TRANSFER OF DNA CODING FOR CELLULASES FROM CELLULOMONAS TO BACILLUS SUBTILIS BY PROTOPLAST FUSION

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BY

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CERTIFICATE

Certified that the work incorporated in the thesis "Transfer of DNA coding for cellulases from Cellulomonas to Bacillus subtilis by protoplast fusion" submitted by Mr. D.V. Gokhale was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Prof. D.N. Deobagkar

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ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CMC	Carboxymethylcellulose
DABT	Dimethyl amino azobenzene
DNS	3, 5 Dinitrosalicylic acid
EDTA	Ethylene diamine tetra-acetic acid
HEC	Hydroxyethyl-cellulose
NCIM	National Collection of Industrial Microorganisms
POP	2,5-diphenyl-1,3-oxazole
POPOP	1,4-bis-[-2-(4-methyl-5-phenyl) 3-oxazolybenzene
PVP	Polyvinyl pyrolidone
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate

SSC

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ABSTRACT

Abstract of the thesis submitted to the University of Poona for the degree of Doctor of Philosophy in Chemistry (Biochemistry)

Name of the Research Student : Mr. D.V. Gokhale

Name of the Guide : Prof. D.N. Deobagkar

Title of the Thesis : Transfer of DNA coding for cellulases from Cellulomonas spp. to Bacillus subtilis by Protoplast Fusion.

Place of Research : Biochemical Sciences Division, National Chemical Laboratory Pune - 411 008.

With the advent of genetic engineering and recombinant DNA technology, prospects of improving industrially important bacterial strains have become more promising . Apart from improving the existing biochemical pathways for production of end products through mutations in the genes concerned, or in other relevent genes, or by the selection of the better strain by enrichment, it has been possible to introduce a new gene from another organism so that its expression not only complements the existing phenotype but also adds new desirable phenotype in certain cases. However, the technology has been established mostly employing tailor cut recipients which are normally deficient in recombination and restriction modification sytems. Manipulations of naturally occuring microbes which are used in industries is thus not easily feasible by genetic engineering technology also. Further, even in the established genetic engineering systems, it has not been possible to stably tranfer and express DNA (genes) coding for a phenotype involving synergistic expression of more than one gene. This situation led us to use yet another approach to manipulate bacterial cells for transfer and expression of foreign genes. Unlike in the case of animal and plant cells, bacterial protoplast fusion and its mechanism were reported as late as 1976(Fodor and Alfoldy, 1976; Gabor and Hotchkiss; 1979).

Both intraspecies and interspecies fusion in bacteria was demonstrated. It was also established that stable recombinants are generated through such cell engineering. Such cell engineering however, has not been reported using unrelated bacteria from different genera. Engineered and properly selected bacteria could be furhter manipulated using recombinant DNA technology.

This thesis presents evidences for stable transfer and expression of phenotypes involving multiple genes between organisms from unrelated genera, viz., Cellulomonas and Bacillus. The phenotype studied was cellulose degradation which involves at least three types of enzymes: endoglucanase, exoglucanase and β- glucosidase. Several bacteria have been found to produce cellulolytic enzymes. Cellulomonas produces active cellulase complex but in relatively low amounts. Both qualitative and quantitative improvements of the desired enzymes could be achieved by introducing various kinds of mutations in concerned genes. Application of recombinant DNA technology in the process of conversion of cellulose to glucose has been recently initiated in various research groups worldwide. Whittle et al (1982) first isolated a cellulase gene from Cellulomonas fimi and cloned in E.coli successfully . Later, cellulase genes of other organisms like Clostridium (Cornet et al ,1983), Bacillus (Sashihara et al, 1984), Thermomonospora (Collmer and Wilson, 1983) and Streptomyces (Coppolecchia et al, 1987) have been cloned E.coli. Since cellulolytic phenotype involves expression and activity of more than one gene product, simple mutations or transfer of individual isolated genes are not always enough in improving the quality and quantity of cellulase system.

The first part of the thesis deals with optimization of conditions to obtain protoplast from Cellulomonas and B.subtilis, their regeneration and fusion. Various stable hybrids were selected for their cellulase producing phenotype (from Cellulomonas) and auxotrophic markers (from the other parent B.subtilis). The isolated hybrids exhibited various morphologies ranging from

bacillus type rods to cocci and chains thereof and also Gram positive and and Gram negative cell walls. Among the Gram positive hybrids, though all of them were selected for cellulase production (cel^+), not all were capable of secreting significant levels of cellulases in the medium. These hybrids showed sporulation.

In the second part of the thesis, biochemical and molecular biological characterization of the stable hybrids is described. (A) Initially, the hybrid status of the isolates was further confirmed by DNA:DNA hybridization in colony blot and dot blot techniques using 32P labelled parental DNAs as probes. Since the reversion frequency of the hybrids in terms of $\underline{Cel}^+$ phenotype was found to be very low, (10^{-6}), and since these genes were stably expressed in the hybrid with Gram positive, sporulating phenotype, it was of interest to find out the methylation status of DNA in the hybrids. Using various restriction endonucleases and iso- schizomers to distinguish between methylated (5mC and 6mA) and unmethylated Cytosine and Adenine, it was found that there was a significant decrease in the amount of methylated nucleotides in hybrid DNAs. This observation was further confirmed by employing enzyme linked immunoassay (amplified by biotin-avadin interaction) using antibodies raised against 5mC and 6mA. These results essentially reflect on possible changes that take place during protoplast fusion and regeneration affecting restriction- modification system of the fusants and thus enabling only such hybrids to be selected. (B) One of these stable, Gram positive, sporulating and $\underline{Cel}^+$ hybrid, viz. Bs/C 005 was further studied. This hybrid was used in studies involving induction of cellulases and xylanases, intracellular and extracellular levels of these enzymes and utilization of various cellulosic substrates and simple sugars. the first striking observation was that β - glucosidase which is always cell bound/intracellular in Cellulomonas, was secreted out by Bs/C 005 when grown on insoluble cellulosic substrates. When grown on various cellulosic substrates the hybrid showed marked differences in the levels of intracellular and extracellular cellulases as compared to those of Cellulomonas. In Cellulomonas both cellulase

and xylanase were induced by any cellulosic or hemicellulosic substrate. On the other hand, in case of Bs/C 005, carboxymethylcellulose induced only endoglucanase and xylan induced only xylanase. This indicated that in the hybrid, resulting recombination that occured during fusion and regeneration, has led to reorganization of the regulatory sequences on Cellulomonas genomic component such that the induction of these enzymes could be seggregated. Alternatively, recombination might have led to seggregation of the two related operons altogether. In any event, the changed organization of these sets of genes seems to be stably maintained in the hybrid. These hybrid strains thus will serve as the source of genes in studying organization and regulation of glucanases in bacteria. These results also showed that Cellulomonas harbours separate xylanase, and not the same enzyme exhibiting endoglucanase and xylanase activities as reported in case of fungi. Polyacrylamide gel electrophoresis in combination with zymogram staining for endoglucanase showed that the isoenzyme patterns were different in Cellulomonas and the hybrid. Cellulomonas has two fast moving bands and two slow moving bands, while Bs/C 005 showed three major activity bands of which one was very slow moving.

The cell bound/intracellular β-glucosidase of Cellulomonas was found to be secreted by the hybrid in presence of insoluble cellulosic substrates; whereas, it was intracellular in presence of soluble or low molecular substrates. When compared with Cellulomonas enzyme, the extracellular β-glucosidase of the hybrid exhibited altered parameters like increase in thermostability and optimum temperature for activity. Cellulomonas -glucosidase was inactivate to 95% of activity in 2 min at 60°C, whereas upto 30 min pretreatment at 60°C, Bs/C 005 enzyme retained 50% activity. Maximum enzyme activity was obtained in vitro at 45°C in case of Cellulomonas enzyme, while in the later case it was at 60°C.

In a separate set of experiments, expression of α- amylase gene was studied in fusion products between B. subtilis (amylase$^+$, leu$^-$,

his$^-$, ala$^-$, trp$^-$, ura$^-$, spo$^+$, amp^s, $HgCl_2$) and Zymomonas mobilis (amylase$^-$, spo$^-$, amp^r, $HgCl_2$). Two of the stable hybrids obtained were selected on a minimal salt medium containing starch as sole carbon source and ampicillin. Distribution of $HgCl_2$ resitant marker was analyzed subsequently in the hybrid BZ1 and BZ2. Extra cellular -amylase production and activity were studied. Enzyme produced by BZ1 and BZ2 was found to be altered in reaction parameters like pH and temprature. Hybrid enzyme exhibited higher activity at lower pH (pH 4.5 to 5.0) and higher temperature (65°C) compared to B. subtilis α-amylase activity (pH 5.5 , 45°C). BZ1 and BZ2 enzymes also exhibited improved thermo tolerence retaining 95% activity even after incubation of enzyme at 50°C for 1h, whereas the B. subtilis enzyme lost its 90% activity within 30 minutes at 50°C.

Significance of these observations in manipulating multigene regulated phenotype is discussed. Possibility of obtaining enzyme with changed desirable properties by cell engineering are also discussed. It is suggested that appropriate combination of these techniques with recombinant DNA technology will be of great significance in biotechnology of microbial products and their improvements. The strains generated in this work also will help in basic research to unravel the organization, structure and regulation of these different gulcanase genes and their interactions.

CHAPTER 1

BACTERIAL CELL ENGINEERING AND BIOTECHNOLOGY OF CELLULOSE DEGRADATION : A GENERAL REVIEW

1.1. Introduction

Industrial strain improvement involves the development and modification of organisms used in the production of commercially important fermentation products with the aim to reduce the production cost. The traditional approach to strain improvement is mutagenesis of a suitable strain followed by random screening of large number of isolates. Though this approach has undoubtedly been successful and has benefited as evident from its application in recent advances in basic sciences and technology (Rowlands, 1984), random screening is labourious in order to detect rare, improved, desirable strains. Genetic manipulation through recombinant DNA technology has helped in constructing special organisms, mostly bacteria and fungi which could synthesize specific new products. Transfer and expression of genes in plants (Vaeck et al. 1987; Della-Goppa et al. 1987; Nelson et al., 1988) and animal cells (Church, 1987) and their propagation in new progeny (transgenic animals and plants) have been demonstrated. Improvement of properties in useful organisms or direct gene manipulation in human genome for correction or addition of new character does not seem to be a distant goal. The recombinant DNA technology in general allows isolation and manipulation of specific genes such that through a vector molecule they could be introduced into another organisms where they either complement existing functions or add to their phenotype. Construction of strains which synthesize products that are not normal constituents of microbial cells has been possible with the advent of this technology. The first demonstration of chemical synthesis, cloning and functional expression of human growth hormone Somatostatin by Itakura et al (1977) was followed by number of reports as the technology was refined with time (Davies, 1988; Kane and Hartley, 1988).

The commercially available enzymes are the normal constituents of cells which produce them. Microorganims such as Bacillus and filamentous fungi are normally used for commercial production of

enzymes like amylase and proteases. Hyper producers of such enzymes have been conventionally derived through mutations. There have been many reports of cloning of structural genes of these industrially important enzymes recently so that one can now attempt production ofthese enzymes in large amounts in related hosts. Extrachromosomal high copy number of autonomously replicating DNA molecules harbouring such genes help in increased production of their products.

Most established fermentation products, such as antibiotics and some commercial enzymes are multigenic in nature. Hence there is no guarantee that all the biosynthetic units will be encoded in an operon or are clustered. This makes the cloning and assembly of genes into an operative biosynthetic unit on a vector an arduous and lengthy task. Cellulose degrading phenotype is another multigenic system which involves synergistic expression of multiple genes and activities of their products. Application of recombinant DNA technology to hydrolysis of cellulose to glucose has been initiated. Successful cloning of individual genes corresponding to components of cellulase system has been reported (Cornet et al., 1983; Whittle et al, 1982), however, none of the recombinant strains as expected, has complete cellulolytic phenotype.

Another disadvantage of recombinant DNA technology while introducing desirable gene into a naturally occuring industrial microbe is that the cloned fragment of DNA might not be compatible with the restriction modification system of host organism, and hence could be rejected. This problem could be overcome by isolating restriction minus mutant strains of the recipient, which in turn is laborious process. Further such a phenotype might affect original properties of recipient organism. Added to this is a problem of plasmid instability in genetically engineered strains used in industrial fermentation. The plasmid instability is due to either structural instability (Nugent et al., 1983) or seggregative instability (Primorse et al., 1983) resulting in progressive loss of productivity. Preliminary results of Rogers

et al (1984) showed that Zymomonas mobilis could express Aspergillus niger glucoamylase activity but the transconjugants of Z. mobilis were extremely unstable and lost the ability to grow on starch after only six generations. In the same experiment, it was also suggested that restriction endonucleases in Z. mobilis might have contributed in eliminating the recombinant plasmids.

Apart from genetic engineering and recombinant DNA technology, cell engineering through protoplast fusion in fungi and bacteria has been exploited for transfer of relatively large segments of genomic DNA within related species (Godfrey, et al., 1978) or within same species (Fodor and Alfoldi, 1976, 1979). Early reports by Fodor and Alfoldi (1976) have established that intraspecies protoplast fusion resulted in stable recombinants. In fact, this technique has been used in generating genetic maps of organisms in which natural process of conjugation does not occur (Baltz, 1978). The technique also helped to overcome the noncompatibility barriers between mating types, species and also genera (Hopwood, 1981; Minuth and Esser, 1983; Peberdy, 1979). Protoplast fusion induced by polyethylene glycol has been demonstrated in several eukaryotic microorganims and in prokaryotic systems. Fusion of mammalian cells (Pontecorvo, 1977) or protoplasts from plants (Kao and Michalyluk, 1974) or fungi (Anne and Peberdy, 1976; Ferenczy et al., 1975) has been shown to be induced by polyethylene glycol. Use of PEG in bacteiral protoplst fusion was first reported by Fodor and Alfoldi (1976) and Schaeffer et al., (1976) in experiments invloving B. megaterium and B.subtilis respectively. As seen from literature, surprisingly protoplast fusion technique in cell engineering has been used much earlier for cells from higher organisms ascompared to the simpler prokaryotic microbes. However, since then the first reports of intra- (Fodor and Alfoldi, 1976) and interspecies (Ochi and Katz, 1978) protoplast fusion appeared, and a number of related reports have accumulated involving application of this technique in strain improvement mainly in two genera, Bacillus and Streptomyces. Various aspects of protoplast generation, their stability, regeneration and fusion condition are

reviewed in the following section.

1.2. Bacterial cell engineering through protoplast fusion

There are known genetic transfer mechanisms like trasduction, transformation, conjugation, sexduction and transfection operating in bacteria. These processes are unidirectional involving the transfer of only DNA from donor into recipient organisms or uptake of pure DNA. The primary products in these cases are the bacterial cells in which foreign DNA has been introduced, and in some cases integrated in the recipient genome.

Artificially induced somatic cell fusion is a useful technique in studying the genetics of prokaryotic as well as eukaryotic organisms in which no other mechanisms to exchange genetic principles have been known. Protoplasts from plant cells and fungi can be induced to fuse (Ferenczy et al., 1975; Powor et al., 1970) with the help of fusogen like PEG or by electrical field (Zimmerman, 1983). The primary products of these types of fusions are cells containing mixed protoplasm with two or three nuclei i.e. complete genome from both the parents.

Protoplast fusion induced by PEG has been demonstrated in several eukaryotes (Anne and Peberdy, 1976; Jones et al., 1976) and in prokaryotic systems (Fodor and Alfolodi, 1976; Hotchkiss and Gabor, 1980). This technique appears to have great potential to effect genetic recombination in prokaryotic and eukaryotic microbes particularly in those organisms lacking classical recombination systems. Number of reports on protoplast fusion in fungi indicated its wide applicability to eukaryotic organisms. Removal of cell wall of fungal cells not only helps in protoplast fusion but also seems to eliminate the species barrier and allows fusion even between cells of two different species. This point has been well demonstrated in protoplast fusion experiments in Saccharomyces cerevisiae (Svoboda, 1978), Schizosaccharomyces pombe (Sipinzki and Ferenczy, 1977) and Saccharomyces lipolytica (Stahl, 1978).

Attempts to obtain genetic recombination through conjugation in Candida tropicalis were unsuccessful (Fournier et al., 1977; Vallin and Ferenczy, 1978). Similarly the isolation of heterokaryons by hyphal anastomoses in Cephalosporium acremonium faced a problem since such heterokaryons could not be separated (Hamlyn and Ball, 1979). On the other hand, the recombinant isolated by intraspecies protoplast fusion in Cephalosporium when used in genetic analysis were titre tested for cephalosporin C and one recombinant was found to show 40% improvement in antibiotic production (Hamlyn and Ball, 1979). This indicated the potential value of protoplast fusion in strain breeding programme. Brodshaw and Peberdy (1984) demonstrated that the hybrids between A. nidulans and A. ruguloses did not produce detectable levels of antibiotic but some seggregants obtained after induced haploidization of the hybrids produced significantly more penicillin than A. nidulans parent. This indicated that the seggregation of one or more genes regulating penicillin biosynthesis must have occured. These results also suggested that the interspecies hybridization mediated by protoplast fusion could generate strains (recombinants) with increased antibiotic production.

As mentioned previously, protoplast fusion can also be used in genetic analysis of bacteria in which no genetic transfer mechanisms have been successful. Linkage maps of the genomes of S. aureus (Stahl and Pattee , 1983), Streptomyces spp. (Baltz, 1980; Hopwood et al., 1977) and mesophilic bacilli (Gaber and Hotchkiss, 1979; Schaeffer et al., 1976) have been generated using protoplast fusion technique. In B. megaterium, an early attempt to map genes by protoplast fusion was not successful probably becasue recovery of recombinants showed strong and variable bias (Fodor and Alfoldi, 1976). Very recently Chen et al (1986) have shown that chromosomal mapping in B. stereothermophilus could be done by protoplast fusion.

Bacillus and Streptomyces are the two genera among the most

imoportant groups of industrial microorganims. Bacilli produce variety of enzymes i.e. proteases and amylases and also few antibiotics of minor commercial significance (bacitracin and gramicidins). Streptomyces produce at least one important enzyme, glucose isomerase, and great majority of naturally produced antibiotics. Two other genera, Micromonospora and Brevibacterium are noted for the production of several antibiotics and amino acids respectively. Not surprisingly, therefore, these organisms were mainly used in early protoplast fusion experiments in an attempt to improve their desirable phenotype. Very recently, protoplast fusion in Brevibacteria was carried out to improve the glucose metabolising activity of industrial lysine producer (Karasawa et al., 1986). They also showed that lysine requiring recombinant obtained by protoplast fusion accumulated threonine with an increased yield. Yanase et al (1985) attempted the protoplast fusion in Z. mobilis using raffinose and obtained the fusants at a relatively higher frequency of 10^{-4}. One of the fusion had regained the original ability of raffinose fermentation. They further claimed that protoplast fusion in ethanol producing Z. mobilis could be useful for genetic improvement of Z. mobilis.

Conditions for isolation of healthy and functional protoplasts from various bacteria, however, are not identical and need to be established for individual organisms. Efficiency of fusion and appropriate phenotype transfer, therefore, is dependent on efficiency protoplast formation and their regeneration.

1.2.1. Protoplast formation

Specific conditions for protoplast isolation have been developed in plant cells (Kao et al., 1974; Power et al., 1970; Tanaka et al 1984), fungi (Gold et al., 1983, Picatoggio et al., 1983) and bacteria (Fodor and Alfoldi, 1976; Gasson, 1980; Hopwood, 1981; Weiss, 1976) including aerotolerant anaerobes such as Clostridia (Allcock et al., 1982; Knowlton et al., 1984; Minton and Morris, 1983).

Protoplasts of bacteria are readily obtained from Gram positive organisms such as B. subtilis and B. megaterium using lysozyme to digest the cell wall (Ghysen, et al., 1966; Schaeffer et.al., 1976). Novick et al (1980) have used lysostaphin instead of lysozyme for obtaining protoplasts from Streptomyces. Streptomyces being mycelial differ from bacilli and Staphylococci and differentiates into aerial phage consisting of chains of spores under appropriate conditions. They are readily protoplasted with lysozyme. (Douglass et al., 1958; Okanishi et al., 1974; Sagara et al., 1971) although specific conditions of cultivation have been shown to be important in some instances.

The physiological status of organism at the time of protoplasting is the major factor in determining protoplast yield. It has been shown that the culture in exponential growth phase gave the highest protoplast yield. (Baltz, 1978; Okanishi et al., 1974; Peberdy et al., 1976; Shahin, 1972). The grwoth medium also has important effect in protoplast formation. Sagara et al (1971) found that Streptomyces mycelium subcultured into the medium containing enough glycine was more sensitive to lysozyme than the mycelium grown in absence of glycine. The levels of glycine vary from 0.8 to 3.5% in different species (Baltz, 1978; Hopwood et al., 1977). The significance of glycin in increasing the susceptibility of mycelium to lysozyme has not been resolved. Some species of Streptomyces grown in presence of glycine are protoplasted even without lysozyme treatment (Hopwood, 1981). Rodicio et al (1978) observed that very young mycelia of some species of Streptomyces even when grown in absence of added glycine were converted to protoplast after treatment with lysozyme. Okanishi et al (1974) when used a mixture of lysozyme and lytic enzyme No.1 from Cytophaga observed rapid protoplast formation than by lysozyme alone. These observations necessarily suggest that the variations in cell wall composition affect the protoplast formation and differences in response to various enzymes.

Protoplast isolation from Gram negative organisms is usually more difficult due to presence of more complex cell envelope (Costerton et al., 1974). A thin layer of peptydoglycan which is sensitive to degradation by lysozyme is sandwiched between the inner membrane and the outer envelope made up of the giant macropolymer called a lipopolysaccharide. The peptydoglycan is shielded from the attack of lysozyme by this outer envelope. The earlier attempts to isolate protoplasts from Gram negative organisms were unsuccessful, wherein formation of osmotically sensitive bodies which retain part of the cell wall were observed. These osmotically sensitive bodies were called the sphaeroplasts. Weiss (1976) described a procedure whereby combination of lysozyme and EDTA convert E.coli cells to true protoplasts. Another procedure described by Rodicio et al (1978) for isolating true protoplasts described use of antibiotic fosfomycin in grwoth medium which inhibits cell wall synthesis. However, this procedure was not suitable for obtaining good yield of protoplasts from another Gram negative organism Serratia marcescens. The standard lysozyme-EDTA method of Weiss (1976) sometimes does not work with some Gram negative bacteria like Z. mobilis as shown by Yanase et al (1985). They cultured Z. mobilis in medium containing pecillin G or glycine to generate sphaeroplasts and observed that more than 99.99% of the cells have been converted to sphaeroplasts after 10 hours. Very recently, a highly efficient procedure for quantitative conversion of E.coli cells to sphaeroplasts has been developed by Marvin and Witholt (1987) in which very less concentration of lysozyme was used.

1.2.2. Protoplast regeneration

For any genetic work on protoplast fusion or protoplast transformation, successful regeneration of protoplasts into normal cell is essential in order to give progeny which could be genetically characterised. The minimum requirement for the regeneration of protoplsts is the presence of osmotic stabilizer in the solid media. For regeneration of protoplasts of yeast and

Gram positive bacteria, an osmotic stabilizers such as 0.6 M KCl (Ferenczy and Maraz, 1977), 0.5 M Nacl (Okanishi et al., 1974), 0.5 sodium succinate (Kaneko and Sakaguchi, 1979), 20% sucrose (Akamatsu and Sekiguchi, 1981) or 16% sorbitol (van Solingen and vander Platt, 1977) have been used. It has been found that the regeneration of B. subtilis protoplasts occurs efficiently when plated on solid media such as 25% gelatin or higher concentration of agar (2 - 2.5%) containing 2% gelatin or serum (Gabor and Hotchkiss, 1979). Okanishi et al (1974) stressed the importance of the concentrations of constituents used for regeneration. In case of Str. purvulus the protoplast regeneration frequency was observed to be 5 times greater when 15% sucrose was used as an osmotic stabilizer (Ochi et al., 1979). Moreover, no regeneration was observed when sucrose was replaced by 0.3 or 0.5 M NaCl. Yanase et al (1985) used different stabilizers for regeneration of sphaeroplasts of Z. mobilis and found that sucrose and sorbitol were most suitable. In addition to osmotic stabilizer 25 mM each of $CaCl_2$ and $MgCl_2$ and low levels of phosphate were found to be necessary for efficient regenration of Str. parvulus protoplasts (Ochi et al., 1979). It has been observed that the regeneration frequency of B. subtilis protoplasts was raised by addition of plasma expanders such as polyvinylpyrolidone, vinyl-pyrolidone and dextran at 3% concentrations to the regenration medium (Akamatsu and Sekiguchi, 1981), but no effect of such plasma expanders were observed on regeneration of Z. mobilis protoplasts (Yanase et al., 1985).

Regeneration frequency is generally affected by various factors. Baltz and Matsushima (1981) obtained 10 fold increase in regeneration of Str. fradae protoplasts when plated on dehydrated regeneration plates (about 20% water loss). Increase osmolarity of the hypertonic regeneration medium is helpful in some species. The temperature at which the culture was grown prior to protoplasting also affects the protoplast regeneration and also the optimum temperature of 29° C has been reported in case of Str. fradae (Baltz and Matsushima, 1981). The effects of concentration

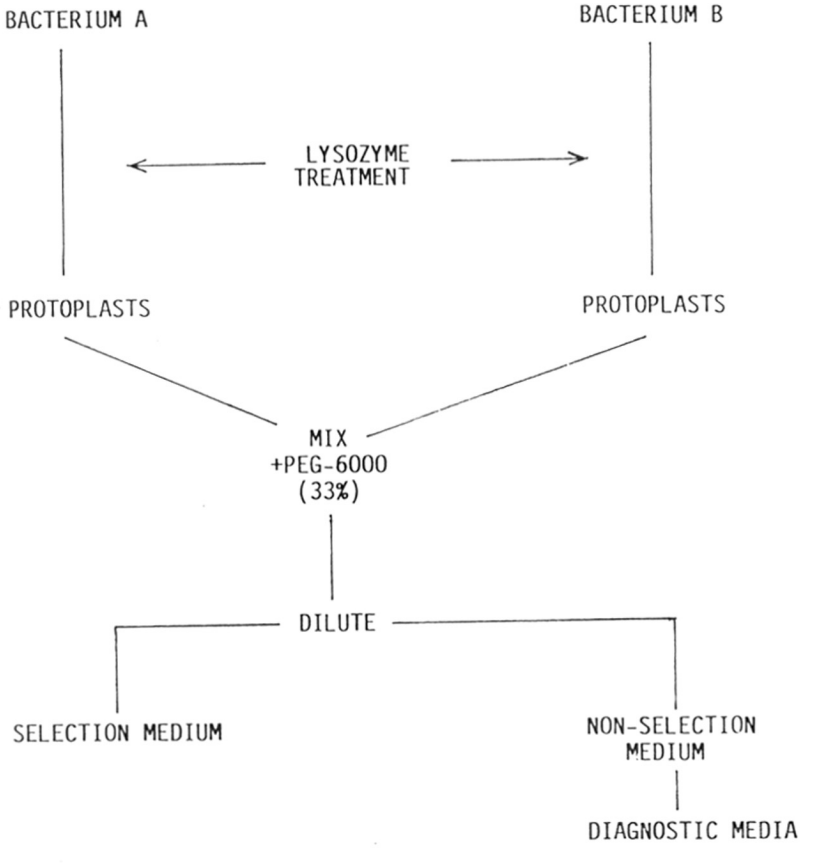
of penicillin G or glycine added at the time of sphaeroplast formation on the regenration of Z. mobilis protoplsts were investigated by Yanase and coworkers (1985). They found that the protoplasts prepared with lower concentration of penicillin G or glycine showed higher regeneration frequency. Casamino acids stimulate the recovery of protoplasts injured by preparation procedure and activate general cellular metabolism and expressed diverse effects on regeneration. Recently, it was found that penicillin treatment was not essential for protoplast formation, however, it was observed that it increased regeneration frequency in Cellulomonas probably by stimulating cell wall synthesis (Kim and Lee, 1985). They also found that sucrose (0.5 M) inclusion in medium at the time of propagation of cells increased regeneration frequency. Landman and colleagues (1968) have reported the B. subtilis protoplasts do not regenerate in liquid medium but trypsin treated protoplasts regenerate in liquid medium containing 10% gelatin. (Decastro- Costa and Landman, 1977). It has been proposed that reversion inhibitory factor is inactivated by gelatin or other hard surfaces or by serum. Hadlaczky et al (1976) reported on the regeneration of protoplasts in liquid media and indicated 3 phases of regeneration sequences such as increase in the size of individual protoplst followed by non-oriented division of protoplast and outgrwoth of bacilliary form.

In general, one can see from available reports dealing with various conditions for obtaining optimum yield of regenerating protoplasts of bacteria that there are more state-of art protocols than general fundamental technique for bacteria as a whole.

1.2.3. Protoplast fusion

The generalised scheme for fusion of bacterial protoplasts has been shown in Fig. 1.1. The procedure involves a use of two parental strains that are genetically marked with complementary auxotrophies of additional selectable markers unique to either parent or generated through prior mutagenesis. Protoplasts of both parental

Fig. 1.1. Schematic Representation of Protoplast Fusion in Bacteria.



strains are mixed in equal numbers and treated with fusogen. PEG induces the aggregation of protoplasts and fusion events occur after the fusogen is washed or diluted away. The PEG treated mixed protoplasts are then plated onto suitable media and the fusion products are recovered by direct or indirect selection. In studies on B. megaterium (Fodor and Alfoldi, 1976), several Streptomyces strains (Baltz, 1978; Ochi et al., 1979) and E.coli (Tsenin, et al., 1978) direct selection method for recovery of fusants has been used. In the case of auxotrophic strains this involved plating of fused protoplasts on the selective regeneration medium lacking the corresponding growth factor. Development of colony on such selective medium depended on the ability of parental genomes within the fusion bodies to complement and generate new phenotype. Complementation could be a result of either recombination between genomes or sometimes, presence of both genomes separately in the same cell. In the later event, one could observe segregation of the corresponding markers in the progeny. The indirect selection for the recovery of recombinants has been used in B. subtilis (Gabor and Hotchkiss, 1979; Hotchkiss and Gabor, 1980; Schaeffer et al., 1976), Streptomyces (Hopwood and Wright, 1979; Hopwood et al., 1977) and Providencia (Coetzee et al., 1979). In this method a protoplast regeneration was allowed to take place on non selective medium and recombinants were selected by subsequent transfers to appropriate diagnostic media, making use of techniques like replica plating from croweded plates. In Streptomyces the recombination frequencies were measured in two ways (Hopwood and Wright, 1978; 1979). In the first case, spores from confluent or subconfluent growth on regeneration plates were harvested and the resulting spores suspension was plated on selective or non selective media. In the second case, a spores from individual regenerated colony were analysed for the presence of recombinants. Hotchkiss and Gabor (1980) have adopted a second method for analysing well separated regenerated colonies obtained after fusion in B. subtilis and confirmed multiple recombinational events during protoplast fusion.

1.2.3.1. Optimal Conditions for protoplast fusion

Protoplast fusion is influenced by a variety of factors. Both in Streptomyces and fungi a concentration of PEG was found to exert opposite effects on fusion process. In Streptomyces, frequency of fusion was found to increase PEG concentrations (Hopwood and Wright, 1979); but thr reverse was observed in P. chrysogenum (Anne and Peberdy, 1975) and A. nidulans (Ferenczy et al., 1976). Hopwood and Wright, (1979) examined whether dimethyl sulphoxide could enhance the fusion in Streptomyces coelicolor but no consistent improvement in recombinant formation was observed. Exposure time of protoplasts to PEG affects the fusion frequency. Only short period of exposure is necessary to induce protoplast fusion; because exposure of protoplasts to PEG for periods upto 60 min did not increase the fusion frequency in P. chrysogenum (Anne and Peberdy, 1975). The brief treatment of 50% PEG 1000 gave 10-20% recombinants in Strepromyces coelicolor (Hopwood and Wright, 1979). In other Streptomyces species there are no reports on systematic studies on optimal conditions for a genetic recombination. Baltz (1978) used PEG-6000 at 36% concentration and found that about 0.3 % of regenrating colonies contained recombinants. In some cases, higher frequencies were obtained by increasing the concentration of PEG 6000 or by using PEG 1000 (Baltz and Matsushima, 1981). Similarly, Ochi and Katz (1978) found high frequencies of colonies containing recombinants when 42% PEG 4000 was used. Later, when different molecular weights of PEG were tested, PEG-6000 was found to be significantly inferior to PEG-4000 and PEG-1540 was found to be inferior to PEG-6000 in the experiments with interspecies protoplast fusion between S. parvulus and S. antibioticus (Ochi et al., 1979).

In first two reports on protoplast fusion in Bacillus spp., 36% PEG-6000 was used and recombinants were obtained at a frequency of 0.5 % (Fodor and Alfoldi, 1976; Schaeffer et al., 1976). Schaeffer and Hotchkiss (1978) observed that PEG polymers 200, 400, 1000, 6000 and 20000 gave very similar number of regenerating colonies

containing prototrophic recombinants, provided that their concentration (w/v) was similar. In Bacillus system, a brief exposure of protoplasts (one minute) to PEG was important. In studies of fusion of L forms of Staphylococcus aureus, 50% PEG was more effective than 30% PEG in inducing recombination. They also found that PEG-6000 appeared to be superior to PEG 4000 and PEG 1000 (Hirachi et al., 1979).

In studies of protoplast fusion in Gram negative bacteria (Coetzee et al., 1979; Tsenin et al., 1978), variations in PEG treatment have not been explored. Tsenin et al (1978) first reported recombination by protoplast fusion in E.coli using 40% PEG-6000. Prototrophic colonies at frequencies of 10^{-3} were obtained with 1% regeneration on selective medium. About 10% of the selected colonies were prototrophs and other were mixtures of parental or recombinant genotypes, some of which continue to segregate. Coetzee et al (1979) reported protoplast fusion in Providencia alcalifaciens in which prototrophic colonies were selected on non selective medium and subsequently replicated on selective plates. Conditions for fusion in Cellulomonas were studied in terms of PEG concentrations and duration of PEG treatment by Kim and Lee (1985). They achieved highest recombination frequency with 40% PEG-3340 while the regenration frequency was decreased as the PEG concentration increased upto 75%. These observations suggest that the type and concentration of PEG used for optimum results probably varies depending on cell size and composition of cell membrane material with which PEG molecules actually interact.

Protoplast fusion when applied to the production of new genotype of industrial microorganisms, counter selection of one of the parental types in a fusion could be advantageous since some classes of recombinants with desired complementation could be enriched which is otherwise not feasible. Alternative approaches other than the use of auxotrophic strains has been employed by various workers. Use of non-viable protoplasts which can function as a vector of genetic material was successfully done by Levi et al.,

(1977) when streptomycin killed B. subtilis protoplasts were fused with streptomycin resistant strain. In this case frequency of fusants obtained was ten fold less compared to that obtained using live protoplasts. In B. megaterium heat killed protoplasts were fused with viable protoplasts but the yield of recombinants was considerably low compared to control fusion of viable protoplasts (Fodor et al., 1978). UV-killed protoplasts of Streptomyces coelicolor were used in fusion experiments by Hopwood and Wright (1981).

1.3. Biochemistry of cellulose degradation

Last decade has witnessed the intensified research worldover for alternate energy sources which could substitute petroleum. Apart from attempts to harness solar energy, possibility of conversion of renewable resources like biomass into useful energy has attained importance for the scientists and industrialists alike. The most readily available sources of biomass are in the form of agriculture and municipal waste residues. The major components of this biomass are cellulose, hemicellulose and lignin. Composition of different cellulosic residues is variable, most of these contain 30 - 40 % cellulose, 15 - 25% hemicellulose and 5 - 15 % lignin. Thus 60 - 70% of the biomass is available as carbohydrate whcich could be converted to fuels and useful chemicals.

Cellulose and hemicellulose could be converted to glucose and xylose respectively by enzymatic hydrolysis. Glucose could then be used in synthesis of various solvents, plastics and other chemicals which are now made from petroleum. Xylose derived from xylans offers potential feedstock for generating food and fuels (Jeffries, 1985). Lignin is noted for its resistance to biodegradation. However, it is well suited to be used for production of low molecular weight phenolic chemicals (Crawford and Crawford, 1980).

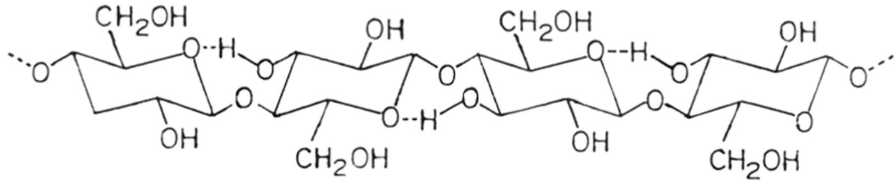
1.3.1. Structure of cellulose, hemicellulose and lignin

Cellulose is highly regular linear polymer of unhydro-D-glucopyranose units joined by β-1, 4-glucosidic bonds. It is a macromolecule with a degree of polymerization upto 10000 with a molecular weight range of 3-5 x 10^5 daltons. In the cellulose chain the unhydroglucose units form a chair like configuration projecting the hydroxyl groups at equatorial position and hydrogen atoms at the axial sites (Fig. 1.2). The long parallel cellulose chains are strongly hydrogen bonded. These chains exist as aggregate to form large crystalline microfibrils which are visible by electron microscopy (Cote, 1977).

Unlike cellulose, hemicelluloses are heteropolymers containing 2 to 4 and rarely 5 to 6 different kinds of sugars. Commonly occuring heteropolymers are L-arabino-D-xylans, L-arabino-D-glucurono-D-xylans, 4-O-methyl-D-glucurono-D-xylans, D-gluco-D-mannans, D-galacto-D-gluco-D-mannans and L-arabino-D-galactans. Hemicelluloses are classified according to type of sugar present. Thus, xylan is a polymer of xylose, mannan of mannose and galactan of galactose. The major hemicellulosic constituents of lignocellulosic waste materials are the hetero-1, 4-β-D-xylans and hetero-1, 4- β -D-mannans. The heteroxylans constitute a major hemicellulosic components of graminae (grass and cereals) and angiosperms (hardwoods). The mannans are more abundant in gymnosperms (soft wood).

Lignin, distinct from cellulose and hemicellulose is widely distributed throughout the plant kingdom except in lichens and mosses (Pearl, 1977). Plant lignins are divided into three major categories which are commonly called as soft wood (gymnosperms), hard wood (dicotyledonous angiosperms) and grass and annual plant (monocotyledonous angiosperms) lignins. It is a polymer synthesized by oxidative polymerization of three substituted cinnapyl alcohols viz. trans-p-coumaryl alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol (Sarkanen and Ludwig, 1971). The

Fig. 1.2. Structure of Cellulose



proportion of these three precursor alcohols differ between angiosperms and gymnosperms. The soft wood contains 8-10% coniferyl alcohol residues (Ander and Eriksson, 1978) whereas angiosperm lignin are frequently derived from equal proportions of coniferyl and sinapyl alcohols.

1.3.2. Factors affecting degradation of cellulosic materials

The lignin sheath and the crystalline nature of cellulose in untreated wood material makes it usually resistant to biodegradation. Cowling (1975) and Cowling and Kirk (1976) have listed the following factors affecting the breakdown of cellulosic materials.

(1) Moisture content of the fibre.

(2) Size and diffusibility of the cellulolytic enzymes and other melecules in relation to the size and surface properties of the gross capillaries, and the space between the macrofibrils and cellulose molecules in the amorphous regions.

(3) The degree of crystallinity of cellulose. Dunlap and coworkers (1976) observed the linear inverse relationship between crystallinity index and digestibility.

(4) The unit cell dimension of cellulose.

(5) The conformation and steric rigidity of the unhydroglucose units.

(6) The degree of polymerization of cellulose. The length of cellulose molecule in the fibre varies over a wide range. α-cellulose contains as many as 10000 - 15000 glucose units per molecules whereas γ-cellulose contains less than 15 glucose units. This variation is expected to affect the rate of hydrolysis considerably, especially of enzymes which cleave the cellulose

molecules by an endwise mechanism.

(7) The nature of substances with which cellulose is associated.

(8) The nature, concentration and distribution of substituent groups. Substituted cellulose derivatives are more susceptible to enzymatic attack as they become more water soluble and less crystalline upto the complete solubility point (Fan et al., 1980).

1.3.3. Pretreatment of cellulose

In order to achieve maximum disruption of cellulose fibre, two types of pretreatments have been generally employed. Depending on the nature of the substrate, the choice is made considering effectiveness and economical factors for a given method. Physical methods include ball milling, hammer milling, boiling, high pressure steam exposure, electron irradiation and gamma irradiation. The chemical pretreatments include a treatment with alkali, acid, sodium hydroxide, solvents and swelling agents. Among these, sodium hydroxide and sulphur dioxide gas pretreatments are more viable chemical methods, the former being useful in disruption of lignin structure, as well as hydration and swelling of cellulose. This decreases the crystallinity in cellulose. A sulphur dioxide method, on the other hand, is effective for agricultural waste with high lignin content. The lignin content of various woods has been found to be decreased after this treatment (Moore et al., 1972). Physical treatment, in general, disrupt crystallinity leading to decrease in particle size so that there is a net increase in the available surface area of substrate for enzymatic attack. The methods used for pretreatment of cellulose are listed in Table 1.1

1.3.4. Biological degradation of cellulose

Native cellulose is degraded by the synergistic action of a group

Table 1.1. Methods for pretreatment of cellulose (Klyosov, 1986)

Mechanical	Chemical	Physical	Biological	Combinations
Ball milling	Phosphoric acid	Steaming	White rot fungi	Steam explosion
Two roll milling	Hydrochloric acid	Wetting	--	High temperature milling
Hammer milling	Sulphuric acid	Pulping	--	Alkali + Ball milling
Vibratory rod	Acetic acid	Freezing/ thawing	--	SO_2 + steaming
Colloid milling	Sodium Hydroxide	radiation	--	Biomechanical pulping
Extrusion	Ammonia			
	SO_2			
	Cadoxen			

of enzymes viz. endo- β -glucanases, exo- β -glucanases and β -glucosidases (Eriksson and Petersson, 1975; Halliwell, 1975). These cellulolytic enzymes are produced by a variety of microorganisms like fungi, actinomycetes, gliding bacteria (myxobacteria) and eubacteria (Goksoyr et al., 1975). Many of them do not grow on native cellulose but do produce cellulolytic enzymes. Such microbes utilize partly degraded cellulose for their growth.

Cellulases are either intracellular (cell bound) or extracellular depending on their location. Enzymes like β-glucosidase acting on cellobiose or low molecular weight cellodextrins are usually located in the periplasmic region, and are true intracellular as in case of Cellulomonas strains (Antheunisse, 1984; Haggett et al., 1978). β -glucosidase in T. reesei (Nanda et al., 1982) is also reported to be trapped in the space between cell wall and cytoplasmic membrane, whereas Aspergilli are known to produce high amounts of extracellular β-glucosidase (Sternberg et al., 1977).

Fungi, in general, have the ability to attack cellulose and produce extracellular cellulolytic enzymes. Among them T. reesei, P. funiculosum, F. solani, A. terreus and S. pulverulentum are the best producers of celluloytic enzymes. Thermophilic fungi, namely Chaetomium thermophile and S. thermophillium produce thermostable enzymes attacking native cellulose. They all decompose cellulose rapidly, but low cellulase activity was detected in their culture filtrate (Mandels, 1975). T. reesei is the most extensively studied fungus for the cellulolytic enzyme production. The mutants of T. reesei (Kawamori et al., 1985; Montenecourt and Eveleigh, 1977; Morikowa et al., 1985; Suh et al., 1986) are currently the best strains for the production of active cellulases. Aspergilli are preferred for the production of β -glucosidase required to achieve saccharification of cellulose. A. awamori (Enari et al., 1975), A. phoenicis (Sternberg et al., 1977) and Aspergillus spp. (Gokhale et al., 1984) have been reported as best sources of β -glucosidase.

Among the bacteria, the major groups that produce cellulolytic enzymes are Clostridium, Cellulomonas, Bacillus and Thermoactinomycetes. Zeikus (1980) has reported that the best producers of cellulolytic enzymes are the members of the genus Clostridium. Clostridium thermocellum produces active cellulases when grown on cellulose (Garcia-Martinez et al., 1980; Ng et al., 1977). The metabolic products of this organism are ethanol, acetic acid and lactic acid (Zeikus, 1980). These biproducts could be produced directly from cellulosic materials using Clostridium thermocellum. Cellulomonas strains produce several different cellulases (Beguin and Essen, 1978; Haggett et al., 1979) and mutants have been isolated which are more efficient than the parental strains in degradation of crystalline cellulose (Choi et al., 1978; Chowdhary et al., 1980). Degradation of highly crystalline cellulose by Bacillus species has not been reported so far, though a few reports could be sited about Bacillus strains capable of hydrolysing carboxymethylcellulose (CMC). The species studied include B. brevis, B. firmis, B. pumulus, B. subtilis (Knosel, 1971), B. polymyxa (Fogarty and Griffin, 1973) and Bacillus strains closely related to B. subtilis (Robson and Chambliss, 1984). Among the actinomycetes, thermophilic filamentus Gram variable bacterium Thermomonospora can grow rapidly on cellulose and it secretes thermostable cellulase complex which attacks both microcrystalline cellulose and CMC at high rates (Hagerdal et al., 1978).

1.3.5. Nature of cellulases

The hydrolysis of native cellulose is a complex process requiring participation of several enzymes. The fungal enzymes produced by Trichoderma, Sporotrichum and Fusarium are most extensively studied. Some bacteria also produce cellulose degrading enzymes but the mechanism of cellulose hydrolysis seems to be different from that of fungi.

In general,there are three major types of celluloytic enzymes produced by fungi.

1. Endoglucanase (1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4)

2. Cellobiohydrolase (1, 4- β -D-glucan cellobiohydrolase, EC 3.2.1.91).

3. B-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21)

1.3.5.1. Endoglucanase

This enzyme hydrolyses β-1, 4-glucosidic linkages randomly. It hydrolyses cellodextrins, phosphoric acid swollen cellulose and substituted celluloses like CMC and HEC. The main products of hydrolysis are cellobiose and cellotriose (Lee and Fan, 1980, Niku-Paavila et al., 1985). It does not act on cellobiose. However, endoglucanase from T. reesei was reported to have some cellobiase activity, but the activity was lower than that expected for a true cellobiose (Ladisch et al., 1980).

The first molecular cloning of cellulase genes from Cellulomonas fimi in E.coli using pBR 322 was reported by Whittle et al (1982). A shot gun library of total genomic DNA fragmented with Bam H1 was constructed in pBR 322 plasmid. Low levels of endoglucanase activity was found in clone, E. coli C 600 pDW1 extracts detected using antibodies against the enzyme. Subsequently, the cellulase coding fragment was subcloned to give pEC1, pEC2 and pEC3 with 6.6 kbp, 5.0 kbp and 5.6 kbp inserts of Cellulomonas fimi DNA respectively (Gilkes et al., 1984a). The 6.6 kbp insert was found to be identical with the cellulase coding insert in pDW1. Nakamura et al (1986) reported the cloning and expression of CMC hydrolysing activity from Cellulomonas uda CB4. The cloned endoglucanase gene was 4.8 kbp Bam H1 fragment of Cellulomonas uda DNA. Wong et al (1986) have sequenced the gene (cen A) coding for endoglucanase

from Cellulomonas fimi. This cen A gene is 1350 bp long encoding 449 amino acids and a stop codon. They found that 31 amino acids leader polypeptide in endoglucanase appeared to be functional in exporting enzyme to periplasm in E.coli.

Attempts were made by many research workers to clone the genes coding for endoglucanse from other organisms like Clostridium (Cornet et al., 1983; Romaniec et al., 1987), Bacillus (Koide et al., 1986; Sashihara et al., 1984), Thermomonospora (Colmer and Wilson, 1983, Ghangas and Wilson 1987) and Streptomyces (Coppolecchia et al., 1987). The genes coding for endoglucanse from Thermomonospora fusca when introduced into S. lividans was found to express a functional endoglucanase which was secreted out by the organism. B. subtilis however, did not show expression of Thermomonospora endoglucanase gene (Ghangas and Wilson, 1987). The endoglucanase genes cel A, cel B, cel C and cel D from Cl. thermocellum were studied in detail and a nucleotide sequence of cel A gene coding for endoglucanse A has been determined (Beguin et al., 1985). Lee and Pack (1987) cloned B. subtilis endoglucanse genes into another Bacillus and the transformants thus obtained were shown to be the overproducers of extracellular enzyme. Very recently, the nucleotide sequence of gene coding for endoglucanase C from Cl. thermocellum has been determined in which coding region contained 1032 bp (Schwarz et al., 1988).

1.3.5.2. Cellobiohydrolase

This enzyme acts on cellulose releasing cellobiose units from nonreducing end of the chain. It does not attack substituted celluloses which reflects a higher substrate specificity than that of endoglucanase. It also hydrolyses cellodextrins but not cellobiose. Reese et al (1950) proposed C1-Cx concept for the mechanism of cellulase synergism and a considerable work was carried out to define active C1 and Cx enzyme components. The cellubiohydrolase was shown to be C1 component in enzyme complex of T. koningii (Wood and McCrae, 1972). It had little affinity for

CMC, but did degrade phosphoric acid-swollen cellulose readily with cellobiose as a principle product of hydrolysis. Though the existance of cellobiohydrolase has been confined to number of cellulolytic fungi; similar types of enzymes have been isolated from cellulolytic bacteria like Cellvibrio gilvus (Storvik et al., 1963), Cellulomonas uda (Nakamura and Kitamura, 1983) and Cl. stercorarium (Nakamura and Kitamura, 1982).

The cloning and expression of exoglucanase gene from Cellulomonas fimi (Gilkes et al., 1984) and its primary nucleotide sequence (O' Neill et al., 1986a) have been reported. In another studies, the same group proposed a leader sequence of 41 amino acids indicating the signal sequence for the exoglucanase from Cellulomonas fimi (O' Neill et al., 1986b). Very recently Curry et al (1988) have demonstrated the expression and secretion of Cellulomonas fimi exoglucanase in S. cereviseae. This yeast exoglucanase was more stable than the C. fimi enzyme both at 60° C and 75° C.

1.3.5.3. β - glucosidase

This enzyme hydrolyses cellobiose and cellooligosaccharides to glucose. It does not attach cellulose or higher cellodextrins. There are two types of β-glucosidases depending on the substrates they use namely cellobiase and aryl- β -glucosidases. They differ in their substrate specificity, physical properties and genetic control of enzyme biosynthesis (Gong and Tsao, 1979). Aryl-β - glucosidases from Chaetomium species, A. faecalis and Stachybotrys atra do not act on cellobiose while β-glucosidases of other fungal and bacterial sources show activity towards both the substrates. This enzyme is widely distributed in plants, filamentous fungi and yeasts, but to date its presence has been described in comparatively few bacteria such as Flavobacterium (Sano et al., 1975), A. faecalis (Han and Srinivasan, 1969), Bacteriodes succinogenes (Forsberg and Groleau, 1982), Cellulomonas fimi (Wakarchuk et al., 1984) and Cl. thermocellum (Ait et al., 1982).

Bacterial β-glucosidase genes from E. adecarboxylata (Armentrout and Brown, 1981) and Cl. thermocellum (Roimaniec et al., 1987; Schwarz et al., 1985), have been isolated and cloned and in one of the studies the high level expression of glucosidase was obtained (Romaniec et al., 1987). Very recently, a gene for thermostable β-glucosidase (cellobiase) was cloned in E. coli and the clones were screened for β-glucosidase activity on the basis of MUC hydrolysis followed by a measurement of glucose released from cellobiose as a test for cellobiase activity (Kadam et al, 1988). They demonstrated that the genetic functions encoding hydrolysis of MUC and cellobiose are associated closely on the Cl. thermocellum chromosome.

1.3.5.4. Glucan glucohydrolases

In addition to the above enzymes, a minor activity of glucan glucohydrolase has been reported in fungi like T. viride (Li et al., 1965), A. niger (King and Vessel, 1969) and P. funiculosum (Wood and McCrae, 1982). This enzyme removes glucose units from non-reducing end of cellulose chain. It is reported to attack also phosphoric acid-swollen cellulose, cellooligosaccharides and CMC.

1.3.6. Regulation of cellulase production

1.3.6.1. Induction

Cellulases are inducible enzymes synthesized only in presence of their substrates. Though specific properties of natural inducer have not yet been understood, cellulose, sophorose and lactose are found to be the best inducers of cellulases (Gong and Tsao, 1979; Mandels et al., 1975; Montenecourt et al., 1979; Nisizava et al., 1971b). Since cellulose is an insoluble macromolecule, the mechanism of induction by cellulose still remains unclear. Induction may occur either by direct contact between microbe and cellulose, or by degradation products of cellulose through low

level constitutive enzymes. It is assumed that low amount of constitutive cellulases are secreted into the medium which generate degradation products of cellulose. These products in effect could then enter the cell and act as inducer (Beguin et al., 1977; Mandels and Reese, 1960).

A number of reports substantiate the hypothesis that soluble degradation products of cellulose induce cellulase. Cellobiose which is formed during hydrolysis of cellulose, is likely candidate for induction of cellulase. It was observed in case of T. reesei (Vaheri et al., 1979) that similar level of cellulase was induced by cellobiose (at lower concentrations) to that by cellulose. Cellobiose concentration in this case was lower than required to cause repression effect. This observation supports the assumption that partial degradation of insoluble cellulose at low levels could generate an inducer of cellulases. Growth on cellobiose has been shown to give similar enhancement of cellulase production in bacterial systems like Cl. thermocellum (Hammerstrom et al., 1955) and Cellulomonas (Beguin et al., 1977).

Sophorose has been shown to be powerful inducer of cellulase. It is active even at very low concentrations with washed mycelium of T. reesei (Sternberg and Mandels, 1980) as well as T. viride (Nisizava et al., 1971a; 1971b) and T. pseudokoningii (Zhu et al., 1982). Stoppok and coworkers (1982) reported that sophorose did not induce endoglucanase in Cellulomonas uda .

1.3.6.2. Repression

Cellulase synthesis in fungi and bacteria has been shown to be repressed by glucose, glycerol or cellobiose. Repression by cellobiose is brought about at relatively higher concentrations, whereas at lower concentrations cellobiose induces cellulases (Mandels and Reese, 1960; Mandels et al., 1962). Similarly sophorose which is the best inducer of cellulase, reduces cellulase synthesis at higher concentrations (Loewenberg and Chapman, 1977;

Nisizava et al., 1972). In conclusion, regulated supply of such carbon sources at low concentration could be used to manipulate increased cellulase production (Vaheri et al., 1979).

The exact mechanism by which these easily metabolisable carbon sources repress cellulase synthesis is not known. Catabolite repression, in some prokaryotes, is due to a decrease in level of cyclic AMP which acts as a necessary effector in the initiation process of the operon transcription (Pastan and Adhya, 1976). The cyclic AMP level of derepressed mutant of Thermomonospora curvata was found to be increased during the growth as compared to that of the wild type strain (Fennigton et al., 1983). In contrast, Zhu and coworkers (1982) suggested that catabolite repression in T. pseudokoningii cultures did not involve cyclic AMP. Similar observation was made by Montenecourt and coworkers (1981) in case of T. reesei. The catabolite repression could be overcome by the selection of proper mutants. Such derepressed mutants have been isolated in case of Trichoderma (Gallo et al., 1978; Montenecourt and Eveleigh, 1977; 1979) as well as in case of Cellulomonas and T. curvata (Choi et al., 1978; Fennington et al., 1983; 1984).

1.3.7. Mechanism of enzymatic hydrolysis of cellulose

Reese et al (1950) proposed C1/Cx concept for the enzymatic hydrolysis of cellulose for the first time. Since then number of models have been proposed (Chang et al., 1981; Enari and Niku-Paavola, 1987; Marsden and Gray, 1986; Wood and McCrae, 1972) to explain cellulase action. All these mechanisms proposed are however, based on mode of action of fungal cellulases. Ramasami and Verachtert (1980) have purified two extracellular, one cell bound endoglucanase and three aryl β-glucosidases from Pseudomonas fluorescens. They further suggested the scheme for mechanism of hydrolysis of cellulose by bacterial cellulases. Schematic representation of cellulase action is given in Fig. 1.3.

Fig.1.3. Schematic Representation of Cellulose Action.

Fig. A.1.3.A : Original C_1 / C_x concept of Reese et al.(1980)

Native cellulose---C_1---	Shorter linear polyanhydro-glucose chains (disordered "activated" cellulose)	---C_x---	Soluble, small molecules, capable of diffusion into the cell. [glucose]n=1-6

Fig. 1.3.B : Sequential action : C_x / C_1 (Wood and McCrae,1972)

Crystalline---C_x--- cellulose	reactive ---C_1 --- cellulose	Cellobiose	---β-glucosidase	---glucose	

Fig. 1.3 C) Concept of enzymatic hydrolysis of cellulose according to Enari (1983).

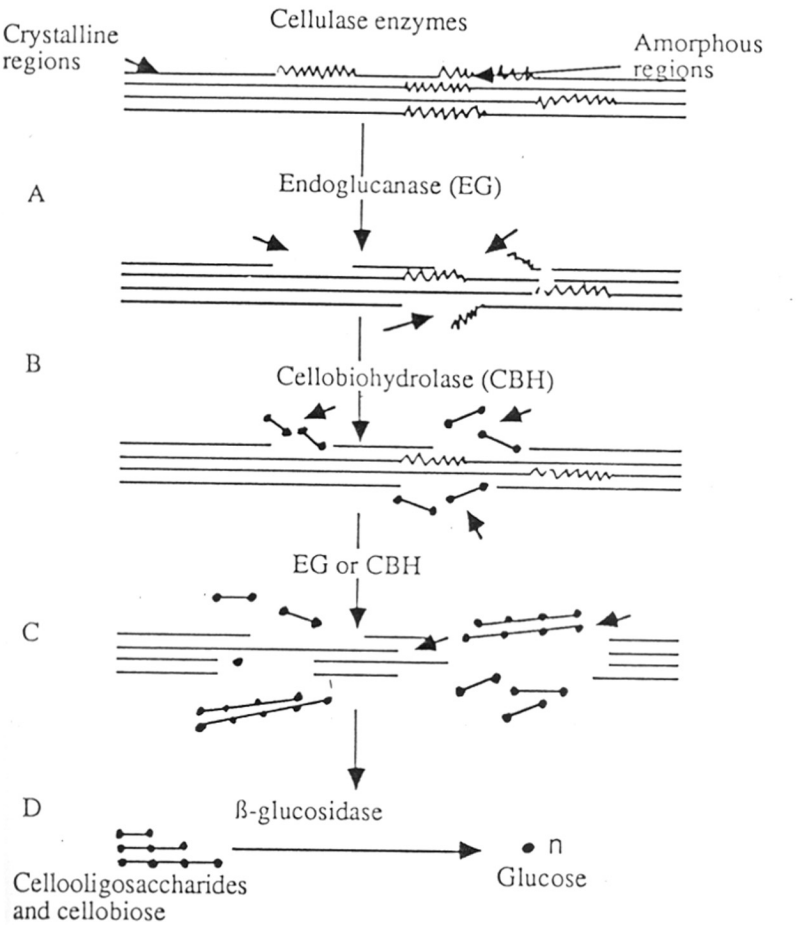


Fig. 1.3. D) Enzymological model for enzymatic hydrolysis of cellulose (Marsden & Gray, 1986)

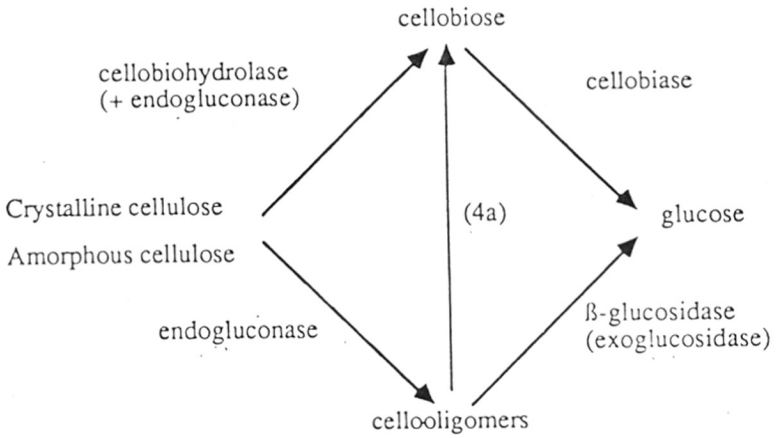
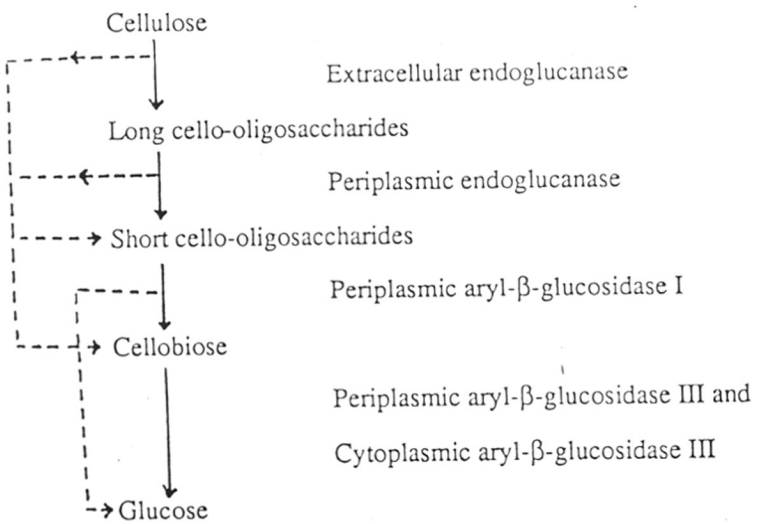


Fig. 1.3. E) Suggested scheme for bacterial cellulose degradation (Ramasamy and Verachtert, 1980).



1.4. Hemicellulose degrading enzymes

1.4.1. Classification of hemicellulases

Hemicellulose is attacked by hydrolytic enzymes referred to as hemicellulolytic enzymes or hemicellulases. These enzymes are classified depending on the nature of their substrates. Thus, L-arabinases are capable of degrading L-arabinose, D-galactanases degrade D-galactans and L-arabino-D-galactan, D-mannanases hydrolyze D-mannans and D-galacto-D-mannans and D-xylanases cleave xylans. D-xylanases are classified into three types.

1.4.1.1 Endo-xylanases (β-1, 4-D-xylan xylanohydrolase, EC 3.2.1.8)

These enzymes act randomly on xylan to produce large amounts of xylo-oligosaccharides of various chain lengths. There are two main types of endoxylanases.

(i) Those that are capable of hydrolysing arabinoxylans and arabinoglucurono-xylans liberating L-arabinose.

(ii) Those that do not liberate L-arabinose from these substrates.

Most D-xylanases constitute the nonarabinose-liberating endoxylanase, and the reaction products are arabino-oligosaccharides of varying degree of polymerization (Takenishi and Tsujisaka, 1973). These enzymes do not hydrolyse xylobiose. Arabinose liberating endoxylanase is produced by Talaromyces byssochlamydoides and this xylanase hydrolyses broad-leaved tree xylan to xylose, arabinose, glucose and traces of xylobiose and xylotriose (Yoshioka et al., 1981).

1.4.1.2. Exo-xylanases (β-1, 4-D-xylan xylohydrolase, EC 3.2.1.91)

These enzymes remove single D-xylose units from non reducing end of xylan chain but are distinguishable from β-xylosidases by virtue of the fact that the configuration of the product is inverted by the action of exo-xylanase but not by β-xylosidases.

1.4.1.3. β-xylosidase or xylobiase (EC 3.2.1.37)

These enzymes hydrolyse disaccharides like xylobiose and also higher xylooligosaccharides with decreasing specific affinity. In addition, these enzymes show high transferase activity (Lachke et al., 1985; Oguntimein and Reilly, 1980).

1.4.2. Xylanase producing microorganisms

These enzymes are widely distributed in prokaryotes as well as in eukaryotes. Most of the fungi are common sources of endoxylanases and the fungal enzyme preparations have been studied more extensively. The endoxylanases have been isolated from strains of Aspergillus (Frederick et al., 1981; John et al., 1979), Trichoderma (Tangnu et al., 1981), Trametes (Kubackova et al., 1975) and Talaromyces (Yoshioka et al., 1981). A. niger has been reported to produce high xylanolytic activities with regard to saccharification of xylan and xylan containing waste materials (Conrad, 1981). A mutant of A. fumigatus secreated high levels of xylanases in to growth medium when grown on hay or Straw (Stewart et al., 1983).

Fungi are better producers of xylanases and therefore, are more extensively studied compared to bacterial xylanases. Most recent studies in bacterial xylanases are restricted to the genera of Bacillus and Streptomyces. The list of strains coming under these two genera and producing xylanases is given in Table 1.2. The highly cellulolytic bacterium Cellulomonas spp. also produces xylan

Table 1.2 Xylanase from prokaryotes

Source	Type of action	References
Bacillus subtilis	Endo	Takahashi and Hashimota (1963); Inaoka (1961); Roger et al. (1983)
Bacillus sp.	Endo	Fogarty and Ward (1973).
Bacillus circulans WL-12	Endo	Esteban et al. (1982)
Bacillus pumilis	Endo	Panbangred et al. (1983)
Bacillus pumilis	Exo	Kersters-Hilderson et al. (1969)
Bacillus polymyxa	NS	Fogarty and Griffin (1973).
Bacillus sp. (Alkalophilic)	Endo or Exo	Horikoshi and Atsukawa (1973)
Bacillus sp. (Alkalophilic and Thermophilic)	NS	Okazaki et al (1984)
Bacillus sp. 11-1S (Thermophilic & Acidophilic)	Endo	Uchino and Nakane (1981)
Cellvibrio fulvus	NS	Sorenson (1957)
Streptomyces vividochromogenes	Endo	Ikeda et al. (1972)
Streptomyces exfoliatus	Endo	Sreenath and Joseph (1982)
Strepromyces sp. E-86	Endo	Kusakabe et al. (1977).
Streptomyces xylophagus	Endo	Iizuka and Kawaminami (1965) Kawaminami and Iizuka (1969); Tangnu et al. (1981).
Streptomyces albogriseolus	NS	Kusakabe et al (1969); Iizuka and Kawaminami (1969).
Streptomyces lividans	Endo	Klupfel et al. (1986) Iizuka and Kawaminami (1969).

NS : Not studied.

Table 1.2. continued

Source	Type of action	References
Streptomyces sp. 3137	Endo	Marui *et al*. (1985).
Streptomyces sp.		Kusakabe *et al*. (1969); Iizuka and Kawaminami (1969).
Streptomyces QMB-8114	NS	Goldschimid and Perlin (1963).
Streptomyces albus	NS	Sorenson (1957)
Micromonospora chakea	NS	Sorenson (1957).
Sporocytophaga myxococcoides	NS	Clermonont *et al*. (1970)
Microbispora rosea	NS	Kusakabe *et al*. (1969)
Nocardia corallina	NS	Kusakabe *et al*. (1969)

NS : Not studied.

degrading enzymes when grown on cellulosic substrates. The mutants of Cellulomonas, CS 1 (Rickard and Peiris, 1981) and CS 1 - 17 (Peiris et al., 1982) have been shown to be improved over the original parent strain with respect to xylanase and β- xylosidase production.

1.4.3. Induction of xylanases

D-xylanases are produced even in absence of xylan (used as a carbon source) though at low levels and are primarily cell wall bound (Notario et al., 1979). The enzyme was produced constitutively in this case and was further induced when grown on xylan as carbon source (Biely et al., 1980). Xylobiose is also an inducer of xylanase of Cryptococcus albidus and the uptake of xylobiose appeared to be caused by induction of active transport system known as xyloside permease (Kratky and Biely, 1980). In Streptomyces spp. non-metabolisable methyl- β -D-xyloside was found to be a better inducer than xylan (Nakanishi and Yasui, 1980a). Rho et al (1982) observed that xylan, thiocellulose, CMC, cellobiose and lactose could act as inducers of xylanase in wood destroying basidiomycete, Schizophyllum commune. The mutant strain of T. reesei Rut C-30 has been shown to be capable of producing xylanase constitutively. (Tangnu et al., 1981). Other examples of microorganisms producing constitutive xylanases include Str. afghaniensis (Loginova et al., 1981), yeasts of the genus Trichosporon (Stevens and Payne, 1977) and Trametes hirsuta (Kubackova et al., 1975). There is considerable controversy whether these and many other xylanases are produced inductively or constitutively. In general extracellular xylanases are produced when xylan is used as a carbon source. In case of Cryptococcus albidus, majority of xylanase was cell wall associated when grown only on glucose (Notario et al., 1979).

1.4.4. Repression of xylanases

The addition of monosaccharides such as xylose or glucose in the

grwoth medium represses the xylanase production. Nakanishi and Yasui (1980b) showed that the repression of xylanase synthesis in fully induced mycelia of Streptomyces spp. occured when methyl-β -D-xyloside or glucose was added to the culture medium, but cyclic AMP failed to release the glucose repression. On the contrary, Biely (1982) demonstrated that induction of -xylanases of C. albidus by methyl-β -D-xyloside began only after all the glucose in the grwoth medium was utilised. Thus glucose was not able to repress xylanase synthesis entirely in the induced cells, and it was concluded that the repression in non-induced cells was primarily due to a block by glucose in the transport of inducer to the cells.

1.4.5. Cloning of xylanase genes

Genes coding for xylanases and β-xylosidases from Bacillus spp. have been cloned and expressed in E. coli (Bernier et al., 1983; Panbangred et al., 1983; 1985) and their nucleotide sequences been determined (Fukusaki et al., 1984; Paice et al., 1986). Xylanase gene from Bacillus spp. C 125 was cloned in E. coli HB 101 using pBR 322 (Honda et al., 1985). Recombinant plasmid, pCX 311 harbouring E. coli could synthesize extracellular xylanase higher than that produced by parent Bacillus (Honda et al., 1986). Recently, Yang et al (1988) reported the cloning and expression of B. polymyxa xylanase gene in E. coli. A 2.9 kbp Bam H1 - Eco R1 fragment was found to code for xylanase activity. This recombinant gene expressed xylanase in absence of xylan, but was repressed by glucose or xylose. The molecular weight of this xylanase expressed by cloned gene was found to be 48000. In general, molecular weight of bacterial xylanases range from 6000 daltons from Chainia spp. (Bastawde, 1987) to 85000 daltons from B. circulans (Esteban et al., 1982). A gene from Str. lividans coding for xylanase was self cloned in xylanase and endocellulase negative double mutant of Str. lividans using multicopy plasmid pIJ 702 (Kluepfel et al., 1986; Mondou et al., 1987). These three clones harbouring 2 kbp DNA fragment as determined by restriction mapping secreted xylanase of

molecular weight 43,000. This DNA fragment carried a complete structural gene for xylanase with its promoter and also the regulatory sequences as evidenced from its induction by xylan. As high as 60 fold higher than the parental strain upto 380 IU/ml of xylanase could be obtained from this engineered organism. Recently in our laboratory, xylanase gene containing 1.2 kbp long Bam H1 fragment of genomic DNA of Cellulomonas spp. has been cloned in pUC 18 and E.coli JM 83 harbouring this plasmid produces extracellular xylanase after induction by xylan (unpublished data from our laboratory).

1.5. Summary and Scope of work

As discussed in the above review, it is evident that phenotypes like cellulose degradation are effected through synergestic action of a cascade of gene products. Such phenotypes are not easy to manipulate. Further, if one wishes to complement this phenotype with other desirable properties, conventional recombinant DNA technology does not assure success. This is because, conventional technology has been developed using tailor cut recipient bacteria like E. coli HB101 or B. subtilis derivatives lacking restriction modification system and recombination. Naturally occurring and industrially important bacteria do not satisfy these requirements. Introduction of foreign genes and their stable expression therefore, has been a challenge in this area of microbial biotechnology. Magnitude of this problem has apparently increased in cases where the phenotype is governed by synergistic expression and activities of more than one independent genes. Though individual genes of the nitrogen fixation cascade or cellulase complex have been isolated and sequenced, their transfer to conventional recipient microbes has not resulted in the phenotypes as nitrogen fixation or complete saccharification of cellulose. Manipulating industrially important bacteria therefore has not been attempted.

Protoplast fusion technique, on the other hand, has been though

effectively used in intra- and inter- species bacterial fusion, has not been considered as a technique to transfer/exchange such complex genotypes. Potential of this technique in generating stable hybrid phenotypes is evident from a fact that multiple recombinational events do occur in stable interspecies hybrids. Application of this technique to generate intergeneric hybrids has not been visualised by others.

It is from this point that present work was undertaken, first to check the possibility of exchange/transfer of desired complex phenotypes through intergeneric protoplast fusion among bacteria. Using cellulolytic, xylanolytic, Gram variable Cellulomonas(NCIM 2353) and auxotrophic sporulating, Gram positive B.subtilis (NCIM 2255) hybrids were obtained harbouring cellulase genes and gram positive phenotypes. These hybrids not only could utilise cellulose or xylan, but also secreted out the specific enzymes. Microbiological, biochemical and molecular biological characterization of these hybrids was attempted. Detailed studies on one of these stable intergeneric hybrids with reference to substrate dependence, regulation of expression of cellulases and xylanases as well as nature of these enzymes in the hybrid have been reported in this thesis.

In a separate attempt to establish applicability of protoplast fusion technique in bacterial cell engineering, isolation and characterization of stable hybrids between amylolytic B.subtilis and Gram negative Z.mobilis was attempted and is described at the end. Change in the properties of specific enzymes in the hybrid organisms has been studied and its significance has also been discussed.

Last, but not least, it has been demonstrated through the work reported herein that the recombination events could generate different organisms hitherto not seen in nature which could be used to understand evolutionary significance of the diverse specialsed features and problems in complementing them in a single organism.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Special chemicals used were procurred from various sources as given below.

Sigma Chemical Company

Agarose, cellobiose, carboxymethyl cellulose (Na salt), Amino acids, RNase A, S1 nuclease, Proteinase K (nucleus free), Lysozyme, bovine serum albumin, Tris (hydroxymethyl) amino methane, N,N'-methylene bis acrylamide (Bis).

Difco Laboratories, U.S.A.

Bacto peptone, Agar (Bacto), Yeast extract, Bacto tryptone.

Karl Schleicher and Schull Co., W. Germany

Cellulose-123 powder

Riedel Dehaen, W. Germany

3,5-dinitrosalicylic acid

Fluka AG, Switzerland

Larchwood xylan

Koch-Light laboratories, U.K

p-Nitrophenyl- β -D-glucopyranoside (pNPG)

BARC, Bombay, India

32p- labelled phosphoric acid

Polysciences Inc., USA

Polyethylene glycol-6000

Brown Co., Berlin, New Hampshire

Solka Floc SW-40

Pierce Chemical Co., U.S.A.

Acrylamide

Pall Ultrafine Filtration Corp., New York, USA

Nylon based nitrocellulose membrane

Kodak Co.,

X-ray films type X-O-MAT.

Vector Laboratories, Burlingame, U.S.A.

Biotinylated goat anti-rabbit IgG, Biotinylated peroxidase and Avidin-DH.

New England Biolabs

Restriction endonucleases, DNA polymerase

Boehringer Mannheim GmbH

Alkaline phosphatase

Pharmacia Fine Chemicals, Uppsala

Sephadex G-50

All the other chemicals were of analytical grade. The chemicals i.e. salts, acids and solvents were from BDH.

2.2. Nutrient media, solutions and buffers

Luria-Bertani medium (LB)

10.0 g tryptone, 5.0 g yeast extract, 5.0 g sodium chloride in a final volume of 1 litre. pH was adjusted to 7.2.

M9 minimal medium

Solution 1 : 6.0 g sodium phosphate dibasic, 3.0 g potassium phosphate dibasic, 5.0 g sodium chloride and 1.0 g ammonium chloride.

Solution 2 : 1 mM magnesium sulphate

Solution 3 : 0.1 mM calcium chloride

All the above three solutions were sterilized separately and then mixed in a final volume of 1 litre. Glucose or cellulose-123 powder was added at a concentration of 1.0 or 0.5% respectively.

Protoplast regeneration medium

M9 minimal medium or LB with 0.5 M sucrose and 0.8 % Bacto-agar was used as regeneration medium.

Dubos medium (Dubos, 1928)

0.5 g sodium nitrate, 1.0 g potassium phosphate dibasic, 0.5 g potassium chloride, 0.5 g magnesium sulphate and 0.5 g yeast extract in a final volume of 1 litre. pH was adjusted to 7.2.

When glucose, cellobiose, xylose, maltose, lactose or glycerol were added as carbon sources, medium was sterilized separately and sterile solutions of these sugars were added separately in appropriate amounts. All other carbon sources like cellulose-123, Avicel etc. were added to medium prior to autoclaving.

Solid Media

All the solid media were prepared by the addition of 2% Bacto- agar except for protoplast regeneration medium where 0.8% Bacto- agar was used.

Amino acid solutions

All amino acid solutions were prepared in distilled water to final concentration of 5 mg/ml. They were separately autoclaved and added to the media in appropriate q uantities. The stock solutions were stored at 4°C.

Polyethylene glycol solution

33% (w/v) of PEG-6000 solution was prepared in protoplast buffer and autoclaved at 121° C for 20 min.

Sucrose dye for DNA gels

70% sucrose, 100mM EDTA, 1 mg % Bromophenol blue.

Ethydium bromide

A solution of 1 mg/ml was prepared in distilled water or in 1x Tris-acetate buffer pH 7.8. Solution was stored at 5° C in dark brown bottle.

Denaturing solution for colony and DNA blots

1.5 M NaCl, 0.5 M NaOH.

Neutralizing solution for colony and DNA blot

1.0 M Tris- HCl (pH 7.4), 1.5 M NaCl.

Denhardt's solution (50 x)

1% BSA, 1% Ficoll, 1% PVP

Prehybridization Solution

5x Denhardt's solution, 5x SSC (pH 7.0), 50 mM sodium phosphate buffer (pH 7.0), 0.1 % SDS, 50% formamide, 0.25% denatured calf thymus DNA.

Hybridization solution

Prehybridization solution containing denatured radiolabelled probe DNA.

D-19 Developer

22% Metol, 7.2% sodium sulphate anhydrous, 0.88% hydroquinone, 4.8% sodium carbonate and 0.4% Potassium bromide.

Scintillation fluid

0.5% PPO, 0.05 % POPOP prepared in toluene.

RNase A

solution (10 mg/ml) was prepared in 0.15 M NaCl. The solution was

kept in boiling water bath for 10 min to make it free from DNase.

DNase I

The solution (1 mg/ml) was prepared in 50% glycerol containing 0.15 M NaCl.

CMC solution

1.0 g of CMC was dissolved in 100 ml of citrate phosphate buffer, pH 6.8. It was stored frozen in aliquots.

Xylan solution (1%)

2.0 g of Larchwood xylan was added in citrate phosphate buffer, pH 6.8. The suspension was kept on rotary shaker for overnight and then centrifuged at 5000 rpm for 10 min at 4° C. The supernatant was used as soluble xylan for determination of xylanase activity.

pNPG solution

The solution of 1mg/ml was prepared in citrate phosphate buffer, pH 6.8. This solution was prepared just before the time of assay of β-glucosidase activity.

Protoplast buffer

0.1 M Tris-HCl (pH 7.5) containing 0.01 M magnesium chloride, 0.1 mM EDTA and 0.5 M sucrose.

TE buffer (pH 7.4)

10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (pH 8.0)

TE buffer (pH 8.0)

1 mM EDTA (pH 8.0) and 10 mM Tris-HCl (pH 8.0)

Buffers for restriction enzymes (10x)

Bam HI buffer

Tric-HCl	200 mM
MgCl	70 mM
NaCl	10 mM
BmSH	20 mM

Msp I Hpa II buffer

Tris HCl (pH 7.4)	100 mM
KCl	60 mM
MgCl	100 mM
DTT	10 mM
BSA	1 mg/ml

Hae III buffer

Tris HCL (pH 7.4)	500 mM
NaCl	500 mM
MgCl	60 mM
BmSH	60 mM
BSA	100 ug/ml

Sau 3 A I buffer

Tris HCl (pH 7.5)	60 mM
NaCl	500 mM
MgCl	60 mM
BSA	500 ug/ml

Eco R I buffer

Tris HCl (pH 7.5)	500 mM
MgCl	100 mM
NaCl	500 mM
DTT	10 mM

Mbo I buffer

Tris HCl (pH 7.4)	500 mM
MgCl	100 mM
NaCl	500 mM

Sma I buffer

Tris HCl (pH 8.0)	150 mM
MgCl	6 mM
KCl	15 mM

TAE buffer (DNA gel buffer, 10x, pH 7.8)

0.5 M Tris-acetate, 0,2 M sodium acetate, 0.02 M EDTA.

Tris-glycine (pH 8.3)

The buffer solution contained 0.69 g Tris and 2.88 g glycine in 1 litre of distilled water. The pH of the buffer was 8.3.

DNA blot washing buffer (low stringency)

2 x SSCP (60 ml 5.0 M NaCl, 30 ml 1.0 M sodium citrate, 50 ml 1.0M phosphate buffer pH 7.0) with 0.1 % SDS.

DNA blot washing buffer (higher stringency)

0.1x SSCP with 0.1% SDS

TBS buffer (Tris buffer saline)

10 mM Tris-HCl (pH 7.5), 0.14 M sodium chloride.

SSC (20x)

3.0 M NaCl, 0.3 M Tri-sodium citrate

Citrate phosphate buffer (pH 6.8)

9.1 ml of 0.1 M citric acid was mixed with 40.9 ml of 0.2 M sodium phosphate dibasic and final volume was made to 100 ml.

2.3. Bacteriological techniques

2.3.1. Bacterial strains used for studies

The bacterial strains used in this study are listed below in Table 2.1.

2.3.2. Maintenance of stock cultures

Cellulomonas spp. NCIM 2353 and B. subtilis NCIM 2255 and B. subtilis NCIM 2635 were grown on LB agar slants at 30 C. All hybrids were grown on Dubos medium agar slants with 0.5% cellulose-123 powder as sole carbon source and 0.05% yeast extract. The cultures on slants were preserved at 4 C and were periodically transferred every two months on freshly prepared media.

The cultures were also maintained by lyophilization method. Cultures were grown in respective liquid media for 24 h at 30 C on rotary shaker. Cells were centrifuged and washed twice with saline. Finally cells were resuspended in 10 % skim milk and lyopholized in vials using Edwards lyopholizer. These vials were preserved at 4 C and served as master stock cultures. To check wheter cultures still retained their characteristic, the lyopholized culture was grown on Dubos medium with cellulose-123 as substrate and tested for its cellulolytic activity.

2.3.3. Preparation of seed culture

The purpose of this step was to generate vigorous and uniform cell mass. One loopful of cells from stock culture was inoculated into LB or Dubos minimal salt medium containing 0.1% cellobiose and 0.05% yeast extract and incubated for 24 h or 48 h at 30 C in an orbital shaker (200 rpm). The cells were centrifuged at 5000 rpm in table top centrifuge and washed twice with sterile distilled water and resuspended in small volume of sterile distilled water. This suspension was used as inoculum (10% v/v) in the subsequent culture propagation.

2.3.4 Growth of culture for DNA isolation

Table 2.1 Bacterial strains used for studies

Strain	Genotype and Phenotype
Cellulomonas spp. NCIM 2353	Cel^{+}, Thi^{-}, Kan^{R}
B. subtilis NCIM 2255	Ala^{-}, His^{-}, Leu^{-}, Trp^{-}, Ura^{-} and Cel^{-}
B. subtilis NCIM 2635	$Indole^{-}$, Trp^{-}, Cel^{-}
Bs/C 003	prototrophic hybrid, Cel^{+}
Bs/C 005	prototrophic hybrid, Cel^{+}
Bs/C 007	prototrophic hybrid, Cel^{+}

The culture for DNA isolation was grown in 500 ml Erlenmeyer flask containing 100 ml LB as described above. The cells were then harvested by centrifugation at 4° C, and 10,000 rpm in Sorvall RC 5B centrifuge using SS 34 rotor. The pelleted cells were washed twice with sterile distilled water and this pellet was used for DNA isolation.

2.3.5. Growth of culture for enzyme production

The shake flask culture for enzyme production was grown in 250 ml conical flask containing 50 ml or 100 ml Dubos medium with specified amounts of cellulosic sources. A flask was inoculated with seed culture (10%) and incubated at 30° C on orbital shaker (200 rpm). Sample for determining enzyme activities were removed after different time intervals. Supernatant after centrifugation at 5000 rpm in SS-34 rotor for 20 min in Sorvall RC 5B centrifuge was used for determining extracellular cellulolytic activities and protein content. Care was taken that the supernatant was free of bacterial cells. The pellet was washed twice with distilled water, suspended in known volume of citrate phosphate buffer (0.05 M, pH 6.8) and was sonicated at 35 Kc for 3 min in Virsonic sonicator with cooling in ice. The cell debris was removed by centrifugation at 4° C. The cell free extract was used to determine protein content and intracellular cellulolytic activities.

2.4. Protoplast fusion and selection of hybrids

2.4.1. Protoplast isolation

Both Cellulomonas spp. NCIM 2353, B. subtilis NCIM 2255 and B. subtilis NCIM 2635 were grown in LB for overnight at 30° C on rotary shaker. The cells were harvested by centrifugation at 3000 rpm on table top centrifuge. The pellet was resuspended in sterile distilled water (10 ml) and samples were plated on LB agar medium or M9 minimal agar medium with cellulose after appropriate dilution to determine initial number of cells. Remaining cells were again

pelleted and washed twice with protoplast buffer without sucrose. Cells were resuspended in 2 ml of protoplast buffer containing lysozyme (1 mg/ml) and incubated at 30° C for 2 h. The suspension was centrifuged at room temperature for 20 minutes at 3000 rpm and the protoplasts were resuspended in original volume of protoplast buffer. The formation of protoplasts was confirmed either by microscopic examination or by plating after giving osmotic shock.

2.4.2. Protoplast regeneration

Protoplasts formed after lysozyme treatment were plated on LB agar or M9 minimal agar regeneration medium. The plates were incubated at 30° C for 3-4 days and colonies were counted on colony counter.

2.4.3. Protoplast fusion

Protoplast fusion was carried out according to the method described by Fodor and Alfoldi (1976) and Schaeffer et al (1976) with slight modification. All the operations were carried out under sterile conditions. Equal number of protoplasts of each of the two organisms used were mixed and centrifuged at 3000 rpm at 30° C for 15 minutes followed by washing with protoplast buffer. The washed protoplasts were resuspended in 2 ml of 33% PEG-6000 in protoplast buffer. The fusion mixture was gently mixed and incubated for 2 min at room temperature. It was then diluted to 15 ml with protoplast buffer and centrifuged at 3000 rpm for 10 min. These protoplasts were then gently suspended in 2 ml of protoplast buffer and were plated out on different regeneration media after appropriate dilution when necessary. The plates were incubated at 30° C for 3-4 days and colonies on each plate were counted.

2.4.4. Selection of hybrids

The colonies of the fusion products grown on M9 minimal medium with cellulose-123 as sole carbon source were subcultured and isolated

single colonies from these plates were then propagated on the same growth medium successively for at least 5 times. Finally colonies growing on this medium were used in further studies and maintained in the selection medium. The isolates were named as Bs/C and numbered serially as, Bs/C 001, Bs/C 002 etc.

2.4.5. Gram staining

All the cultures were grown overnight in LB and were harvested by centrifugatiion at 3000 rpm for 20 min. Cells were washed with distilled water, fixed on slide and were further treated for Gram staining. Slides were observed under light microscope.

2.4.6. Scanning electron microscopy

Bacterial cultures were grown overnight in LB at 30° C and harvested by centrifugation at 3000 rpm for 20 min. Cells were washed with distilled water and fixed in 3% glutaraldehide at 4° C overnight. Fixed cells were washed with distilled water twice and suspended in minimal volume of distilled water. They were spread on aluminium foil to form a thin film. After gold sputtering, specimens were observed in Cambridge Stereoscan Model 150 electron microscope and photographed.

2.5. **Characterization of DNA**

2.5.1. Isolation of DNA

The isolation of DNA from the cells was carried out by phenol isoamyl alcohol extraction method described by Miura (1967). The cells were washed once with TE (pH 7.4) buffer. Pellet was resuspended in protoplast buffer (without sucrose) containing freshly prepared lysozyme (1 mg/ml) and incubated at room temperature for 2 h. The cells were then lysed with SDS (1%) and treated with self digested proteinase K for 1 h at 37° C. This was followed by extraction with phenol equilibrited with Tris-HCl

buffer until no interphase was obtained, and aqueous phase was collected. This was further extracted with chloroform: isoamyl alcohol (24 : 1, v/v) until no interphase was seen. Aspirated aqueous supernatant was kept on ice and after adding sodium chloride (final concentration 100 mM), DNA was precipitated by addition of 2 vols of chilled ethanol. The precipitate was dissolved in TE buffer and contaminating RNA was removed by digestion with DNase free pancreatic RNase A (100 µg/ml). Following phenol and chloroform : isoamyl alcohol extractions, DNA was precipitated with chilled ethanol. RNA free DNA was dissolved in TE (pH 7.4) buffer and was quantitated spectrophotometrically. DNA was stored at 4° C.

2.5.2. In vitro radiolabelling of DNA (Nick Translation)

DNA from Cellulomonas and B. subtilis were labelled in vitro using d[α^{32}-P] CTP according to the method of Rigby et.al (1977) in a nick translation system. 8 pmoles of d[α^{32}-P] CTP (25 uC/assay) along with 1 nmole of cold dCTP and 10 n moles each other dNTPs were added in the reaction mixture of 50 ul containing final concentrations of 50 mM Tris-HCl (pH 7.4), 5 mM MgCl , 50 ug/ml BSA, 10 mM 2-mercaptoethanol, 1 ug DNA, 0.1 ng DNase I and 5 units of DNA polymerase I. Incubation was carried out at 14°C for 2.5 h and free dNTPs were removed by gel filteration over sephadex G-50 (3 ml bed volume column) in a Tris-EDTA (pH 8.0) buffer. Fractions (200 ul) were collected and Cerenkov counts of these fractions were measured in Kontron liquid scintillation counter.

Radiolabelled DNA containing fractions (leading peak) were pooled together, and trichloroacetic acid (TCA) precipitable radioactivity in DNA was estimated using a small aliquot of pooled fraction. Total incorporated d[α^{32}-P] CMP was thus calculated.

2.5.3. TCA precipitation to determine incorporation of 32-P in the DNA

10 ul sample from the pooled rediolabelled DNA containing

fractions from nick translation assay was diluted ten times with distilled water. 10 ul of this diluted sample was spotted on GF-C filters (Whatman, 2.2 cm disks) as a control, while remaining was divided into fractions of 30 ul each. To each of these aliquots 50 ug of BSA was added. After adding equal volume of 10% TCA each sample was mixed and kept on ice for atleast half an hour. These samples were then vaccum filtered using GF-C, washed twice with chilled TCA (5%) and chilled absolute alcohol. Air dried filters were transferred into scintillation fluid and counted in Kontron liquid scintillation counter. Actual precipitable counts were estimated comparing total control counts.

2.5.4. DNA blotting and hybridization

2.5.4.1. Colony blotting

Colony blotting and hybridization were carried out by the method of Gruensten and Hogness (1975). Both parental and hybrid organisms were grown on LB agar plate. The grown colonies were blotted onto Biodyne A nylon membrane filter by putting the paper on the plate for 2 min. The membrane along with the colonies was processed in denaturing solution for 5 min and subsequently in neutralizing solution for 5 min (three changes). When still wet, DNA was immobilized also by exposing this blot to short wave length UV by a hand torch at a distance of 6 cm. and baked for 1.5 h at 80° C. This was stored dry at 4° C until used.

2.5.4.2. DNA dot blot preparation

Dot blots were prepared by immobilizing known amounts of denatured DNAs on Biodyne A nylon membrane filter. These were further treated with 1.5 M NaCl in 0.5 N NaOH, followed by 1.5 M NaCl in 0.5 M Tris-HCl (pH 7.4) for 5 min. The membrane was air dried, baked at 80° C for 1 h and hybridization with labelled DNA probe was carried out as explained below.

2.5.4.3. Hybridization

Biodyne paper with blotted colonies or with DNA blots as described above was transferred into hybridization solution and incubated at 42° C for 1 h. Denatured radiolabelled probe DNA was added into hybridization mixture in the same bag for 24 h. Filter was washed thrice with 2x SSC and 0.1% SDS solution for 5 min each at room temperature. Subsequent washes were given either in 0.5x SSC and 0.1% SDS solution for 15 min at 45°C (twice) [low stringency]; or at 55°C (medium stringency] or at 65° C with 0.2x SSC and 0.1% SDS (high stringency). Filters were then air dried, wrapped in Saran wrap and exposed to X -ray films (kodak).

2.5.5. Restriction enzyme digestion and agarose gel electrophoresis.

Purified DNA preparations from the parental and hybrid strains were subjected to digestion by the restriction endonuclease enzymes Msp I, Hpa II, Hae III, Sau 3A, Sma I and Bam H1 under the conditions specified by the supplier. Bacteriophage DNA was used as a positive control to check the activities of these restriction enzymes. Digested DNAse were electrophoresed in 0.8% agarose gels in TAE buffer (pH 7.8). Gels were stained with ethidium bromide (2 ug/ml) washed and DNAse were visualized as fluorescent bands on UV transilluminator. Gels were photographed using Polaroid camera on a film T57.

2.5.6. Immunochemical assay for detection of 5 mC and 6 mA.

Desired amounts of DNA in 1 ul of TE buffer (pH 7.2) were spotted onto BA-85 nitrocellulose paper. The paper was baked at 60° C for 4 - 6 h and the assay was carried out as described by Achwal and Chandra (1982) and Achwal et al (1983). The paper was placed in petri dish and flooded with 10 ml of TBS containing 100 - 120 ug of antibodies to 5 mC or to 6 mA. The paper was left in the solution at room temperature (6-8 h) and then washed extensively

in TBS. It was then incubated in biotinylated goat anti-rabbit IgG, and subsequently in a complex of biotinylated peroxidase and avidin-DH. The paper was then washed extensively and stained for peroxidase using a substrate solution containing 0.1% DABT and 0.2% H_2O_2 (Hsu et al., 1981a; 1981b). The paper strip was then washed and dried. The visualised stained dots were photographed. Non-specific binding of antibodies was negligible. Known positive control DNAse were used on the same paper in each experiments.

2.6. **Analytical techniques**

2.6.1. Determination of reducing sugars

Reducing sugars in culture broths and in enzyme assay mixtures were estimated by dinitrosalicylic acid (DNS) method described by Fischer and Stein (1961). One ml sample was treated with 1 ml of DNS reagent and then heated for 5 min in boiling water bath. The tubes were cooled under running tap water. Final volume of reaction mixture was made to 12 ml with distilled water and the optical density was measured at 540 nm on Beckman spectrophotometer, Model 25.

2.6.2. Determination of Protein

Protein estimation in the samples was carried out using Folin-Ciocalteu reagent as described by Lowry et al (1951). Crystalline bovine serum albumin (1 mg/ml) solution was used as the standard.

2.6.3. Determination of enzyme activities

Enzymatic activities assayed were carboxymethylcellulase (CMCase), xylanase, β- gluocosidase and filter paper degrading activity (FPA).

2.6.3.1. Endoglucanase activity (CMCase)

Endoglucanase activity was assayed according to the method described by Mandels et al (1974). 0.5 ml of appropriately diluted culture filtrate or cell extract was mixed with 0.5 ml of 1% CMC solution. The reaction mixutre was incubated for 30 min at 50°C and reaction was stopped by the addition of 1 ml of DNS reagent. Reducing sugars released were measured as glucose equivalents. Assays were carried out using enzyme solutions diluted to give a value between 0.1 to 0.5 mg of reducing sugar equivalent.

A unit of endoglucanase activity equivalent to 0.5 mg of reducing sugars released as glucose equivalents in 30 min (Reese unit) and is equivalent to 0.092 IU.

2.6.3.2. Filter paper degrading activity

Filter paper degrading activity was determined by the method of Mandels et al (1974). To 50 mg of Whatman No.1 filter paper was added 1 ml of citrate phosphate buffer (0.05 M, pH 6.8) and 1 ml of enzyme solution. The mixture was incubated at 50° C for 60 min and the reaction was terminated by the addition of 1 ml of DNS reagent. The reducing sugars released were determined as glucose equivalents.

A unit of filter paper activity was equivalent to 0.5 mg of reducing sugars released as glucose equivalents in 60 min (equivalent to 0.046 IU).

2.6.3.3. Xylanase activity

Xylanase activity was assayed according to Bucht and Eriksson (1968) after slight modification. 0.5 ml of suitably diluted enzyme solution was mixed with 0.5 ml of 1% soluble xylan. Reaction mixture was incubated for 30 min at 50° C and reducing sugars released were estimated as xylose equivalents by DNS method. Assays were carried out using appropriately diluted enzyme to give 0.2 to 0.5 mg of reducing sugars as xylose equivalents.

A unit of xylanase activity is equivalent to 0.5 mg of reducing sugars as xylose equivalents produced in 30 min (equivalent to 0.11 IU).

2.6.3.4. β-Glucosidase activity

β-Glucosidase activity was determined using pNPG as substrate by modified method of Eberhart (1961). The assay mixture consisted of 0.9 ml of substrate (1 mg/ml) and 0.1 ml of enzyme solution. This was incubated at 50° C for 30 min and the reaction was stopped by the addition of 2 ml of sodium carbonate (2%). The amount of p-nitrophenol released was calculated from the absorbancy index of 18.5 cm /mole for p-nitrophenol at 410 nm. The enzyme dilution used was such as to release 0.01 to 0.07 umoles of p-nitrophenol from pNPG which is in the linear sensitive range of this assay.

A unit of β-glucosidase activity is expressed as 1 umole of p-nitrophenol produced from pNPG in 30 min (equivalent to 0.033 IU).

2.6.4. Polyacrylamide gel electrophoresis

Slab gel electrophoresis was performed at pH 8.3 accoriding to Davis (1946) using 7.5% acrylamide gel. Electrophoresis was carried out at 20 mA and 80 V for 4-6 h at 4° C until the dye, bromophenol blue reached the bottom of the gel. After electrophoresis was over, gels were processed for specific zymogram staining as described below.

2.6.5. Zymogram staining for endoglucanase and xylanase

Activity staining for endoglucanase and xylanase was done according to the method of Mackenzie and Williams (1984). Gels containing agar (2%) and CMC (0.1%) or soluble xylan (0.2%) were cast on the glass plates. Aliquotes of 5 ul enzyme were placed on the substrate gels and incubated at 50°C for 15 min. After incubation, substrate gels were washed with 1.0 M NaCl and kept in congo red (0.1%) solution for 30 min at room temp. Finally they were washed with 1.M NaCl until the hydrolysis zones were visible. The gels were placed in acetic acid (5.0%).

The polyacrylamide slab gel after electrophoresis was laid on the top of the substrate gel and the test tube was rolled on the top of the acrylamide gel to ensure the removal of air bubbles. To prevent evaporation, the whole asembly was placed in a tray containing water and incubated at 50° C for 30 min. The acrylamide gel was separated from the substrate gel and the substrate gel was processed further for activity staining as described previously.

CHAPTER 3

ISOLATION OF INTERGENERIC HYBRIDS BY PROTOPLAST FUSION BETWEEN *CELLULOMONAS* AND *B. SUBTILIS*

3.1. Introduction

Bacterial protoplast fusion was first reported by Fodor and Alfoldi (1976); and has been since employed as a means of genetic recombination in bacteria (Hopwood, 1981). This technique allows the transfer of large segments of DNA or the whole genome from one organism to another. Various aspects of protoplast fusion technique and its application have been reviewed in chapter 1. Many intraspecies and interspecies bacterial protoplast fusions have been reported in the literature (Fleischer and Vary, 1985; Kaneko and Sakaguchi, 1979; Peberdy, 1980). Among number of other traditional methods used in strain improvement, mutagenesis and recombinant DNA technology have been employed in most of the systems, but they have certain limitations. For example, transfer of phenotypes involving multigenic components like nitrogen fixation, and their expression in other microbes by recombinant DNA technology seems to be difficult. Such multigenic transfers and expression could be achieved with the help of protoplast fusion since large part of genome could be stably transferred into recipient. The phenotypes like nitrogen fixation and cellulose degradation are multigenic in nature which involved synergistic expression and action of component enzymes. One of the advantages of protoplast fusion technique is that it allows genetic analysis of those microbes in which conjugation analysis is not feasible. Application of this technique in engineering bacteria belonging to different genera, however, was not documented until 1984. In order to investigate the feasibility of such intergeneric gene transfer through protoplast fusion, cellulose degrading *Cellulomonas* and non- cellulolytic Gram positive sporulating *B. subtilis* were chosen as parental organisms in this work. The *Cellulomonas* was chosen as a donor of Cel^+ phenotype because this organism is under increasing investigations world wide due to its ability to produce both cellulases and hemicellulases extracellularly. Genetics of *Cellulomonas*, however, is poorly understood except a few markers on it. *B. subtilis* used as recipient of Cel^+ phenotype was Spo^+, and harboured number of auxotrophic markers. *B. subtilis*

derivative hosts are widely used in fermentation industries particularly for the production of exoenzymes using recombinant DNA technology. In addition, genetics of B. subtilis is relatively well understood.

In this chapter, isolation of stable hybrid organisms through protoplast fusion between Cellulomonas and B. subtilis (NCIM 2255) has been described. Conditions for generation of protoplasts and their regeneration as well as biochemical and molecular biological analysis of hybrids in comparison with parents is also reported.

General methods employed are described in Chapter 2. Specific experimental conditions if different are indicated under corresponding results/figures/tables.

3.2. Results

3.2.1. Protoplast isolation

The optimum conditions for protoplast formation for bacterial cells used were as follows. The cells of Cellulomonas NCIM 2353 B. subtilis NCIM 2255, B. subtilis NCIM 2635 and B. cereus were harvested just before onset of stationary phase and used for protoplast isolation. These were treated separately with lysozyme (1mg/ml) prepared in protoplast buffer. Requirement of EDTA has been shown to be essential for the formation of protoplasts from Gram negative organisms (Weiss, 1976), and therefore protoplast buffer was supplemented with EDTA (0.1 mM) to isolated protoplasts from Cellulomonas spp. The higher concentration of lysozyme was used to reduce the incubation time for protoplast formation and to obtain in short time viable protoplasts. Table 3.1 gives the protoplast formation frequencies of strains used. Under these conditions almost all cells were converted to protoplast in case of Bacillus strains. However, protoplast formation frrequency was 98% for Cellulomonas species. The formation of protoplasts was monitored by their lysis after hypotonic shock and thus decrease in number of viable cells as described in Chapter 2.

Table 3.1 Protoplast formation and regeneration frequency

Organism	Starting no. of cells (Z)	Total no. of colonies (X)	No.of colonies after osmotic shock (Y)	Protoplast formation frequency * (%)	Protoplast regeneration frequency ** (%)
Cellulomonas spp. NCIM 2353	1.2×10^{10}	5.8×10^{8}	2.5×10^{8}	97.9	2.75
B. subtilis NCIM 2255	1.5×10^{10}	2×10^{8}	ND	100	1.3
B. subtilis NCIM 2635	1.3×10^{10}	1.6×10^{8}	ND	100	1.2

ND : Not detected

Z : Total number of cells

X : Total number of colonies from regenerated protoplasts and non protoplasted cells

Y : Colonies from non-protoplasted cells which resisted lysis upon osmotic shock

* : Protoplast formation frequency = Z - Y / Z x 100

** : Protoplast regeneration frequency = X - Y / Z x 100

3.2.2. Protoplast regeneration

The protoplasts obtained under optimum conditions were centrifuged at low speed to avoid their disruption and washed twice with protoplast buffer to remove lysozyme. These protoplasts were spread onto the regenerating medium consisted of LB with 0.5 M sucrose and 0.8% agar. Visible colonies appeared after 4 days of incubation in case of Cellulomonas while B. subtilis protoplasts regenerated after 48 h to give clear visible colonies. The regeneration frequencies were low as indicated in Table 3.1 which were 2.75% for Cellulomonas and 1.25% for B. subtilis. The protoplasts from Cellulomonas and B. subtilis were mixed and treated with PEG. After 3 min, the PEG was removed by washing with protoplast buffer. The frequency of regeneration of these treated mixed protoplasts were estimated in LB regeneration medium and was found to be 1 - 1.25%.

3.2.3. Protoplst fusion and selection of hybrids

The protoplast fusion was carried out by mixing identical number of protoplasts from each strain and the fusion was performed as described in Chapter 2. After removal of PEG, the mixed treated protoplasts were plated onto minimal regeneration medium with cellulose-123 as sole carbon source. Both the parental strains did not grow on the selection medium. Similarly, the regeneration frequency of mixed protoplasts after PEG treatment was also determined. About 18-20 colonies were recovered from all the plates. These single colonies were propagated under selection pressure and further subcultured on minimal medium with cellulose-123. Some colonies continued to grow after repeated subcultures. These prototrophic colonies were taken for further studies. Since our interest was only to look for the progeny with Cel^+ phenotype, only colonies grown on cellulose-123 showing Gram positive property were maintained. There were a few Cel^+ , Gram negative colonies which were lost during repeated subcultures. Of

the total colonies recovered, only four fusants were stably maintained. They were BS/C 003, BS/C 004, Bs/C 005 and BS/C 007.

3.2.4. Seggregational analysis of hybrids

Frequency of transfer of various parental markers in hybrids was calculated and their linkage was estimated. As seen from data in Table 3.2, Cel^+ and trp^+ markers in Cellulomonas seemed to be closely linked. Number of stable hybrids with prototrophic and cellulase phenotype thus was the least. These results indicated that in stable hybrids recombination between genomes of Cellulomonas and B. subtilis did occur. Further, this technique could be utilized to map the genome of Cellulomonas whose genetics is least worked out so far. These observations also indicated that only part of Cellulomonas genome was present in hybrids and that in different hybrids, this component was not necessarily identical.

3.2.5. Morphologies of the hybrids

The colonies recovered were purified by dilution plate method and representative colonies were selected and taken for further studies. Variations in colony morphologies as well as in shape and size of the hybrids were observed. Table 3.3 gives the detail studies on the morphology of the hybrids observed under light microscope after Gram staining. Some hybrids were bigger than Bacillus with clear indication of sporulation (Fig.3.1) Some were Gram negative rods either smaller or bigger than Cellulomonas and definitely smaller than Bacillus. One of the hybrids BS/C 004 showed very thick rod with three times shape of Bacillus. The fusant BS/C 007 showed chains of cocci and formed dry colony on LB or Dubos medium. In liquid culture, this hybrid formed big pellets which settled down immediately to give clear liquid medium. The pelletes were found to be aggregates of cells.

Table 3.2. Frequency of transfer of various genomic markers in fused cells

Medium	Genotype	Total no. of colonies	* Regeneration frequency of fused cells out of	
			Total cells (10^{12})	regenerating mixed protoplasts (1.25×10^{10})
M9+ cellulose	Cel^+, Ala^+, Thi^+, Leu^+, His^+, Trp^+	23	2.3×10^{-11}	1.8×10^{-9}
M9+ cellulose Ala , Thi	Cel^+, Leu^+, His^+, Trp^+	25	2.5×10^{-11}	2.0×10^{-9}
M9+ cellulose Ala , Thi , Leu , His	Cel^+, Trp^+	5×10^{6}	0.5×10^{-5}	4.3×10^{-4}
M9+ cellulose Ala , Thi , Leu , Trp	Cel^+, His^+,	1×10^{5}	1×10^{-7}	8×10^{-6}

* : Results are comparable in three independent experiments. Calculations as explained in Chapter 2.

Table 3.3 Properties of the hybrids

Organism	Morphology	Cellulase positivity
A) *Cellulomonas* x *B. subtilis* NCIM 2255		
Bs/C 001	Gram +ve short rods but bigger than *Cellulomonas*	+
Bs/C 002	Gram +ve thick rod like *Bacillus* sporulation observed	+
Bs/C 003	Gram +ve rod like *Bacillus*	++
Bs/C 004	Gram +ve rods thicker and bigger than *Bacillus* Spo	++
Bs/C 005	Gram +ve small rods Spo	++++
Bs/C 006	Gram +ve *Bacillus* type	+
Bs/C 007	Gram +ve cocci in chains	+

Strains were grown in LB for 48 hours and then taken for Gram staining. The observations were made under light microscope under oil immersion.

++++ good activity of endoglucanase

++ moderate activity

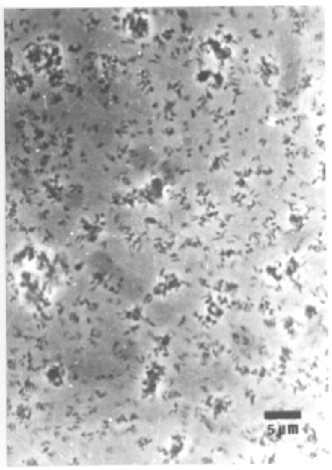
+ activity may or may not be detected

Table 3.3 continued

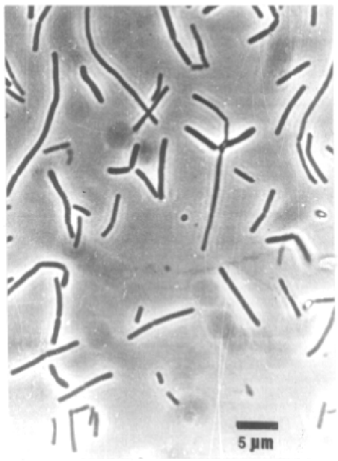
Organism	Morphology	Cellulase positivity
B) *Cellulomonas* x *B. subtilis* NCIM 2635		
Bs/C 001	Gram +ve, long rods thinner and larger than *Bacillus*	-
Bs/C 002	Gram -ve, very long rods	+
Bs/C 003	Gram +ve, chain formation observed	+
Bs/C 004	Gram +ve, very thin rods	++
Bs/C 005	Gram -ve, *Cellulomonas* like rods	-
Bs/C 006	Gram +ve rods, sporulation observed	++
Bs/C 007	Gram +ve, thick rods than *Bacillus*	-
Bs/C 008	Gram +ve, sporulating very thick rods,	+
Bs/C 009	Gram +ve rods like *Bacillus*	-

Fig. 3.1. Morphology of hybrids

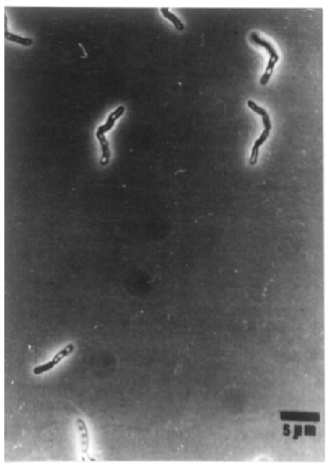
I. Light Microscopic pictures of Cellulomonas spp. (A) B. cereus (B), Hybrid 1(C) and Hybrid 2(D).



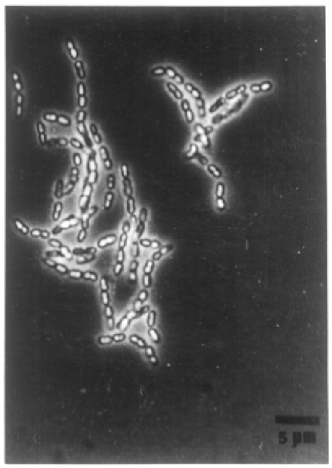
A



B



C

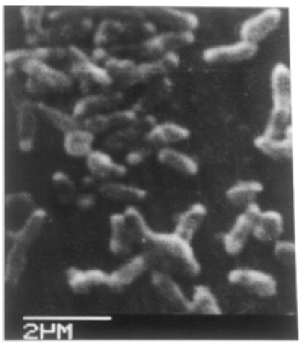


D

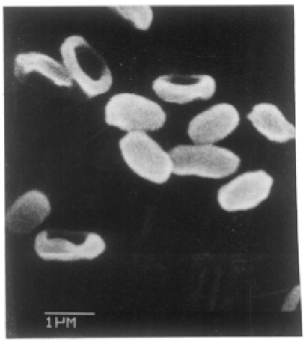
Fig. 3.1(I)

Fig. 3.1. Morphology of hybrids contd.

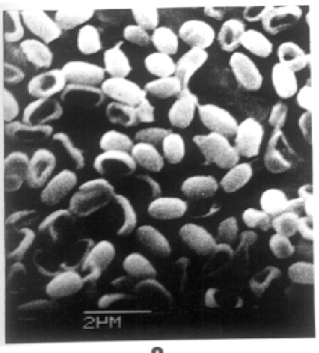
II. Scanning electron microscopic (SEM) pictures of Cellulomonas (A), B. subtilis, NCIM 2255 (B) Hybrid Bs/C 005(C) and Bs/C 007(D)



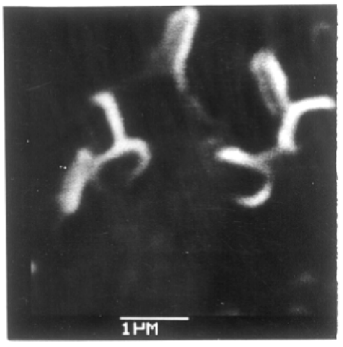
A



B



C



D

Fig. 3.1(II)

3.2.6 Testing of Cel$^+$ phenotype of the hybrids

All the hybrids were tested for their production of cellulolytic activity (only endoglucanase). Initially the innolculum was built up in Dubos medium with cellobiose as substrate and the cells obtained were innoculated into Dubos medium with cellulose-123 as substrate. After 5 days of incubation at 30° C, extracellular as well as intracellular endoglucanase activity was detected. As shown in Table 3.3 it was observed that only Bs/C 005 gave significant levels of endoglucanase, both extracellularly and intracellularly. Some of these hybrids gave detectable levels of endoglucanase but this could not be revived during repeated subcultures. Since Bs/C 005 gave comparatively better endoglucanase activity, this was screened for the ability to produce other enzymes like xylanase and β-glucosidase. Table 3.4 showed that low but detectable xylanase activity was found in cultured filtrate of cellulose-123 grown Bs/C 005 when compared to Cellulomonas. Additionally extracellular β-glucosidase was detected in case of this hybrid indicating in Bacillus environment, β-glucosidase distribution was changed as compared to that in Cellulomonas.

3.2.7 Reversion analysis of hybrids

The hybrids were grown overnight in LB and the cells were pelleted. They were washed with saline and after appropriate dilution were plated onto minimal medium with cellulose-123 but not containing amino acids (Table 3.5). This experiment was performed to demonstrate whether hybrids could seggregate in absence of selection pressure giving rise to auxotrophic cells. As seen from the Table 3.5, no revertants were detected in case of Bs/C 005 and BS/C 007 even when they were grown in rich medium in absence of cellulosic substrate. Hybrids BS/C 003 and Bs/C 004 on the contrary showed that more than 90% cells lost the Cel$^+$ phenotype on growing them in absence of cellulose. However, as under conditions of this experiment, significant decrease in number of

Table 3.4 Production of extracellular enzymes by *Cellulomonas* and Bs/C 005 in presence of cellulose-123.

Organism	Enzyme activity (units/ml) *			
	FPase	CMCase	Xylanase	-glucosidase
Cellulomonas spp. NCIM 2353	0.01	1.0	21.0	ND
Hybrid Bs/C 005	0.015	1.15	6.0	1.0
B. subtilis NCIM 2255	-	-	-	-

ND : Not detected

* Units as defined in Chapter 2.

Table 3.5 Reversion of the hybrids

Organism	Colonies on		Reversion
	LB	Minimal medium + cellulose-123	(%)
Bs/C 003	100 ± 8	8 ± 3	92%
Bs/C 004	60 ± 6	5 ± 1	90%
Bs/C 005	70 ± 5	72 ± 2	-
Bs/C 007	78 ± 4	80 ± 5	-

Individual colonies were grown on LB medium (rich medium). After 20 h of incubation at 30 C, the fully grown cultures were washed with saline. They were diluted and plated onto both LB agar and minimal medium agar plates. This experiment was performed to find whether the primary colonies selected were composed of stable prototrophic bacteria, or they gave rise to auxotrophic bacteria due to seggregation after passage through rich medium.

cells growing on Dubos medium with cellulose could be due to loss of other markers leading to single or multiple auxotrophic and hence reversion. Individual auxotrophic markers were not analysed further. Nevertheless, compared to these two hybrids, Bs/C 005 and BS/C 007 indeed were stable even with respect to entire complementary DNA and there was no loss of even a part of foreign DNA.

3.2.8. Stability of hybrids

The hybrid was tested for maintenance of parental phenotypes when grown with or without selection pressure. In order to check stability of Cel^+ phenotype, Bs/C 005 grown in absence of cellulosic substrates (in rich medium) was subsequently grown on Dubos medium with cellulose-123. Simultaneously Bs/C 005 innoculum prepared in cellobiose containing Dubos medium were also grown in Dubos medium with cellulose. Keeping the innoculum size approximately identical cellulase activities were estimated at identical time points. There were no significant difference in the amount of enzyme activities in these two cultures (Table 3.6) indicating stability of Cel^+ phenotype in Bs/C 005. On the contrary, LB grown cells produced slightly increased levels of cellulolytic enzymes probably because more number of cells in the innoculum obtained in LB. These observations further comfirmed stable maintenance of cellulose degrading property in Bs/C 005.

3.2.9. Colony blot and DNA hybridization

To establish the hybrid status, DNAs from BS/C 003, Bs/C 005 and Bs/C 007 were analysed for homology with the parental DNA in a colony hybridization or DNA dot hybridization experiments as described in Chapter 2. Spectral properties of DNase are summarised in Table 3.7. Different amounts of DNA were immobilized on nitrocellulose membrane . There was very little hybridization between Cellulomonas and B. subtilis DNAs. (Fig. 3.2). However, relatively more hybridization was observed when radiolabelled B.

Table 3.6 Stability of Cel in hybrid Bs/C 005

	Enzyme activities (units/ml)		
Inoculum used	FP activity	CMCase activity	β-glucosidase activity
LB grown	0.025	1.25	1.05
Cellobiose brown	0.015	1.10	1.0

Activites were tested after 7 days of growth on Cellulose-123 using inoculum as mentioned above.

Table 3.7 Spectral Properties of DNA

Organism	A 260 / A 280
Cellulomonas spp. NCIM 2353	1.65
B. subtilis NCIM 2255	1.77
Hybrid Bs/C 005	1.68
Hybrid Bs/C 007	1.80

Fig. 3.2. DNA hybridization analysis

I. DNA dot hybridization : DNA from Cellulomonas was labell with 32p-dCTP in vitro and was used as probe

1. Bs/C 003
2. Bs/C 005
3. Bs/C 007
4. B. subtilis NCIM 2255
5. Cellulomonas spp

II. Colony hybridization : 32p-labelled B. subtilis NCIM 2255 DNA was used as probe

1. Cellulomonas spp.
2. Hybrid Bs/C 003
3. B.subtilis NCIM 2255
4. Hybrid Bs/C 005

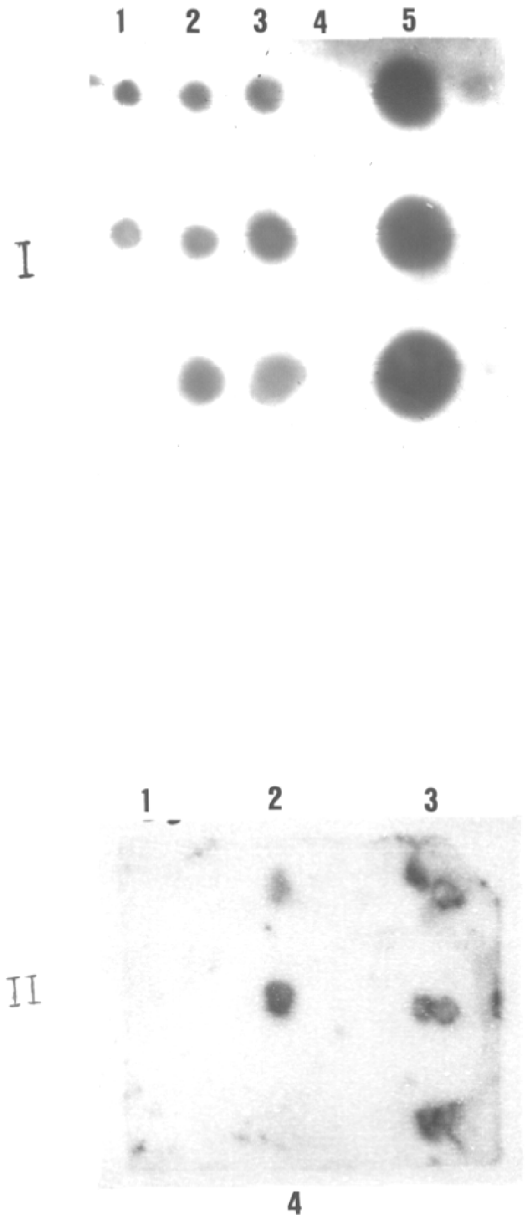


Fig. 3.2

subtilis DNA in limiting concentration was used as probe. Hybrid DNAs exhibited homology to both parental DNAs significantly. As could be seen from the Fig. 3.2, relative amounts of Cellulomonas homologous sequences were more in the DNA of BS/C 007 compared to other two hybrids; irrespective of expression levels of cellulases. This could be due to presence of Cellulomonas DNA other than cel^+ genes present exclusively in BS/C 007 hybrid.

3.2.10 Methylation of DNA in hybrids

By employing specific isochizomer pairs of restriction endonucleases as well as some specific restriction enzymes, susceptibility pattern of hybrid DNAs were compared to those of parental DNAs. As seen from Fig. 3.3, hybrid DNAs (Bs/C 005 and Bs/C 007) were more extensively broken down as compared to B. subtilis and Cellulomonas DNAs under identical reaction conditions. Simultaneously, bacteriophage lambda DNA was degraded to check the enzyme activities. Comparing the digestion patterns, one could conclude that specific restriction sites were now available more freely in hybrid DNAs possibly due to removal/absence of methylation at those sites.

This possibility was confirmed in a separate DNA dot detection of total methylated 5 mC or 6 mA content using specific antibodies. As seen from the Fig. 3.4 Cellulomonas DNA contained high amount of both 5mC and 6 mA. Bs/C 005 DNA, on the other hand, showed less quantities of both 5 mC and 6 mA than either of the parenetal DNA. Presence of relatively increased more 6 mA in Bs/C 007 could be due to methylation of `A´ at sites other than those recognised by the enzymes used and also due to more amount of Cellulomonas DNA component in BS/C 007 as indicated in DNA dot hybridization experiments.

Fig. 3.3 Restriction endonuclease sensitivity pattern of parentals and hybrid DNAs.

2 ug of each DNA was digested with individual enzyme with excess of enzyme and electrophoresed on 1% agarose gel in Tris-acetate-EDTA buffer. The electrophoresis was carried out at 60 V until the marker dye front (BPB) was moved upto 6 cm. Bacteriophage lambada DNA was digested under identical conditions to check the enzyme activity.

C. Control without enzyme

1. Msp I C C↓G G and C C*↓G G
2. Hpa II C C↓G G and *C C↓G G
3. Hae III G G↓C C
4. Mbo I G↓A T C
5. Sau 3 A I G↓A T C and G↓A* T C
6. Sma I C C C↓G G G

M1. Lambda Hind III DNA fragments

M2. Lambda Eco R I DNA fragments

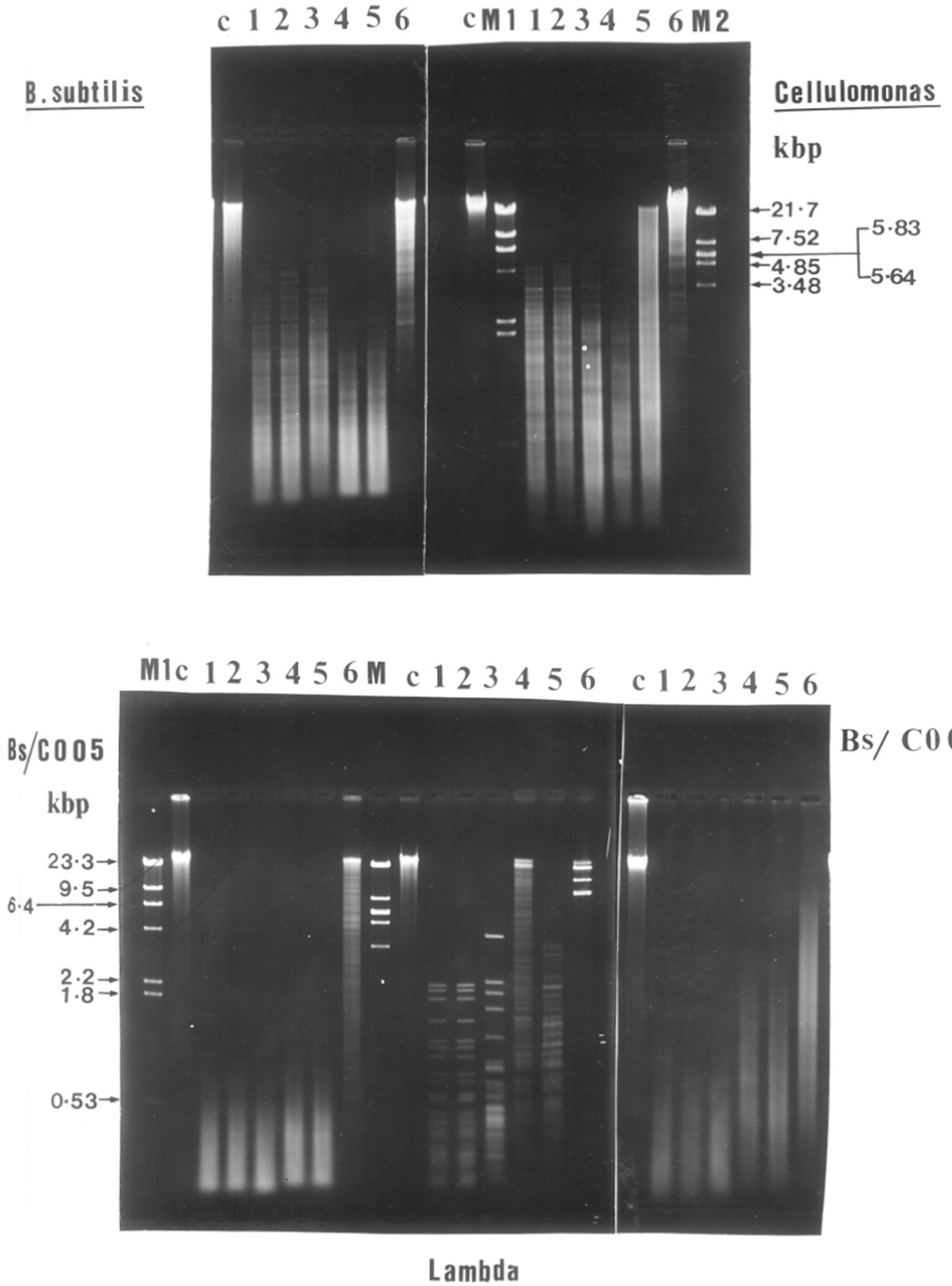

c 1 2 3 4 5 6
cM1 1 2 3 4 5 6 M2
B. subtilis
Cellulomonas
kbp
21·7
7·52
4·85
3·48
5·83
5·64
M1c 1 2 3 4 5 6 M
c 1 2 3 4 5 6
c 1 2 3 4 5 6
Bs/C005
Bs/ C007
kbp
23·3
9·5
6·4
4·2
2·2
1·8
0·53
Lambda

Fig. 3.3

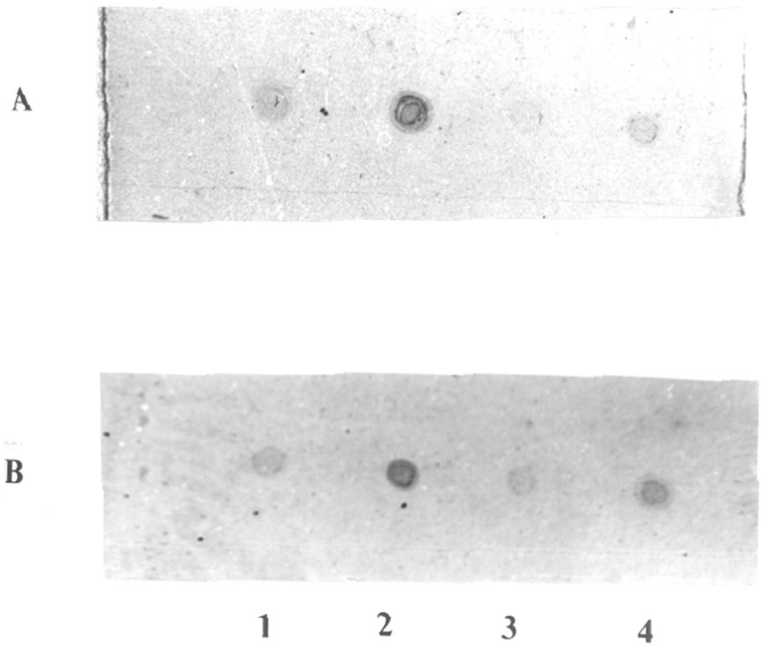


Fig.3.4

3.3. Discussion

Though conditions for complete protoplasting were standardized, the regeneration frequencies of protoplsts of both Cellulomonas and Bacillus strains were very low. This might be due to the high concentration of the lysozyme used for protoplast isolation which has been reported to lower the regeneration frequency (Kim and Lee, 1985). It could be attributed also to the absence of supplements like plasma expanders in the regeneration medium. The plasma expanders are known to enhance the regeneration frequency of protoplasts of bacteria (Hopwood et al., 1981a).

Protoplast fusion technique has attracted a great deal of interest in order to improve properties of microorganisms. Earlier reports on intraspecies hybrids in Bacillus (Fodor and Alfoldi, 1976; Schaeffer et al., 1976); Streptomyces (Baltz, 1979); Brevibacterium (Kaneco and Sekiguchi, 1979) etc. created an impression that success of hybrid formation by protoplast fusion depended on the taxonomic proximity of the strains (Lewis, 1969). Studies on interspecies protoplast fusion in Streptococcus (Okamoto et al., 1985 and Streptomyces (Hopwood, 1981) have reported successful isolation of hybrids. However, results presented here form the first report of successful isolation of stable intergeneric bacterial protoplast fusion hybrids (See also Gokhale et al., 1984). Following this, reports on intergeneric protoplast fusion from other laboratories (Chen et al, 1987; Prakash and Cummings, 1988) proved unequivocally that success of hybrid formation, even though with less frequency, does not require closely related strains.

Cellulomonas spp. and B. subtilis are very different in physiology and morphology and can be differentiated taxonomically. Cellulomonas is Gram variable cellulolytic microbe, while B. subtilis NCIM 2255 is Gram positive, sporulating rod with five auxotrophic markers. Fusion products were recovered on M9 minimal

medium with cellulose -123 as sole carbon source. The purpose of using this selection medium was to recover the hybrids which have only Cel properties. The fusants showed mixed physiological phenotypes of both parents such as Gram reaction, sporulation character and total complementation of the auxotrophic markers. A prototrophic nature of the fusion product indicated that genetic recombination (multiple) had occured between chromosomal DNAs of Cellulomonas and B. subtilis resulting in intergeneric recombinants. Such multiple recombinations were observed in intra and inter species fusion in Bacillus (Hopwood and Wright, 1978; Schaeffer et al., 1976).

Another problem that is often encountered in the application of protoplast fusion technique is the instability of fusion products. Results of genetic analysis of Streptomyces spp. (Hopwood , 1981; Ochi et al., 1979) and Bacillus spp. (Hotchkiss and Gabor, 1980; Sanchez-Rivaz et al, 1982) have shown that deploids or true prototrophic recombinants could be formed after protoplast fusion, in which heterodeploids are unstable which gave rise to parental seggregants, while haploid recombinants maintained genetic stability. In this study, I examined the morphological characteristics and also cellulose degrading activities of a few hybrids of Cellulomonas and B. subtilis after multiple transfers in rich medium. It was found that these hybrids were highly stable, and they retained properties of both parental strains. They have not lost these properties for last four years. Though the hybrids Bs/C 003, Bs/C 005 and Bs/C 007 grew well in cellulose - 123 medium, only Bs/C 005 exhibited enhanced cellulose degrading activity. Observations reported here that hybrids with different genotypes and morphologies were obtained indicates that the amount of genomic DNA of parents in all these hybrids was not similar. Further, it is also ruled out that eitire genomes of both the parents are retained in the hybrids. Occurrence of recombination is supported from the stability of hybrids, Bs/C 005 and Bs/C 007 which did not revert back to give any auxotrophic progeny. Interestingly thus a new microbe was generated exhibiting

cellulolytic property along with Gram positive sporulating phenotype. It is noteworthy that significantly large genomic fragments of Cellulomonas was retained and expressed in Bacillus environment.

While manipulating industrially useful microorganisms by introducing other complementary foreign genes into them, one comes across problem of restriction modification incompatibility barrier. Donor DNA need not be always stably accepted by recipient organism, thus not allowing expression of the desired foreign gene. DNAs of B. subtilis and Cellulomonas differ significantly in methylation levels, though specific restriction endonucleases in the strains used in this work have not been reported. One striking observation was that the susceptibility of genomic DNA in the stable hybrids to different restriction endonucleases increased significantly as compared to that of parental DNAs. Quantitative detection of 5 mC and 6 mA indeed confirmed this direction. This change might be a reason for adaptable stability of two different DNAs in the hybrids. However, whether this adaptation takes place during fusion and/or only adapted hybrids are selected is not understood; nor is it clear as to how this temporary complemntation is brought about. Newvertheless, results presented here suggest a very useful technique for tranasferring cloned genes into another desired microbe through protoplast fusion, wherein very few that stable hybrids would fulfil the requirement of new phenotype complementation.

Protoplast fusion thus helps in transfer of multigenic phenotypes across the barrier of genera. Interspecies protoplast fusion have yielded not only recombinant phenotypes but in some events have resulted in an additional new phenotype. For example, the possibility of discovering new antibiotics by recombination between structural and regulatory genes for antibiotic synthesis and thus activiating the silent genes would also be possible (Hopwood, 1981b). It is also likely therefore that the parental phenotypes are changed in the stable hybrids. Observation described earlier

show that hitherto cell bound β-glucosidase of Cellulomonas was found to be extracellular in the case of hybrid Bs/C 005. This approach of cell engineering thus could be used even for screening enzymes with new desirable properties, like hyperproduction, thermostability and extracellular secretion. Possibility that particular recombinational events might cause such changes is very strong, since different length of genomic fragments are found to be shared in the hybrids. The hybrid Bs/C 005 is exhibited cellulase phenotype also showed presence of active xylanase gene from Cellulomonas. In Cellulomonas cellulose as well as hemicellulose induced both cellulase and xylanase. It was of interest therefore to analyse substrate dependent induction of these enzymes in hybrid; since it is likely to obtain rearranged or changed organization of these genes in the hybrid genome. Indeed it was seen that relative levels of xylanase produced in Cellulomonas and Bs/C 005 in presence of cellulose - 123 were significantly different. protoplast fusion technique thus could be used in bacterial strains improvement, or even to transfer desirable complex phenotype to other organism belonging to different genera. Proper selection could help in obtaining even rare events of such intergeneric genomic complementation.

CHAPTER 4

CELLULOSE AND HEMICELLULOSE DEGRADING ENZYMES OF THE HYBRID Bs/C 005

4.1. Introduction

In the last three to four decades, much efforts have gone into the study of cellulolytic enzymes as potential means for obtaining energy, chemicals and single cell protein from abundant, renewable resources like cellulose. In this regard, cellulase system of the fungus T. reesei has been extensively studied. Several bacteria from various genera have also been known to produce cellulolytic enzymes. Among these bacteria, the best studied are Thermoactinomycetes (Hagerdal et al., 1978), Clostridium (Ng and Zeikus, 1981a, 1981b); Bacillus (Chan and Au, 1987; Robson and Chambliss, 1984) and Cellulomonas (Ham and Srinivasan, 1968; Stoppok et.al., 1982). Cellulomonas species are known as active producers of cellulase complex. Cellulomonas is usally found associated with agricultural waste and could be easily isolated (Han and Srinivasan, 1968). This organism, therefore, has been studied extensively with respect to cellulolytic enzyme production (Langsford et al., 1984). Mutants of Cellulomonas spp. with higher productivities of cellulase and β-glucosidase enzymes were isolated (Choi et al., 1978; Haggett et al., 1978). Some of these mutants have been shown to degrade crystalline cellulose more efficiently compared to parental strain (Haggett et.al, 1979).

The enzymatic hydrolysis of cellulose involves at least three types of enzymes, endo-1, 4- β -glucanase, exo-1,4- β -glucanase and β-glucosidase. Mode of action of individual enzymes has been described in Chapter I. Though Cellulomonas secretes both exoglucanase and endoglucanase enzymes, β-glucosidase enzyme remains either cell bound or intracellular (Gilkes et al., 1984a; Haggett et.al., 1978; Langsford et al., 1984). These enzymes have been further purified and characterize with reference to their substrate specificity and isoenzyme patterns (Prasertsan and Doelle, 1986; Thayer et al., 1984). Xylan degrading enzymes have also been studied with respect to their production, localization

and regulation (Rickard and Laughlin, 1980; Rapp and Wagner, 1986). The cloning and expression of cellulase genes from Cellulomonas fimi was first reported by Whittle et al (1982). The individual genes coding for endoglucanase and exoglucanase were cloned in E.coli (O'Neill et.al., 1986a; 1986b; Wong et al., 1986) which have been subsequently transferred and expressed in S. cerevisiae with an intention to engineer yeast strain capable of utilising cellulose as main carbon source for ethanol production (Curry et al., 1988). The transformed yeast was found to produce extracellular exoglucanase. Till today the transfer and expression of all genes coding for cellulase complex into a non-cellulolytic microbe has not been achieved. Our first report on isolation of intergeneric hybrids between Cellulomonas spp. and B. subtilis (Gokhale et al., 1984), demonstrated that such multigenic transfers and their coherent expression could be possible by way of protoplast fusion.

This Chapter deals with further characterization of one of these hybrids, Bs/C 005. Growth and enzyme production by Bs/C 005 in presence of different cellulosic and non-cellulosic substrates have been studied. Extracellular β-glucosidase activity secreted by Bs/C 005 was analysed further in relation to pH and temperature optima and stability at high temperature, as compared to Cellulomonas β-glucosidase. Isoenzyme pattern analysis of endoglucanase and xylanase produced by Cellulomonas and Bs/C 005 was studied.

4.2. Results

4.2.1. Effect of different cellulosic sources on extracellular enzyme production.

During the screening of hybrids for cellulolytic enzyme activities, only one hybrid, Bs/C 005, was found to secrete significant levels of enzyme in the growth medium. In comparison to Cellulomonas , Bs/C 005 showed singificant differences in endoglucanase, xylanase

and β-glucosidase levels during the growth on cellulose-123 powder as substrate. Therefore, changes in the distribution and levels of these enzymes during growth on various cellulosic substrates were studied. Data on the amounts of extracellular enzymes produced by both Cellulomonas and Bs/C 005 on different cellulosic substrates are given in the Table 4.1.

Cellulose-123 powder, Avicel and Solka Floc induced endoglucanse activity in either strain but level of endoglucanse activity in Bs/C 005 was always a little higher. CMC was poor inducer of this enzyme in either strain. Significantly, xylan induced endoglucanase in Cellulomonas but not in the hybrid.

As can be seen (Table 4.1), xylanase activity was induced in Cellulomonas by all the substrates used, though relatively higher xylanase activity was detected in culture filtrates of Cellulomonas when grwon on xylan. In Bs/C 005, cellulosic substrates like cellulose-123, Avicel were found to be poor inducer of xylanase while 5 and 10 fold increrase in xylanase levels were observed when the hybrid was grown on either xylan and solka floc respectively. Solka Floc gave highest yields of xylanase. CMC did not induce xylanase in the hybrid.

Regardless of the cellulosic substrates used, β-glucosidase in Cellulomonas was always intracellular. In contrast, depending upon the nature of substrate used, the hybrid produced major fraction of β-glucosidase in the growth medium. Cellulose-123 and Avicel were found to be better inducer of extracellular β - glucosidase in Bs/C 005.

4.2.2. Intracellular enzymes of Cellulomonas and Bs/C 005.

Cells grwon on different cellulosic substrates were harvested by centrifugation, washed and suspended in minimal volume of citrate phosphate buffer, pH 6.8. Cells were sonicated and each sonicated extract after centrifugation was used as source of intracellular

Table 4.1 Extracellular enzyme activities of *Cellulomonas* spp. and the hybrid Bs/C 005 on different cellulosic substrates.

Substrate (0.5%)	Enzyme activities (units/ml) *					
	Cellulomonas spp.			Bs/C 005		
	CMcase	Xylanase	β-glucosidase	CMcase	xylanase	β-glucosidase
Cellulose-123	1.0	21.0	ND	1.25	6.0	1.0
Avicel	0.9	21.0	ND	1.20	2.3	0.6
Solka Floc	0.9	19.0	ND	1.20	40.0	0.2
Xylan	0.66	26.0	ND	ND	23.0	0.15
CMC	0.5	5.0	ND	0.66	ND	ND

ND : Not detected

* Units as defined in Materials and Methods (Chapter 2).

enzyme. Intracellular activities of different enzymes of Cellulomonas and Bs/C 005 grown in presence of different cellulosic substrates when compared (Table 4.2), it was seen that specific activity of endoglucanase and β- glucosidase of the hybrid were higher in cellulose-123, Avicel and Solka Floc grown cultures. Similar to the results obtained for extracellular enzymes, in the case of hybrid organism, xylanase activity in CMC grown cells and endoglucanase activity in xylan grown cells could not be detected (Fig. 4.1). Xylanase activity of Bs/C 005 was comparatively more than that of Cellulomonas when grown in presence of Solka Floc or xylan. Among these substrates xylan was the poorest inducer of glucosidase.

4.2.3. Intracellular enzyme activities of Cellulomonas and hybrid grown on simple carbon sources

Cells grown for 96 h in presence of specific sugars/glycerol were centrifuged, washed twice and suspended in minimal volume of citrate phosphate buffer. After sonication followed by centrifugation various enzymatic activities were assayed as described in Chapter 2. The results are summarised in Table 4.3. When grown on cellobiose, endoglucanase and xylanase activities were detected only in Cellulomonas, while β- glucosidase activity was observed both in Cellulomonas and Bs/C 005 in presence of all the sugars tested. β-glucosidase activity in presence of almost all sugars was higher in Cellulomonas than that in the hybrid except in presence of lactose wherein the relative amounts were more in the hybrid cells. At the end of 4 days growth no reducing sugar could be detected in the culture filtrate indicating complete utilization by both organisms.

4.2.4. Profile of growth and enzyme production by Cellulomonas and Bs/C 005 in presence of cellulose-123

Cellulomonas and Bs/C 005 were grown in Dubos medium in cellulose 123 (0.5%) at 30 C. The method of cultivation is described in

Table 4.2 Intracellular enzyme activities of *Cellulomonas* spp. and the hybrid Bs/C 005 on different cellulosic substrates.

Substrate (0.5%)	Enzyme activities (units/mg protein) *					
	Cellulomonas spp.			Bs/C 005		
	CMcase	xylanase	β-glucosidase	CMcase	xylanase	β-glucosidase
Cellulose-123	1.9	12.9	0.53	6.1	2.39	2.24
Avicel	2.2	5.3	0.74	5.7	1.20	2.00
Solka Floc	0.99	5.37	0.65	2.9	8.50	1.25
Xylan	0.86	6.1	0.41	ND	14.2	0.35
CMC	4.8	2.43	3.29	2.58	ND	3.2

ND : Not detected

* Units as defined in Materials and Methods (Chapter 2).

Fig. 4.1. Differential Induction of enzymes in Bs/C 005. Using dot zymogram staining method, xylanase (A) and endoglucanase (B) activities were detected.

1. Extracellular enzyme from Cellulomonas spp. grown on CMC.
2. Extracellular enzyme from Bs/C 005 grown on CMC.
3. Intracellular enzyme from Bs/C 005 grown on CMC.
4. Extracellular enzyme of Bs/C 005 grown on xylan.

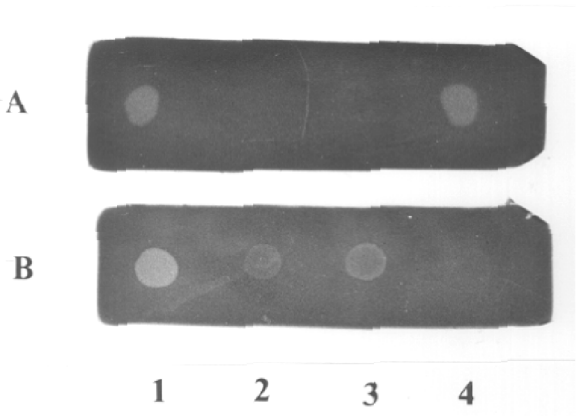


Fig. 4.1

Fig. 3.4. Immunochemical detection of 5 mC and 6mA in DNA

50 ng of individual DNA was immobilized on nitrocellulose BA-85 membrane and was stainied for 5 mC or 6 mA content using specific antibodies (purified IgG fraction) as described in Chapter 2.

A. 5 mC detection
B. 6 mA detection

1. B. subtilis NCIM 2255
2. Cellulomonas spp.
2. Hybrid Bs/C 005
4. Hybrid Bs/C 007

Table 4.3. Intracellular enzyme activities of *Cellulomonas* spp. and the hybrid Bs/C 005 on various carbon sources

Substrate (0.2%)	Enzyme activities (units/mg protein) *					
	Cellulomonas spp.			Bs/C 005		
	CMcase	xylanase	β-glucosidase	CMcase	xylanase	β-glucosidase
Glucose	ND	ND	0.50	ND	ND	0.27
Maltose	ND	ND	0.54	ND	ND	0.47
Lactose	ND	ND	0.60	ND	ND	0.80
Glycerol	ND	ND	0.50	ND	ND	0.35
Xylose	ND	ND	0.20	ND	ND	0.26
Cellobiose	0.8	1.0	2.7	ND	ND	1.7

ND : Not detected

Chapter 2. Samples were drawn at intervals as shown in appropriate figures. Parameters studied included growth (viable counts), endoglucanase, xylanase and β-glucosidase activities, and soluble protein in extracellular filtrate. These cultures were also grown separately on nutrient broth.

4.2.4.1 Growth on nutrient broth

The growth patterns of Cellulomonas, B. subtilis and Bs/C 005 in nutrient broth were compared (Fig. 4.2). The growth rate of Cellulomonas was low as compared to B. subtilis. The hybrid, Bs/C 005 however, exhibited higher growth rate than either of the parents.

4.2.4.2 Growth and soluble protein

From Fig. 4.3, it is apparent that the population of viable cells of both Cellulomonas and Bs/C 005 increased upto 2 days after which this increase was not significant. B. subtilis did not grow in this medium. The number of viable cells of Bs/C 005 were comparatively more than that of Cellulomonas. Since there was no decrease in the viable cells observed in both strains the possibility of release of cell bound β-glucosidase of Bs/C 005 was ruled out. As shown in Fig. 4.3, the profile of extracellular protein production by both the organisms resembled each other. The overall extracellular protein productivities of Cellulomonas were 1-1.2 time higher upto 4 days that those observed for Bs/C 005.

4.2.4.3. Endoglucanase, Xylanase and β-glucosidase

As illustrated in Fig. 4.4 A & B, endoglucanase and xylanse activity of Cellulomonas were first detected after 24 h and reached their maximum by 6 days. In case of Bs/C 005, a definite lag of 2 days was observed before the detectable levels of both endoglucanase and xylanase appeared in the culture filtrates. Such

Fig. 4.2. Growth patterns of parental strains and Bs/C 005 on nutrient broth.

Cultures were inoculated (10% inoculum, v/v) in nutrient broth and growth was monitored using Klet Summerson photoelectric colorimeter. The Klettreading was converted to optical density as described by Hawk (1965).

Optical density = Klett reading / 500

Cellulomonas spp : Csp 2353

B. subtilis NCIM 2255: Bs 2255

Hybrid Bs/C 005 : Bs/c 005

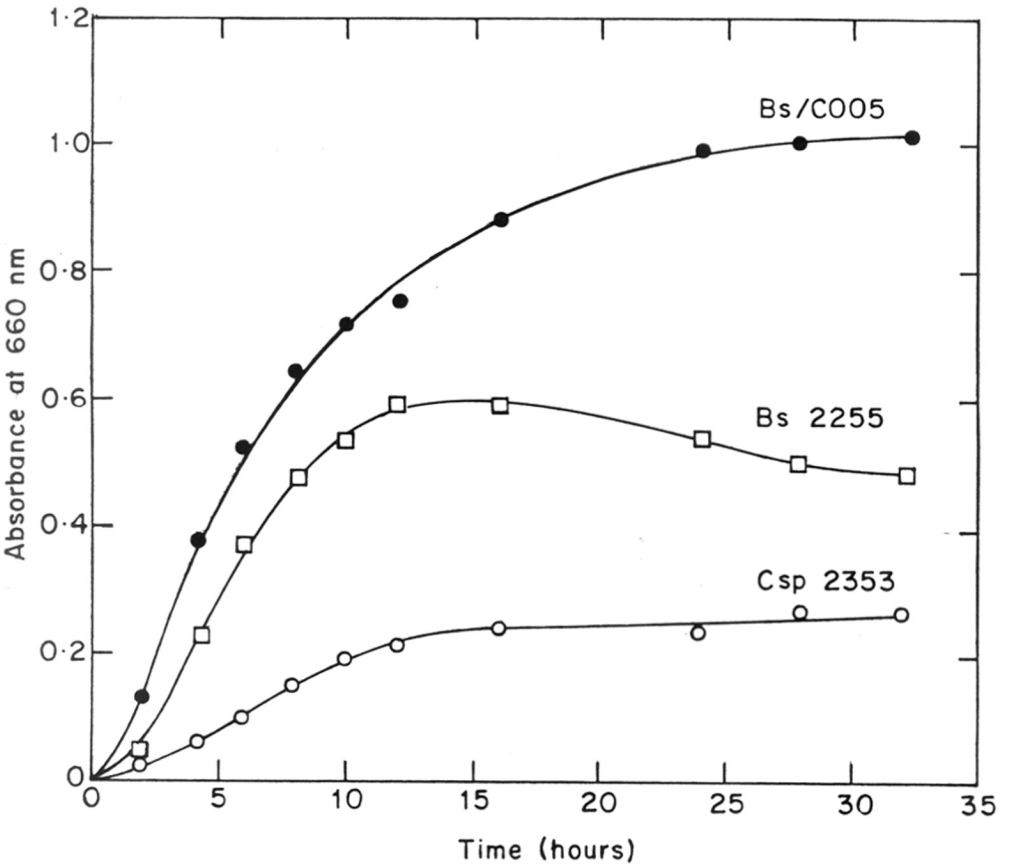
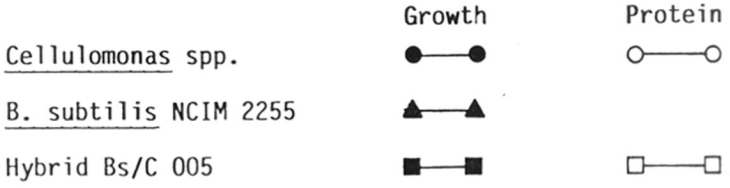

Absorbance at 660 nm
1·2
1·0
0·8
0·6
0·4
0·2
0
0
5
10
15
20
25
30
35
Time (hours)
Bs/C005
Bs 2255
Csp 2353

FIGURE 4·2

Fig. 4.3. Growth and extracellular protein of parental bacteria and Bs/C 005 in Dubos medium with cellulase-123 powder.



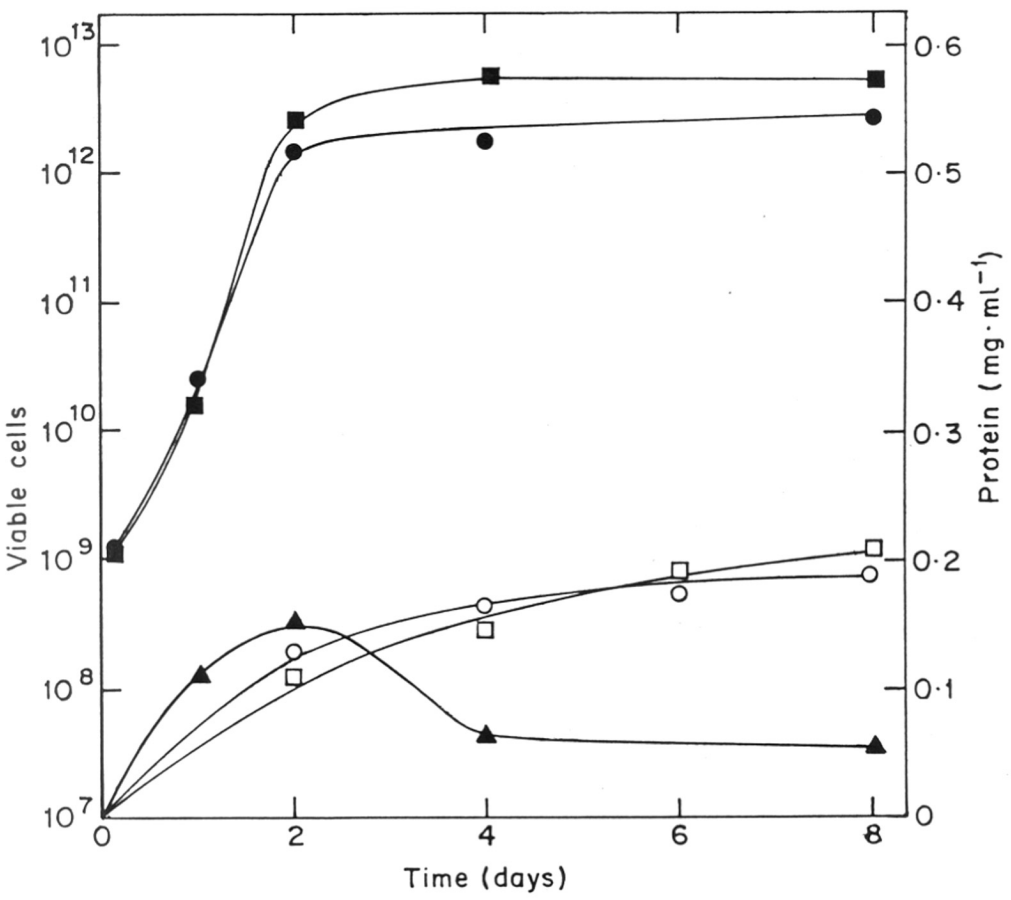

Viable cells
10^{13}
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
0·6
0·5
0·4
0·3
0·2
0·1
0
Protein (mg·ml^{-1})
0
2
4
6
8
Time (days)

FIGURE 4·3

Fig. 4.4. Enzyme activity profiles of cultures grown on cellulose-123 powder.

Open symbols : Cellulomonas spp.

Closed symbols : Bs/C 005

A) Endoglucanase and glucosidase activity

Endoglucanase activity

β-glucosidase activity

B) Xylanase activity

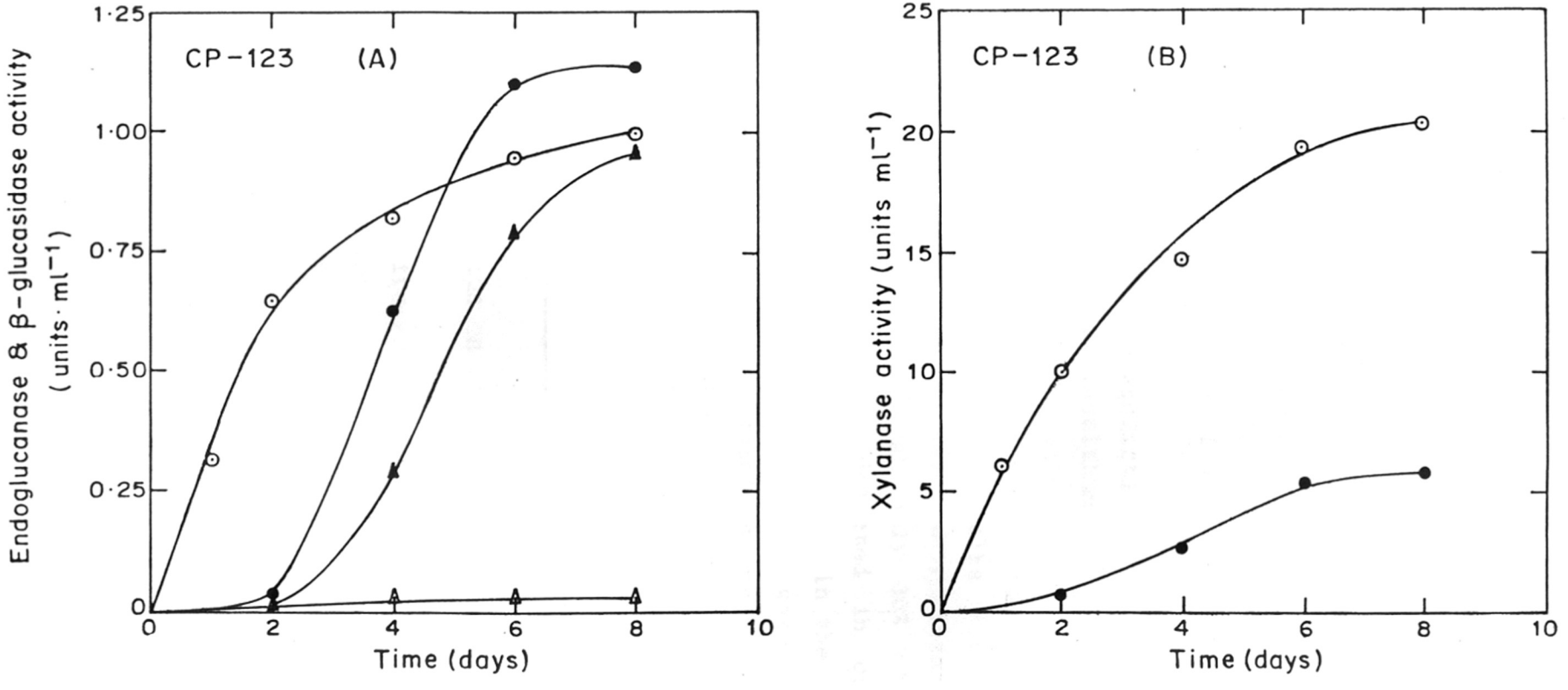

CP-123 (A)
Endoglucanase & β-glucasidase activity (units · ml⁻¹)
1·25
1·00
0·75
0·50
0·25
0
0
2
4
6
8
10
Time (days)
CP-123 (B)
Xylanase activity (units ml⁻¹)
25
20
15
10
5
0
0
2
4
6
8
10
Time (days)

FIGURE 4·4

lag was also observed in β-glucosidase synthesis by Bs/C 005. Followed by sharp increase in endoglucanase and glucosidase activities, these enzyme levels reached their maximum by 6th day in Bs/C 005 culture filtrate. In spite of early lag seen in enzyme production by the hybrid, the relative yields and activities were more than that of Cellulomonas by 6th day of growth.

4.2.5. Profile of extracellular enzyme production by Cellulomonas and Bs/C 005 using Solka Floc as substrate

Fig. 4.5 A and B indicate the profile of enzyme synthesis in both Cellulomonas and Bs/C 005 during growth on Solka Floc. In Cellulomonas, major amounts of endoglucanase and xylanase were produced with 4 days. In Bs/C 005 though only 30% of both endoglucanase and xylanase activity were detected in culture filtrates after 48 h, all xylanase activity came out in the medium within 4 days while majority of endoglucanase activity was observed only after 6 days. A definite lag in β-glucosidase production by Bs/C 005 was observed though the amounts produced were very low. Similar lag in -glucosidase production was observed when Bs/C 005 was grown on other insoluble substrates like Avicel.

4.2.6. Induction and catabolite repression

4.2.6.1. Effect of glucose addition on enzyme production

To examine the effect of catabolite repression, both Cellulomonas and Bs/C 005 were grown in Dubos medium with 0.5% cellulose-123 and 0.1% glucose. The endoglucanase, xylanase and β-glucosidase in culture filtrates were estimated at different time points. These results are given in Fig. 4.6 and 4.7. As shown previously, growth of Cellulomonas and Bs/C 005 on cellulose-123 alone resulted in the production of endoglucanase, xylanase and glucosidase. When grown on glucose alone no extracellular enzymes were detected in spite of the fact that both the bacteria grew well. These results

Fig. 4.5. Enzymatic activity profiles of cultures grown on Solka Floc SW 40

Open symbols : Cellulomonas spp.

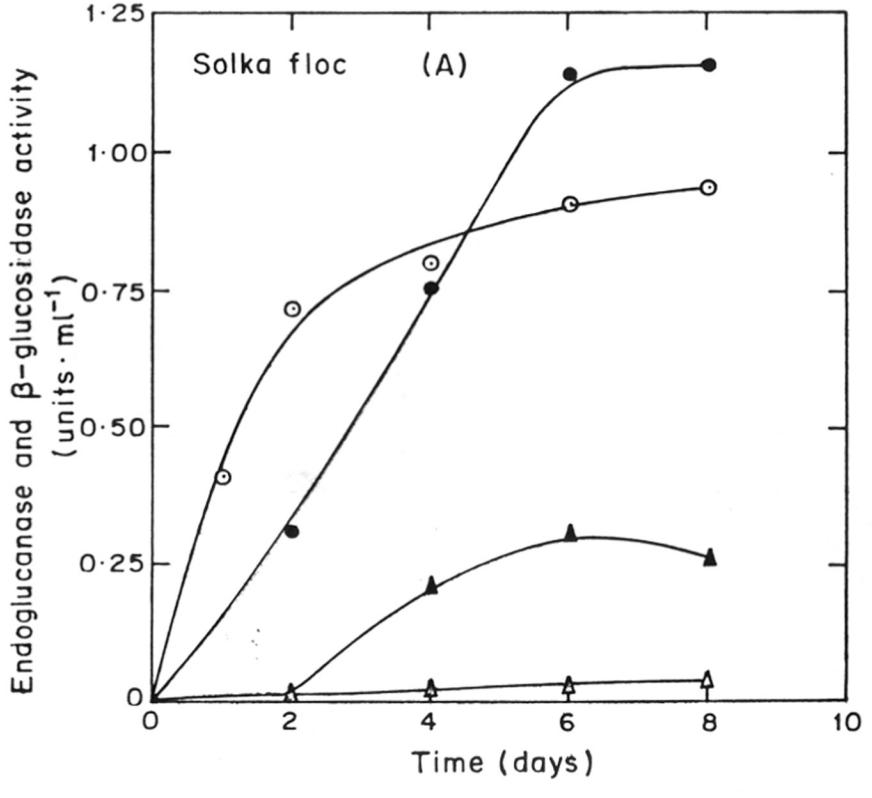
Closed symbols : Bs/C 005

A) Endoglucanase and β-glucosidase activity

Endoglucanase activity ⊙——⊙

β-glucosidase activity △——△

B) Xylanase activity ⊙——⊙


Solka floc (A)
Endoglucanase and β-glucosidase activity (units · ml⁻¹)
1·25
1·00
0·75
0·50
0·25
0
0
2
4
6
8
10
Time (days)

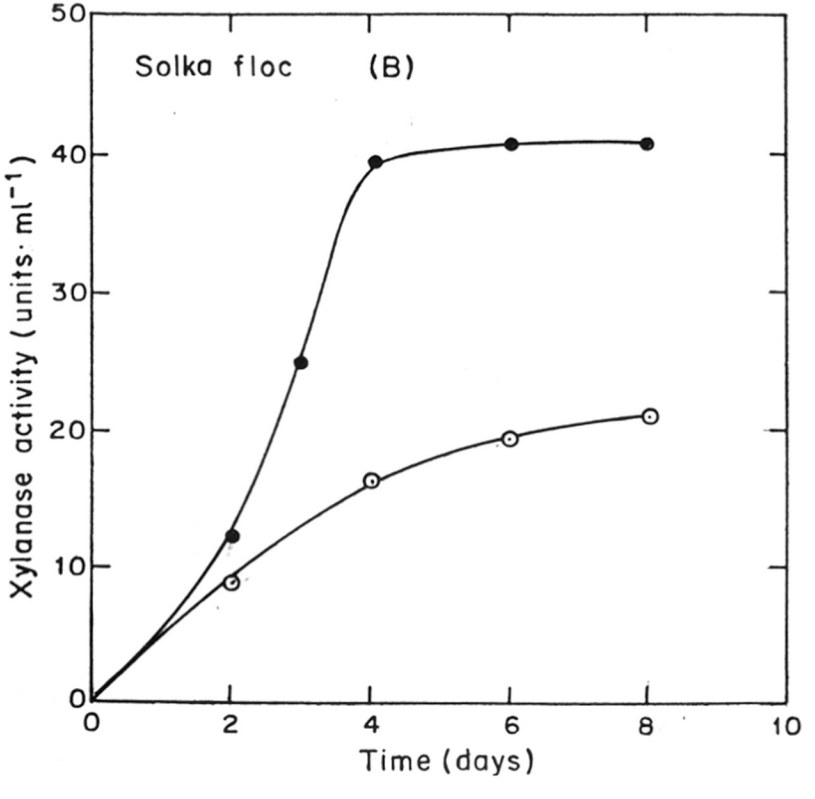

Solka floc (B)
Xylanase activity (units · ml⁻¹)
50
40
30
20
10
0
0
2
4
6
8
10
Time (days)

FIGURE 4·5

Fig. 4.6. Effect of addition of glucose and cellobiose on enzyme production in Cellulomonas spp.

A) Endoglucanase activity

B) Xylanase activity

1. Culture grown on cellulose-123 ⊙——⊙
2. Culture grown on cellulose 123 + glucose □——□
3. Culture grown on cellulose-123 + cellobiose △——△

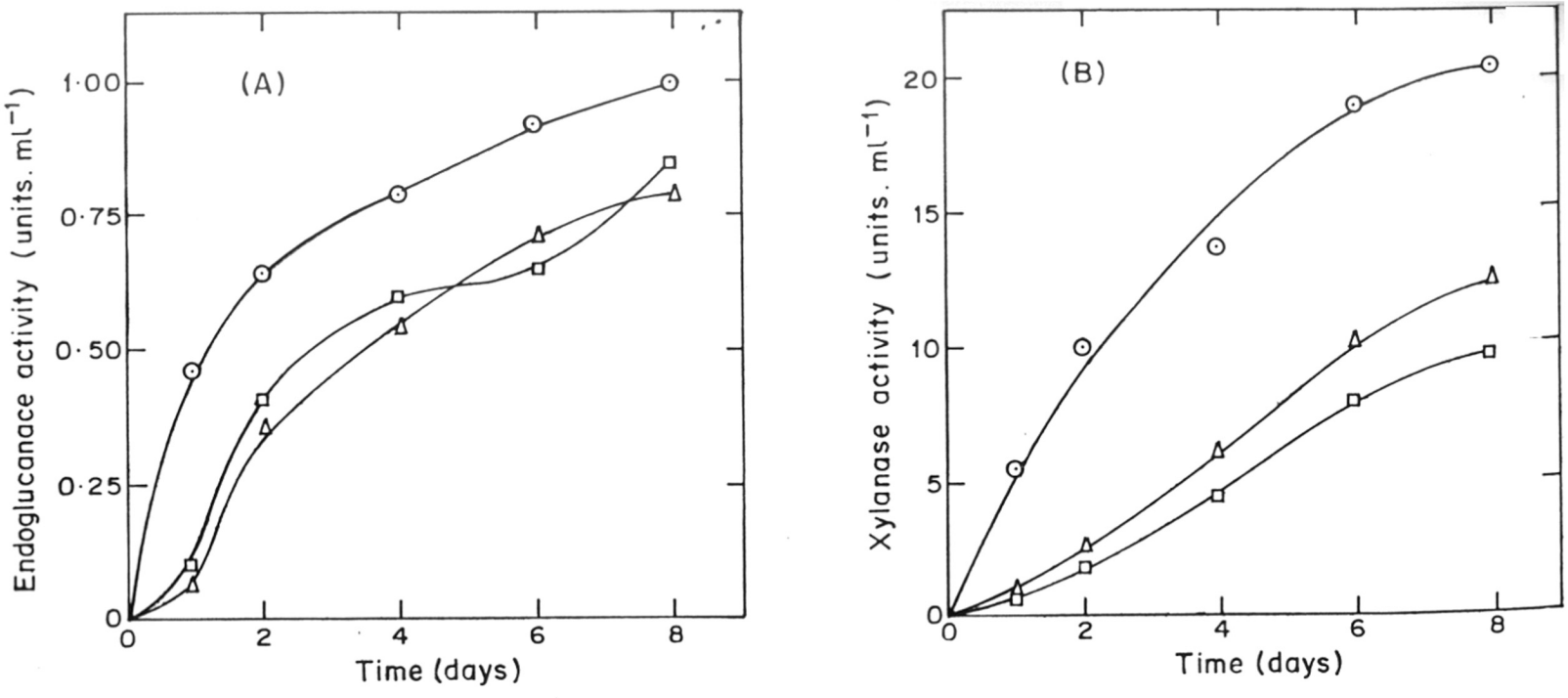

(A)
Endoglucanace activity (units. ml^{-1})
1·00
0·75
0·50
0·25
0
0
2
4
6
8
Time (days)
(B)
Xylanase activity (units. ml^{-1})
20
15
10
5
0
0
2
4
6
8
Time (days)

FIGURE 4·6

Fig. 4.7. Effect of addition of glucose and cellobiose on enzyme production in Bs/C 005

A) Endoglucanase activity

B) Xylanase activity

C) β-glucosidase activity

1. Culture grown on cellulose-123 ⊙——⊙
2. Culture grown on cellulose-123 + glucose □——□
3. Culture grown on cellulose-123 + cellobiose △——△

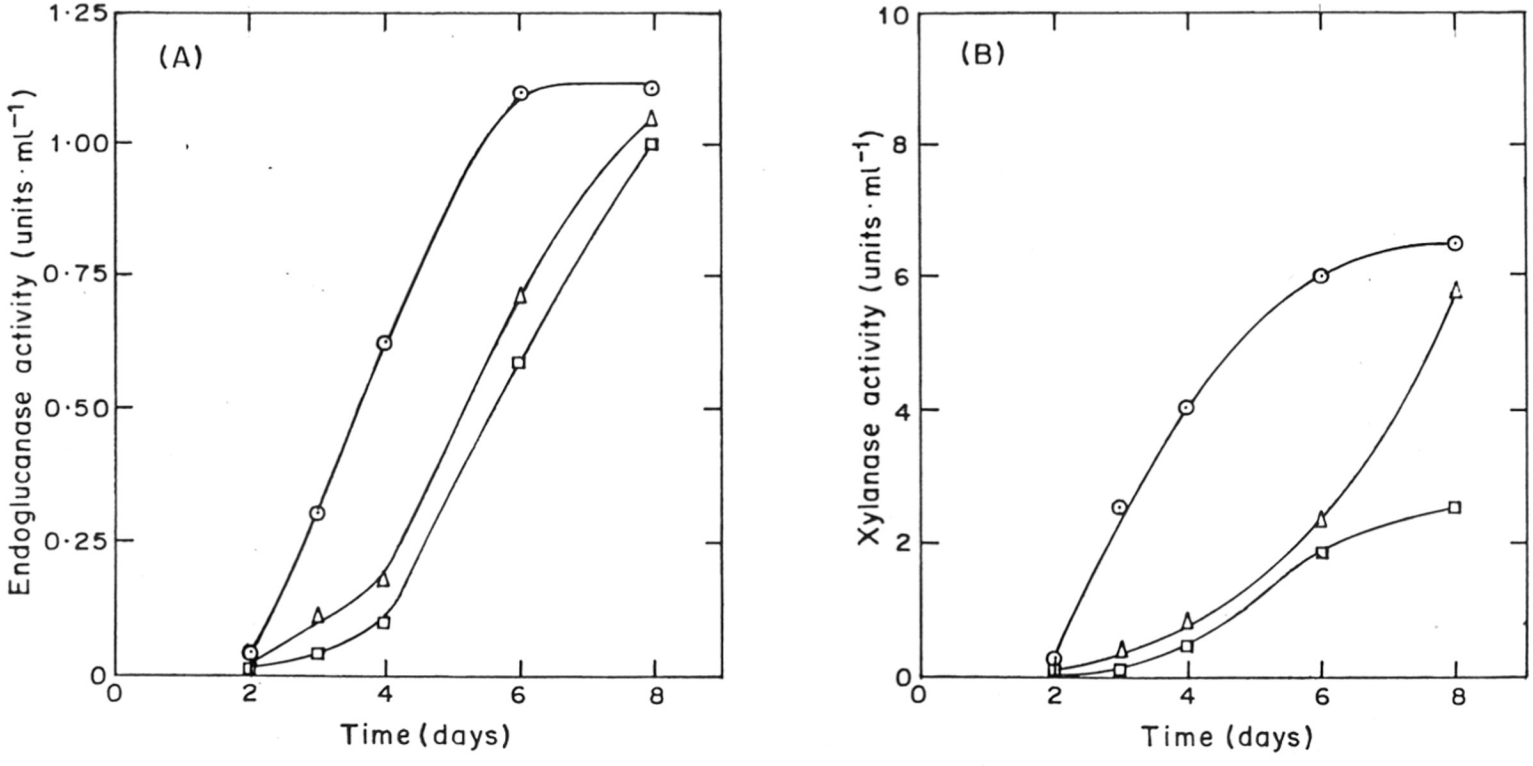

(A)
Endoglucanase activity (units · ml⁻¹)
1·25
1·00
0·75
0·50
0·25
0
0
2
4
6
8
Time (days)
(B)
Xylanase activity (units · ml⁻¹)
10
8
6
4
2
0
0
2
4
6
8
Time (days)

FIGURE 4·7

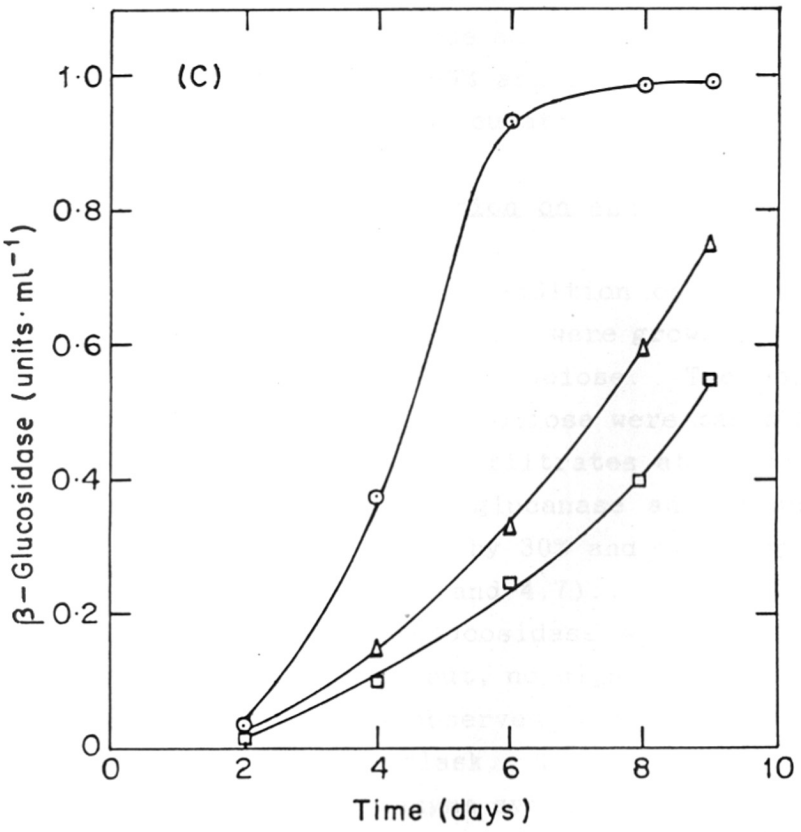


FIGURE 4·7

indicated possible regulation of enzyme production by catabolite repression. In case of Cellulomonas, glucose addition resulted in the reduction of endoglucanase and xylanase by 30% and 50% respectively. Though repressed in the initial stages, endoglucanase levels in Bs/C 005 at the end of 8 days were similar to those in absence of glucose. However, glucose addition reduced the xylanase and β-glucosidase activities by 60% and 30% as compared with the control even after the 8 days of incubation.

4.2.6.2. Effect of cellobiose addition on enzyme production

To examine the effect of cellobiose addition on the synthesis of these enzymes, Cellulomonas and Bs/C 005 were grown in Dubos medium with 0.5% cellulose-123 and 0.1% cellobiose. The enzyme levels obtained without the addition of cellobiose were taken as control. Activities were detected in culture filtrates at appropriate time points. In Cellulomonas, the endoglucanase and xylanase showed consistent reduction in the levels by 30% and 50% respectively as compared to the control (Fig. 4.6 and 4.7). In case of Bs/C 005, endoglucanase, xylanase and β-glucosidase were detected in low levels upto 6 days of incubation but, no significant difference in the levels of these enzymes was observed on 8th day as compared to the levels obtained in control flask. This observation indicated that cellobiose repressed the enzyme synthesis at early stages and this effect was eliminated after prolonged incubation.

4.2.7 Characterization of β-glucosidase of Cellulomonas and Bs/C 005

From previous experiments, it was observed that unlike Cellulomonas, the hybrid Bs/C 005 secreted significant amount of glucosidase in the growth medium during the growth on insoluble substrates such as cellulose 123 and Avicel. This interesting finding led us to characterize β-glucosidases from Cellulomonas and Bs/C 005 in relation to their temperature and pH optima as well as their stabilities towards higher temperature and pH. For these

studies, the effect of temperature and pH on activity and stability of these enzymes was assessed. These experiments were carried out using culture filtrates of Bs/C 005 and cell extracts of both Cellulomonas and Bs/C 005 grown on cellulose-123 for 7 days in shake flasks.

4.2.7.1. Effect of temperature on enzyme activity and stability

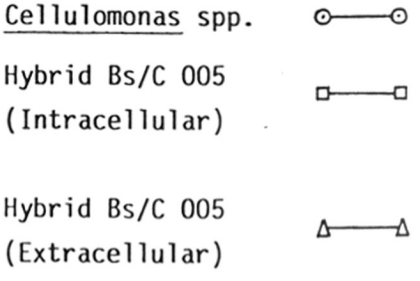
β-glucosidase assay was carried out at various temperatures ranging from 30° to 80° C and at optimum pH to determine the temperature at which the enzyme was most active. It is seen from Fig. 4.8, that β-glucosidase of Cellulomonas exhibited highest activity at 45° C while extracellular β-glucosidase of Bs/C 005 was most active at 60° C. Optimum temperature for intracellular β-glucosidase of Bs/C 005 was 50° C. The thermal stability of the enzyme was determined by preincubating the enzyme solutions at the specified temperatures for 30 min and then assaying the activity at their optimum temperature. It is observed from Fig.4.9 that Cellulomonas β-glucosidase retained only 65% and 15% activity at the end of 20 min at 50° C and 55° C respectively and lost 100% activity at 60° C within 5 min. In contrast, Bs/C 005 extracellular β-glucosidase showed only 50% inactivation at 60° C and 95% inactivation at 70° C after 20 min. The thermostability of intracellular β-glucosidase of Bs/C 005 was not checked.

4.2.7.2. Optimum pH and pH stability studies

To determine the optimum pH of enzyme solution, a standard β-glucosidase assay was performed in buffers of different pH ranging from pH 3.0 - pH 8.0. The assay was carried out at optimum temperature. From Fig. 4.10, it was observed that β- glucosidase of Cellulomonas, extracellular β-glucosidase of Bs/C 005 and intracellular β-glucosidase of Bs/C 005 exhibited optimum pH of 6.0, 5.0 and 5.5 respectively.

pH stability experiments were performed with Cellulomonas β-

Fig. 4.8. Optimum temperature for β-glucosidase



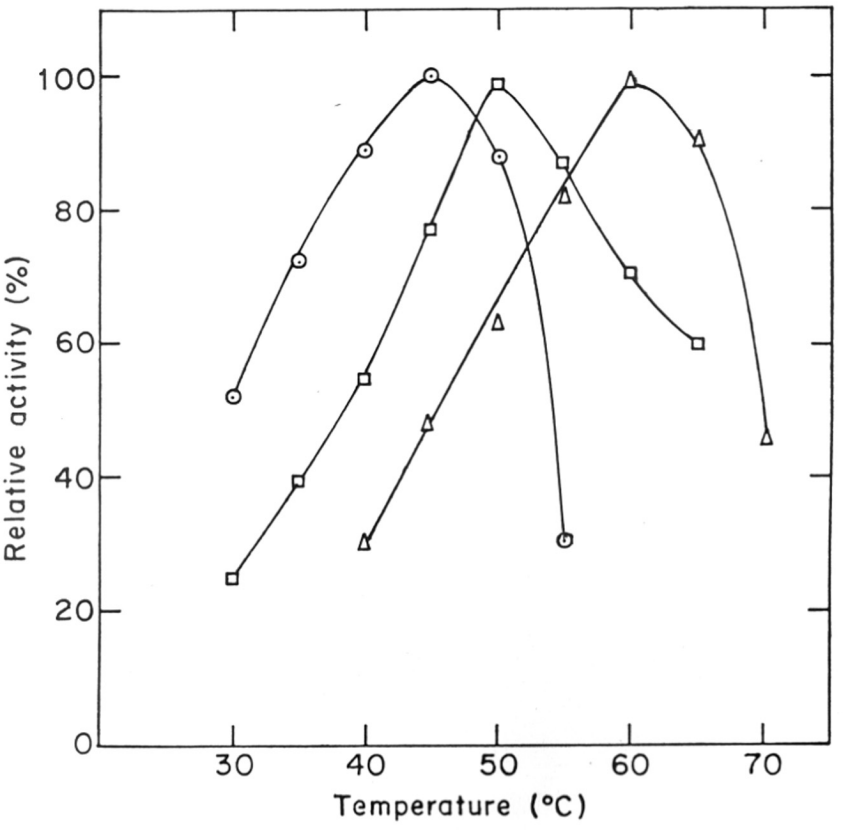
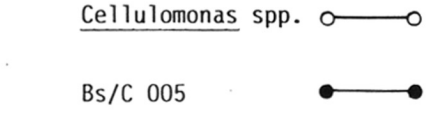

Relative activity (%)
100
80
60
40
20
0
30
40
50
60
70
Temperature (°C)

FIGURE 4·8

Fig. 4.9. continued

(E) Enzymes were preincubated at different temperatures for 5 min and then assayed for β-glucosidase activity under standard conditions.



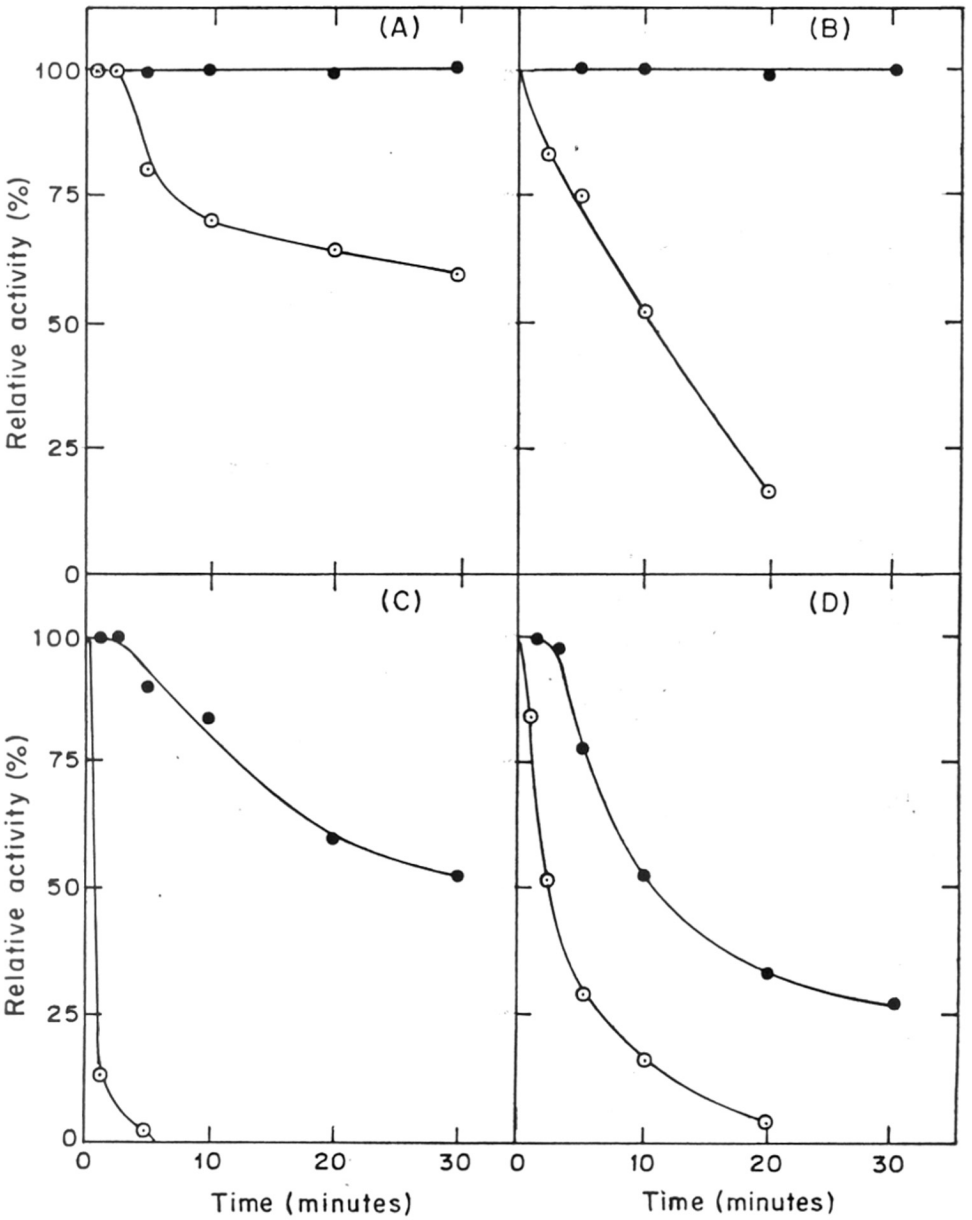
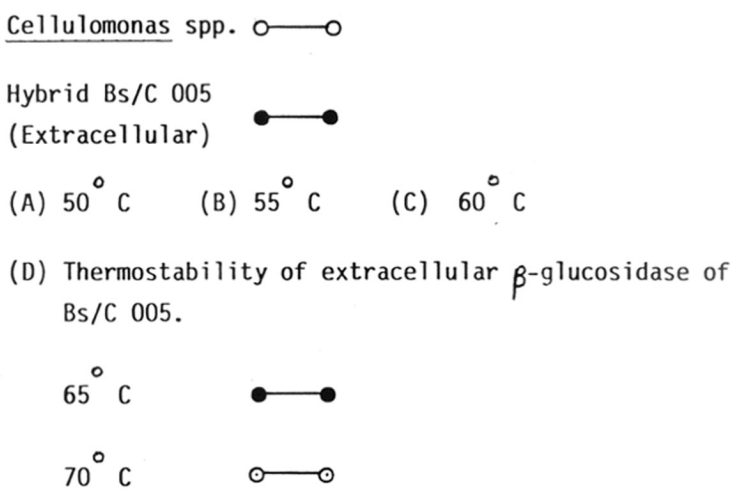


FIGURE 4·9

Fig. 4.9. Thermostability of β-glucosidase



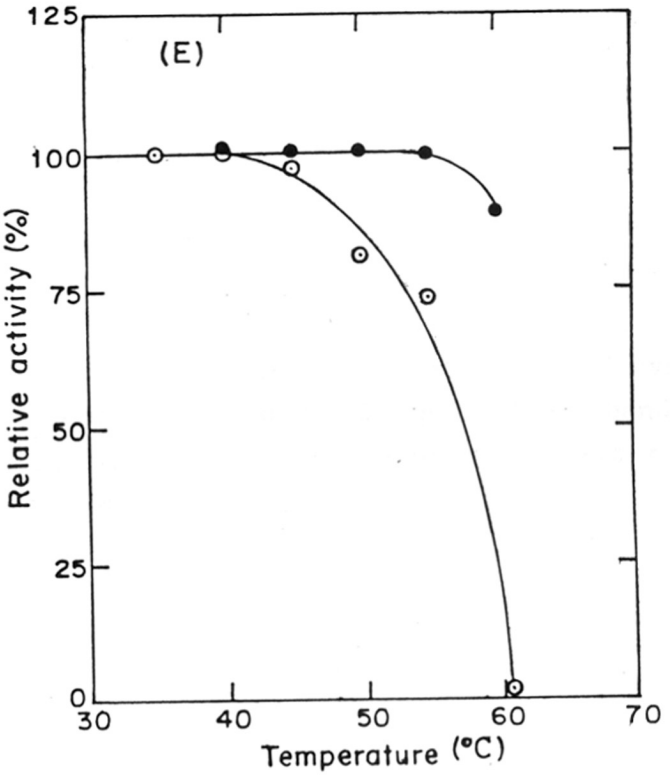

125
(E)
100
75
50
25
0
Relative activity (%)
30
40
50
60
70
Temperature (°C)

FIGURE 4·9

glucosidase and extracellular β-glucosidase of Bs/C 005. The enzyme solutions were preincubated in buffers of different pH at 30°C (for Cellulomonas) and 40°C, for Bs/C 005. The assays were carried out at their respective optimum temperatures and pH. Fig. 4.11 shows that β-glucosidase of Bs/C 005 was stable between pH 4.5 - pH 7.0 while β-glucosidase of Cellulomonas showed stability bwetween pH 5.0 - pH 6.5.

4.2.8. Resolution of endoglucanase activity by PAGE

Cellulomonas and Bs/C 005 were grown on cellulose-123 and the extracellular filtrate was concentrated 10 fold by lypholisation. Identical amounts of protein were loaded and PAGE was run as described in section . The PAGE in combination with zymogram staining for endoglucanase yielded 5 activity bands of endoglucanase for Cellulomonas, two of these were fast moving. Presence of 4 endoglucanase activity bands in Bs/C 005 was apparent.(Fig. 4.12) One of these bands was slow moving and unique to Bs/C 005. Two of these bands present in Cellulomonas were not found in Bs/C 005.

Fig. 4.10 Determination of optimum pH for β-glucosidase

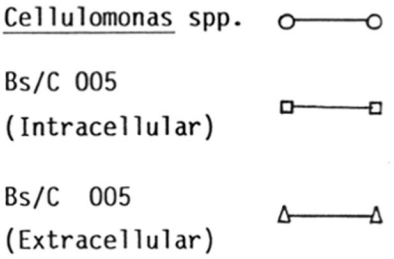
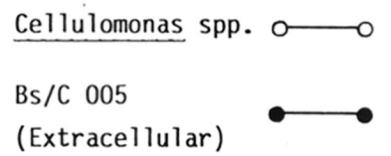


Fig. 4.11. pH stability of β-glucosidase



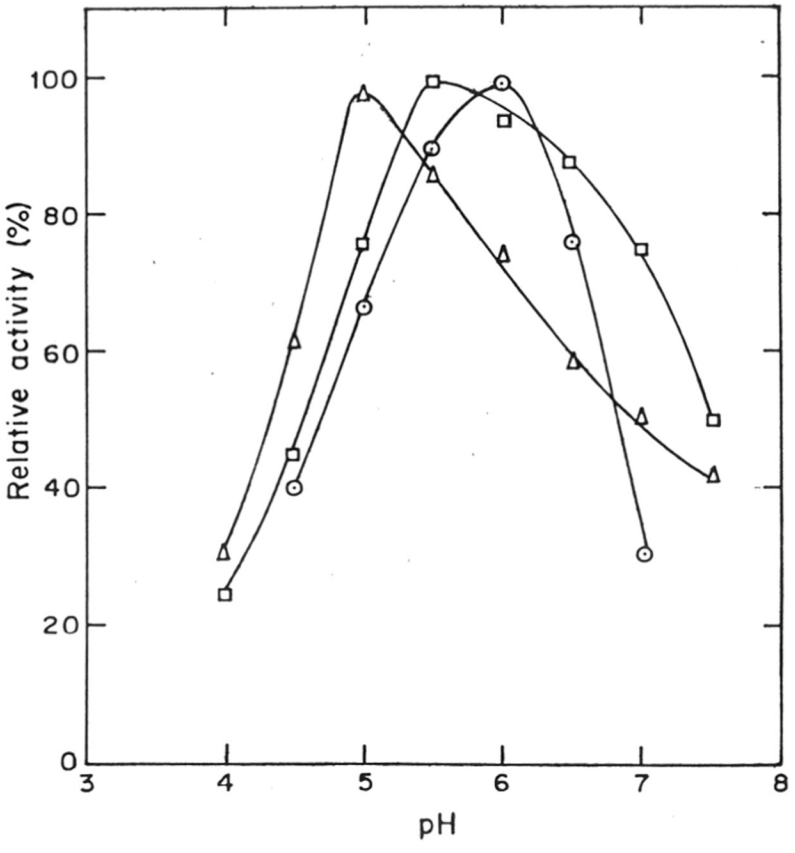


FIGURE 4·10

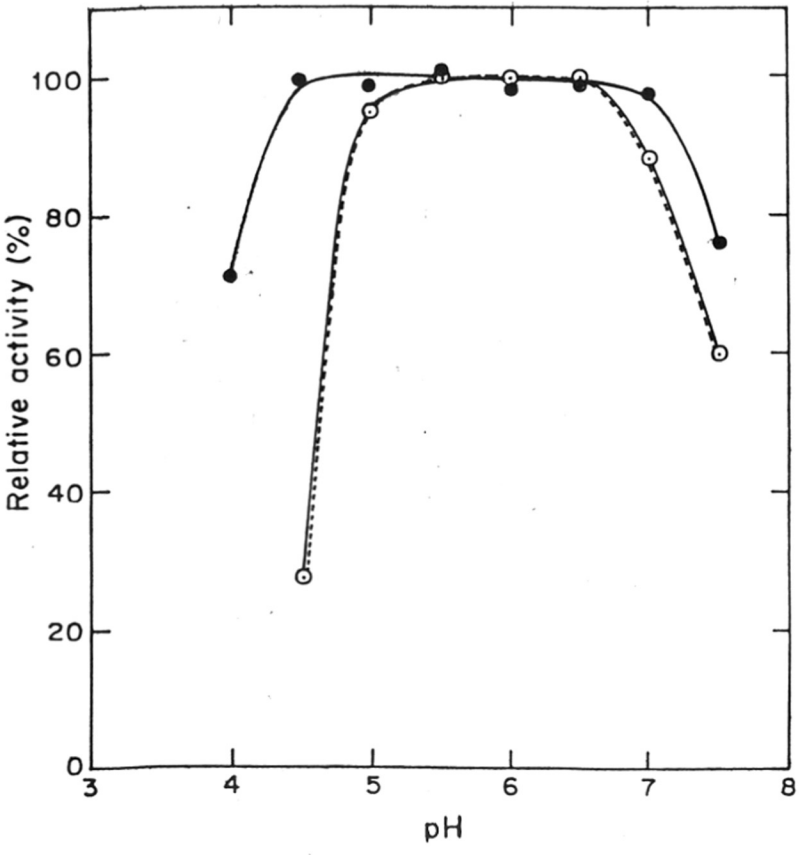


FIGURE 4·11

Fig. 4.12 Zymogram staining of endoglucanase on PAGE

Cultures were grown on cellulose-123 and samples were harvested after 8 days. These were lyophilized and redissolved in citrate phosphate buffer to yield 20 fold concentration. 40 ug of protein were applied to each lane of the gel and following electrophoresis the procedure for staining for eldoglucanase activity was followed as described in Chapter 2.

A) Cellulomonas culture filtrate

B) Hybrid Bs/C 005 culture filtrate

A B

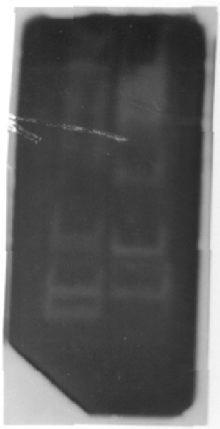


Fig. 4·12.

4.3. Discussion

Attempts to clone and express the cellulase genes from Cellulomonas and other bacterial strains in E. coli have demonstrated limited success. Subsequently, coexpression of exoglucanase and endoglucanase genes in other heterologous hosts such as S. cerevisiae has been reported (Wong et al., 1988). Conventionally, isolation of individual genes for a single functional polypeptide and its transfer and expression in other microbes has been feasible through recombinant DNA technology. However, as has been evident from phenotypes like nitrogen fixation and cellulose degradation, transfer and expression of DNA coding for such complex phenotypes into other unrelated microbes has not been successful.

Problems in such experiments have been of two major types. One, that the final phenotypes are result of synergenstic expression of more than one gene which is difficult to achieve through individual gene cloning, and two, that if the recipient organism were to be an existing industrially useful microbe then the compatability of restriction modification system of the donor and the recipient need not necessarily match.

As has been demonstrated and described in chapter III, protoplast fusion technique could be used to obtain B. subtilis cells harbouring complete cellulase complex along with xylanase activity. Interestingly, all these enzymes, including β- glucosidase were secreted out by this BS/C 005, whereas β- glucosidase is always cell bound in case of Cellulomonas (Choi et al., 1978). As a matter of fact, no other bacterial extracellular β-glucosidase has been reported except one recent report of Bronnenmeier and Staudenbauer (1988). Further, it was observed that secretion of β-glucosidase by Bs/C 005 was dependent on the nature of substrate; there being no detectable extracellular β-glucosidase in presence of simple carbon sources or cellobiose. Recently, multiple genes for β- glucosidase in Clostridium have been reported (Grabnitz & Staudenbauer, 1988). Whether there exist more

than one β - glucosidase gene in Cellulomonas and whether they are segregated in this hybrid could be studied by molecular cloning of β- glucosidase genes from Bs/C 005. Presence of signal polypeptide regions with -glucosidase gene in Bs/C 005 could be confirmed only after analysing the cloned β-glucosidase gene from this organism.

Secretion of proteins by B. subtilis and the role of signal peptide in such protein secretion has been well documented (Robbins et al.,1984; Vasantha et al., 1984). Conversion of hitherto, cell bound β-glucosidase into secretory protein by transfer of its gene(s) in B. subtilis could be due to fortuitous recombinational event close to signal peptide coding region during protoplst fusion. Occurrence of multiple stable recombinational events in protoplast fusion hybrids has been well documented in case of B. subtilis and S. aureus (Gabor and Hotchkiss, 1982; Stahl and Pattee, 1983). Stable and multiple recombinations during intergeneric protoplast fusion between B. subtilis and Cellulomonas spp. has been evident from the different recombinational frequencies between various markers used as demonstrated in the previous chapter. It is however, not established yet whether only a few of such recombinants are finally stable and are selected. Variations in properties of different stable hybrids support the probability of recombination taking place randomly but selection of stable recombinants is a function of further stability of such recombinant genomes.

The enzyme induction pattern in Cellulomonas spp. reveals that regardless of the cellulosic substrate used, all enzymes except β-glucosidase were secreted into the growth medium.. Choi et.al (1978) have reported that endoglucanase was predominantly cell bound in Cellulomonas during growth on insoluble substrates. However, we observed that majority of endoglucanase activity on insoluble substrates was extracellular in Cellulomonas. The major fraction of xylanase activity of Cellulomonas was also extracellular, comfirming the observations of Rickard and Laughlin

(1980) and Peiris et.al (1982). Increased levels of xylanase activity in presence of xylan supports the concept that Cellulomonas strains do possess true xylanase which is induced only by xylan. In Cellulomonas, celllulase and xylanase activities are induced by both cellulose and hemicellulose. In comparison to this, Bs/C 005 exhibited differences in enzyme induction pattern when grown on different cellulosic substrates. Xylanase activity of Bs/C 005 was mainly induced either by Solka Floc or by xylan. The small amount of xylanase activity observed in Bs/C 005 when grown on cellulose-123 and Avicel was due to hemicellulosic fractions present in these substrates. Xylan did not induce endoglucanase and CMC did not induce xylanase in Bs/C 005 suggesting that xylanse induction was totally dependent on xylan. These results suggest the possibility of segregation of xylanase gene in Bs/C 005 from the rest of cellulase complex as result of recombination during protoplast fusion. These observations also confirm the deduction that Cellulomonas spp. harbours true xylanase which is induced by xylan. No xylanase gene from Cellulomonas as yet been characterized. The use of isolated xylanase gene of Cellulomonas could help in resolving this observation regarding segregation of cellulase genes in stable hybrids.

Another way of interpreting the induction profile of xylanase and endoglucanase in Cellulomonas and Bs/C 005 could be that xylanase genes are specifically induced by xylan in both cases. Observed xylanase activity in Cellulomonas in presence of CMC could be due to endoglucanase protein which also acts on xylan. This suggestion is based on the reports of Gilkes et.al (1984b) where molecular clone identified as of Cellulomonas exoglucanase (pEC1) coding for 56 kd protein also exhibited high xylanase activity. Another clone pEC2 coding for 58 kd protein, on the other hand, exhibited endoglucanase and also lichenan degrading activity. It is therefore, visualizwed that the certain genome sequences code for a polypeptide having activity recognition domains of different glucanases. However, their presentation for interaction with

specific substrates depends on conformation and the folding of these polypeptides.

In presence of sugars like glucose, maltose, lactose, only intracellular β-glucosidase was induced both in Cellulomonas and Bs/C 005. Cellobiose induced all other enzymes in Cellulomonas but only intracellularly, whereas none in Bs/C 005. Induction of cellulases thus depends on cellulosic substrates. β- Glucosidase, on the other hand appeared to be constitutive in Cellulomonas and its levels further increased when Cellulomonas were grown in either in CMC or cellobiose. Constitutive nature of β-glucosidase has also been reported in C. uda by Stoppok et.al (1982). The number of viable cells of Bs/C 005 did not decline upto 8 days of incubation and therefore, extracellular β- glucosidase activity did not seem to be a result of cell lysis.

Extracellular protein production could be detected after 24 h incubation and continued upto approximately 4 days for Cellulomonas and 6 days for Bs/C 005. Levels of extracellular endoglucanase and xylanase in case of Cellulomonas were low in first 48 h. These enzymes appeared extracellularly in case of Bs/C 005 only after 48 h. One thus cannot necessarily conclude that extracellular protein production precedes enzyme production. The inability to detect enzyme activities at the early growth phase may be due to the fact that the enzyme assays are not as sensitive as protein assay. The reason for long lag before enzyme production by Bs/C 005 was not understood. This lag however, was not observed when Solka Floc was used as a substrate. Cellulase enzymes have been known to be adsorbed on insoluble cellulosic substrates and hence they are removed from the culture supernatants prior to assay. This could be one of the reasons for inability to detect extracellular activities by standard assay methods in the early period of incubation when the high levels of insoluble substrates still exist in the medium. Similar phenomena has been described in case of Trichoderma by Su (1976). Cellulase production by Cellulomonas appeared to be growth associated with most of the activity detected

during the later phase of growth; the observation which has also been reported in Trichoderma cultures (Andreotti et.al., 1977) and in Cellulomonas spp. (Vladul - Talor et al., 1986).

Extracellular cellulase production by Cellulomonas is repressed by readily assimilated carbon sources, similar to those in case of other bacteria and fungi. (Berg et al., 1972; Mandels, 1975). Overall levels of these enzymes were indeed repressed in presence of glucose both in Cellulomonas and hybrid. Endoglucanase, however, was derepressed faster in Bs/C 005. Xylanase, on the other hand, was significantly represed both in Cellulomonas and Bs/C 005 in presence of glucose. The extent of xylanse repression by cellobiose was relatively more in Cellulomonas. Apparently, the week inducing power of cellobiose is due to the catabolite repression which counteracts the inducing activity of cellobiose as reported in T. reesei (Mandels and Reese, 1960). The mutants of Cellulomonas insensitive to catabolite repression have been isolated so as to get higher productivities of enzyme in presence of glucose and cellobiose (Beguin and Essen, 1977; Choudhary et al., 1980; Stewart and Leatherwood, 1976). B. subtilis DLG strain was reported to be free from catabolite repression of glucanase production (Robson and Chambliss, 1984). Lee and Pack, (1987) have shown that transformants of both B. subtilis RM 125 and B. megaterium (pCK 98) produce very high level of endoglucanse during the exponential growth period and the production was not repressed by glucose or cellobiose. Such engineered strains offer advantages over the other cellulase producers such as Trichoderma (Mandels, 1981) and C. uda (Stoppok et al., 1982) in which catabolite repression is common.

In general, extracellular enzymes are more stable under extreme physical conditions such as higher temperatures and they also have fairly broad pH tolerance (Frost and Moss, 1987). These properties have partly been attributed to post-translational modification such as glycosylations. These glycosylated enzymes (especially N-linked glycosylation) are protected from the attack of proteases in

Trichoderma and Cellulomonas (Langsford et al., 1987; Merivuori et al., 1987). It was therefore not surprising to find that extracellular β-glucosidase of Bs/C 005 was more thermostable than that of intracellular β-glucosidase of Cellulomonas. Thus glycosylation if any and its nature in glucosidase of Bs/C 005 needs to be investigated. The shift in pH optimum towards acidic side in extracellular β-glucosidase of Bs/C 005 could be due to many other reasons. Studies on β- glucosidase from thermophilic bacteria including Cl. thermocellum (Ait et al., 1979; 1982); Thermomonospora spp. (Hagerdal et al., 1980) and Thermoanaerobacter ethanolicus (Mitchell et al., 1982) have demonstrated enhanced thermostability compared to β-glucosidases from mesophiles. Product inhibition and thermal inactivation of -glucosidase constitute two major barriers to the realisation of enzymatic hydrolysis of cellulose as commercial process (Woodward and Wiseman, 1982). Use of such thermostable β-glucosidase preparation in the enzymatic hydrolysis of cellulose might enhance the reaction rate, reduce contamination hazards and prolong period of reaction time. This is reflected through increase of interest in isolation of thermophilic microorganisms producing industrially important enzymes. Attempts have been made to clone the gene for thermostable β-glucosidase from Cl. thermocellum (Kadam et al., 1988) and obtained overproduction of enzyme to be used for cellulose hydrolysis.

Protoplast fusion seems to alter some of the properties of proteins, and desirable changes if any like increased thermostability could be of major interest in cell engineering. Changes if any in terms of addition of peptides responsible for the observed new properties need to be identified so that one could correlate the structural and functional alterations.

Changed regulation of expression of cellulases in the hybrid as compared to Cellulomonas further confirm occurrence of rearrangement of DNA through multiple recombinational events during protoplast fusion. This technique thus offers relatively easy way

for cell engineering and to generate new desirable combinations of phenotypes. Applications of this technique to all the bacteria, however, cannot be guaranteed at this stage. It is necessary to point out that subsequent to our first report on isolation of stable intergeneric bacterial protoplast fusion hybrids (Gokhale et al., 1984), few reports on intergeneric protoplast fusion in bacterial systems have appeared (Chen et al., 1987; Prakash and Cummings, 1988; Van der Vossen et al., 1988). Involvement of complete genome or part of it in recombination, the base composition of each bacterium, selection system used and also the characters used for complementation could decide the extent of success in the experiments. Choosing a single important enzyme and finding changes if any in its properties under hybrid environment thus could be one more way of testing applicability of this technique. One such test system involving bacterial α-amylase is described in the following chapter. In any event, protoplast fusion indeed generates new organisms with unnatural genomic complements and thus phenotypes which could be very useful reagents in studying molecular interactions, expression of genes and their regulation, and in some cases also possible evolutionary interactions.

SUMMARY

The results and observations made in the course of this work reported in this thesis led to the following conclusions.

1. Intergeneric protoplast fusion in bacteria could be used as a tool to obtain stable hybrids with desirable phenotypes. This technique thus can be substitute to recombinant DNA techniques in cell engineering by introdicing desirable phenotypes in unrelated bacteria.

2. Genes coding for complex phenotype like cellulose degradation could be stably transferred and also expressed in B. subtilis. This observation distinguished the technique from conventional recombinant DNA technology wherein singly isolated genes could be transferred and expressed in genetically well defined recipient organisms.

3. The hybrid status of the organism was confirmed through complementation of the auxotrophic and selection markers of the parental strain as well as from the DNA hybridization experiments.

4. The component enzyme of cellulose cascade showed changes in the properties when transferred and expressed from Cellulomonas into B. subtilis environment. The induction profiles by various substrates of the component enzymes were different in the hybrid. Catabolite repression patterns of these enzymes were also changed.

5. Reversiion analysis, stable expression of the cellulases and xylanase, maintenance of changed induction properties of these enzymes and stable but variant β-glucosidase with increased temperature optimum and temperature stability in the hybrid Bs/C 005 confirmed that the parental genomes indeed recombined in the hybrid.

The results reported herein thus open a new possibility of manipulating bacteria which are important industrially and which are unrelated taxonomically. This conclusion leads to potential application of this approach to engineering the cells to complement even the complex phenotypes which are resulted from the synergistic expression of multiple genes. Unlike the recipients of genes in recombinant DNA technology, the recipient organism is not required to be restriction minus and/or recombination minus; neither is it required to be completely compatible with the donor bacterium as long as a proper selection system exists to isolate the desirable hybrid organism.

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ANNEXURE

ISOLATION OF STABLE HYBRIDS BETWEEN *B. SUBTILIS* AND *Z. MOBILIS* BY PROTOPLAST FUSION TECHNIQUE

A.1. Introduction

Starch degrading enzymes are widespread in animal, microbial and plant kingdom. Originally the term diastase and amylase were used to describe enzymes capable of degrading alpha-1, 4 linkages in starch molecules, but the systematic studies on action of these enzymes led to the discovery of several new enzymes (Boyer and Ingle, 1972; Fogarty and Griffin, 1973; 1975) from the microbial sources. Starch degrading enzymes have been classified on the basis of their recognition and hydrolysis of specific linkages and their mode of action. The enzymes invloved in starch degradation are listed in Table A.1.

Bacterial alpha amylases hydrolyse alpha-1, 4 glucosidic bonds in amylose, amylopectin and glycogen in an endo fashion. They are all endoacting enzymes and therefore effect a rapid decrease in iodine staining capacity and viscosity of starch solution. The initial products of hydrolysis of amylose are maltose and maltotriose. Maltotriose, being poor substrate for alpha amylase is slowly converted to maltose and glucose (Walker and Whelan, 1960). Hydrolysis of amylopectin by alpha amylase results in the production of series of branched alpha-limit dextrins, glucose and maltose. These dextrins containing four or more glucose residues possess all the alpha-1, 6 glucosidic linkages of the original structure. A presence of alpha-1, 6 glucosidic linkages confer some stability on certain alpha-1, 4 linkages near the branch points thus protecting from the attack of alpha-amylase.

In general, there are two types of alpha amylases namely liquefying amylases and saccharifying amylases. They are distinguishable by their mechanisms of starch degradation. Saccharifying amylase produces twice the amount of reducing sugars as compared to liquefying alpha amylase (Fukumoto, 1963; Pazur and Okada, 1966). B. amyloliquefaciens produces large quantities of liquefying amylase (Walker and Campbell, 1967a;

Table A.1. Classification of starch-degrading enzymes

Bond between -D-Glucopyranosyl residues hydrolysed	Trivial name	Systematic name	EC number
(1 → 4)	α-Amylase	1,4-α-D-glucan glucanohydrolase	3.2.1.1
	β-Amylase	1,4-α-D-glucan maltohydrolase	3.2.1.2
	Glucoamylase	1,4-α-D-Glucan glucohydrolase	3.2.1.3
	α-D-Glucosidase	α-D-Glucoside glucohydrolase	3.2.1.20
	Exo-(1→4)-α-D Glucanase	--	--
	Cyclomaltodextrin	1,4-α-D-Glucan	2.4.1.19
	D-glucano-transferase	4-α-D-(1,4-α-D-glucano)-transferase (Cyclizing)	
(1 → 6)	Pullulanase	Pullulan 6-glucanohydrolase	3.2.1.41
	Isoamylase	Glycogen 6-glucanohydrolase	3.2.1.68
	Amylo-1,6-D-glucosidase	Dextrin 6-α-D-glucosidase	3.2.1.33

1967b) while B. subtilis produces saccharifying amylase (Matsuzaki et al., 1974; Yoneda et al., 1974). These enzymes also differ with respect to their temperature and pH stabilities, activity on various substrates, kinetic constants and energy of activation. These differences do not seem to be due to structural alterations, since they have almost identical primary structures (Borgia and Campbell, 1978).

All alpha-amylases are calcium metallo-enzymes (Fischer and Stein, 1960) having atleast one and upto 10 atoms of calcium per molecule of enzyme. They are stable at extreme pH, temperature conditions and also resistant to attack of protease in presence of calcium (Whitaker, 1972). This amylase after treatment with EDTA (calcium free enzymes) becomes highly susceptible to denaturation by similar treatments (Fischer and Stein, 1960). The molecular weights of amylases vary considerably between 22500 daltons as in case of B. licheniformis amylase (Saito, 1973) to 92000 daltons for Bacteroides amylophilus (McWethy and Hartman, 1977). The pH optima for α-amylase from different sources fall in an acidic region between pH 4.5 and pH 6.5, with the exception of α-amylases from B. acidocaldarius (Buonocore et al., 1976) B. licheniformis (Saito, 1973) possessing maximum activity at pH 3.5 and pH 9.0 respectively.

Recently, in order to achieve bioconversion of starchy material to useful end products like ethanol or antibiotics, attempts are being made to transfer and express amylase gene into suitable recipient organisms. Hurdles in such exercise are usually instability of amylase gene into new unrelated microbe or non- expressibility of this gene in a foreign environment. Attempts to transfer a fungal amylase gene in Z. mobilis reported by Rogers et al (1984) described similar difficulty due to the presence of restriction endonucleases in Z. mobilis which do not allow retention of this foreign gene. Thus amylolytic phenotype could not be transferred in Z. mobilis. This observation in fact represents a general problem in transferring desirable complementary genes into naturally occuring industrial microbes.

Our results described in previous chapters indicate that using protoplast fusion technique one could stably transfer genes from unrelated bacteria. Further, these genes are expressed and such hybrids could be specifically selected. Some of these hybrids exhibit changes in parental phenotype so that a new organism harbours modification in product proteins which could be beneficial in certain cases. Even phenotypes invloving complex multigenic expression like cellulose degradation were successfully transferred from Cellulomonas to B. subtilis. As mentioned earlier, there is no specific information about restriction modification pattern of these two strains used. It was therefore of interest to experiment with known restriction endonuclease harbouring microbe and attempt to transfer a desirable foreign phenotype into it using protoplast fusion approach.

A.2. **Materials and methods**

All the chemicals used were of analytical grade. Soluble starch was procurred from E. Merck. Bacterial strains used were B. subtilis NCIM 2439 (amylase$^+$, Leu$^-$, His$^-$, Ala$^-$, Trp$^-$, Ura$^-$) and Z. mobilis ATCC 10988 (amp^R, tet^R, $HgCl_2^R$, Cam^R, amylase$^-$). Both the strains were maintained on LB agar slants and were periodically subcultured every two months on freshly prepared slants. LB medium was prepared as described in chapter 2. The regeneration media used were either LB with 0.5 M sucrose and 0.8% agar or Dubos minimal medium with 0.5 M sucrose and 0.8% agar. The selection medium was prepared by incorporating ampicillin into Dubos minimal regeneration medium.

Protoplast isolation, regeneration and fusion

Protoplasts of B. subtilis and Z. mobilis were isolated as described earlier in Chapter 2 using log phase cells and lysozyme (1 mg/ml). Formation of protoplasts was monitored by hypotonic shock method as described earlier. Protoplasts were mixed and fusion was carried out at room temperature using PEG - 6000 (33%)

solution. Treated protoplasts were washed appropriately, and were plated on selection medium with starch as sole carbon source. Individual regeneration efficiency were separately estimated using LB agar regeneration medium without antibiotic and starch. Efficiency of protoplast formation and regeneration were calculated.

Analytical methods

Antibiotic resistantance assay was carried out using the inhibition zone assay method. Inhibition zones were measured for comparison of resistance phenotype.

Amylase activity was detected in two different ways. In one type of assay, the zone of hydrolysis on starch agar medium was visualized using iodine staining of starch. Amylase enzyme assay was carried out according to Bernfield (1955) and reducing sugars were estimated by DNS method (Fischer and Stein, 1961). 0.5 ml of enzyme was incubated with 0.5 ml of 1% starch in acetate buffer (0.1 M, pH 5.5) and the incubation was carried out at optimum temperature in each case.

A unit of activity has been defined as the amount of enzyme required to produce 0.5 mg of glucose in 30 min.

Optimum temperature for amylase activity was determined by incubating the reaction mixutre at different temperatures whereas optimum pH was determined by estimating the activity in buffers of different pH at optimum temperature. Stability of the enzyme activity at different temperatures was estimated by preincubating the enzyme solution without substrate followed by estimation of activity using starch under standard conditions. The preincubation was carried out at various temperature ranging from 30° C to 80°C.

A.3. **Results and discussion**

Under the conditions of protoplasting 100% conversion of both B. subtilis and Z. mobilis cells to protoplasts was obtained as determined by using hypotonic shock method (Table A.2). There was little but significant difference in the efficiency of regeneration of protoplasts of B. subtilis and Z. mobilis. Very few hybrids were obtained after regeneration of mixed protoplasts (PEG treated) on selection medium containing starch as sole carbon source and ampicillin. The regenrated colonies were therefore expected to be prototrophic, amylolytic and ampicillin resistant organisms. This phenotype essentially required presence of DNA from both the parental microbes since neither B. subtilis NCIM 2255 nor Z. mobilis alone could grow on this selection medium. These hybrids on the medium containing starch gave rise to easily visible clear zone even without staining with iodine. Expression of amylase activity by the hybrids and in their culture filtrates are visualised after iodine staining (Fig. A·1).

The hybrids between B. subtilis and Z. mobilis were numbered as BZ - 1, BZ - 2, BZ - 3 and BZ - 4. All the four stable hybrids obtained were Gram positive and could sporulate. The isolated hybrids were purified by single colony isolation, and were grown in the Dubos medium containing starch and ampicillin and allowed to sporulate. The spores were regenerated on same medium with agar and the isolated colonies were picked up and used for further studies.

Apart from Gram positive sporulating phenotype, the hybrids distinguish themselves from both B. subtilis and Z. mobilis in size and shape of the cells (Fig. A.2). This observation suggested that not only the individual properties of parents were expressed in the hybrids but also some other phenotypes had been regenerated as a result of interactions of mixed function of both the parents.

The hybrids were selected for ampicillin resistantance which is a

Table A.2. Protoplast formation and regeneration frequency

Organism	Starting no. of cells (Z)	Total no. of colonies (X)	No. of colonies after osmotic shock (Y)	Protoplast formation frequency * (%)	Protoplast regeneration frequency ** (%)
B. subtilis NCIM 2439	1.6×10^{9}	5×10^{7}	ND	100	3.1
Z. mobilis ATCC 10988	1.9×10^{8}	7.2×10^{6}	2.4×10^{5}	99.9	3.6

ND : Not detected

Z : Total number of cells

X : Total number of colonies from regenerated protoplasts and non protoplasted cells

Y : Colonies from non-protoplasted cells which resisted lysis upon osmotic shock

* : Protoplast formation frequency = Z - Y / Z x 100

** : Protoplast regeneration frequency = X - Y / Z x 100

Fig. A.1. Detection of α-amylase activity

1. B. subtilis
   NCIM 2439

2. Hybrid BZ-1

3. Hybrid BZ-2

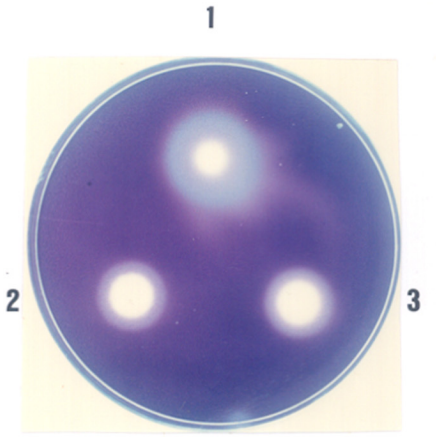
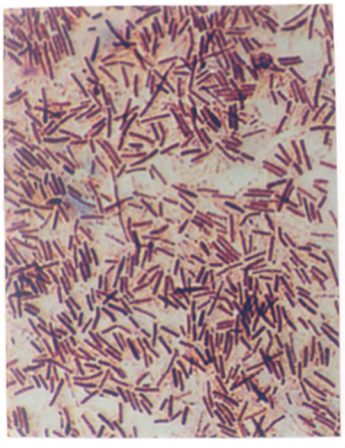


Fig. A.1

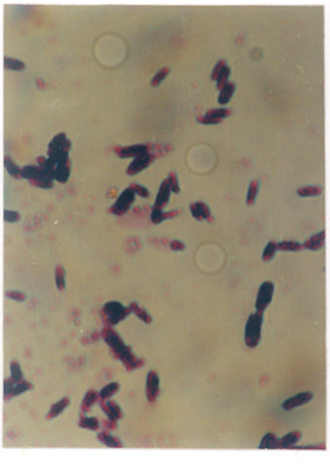
Fig. A.2 Morphology of *B. subtilis*, *Z. mobilis* and their hybrids (Magnification 6000)

1. *B. subtilis* NCIM 2439
2. *Z. mobilis* ATCC 10988
3. Hybrid BZ-1
4. Hybrid BZ-2

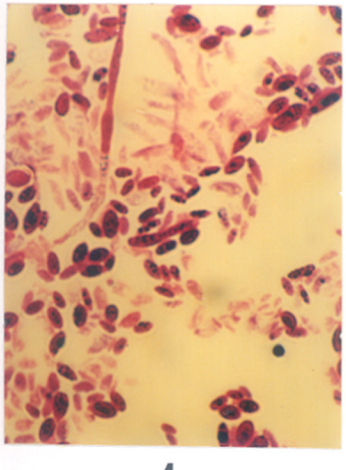


1

2



3



4

Fig. A.2

Z. mobilis phenotype. Z. mobilis is known to harbour many other resistant markers like amp^R, $HgCl_2^R$. Many of these markers are expected to be plasmid borne through ampicillin (Walia et al., 1983) and recently $HgCl_2$ resistance (Ogale and Deobagkar, 1988) are the only two clearly identified markers on specific plasmids so far. The hybrids BZ-1 and BZ-2 were further screened for their resistance to other drugs. In addition to ampicillin, they were found to be resistant to tetracycline and streptomycine, but significant inhibition zone was detected in presence of chloramphenicol and $HgCl_2$ (Table A.3). Z. mobilis is resistant to all the antibiotics used. This observation showed that atleast the low molecular weight ampicillin resistant plasmid is stably expressed in the hybrid which predominantly exhibited B. subtilis features. Z. mobilis is Gram negative organism. Recently, very efficient plasmid transfer by intergeneric protoplast fusion between Streptococcus lactis and B. subtilis was reported by Van der Vossen et al (1988). However, transfer and expression of genes from Gram negative microbe into Gram positive are very rare like in case of shuttle vectors between E. coli and B. subtilis. No protoplast fusion between Gram negative and Gram positive bacteria has been earlier reported.

Amylase activity was detected in culture filtrates of the hybrids grown on starch medium. When compared to B. subtilis NCIM 2439, the relative amount of amylase activities in culture filtrate of all the hybrids was higher (Table A.4). BZ-1 and BZ-2 exhibited more than 2 fold higher activity than B. subtilis parent. B. subtilis α-amylase activity was highest at 40° C and at pH 5.5. Surprisingly it was seen that the optimum temperature for amylase activity of BZ-1 and BZ-2 was increased to 60° C (Fig. A.3). Alongwith the increase in optimum temperature, the enzyme secreted by hybrids exhibited stability at high temperature (Fig. A.4). The increased temperature stability of α-amylase seems to be a result of changed folding or conformation of the protein or possibly to a certain extent due to additional post translational modifications like glycosylation. This analysis is necessary to be carried out before

Table A.3 Drug resistance in hybrids
(inhibition disc zone assay)

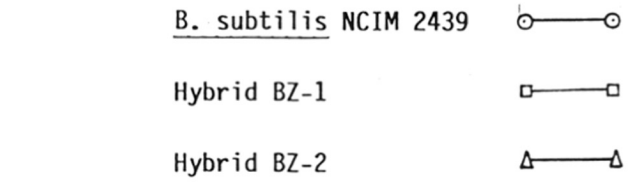
Strain	Inhibition zone in presence of Antibiotics (ug/ml)				
	Amp (50)	Tet (25)	Strept (10)	Cam (15)	HgCl (20)
B. subtilis NCIM 2439	4 mm	7.5 mm	ND	6.4 mm	2.5 mm
Z. mobilis ATCC 10988	ND	ND	ND	ND	ND
Hybrid BZ-1	ND	ND	ND	5.0 mm	5.0 mm
Hybrid BZ-2	ND	ND	ND	4.0 mm	4.0 mm

ND : Not detected.

Table A.4 Amylase activity of *B. subtilis* & hybrids

Strain	Amylase activity (units/ml)
B. subtilis NCIM 2439	7.5
BZ-1	16.3
BZ-2	17.5
BZ-3	10.3
BZ-4	10.5

Fig. A.3. Optimum temperature for α-amylase activity



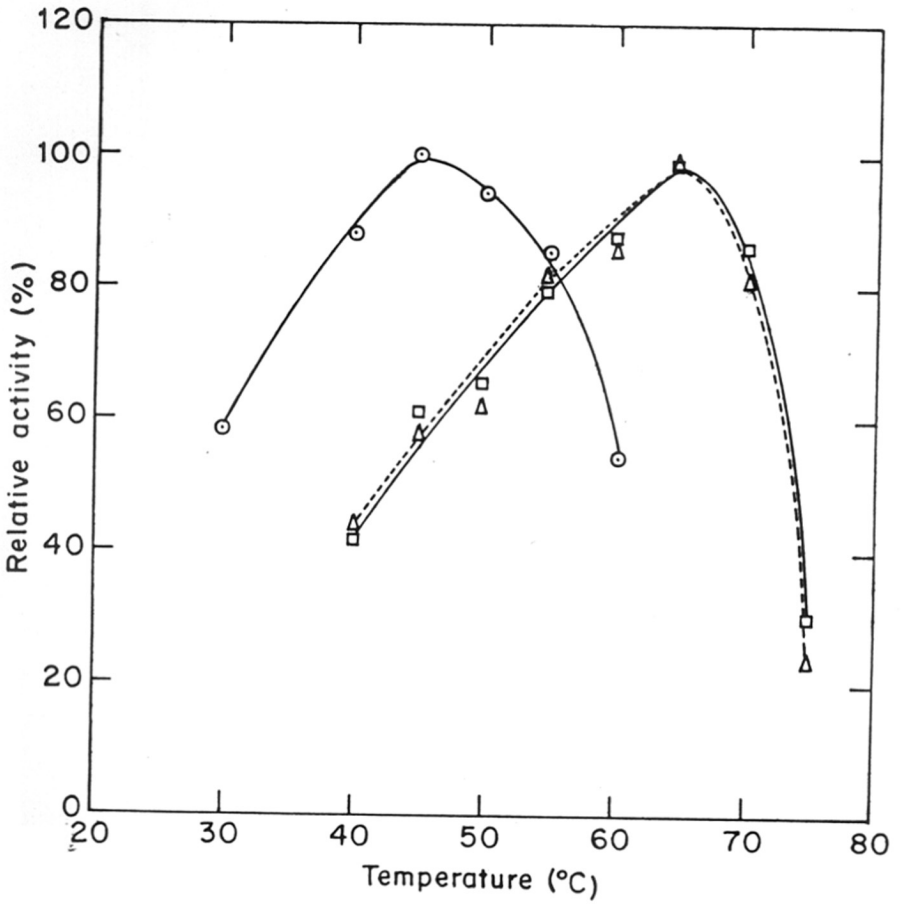

120
100
80
60
40
20
0
Relative activity (%)
20
30
40
50
60
70
80
Temperature (°C)

FIGURE A·3

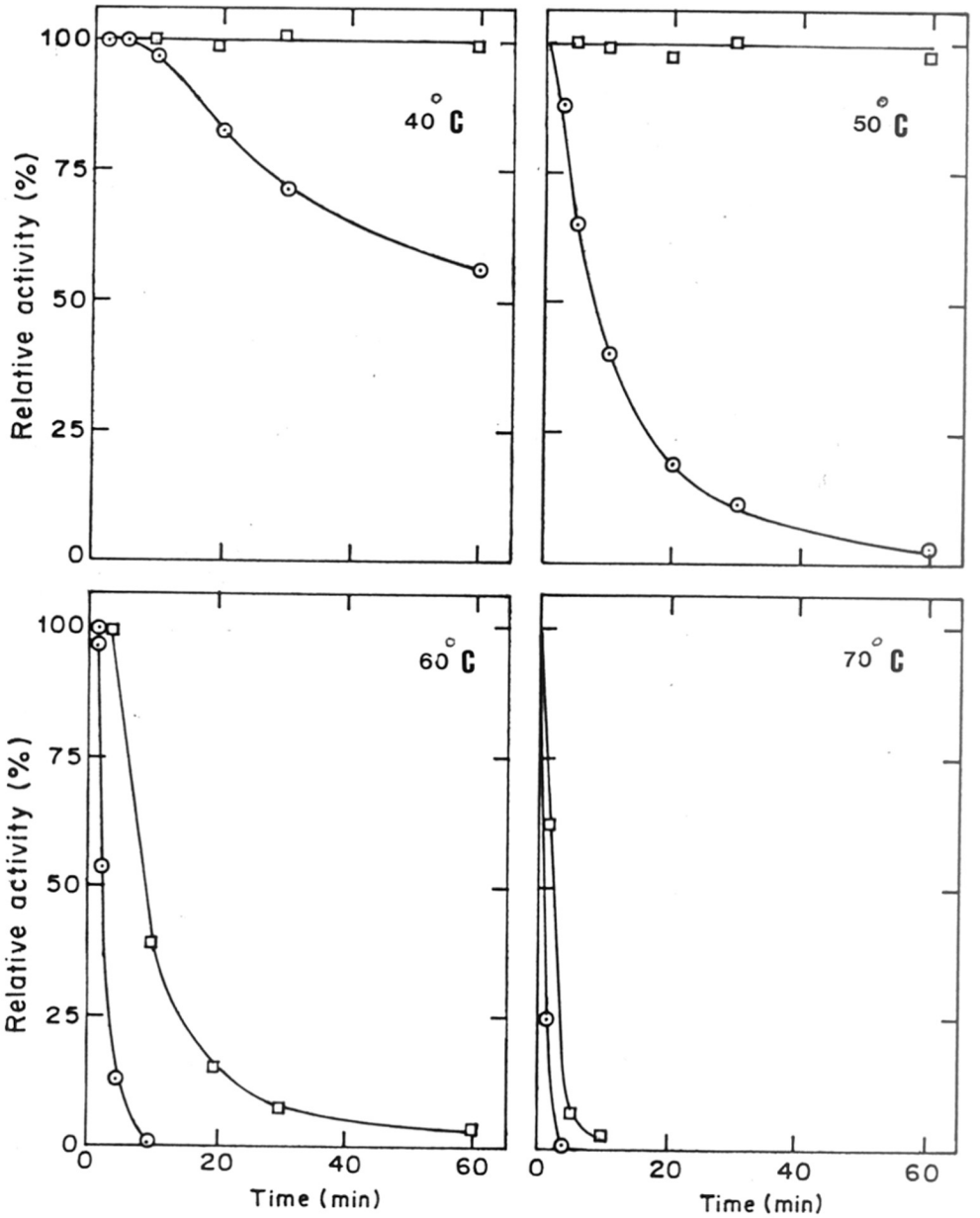
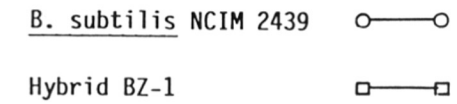

100
75
50
25
0
Relative activity (%)
40° C
50° C
60° C
70° C
0
20
40
60
Time (min)

FIGURE A·4

Fig. A.4. Temperature stability of α-amylase in hybrid BZ-1

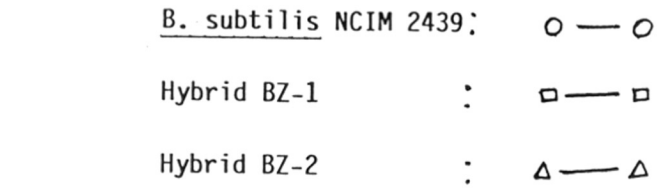


making any firm conclusions as a cause for this change in property of enzyme. In any case, this change is desirable and is useful in actual application of this enzyme in liquefaction or saccharification of starch. Isolation of thermostable enzymes like amylase has been achieved so far by using glutagenesis and recombinant DNA technology for their improved applicability in industrial processes (Oriel and Schwacha, 1988; Tsukagoshi et al., 1984). Occurrence of thermostable α-amylase through protoplast fusion as reported here had a parallel in another set of bacterial protoplast fusion hybrids reported in previous chapter wherein newly appearing thermostable β-glucosidase has been described in case of B. subtilis and Cellulomonas hybrids. Protoplast fusion thus seems to generate change in properties of some of these enzymes and this could be desirable in certain cases.

The hybrid enzyme also exhibited significantly broad pH optima ranging from pH 4.0 to pH 5.5. The interesting point being that the equivalent high activity was obtained even at lower pH (Fig. A.5). Identification of differences in the protein structure in any of the B. subtilis and hybrid enzyme could allow us to relate changes in temperature and pH optima of alpha-amylase.

These observations therefore not only substantiate the hypothesis put forth in previous chapters that desirable complementation between properties of unrelated bacteria belonging to different genera could be achieved through protoplast fusion technique but also demonstrates that through some unknown mechanisms the DNAs from otherwise incompatible organisms could coexist stably. It is therefore of interest to further investigate and isolate a stable hybrid predominantly exhibiting Z. mobilis properties expressing alpha-amylase gene from B. subtilis. The protoplast fusion technology thus has high potential in manipulating naturally occuring industrially important microbes where manipulation by recombinant DNA technology has not yet been successful.

Fig. A.5. Optimum pH for α-amylase



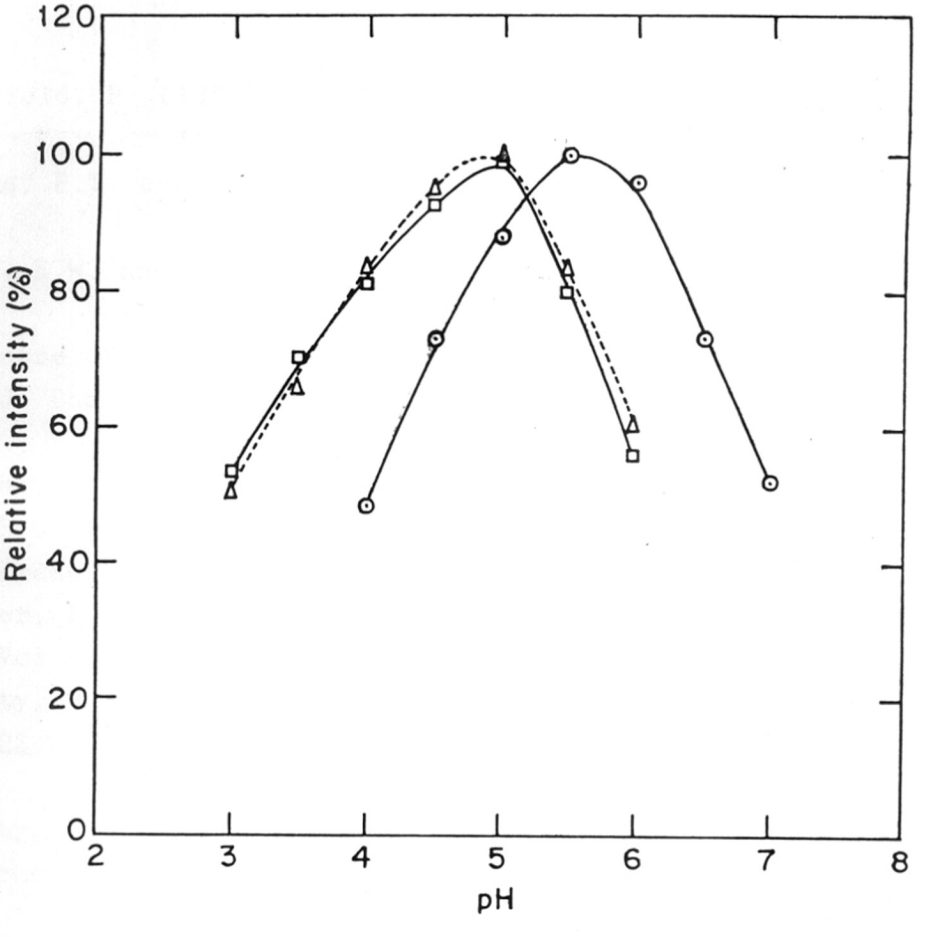


FIGURE A·5

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