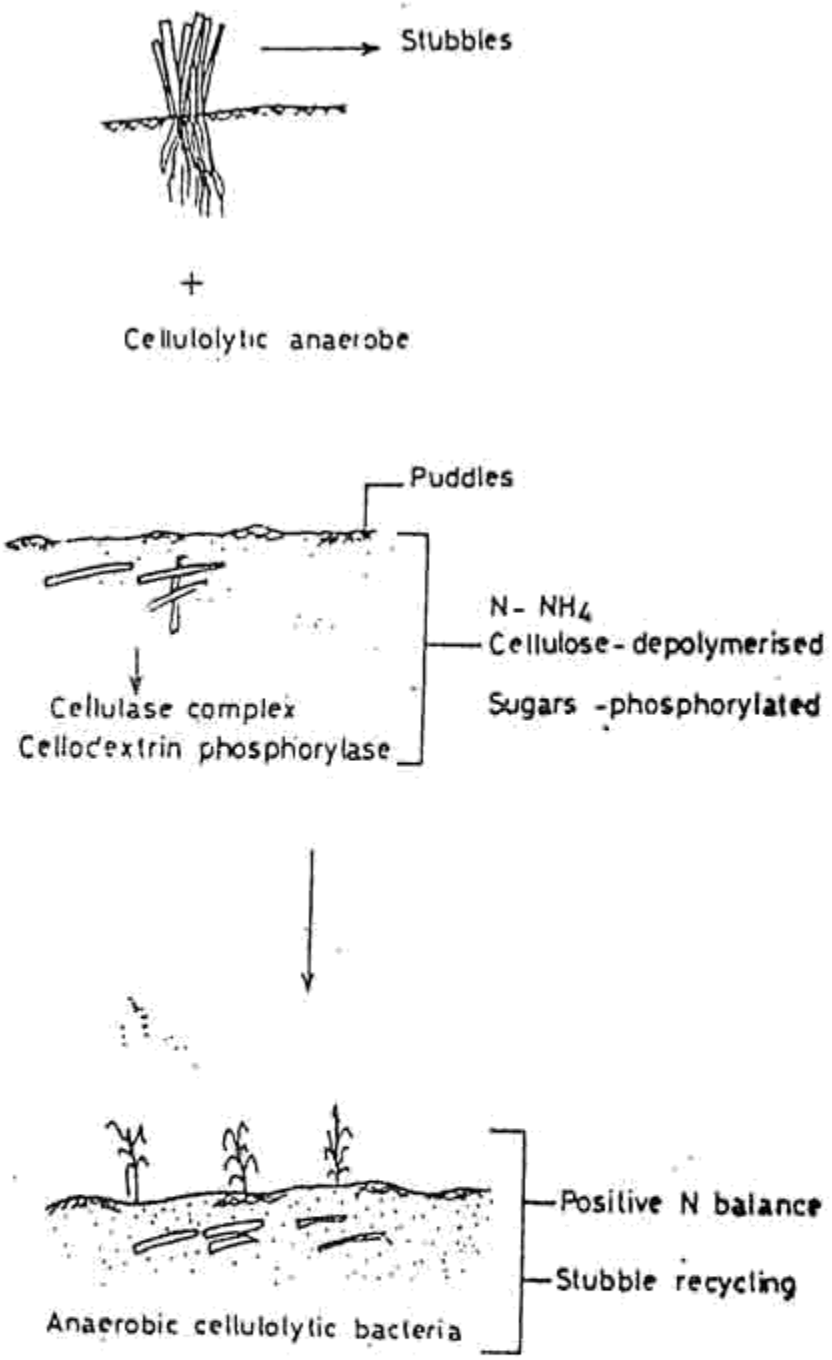


Fig. 4 Nitrogen mobilisation by cellulolytic anaerobes in flooded soil
Straw incorporated in soil acts as suostrate for the microorganisms. The bacteria derives the required biological currency by phosphorylating the substrate through its phosphorylase enzyme and uses this energy for the fixation of nitrogen

present in many cellulases. Majority of the cellulases are modular proteins. All of them have a catalytic domain; many of them have discrete, independently functioning cellulose binding domains (CBDs) that are devoid of hydrolytic activity. They form nine families of related amino acid sequences. One family is restricted to fungi and five to bacteria. The fungal domains are about 35 amino acids long; the bacterial domains range from about 80 to 250 amino acids in length depending on the family. Cellulose binding domains differed in their affinities for cellulose. Some bind to amorphous and some to crystalline cellulose; atleast one binds only to amorphous cellulose and one only to crystalline cellulose. Cellulose binding domains have been differentiated on the basis of the conditions required to desorb them from cellulose into high-affinity and low-affinity types without any measurements of the association constants. Most binding domains are smaller than most catalytic domains; so they may be unable to form effective catalytic folds. The

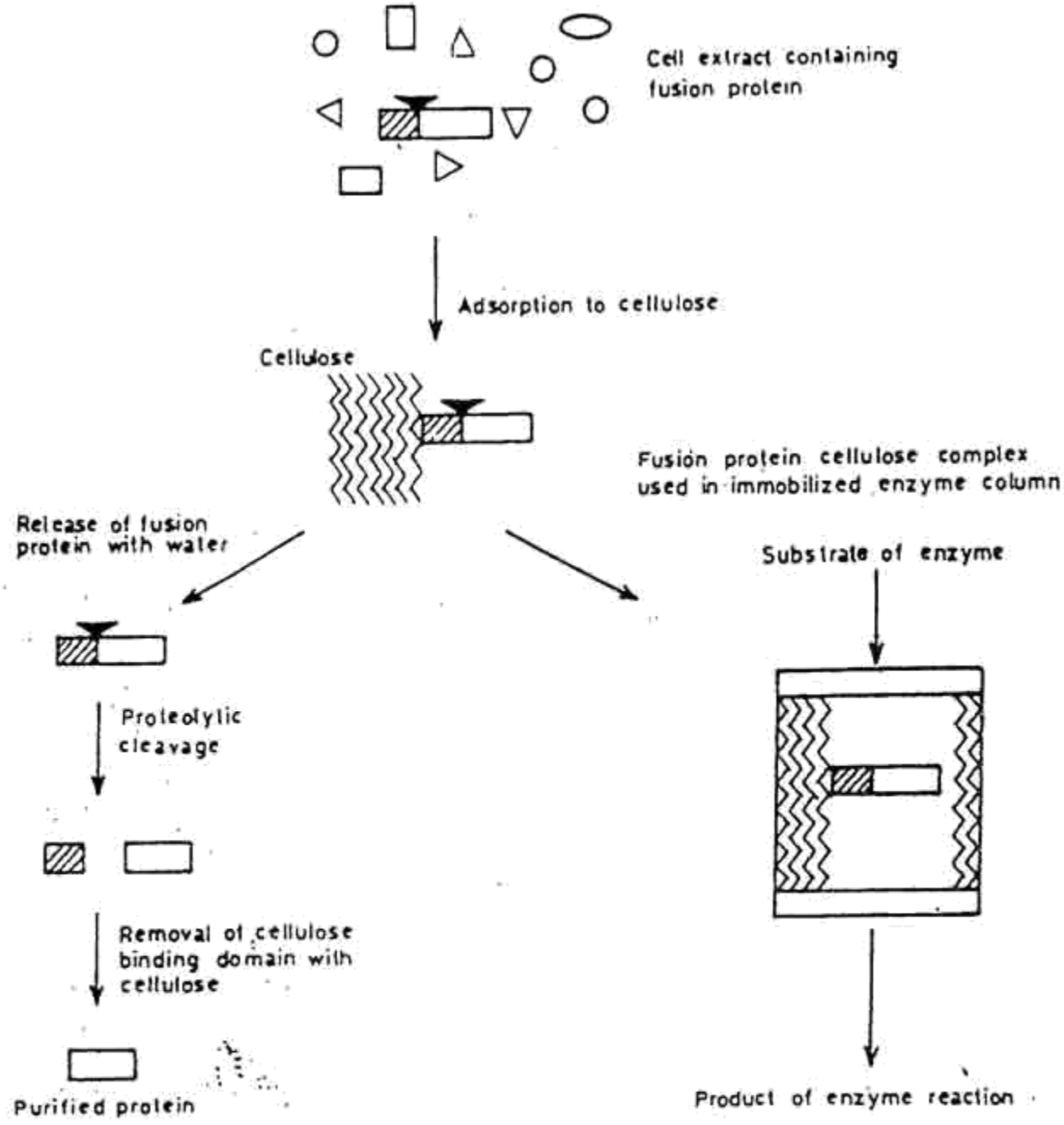


Fig. 5 Applications of cellulose-binding domains in protein purification and enzyme immobilization (Ong *et al.*, 1989)

only similarity between a catalytic and a substrate binding domain occurred in endoglucanase II from *T. reesei*. A sequence of about 100 aminoacids in the middle of the catalytic domain is related to family II cellulose-binding domains (Warren, 1996). Binding of cellulases to cellulose is mediated by an independent domain separated from the catalytic domain by a linker sequence. The cellulase enzyme possesses a short cellulose binding domain which is linked through a hinge region to a larger catalytic domain or core protein. It is a tadpole-shaped enzyme with the head comprising the catalytic domain and the tail comprising the cellulose binding domain. Cellulase activity was greatly impaired by the loss of the binding domain whether or not it is involved in hydrolysis (Din *et al.*, 1991).

Studies on the cellulosomes of *Cl. cellulovorans* by Doi *et al.* (1994) indicated that cellulose binding protein A (Cbp A) is the major nonenzymatic scaffolding protein with several functional domains. The Cbp A interacts with a number of endoglucanases to form an active cellulase enzyme. The presence of functional

domains in Cbp A is based on the derived aminoacid sequence of Cbp A. There is a cellulose binding domain (CBD) with four hydrophilic domains (HLD) and nine hydrophobic domains (HBD).

The single CBD at the N terminus of Cbp A was responsible for binding the cellulosome to the crystalline cellulose substrate (Doi et al. 1994). They also reported that nine endoglucanase binding domains (EBDs) comprised the major part of Cbp A and these EBDs were binding sites for the endoglucanases that were associated with the cellulosome. The fact that the cellulosome binds preferentially to crystalline cellulose and that cellulosomal derived free endoglucanases can only degrade amorphous cellulose suggested that part of the function of the cellulosome is to convert the crystalline cellulose to the amorphous form. Removal of the CBD by proteolysis or genetic manipulation reduced the hydrolytic activity of the catalytic domain on insoluble cellulose but not on soluble derivatives of cellulose. Discrete binding domains were also found in enzymes that hydrolyse other insoluble substrate such as chitinases and amylases. Removal of the binding domains from some of these enzymes also decreased their activities against insoluble substrates. These observations implied a general role for binding domains in the hydrolysis of insoluble polysaccharides (Din *et al.*, 1994). The structural organization of cellulase gene in *Cl. thermocellum*, *C. fimi* and *Aspergillus niger* are shown in Figure 3. Wood *et al.* (1988) detected two immunologically distinct cellobiohydrolases CBH I and CBH II in the extracellular medium of *T. reesei*. It has been proposed that the carboxy terminus of CBH I and the amino terminus of CBH II may represent the cellulose binding domains of these enzymes (Henrissat *et al.*, 1988).

Applications of cellulose binding domains

Adherent nature of cellulolytic bacteria was found to be positively correlated to cellulolysis (Thanga and Ramasamy, 1996). In natural environments such as flooded water ecosystems, these anaerobic cellulolytic bacteria adhered to the available substrates or roots of paddy seedlings and were prevented from wash out due to irrigation. The adherent cellulolytic clostridial cells were also found to fix atmospheric nitrogen deriving their energy by degrading straw, which is incorporated in paddy soil (Figure 4). Further during decomposition of the straw, they release organic acids which aids in solubilization of rock phosphate. As a consequence, the adherent nature not only help the organisms to be in the vicinity of the substrate, but also mobilise nutrients to soil ecosystem and help in nutrient cycle and soil enrichment (Thanga, 1997). Cellulose binding domains have wide application in protein purification and enzyme immobilization. Cellulose-binding domains bind to and are eluted from cellulose under mild conditions and specific reagents are not required. Under appropriate conditions, the binding is strong enough for enzyme immobilization and the cellulose binding domain allows purification and immobilization in a single, simple step as shown in Figure 5 (Ong *et al.*, 1989). Various heterologous enzymes and other biologically active materials have been fused to cellulose binding domains in order to immobilize them to cellulose. Cellulosomal domains may provide an alternative and auxiliary affinity system for a variety of applications. Cohesins can be fused or conjugated to protein A, antibodies, lectins, DNA etc. to form hybrid biomolecules (Bayer *et al.*, 1994). Selected domains, which exhibit a desired activity, specificity or function can be incorporated by crosslinking or by fusion into the components of unrelated affinity systems. The resultant hybrid biomolecules can then be used to mediate the molecular counterparts for a variety of applications.

Future Prospects

A dynamic adsorption-desorption mechanism plays a key role during the hydrolysis reaction and the physico-chemical surface characteristics usually determine the amount of biocatalyst that is bound to the substrate, the rate and time of desorption and the activity of the bound biocatalyst. Little is known about

- ☆ what regulates and controls their adsorption into the substrate ?

☆ how enzymes are employed by microbes in natural ecosystems ? and

☆ how the enzyme components interact while adsorbed on heterogenous surfaces?

Knowledge on these areas would help in the application of adsorption criterion to productive systems. Purification and characterization of these complexes will lead to a better understanding of the intricate enzymatic mechanism through which cellulose is degraded in anoxic environments.

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