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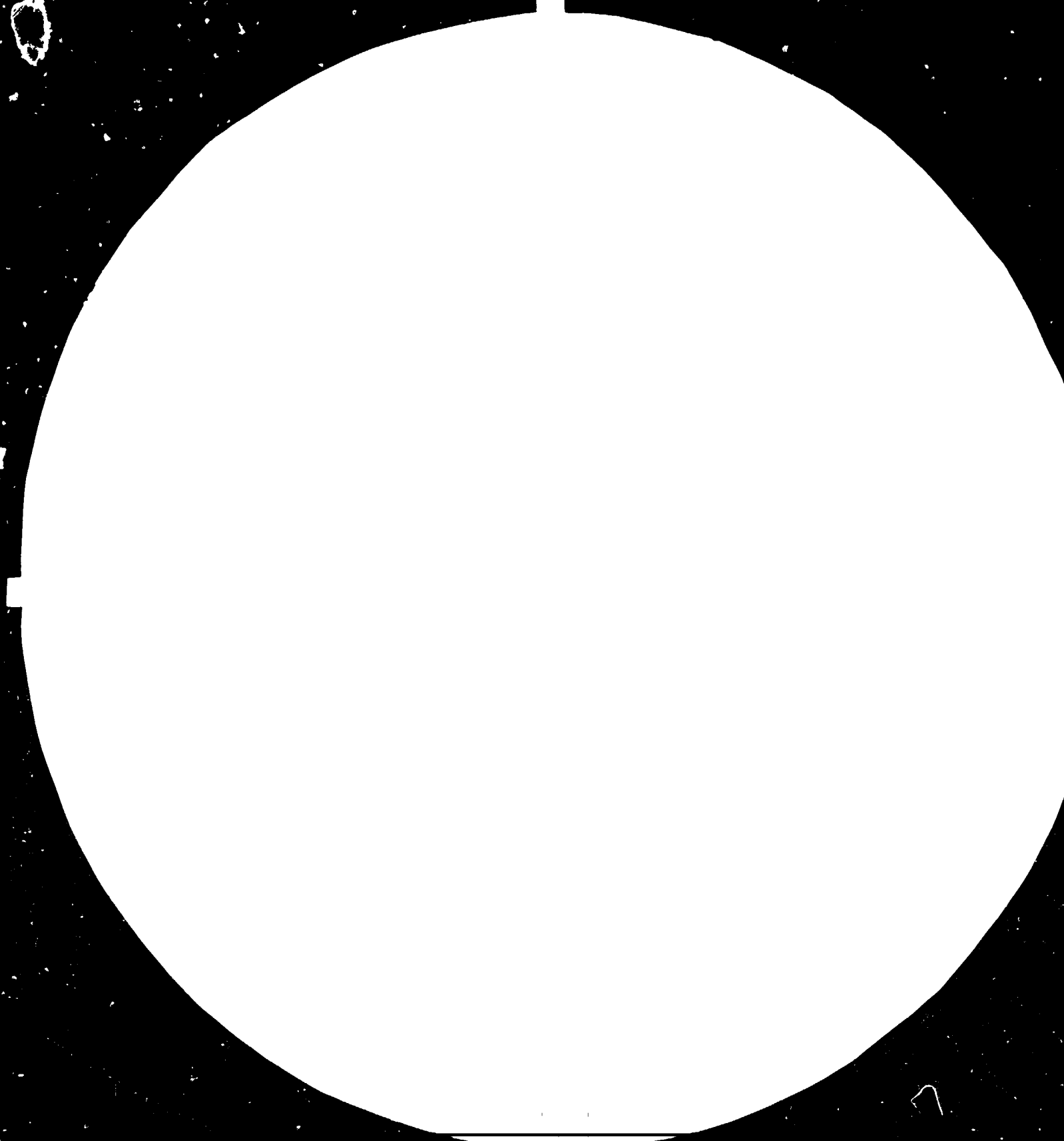

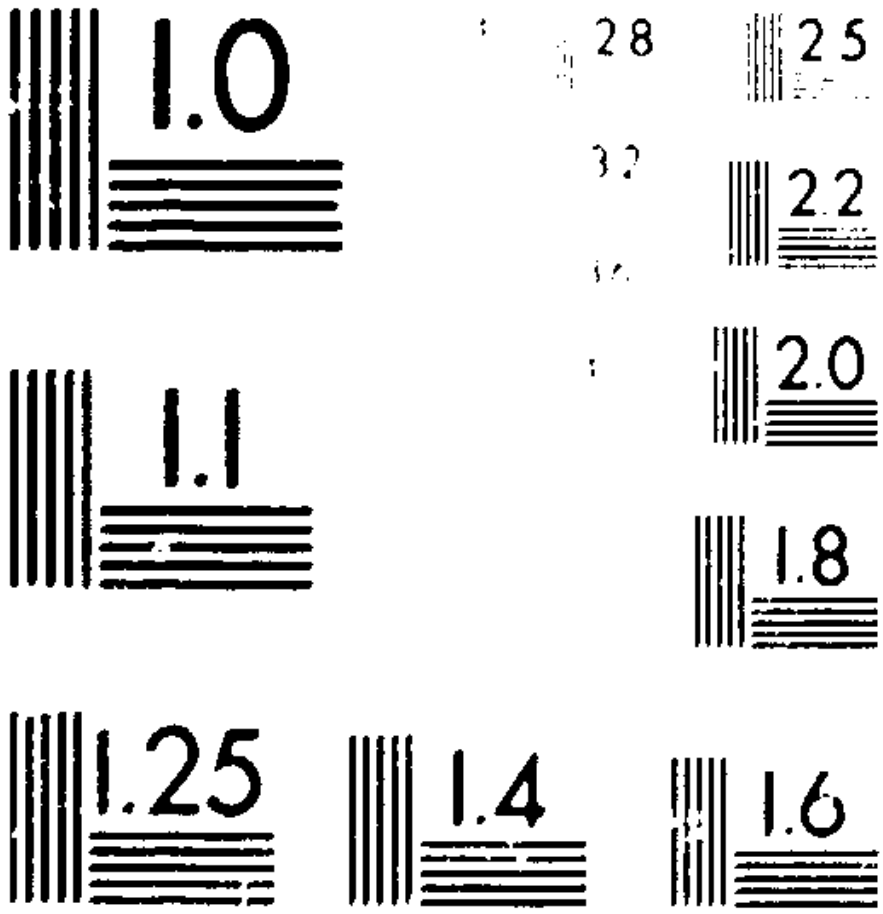

MICROCOPY RESOLUTION TEST CHART

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REPORT ON

MOLECULAR CLONING OF CELLULASE GENES

A PROJECT UNDERTAKEN AT

GENETICS DEPARTMENT, TRINITY COLLEGE,
UNIVERSITY OF DUBLIN, DUBLIN-2, IRELAND

M. Waheed Akhtar

DURING JULY—SEPTEMBER, 1984

SUBMITTED TO

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PREFACE

This work on molecular cloning of cellulase genes was undertaken at Genetics Department, Trinity College, University of Dublin, Dublin-2, Ireland and constitutes a part of our research programme on the breakdown of cellulosic substances by microbial cellulolytic enzymes for use in the production of products like alcohol and protein rich biomass. This research was made possible due to the interest, guidance and help from Dr. David McConnell for which I am highly grateful to him. To be able to obtain substantive results within a period of three months of experimental work was mainly due to availability of all the facilities in his well equipped and productive laboratory of Molecular Genetics at the Trinity College. I am also thankful to Dr. Barbara Cantwell, Dr. Terek Schwartz and all the other colleagues at the Genetics Department for their cooperation and help. I really enjoyed working with them which made my stay at Trinity College very pleasant.

I am highly grateful to the United Nations Industrial Development Organization, Vienna and University Grants Commission of Pakistan, for providing the financial support for this work. I also wish to thank the University of the Punjab and the Government of Pakistan for allowing me to undertake this research programme.

This work was indeed made possible due to the immense patience shown by my wife Seemi, daughter Maryam and son Jananzeb during my stay away at Dublin.

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CONTENTS

	Page
Summary	1
Introduction	2
Experimental	5
Discussion	17
References	20

SUMMARY

This work on molecular cloning of cellulase genes was initiated with ultimate objectives to develop microbial strains especially a eucaryotic like yeast that could breakdown lignocellulosics and hemicelluloses of the agricultural bye-products and to convert the sugars thus obtained into the end products like alcohol and protein rich biomass, preferably in a single process. The experiments done during the three month period of the last summer dealt with cloning of Cellulomonas biazotea eudoglucanase genes in Escherichia coli. Conditions were studied and standardised for the preparation of chromosomal DNA and the plasmids, restriction of the DNAs and ligation and transformation reactions. Congo red dye staining method was employed to screen the transformants for endoglucanase activity. Over 12,000 clones transformed with pUC18 carrying inserts of BamHI restriction fragments of chromosomal DNA were screened and two of these showed the enzyme activity. Further experiments to characterise these genes and to clone the other enzymes involved in the breakdown of cellulosic materials are planned.

INTRODUCTION

Cellulose, the most abundant organic compound in our planet, is of tremendous importance because of its possible utilisation in the production of alcohol, protein rich biomass and other products. In Pakistan output of cellulosic materials like wheat straw, rice straw, cotton wood, baggase, wood wastes, etc., is estimated to be over 40 million tonnes per year. This cellulase which occurs in the form of lignocellulose must first be hydrolysed to glucose and other cellulosic sugars before it can be further utilized.

Hydrolysis of lignocelliulosics can be achieved either by acid or enzymatic treatment. Since the application of enzymes to achieve this objective seems to have much more potential, a lot of work has been done on the breakdown of celluiosic materials by the enzymes produced by a variety of bacterial, yeast or fungal species. It is now known that at least four different enzymatic activities are present in Trichoderma, which is one of the most extensively studied cellulolytic organism(1-3). These are β 1, 4-endoglucanse, β 1, 4-glucan cellobiohydrolase, cellobiase and exo-β 1, 4-glucohydrolase. Organisms belonging to about two dozens of genera are known to produce cellulolytic enzymes(4). Apart from Trichoderma other organisms like Eupenicillium javanicum(5), Scytalidium lignicola(6), Pellicularia filamentosa(7), Thermonospora species(8), Chaetomium cellulolyticum(9), Aspergillus terreus(10) Sporotrichum pulverulentum(11), Phanerochaete chrysosporium(12), Cellulomonas sp.(13) have been studied for the production of cellulolytic enzymes. Apart from production and cellulolytic action of these enzymes, conversion of cellulosic materials directly into alcohol(14,15) or protein rich biomass(16) have been reported.

Inspite of the many studies reported on the enzymatic breakdown of cellulose much remains to be done still in order to make the process economically viable. It is highly desirable that the cellulolytic organism is stable and gives high yields of the various enzymes involved in the breakdown of cellulose and possibly lignin and hemicellulose, which occurs in significant amounts along with cellulose in the native form. The enzymes produced should have high

specific activities, they should be stable at high temperatures and resistant to end product inhibition and physical factors other than temperature as well. It would be far more desirable if the same system is capable of not only breaking down cellulose to glucose but also utilize products in a single process. Although in order to accomplish the above mentioned objectives in a single process the use of mixed culture has been suggested, genetic manipulation of microorganisms seems inevitable.

During the recent years cloning of genes coding for endoglucanase activity has been achieved by a few workers. Two genes from Thermomonospora which code for endoglucanase activity were cloned and expressed in Escherichia coli which resulted in the production of 50 times more cellulase activity than the original strain(17). Similarly genes for cellulases of an alkalophilic Bacillus which were found to be in 2.0 - 2.8 kilobase HindIII fragments were cloned and expressed in E. coli (18). Gilkes et al.(19) isolated and characterized E. coli clones expressing cellulase genes from cellulomonas fimi. Out of the various clones obtained those transformed with the plasmid having a 5.0 kb insert contained high levels of CM-cellulase activity. Cloning and expression of a B. subtilis gene coding for endo-β-1, 3-1,4-glucanase has been transferred to E. coli by molecular cloning using bacteriophage λ and plasmid vectors(20). The gene was contained within a 1.6-kb EcoRI-PvuI DNA fragment. It would be apparent that the reports available dealt with only endoglucanase activity whereas cloning of other cellulolytic enzymes have not been reported yet. Through gene cloning it is possible to develop strains which have high cellulolytic activity and can grow under favourable nutritional and physical conditions. This can be accomplished by isolating genes coding for cellulases on recombinant DNA plasmid or other vectors and then to modify the expression of these genes by current molecular genetic techniques which include coupling the gene to a strong promoter, eliminating operators sensitive to repression and increasing efficiency to translation.

We have been engaged in screening of a variety of microorganisms in order to obtain potent cellulolytic organisms and also on the production, purification and characterization of cellulolytic

enzymes from some of these strains at the Institute of Chemistry, University of the Punjab, Lahore, Pakistan(21-23). As a result of this work it has been possible to develop some fungal and bacterial species through culture enrichment and optimizing fermentation conditions, which have high cellulolytic activity. One of these strains i.e. Cellulomonas biazotea was used as a source of CM-cellulase gene for cloning in E. coli in this research project. This work on molecular cloning and expression of cellulase gene is a part of the overall programme to develop a system for efficient utilization of cellulosic materials through the action of enzymes.

EXPERIMENTAL

Organisms and growth media

The strain of Cellulomonas biazotea was isolated in Pakistan under a programme for the collection and screening of cellulolytic organisms. It was grown in the medium containing 1 ml of the salt mixture of composition (g/100 ml) $NaNO_3$ 5, KH_2PO_4 10, $MgSO_4.7H_2O$ 5, KCl 5, and $FeSO_4.7H_2O$ 0.1 and yeast extract 0.2% and cellobiose 0.5% (pH 7.3). E. coli L5 supE, tonA, $hsdR^-_k$, thr, leu, thi (imm434cIts)(23) was used for cloning and it was grown at 30°C on agar plates containing Vogel as Bonner salts, glucose 0.4%, yeast extract 0.1%, l-leucine (5 mg/100 ml) and carboxymethyl cellulose (CMC)(0.2%). The plates used for screening the clones also contained ampicillin 25 µg/ml. The strain E. coli JM107 lac pro, endAI, gyrA96, thi^-I, hsd[illegible]7, SupE44, relAI, F′, traD36, $ProAB^+$, $lacIq^-Z/M15$(24) was employed to monitor the rate of transformation with pUC plasmids carrying DNA fragment inserts. It was grown at 37°C on the LB agar plate with 0.1 ml overlay of x-gal (5 bromo-4-chloro-3-indolyl-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside), prepared by mixing 30 µl of x-gal(20 mg/ml dimethyl formamide) and 30 µl of IPTG (24 mg/ml water) in 0.1 ml LB broth. Appearance of white colonies among the blue ones on agar plates showed transformation with plasmid carrying DNA inserts.

Screening for cellulolytic organisms

Method for the detection of CM-cellulase activity on agar plates in order to screen cellulolytic organisms and to detect the positive clones was developed. The method employed the respective agar media containing 0.4% CMC. The plates after sufficient growth of the colonies were incubated at 50°C for 2 hours before flooding these with a 0.1% solution of congo red. Cellulolytic colonies showed haloes against a red background as shown in Fig.1. This screening method was employed to detect the positive clones after transformation.

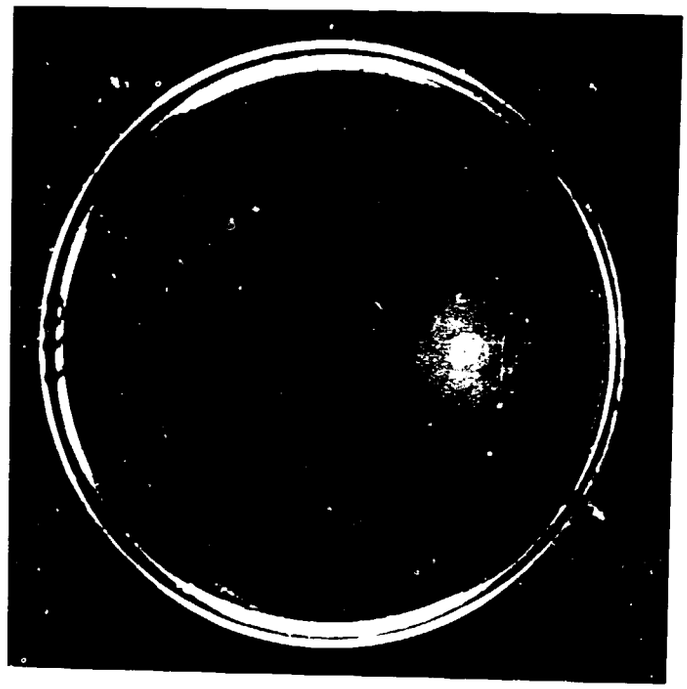

Fig.1. Detection of cellulolytic colonies of Cellulomonas biazotea(left) and a strain of Bacillus subtilis(right) after growing on a CMC-agar plate and staining with congo red.

Preparation and restriction of chromosomal DNA

A loop of cells from a freshly grown slant of C. biazotea was grown overnight in 50 ml medium of the composition given above in 250 ml flask at 30°C with shaking in a Gallenkamp Orbital Incubator Shaker. 10 ml of this culture was then used to inoculate 500 ml medium contained in a 2 L flask and incubated overnight as above. Harvested the cells by centrifugation in a Sorval RC 5B using the rotor GSA at 12,000 rpm for 30 minutes at 4°C. The cells were washed with 250 ml TEN buffer [10mM Tris-HCI (pH 7.6), 1mM EDTA, 10mM NaCl]. The cell slurry thus obtained was lysed and the chromosomal DNA was prepared according to the method described by Rodriguez and Tait(25). The DNA thus prepared was dissolved in TEN buffer and after appropriate dilution it gave a proportion of 1.9 between absorptions at 260 and 280 nm. The same sample showed a single band on agarose electrophoresis(Figs. 2-4).

The chromosomal DNA prepared as above was restricted with the enzymes Sau3AI and BamHI in order to obtain fragments of a suitable size. The reaction mixtures consisted of 4µl DNA (approx. 2ug), 1µl of the 10x buffer as recommended by supplier of the enzyme and 0.05 - 0.5 U of Sau3AI (BRL Cambridge, UK) or 0.2-2.0 U of BamHI (BRL, Cambridge, UK) in a total volume of 10 µl. The reaction mixtures were incubated at 37°C for 8 mins. The Sau3AI reactions were stopped by heating the mixture at 65°C for 10 minutes while those of BamHI were stopped by washing the mixture with TEN buffer equilibrated phenol:$CHCI_3$ (1:1). DNA from the aqueous layer was precipitated with two volumes of absolute alcohol at -20°C after adding 1/10th volume of 3M sodium acetate, centrifuged off the precipitate, washed with 70% alcohol and redissolved in 10µl TEN buffer. After agarose (0.8%) electrophoresis the bands were stained with 0.1% ethidium bromide and photographed over UV plate. As shown in Fig.2 the DNA was restricted efficiently with Sau3AI and BamHI and there was increased breakdown of the DNA with increasing amounts of the enzymes.

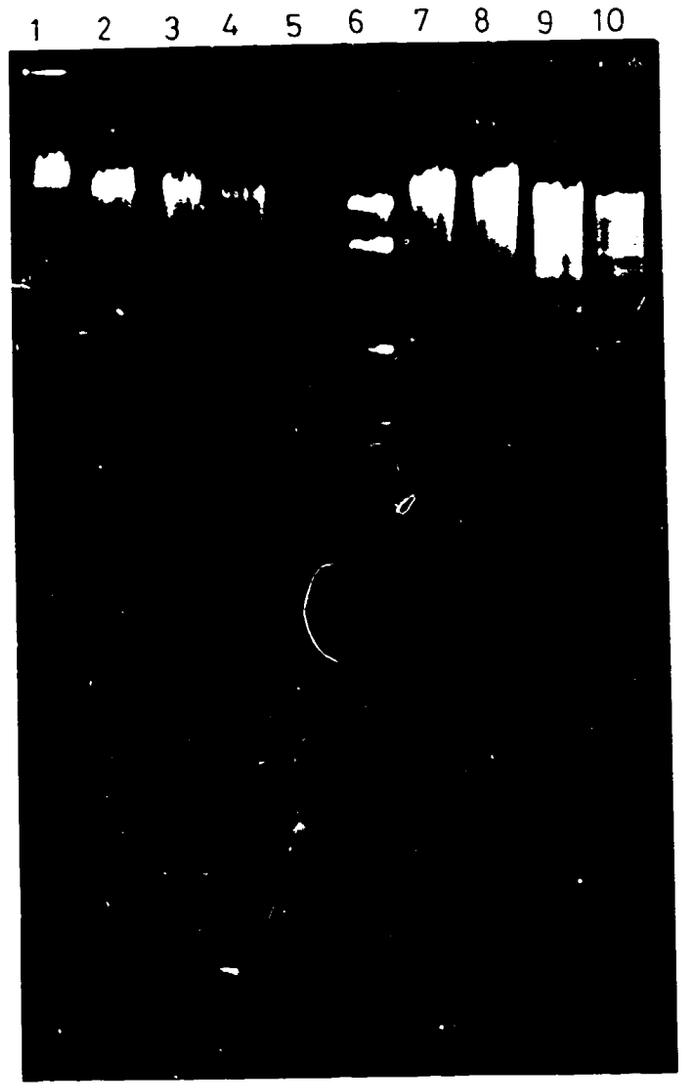

Fig.2. Sau3AI and BamHI restriction digests of Cellulomonas DNA with different levels of the enzymes. Reaction conditions were as given in the text. Lines from left: 1, uncut DNA (approx. 0.5 ug); 2-5, restriction with Sau3AI, 0.05, 0.1, 0.2 and 0.5 U, respectively; 6, ClaI restriction fragments of bacteriophage T_7 with (from top to bottom) 22856, 9980, 4243 and 2858 base pairs; 7-10, restriction with BamHI, 0.2, 0.4, 0.8 and 2.0 U, respectively.

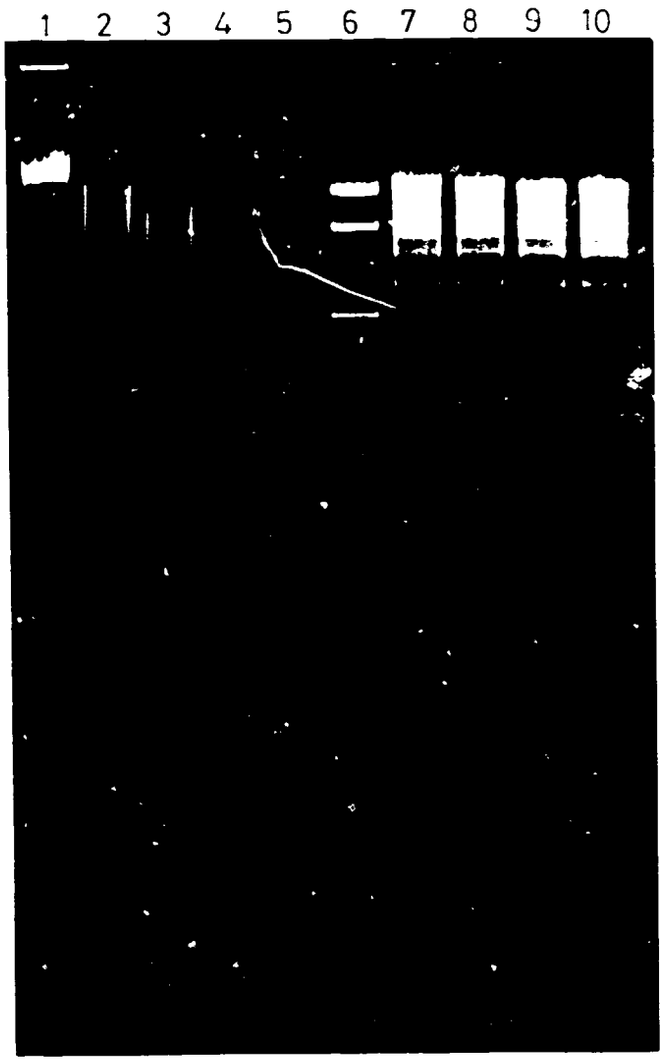

Fig.3. Sau3AI and BamHI restriction digests of Cellulomonas DNA when reacted for different time periods. Reaction conditions were as given in the text. Lanes from Left: 1, uncut DNA (approx. 0.5 μg); 2-5, restriction with Sau3AI for 5, 10, 20 and 40 mins., respectively; 6 ClaI restriction fragments of bacteriophage T_7; 7-10 restriction with BamHI for 5, 10, 20 and 40 mins.

Restriction of the DNA was also done with fixed amounts of the enzymes, i.e. 0.1 U for Sau3AI and 0.5 U for BamHI, for 1.0 μg of the DNA but for time periods of 5,10,20 and 40 mins., while rest of the reaction conditions were the same as described above. The restriction patterns are given in Fig.3 which show increased hydrolysis of the DNA with time, being more marked with Sau3AI digests. The central lane in Figs.2 and 3 is the restriction digest of bacteriophage T_7 with ClaI which give fragments of 22856, 9980, 4243 and 2858 base pairs were used as size markers. The Sau3AI restriction for 20 mins. and that of BamHI for 10-20 mins. yielded much of the DNA in the form of fragments which were in the size range of 3-10 kb pairs which would be suitable for the ligation reactions. These reaction digests after washing with TEN buffer equilibrated phenol and then with chloroform: isoamyl alcohol(24:1), were precipitated with two volumes of absolute alcohol at -20°C. The DNA precipitate after centrifuging off was redissolved in TEN buffer for use in the ligation reactions.

Preparation and Linearisation of plasmids

The plasmids pBR322(26) and pUC18(24) were prepared from the strains of E. coli carrying these for use in the cloning experiments. The organisms were grown overnight in 10 ml LB medium containing 50 μg/ml ampicillin in a test tube at 37°C with sterile air bubbling through it. 500 ml of the LB medium containing 50 μg/ml ampicillin in a 2L flask was inoculated with 5 ml of the bacterial culture and incubated at 37°C and 140RPM in a Gallenkamp Orbital Incubator Shaker till OD_{600} of the culture was 0.4. The plasmid was then amplified by adding 2.5 ml chloramphenicol solution in ethanol (35 mg/ml) to make a final concentration of 170 μg/ml and incubating overnight at 37°C. The cells were then harvested and the plasmid prepared by the alkali lysis method described by Maniatis et al.(27). Milligram quantities of both the plasmids as judged by comparison with standard quantities of DNA on agarose electrophoresis, were thus prepared, each of which showed a number of bands due to supercoiled, open circular, nicked and multimeric forms on agarose electrophoresis (Fig.4). Each of the preparation was rendered into a single band of linearised form on cutting with BamHI under the conditions described above.

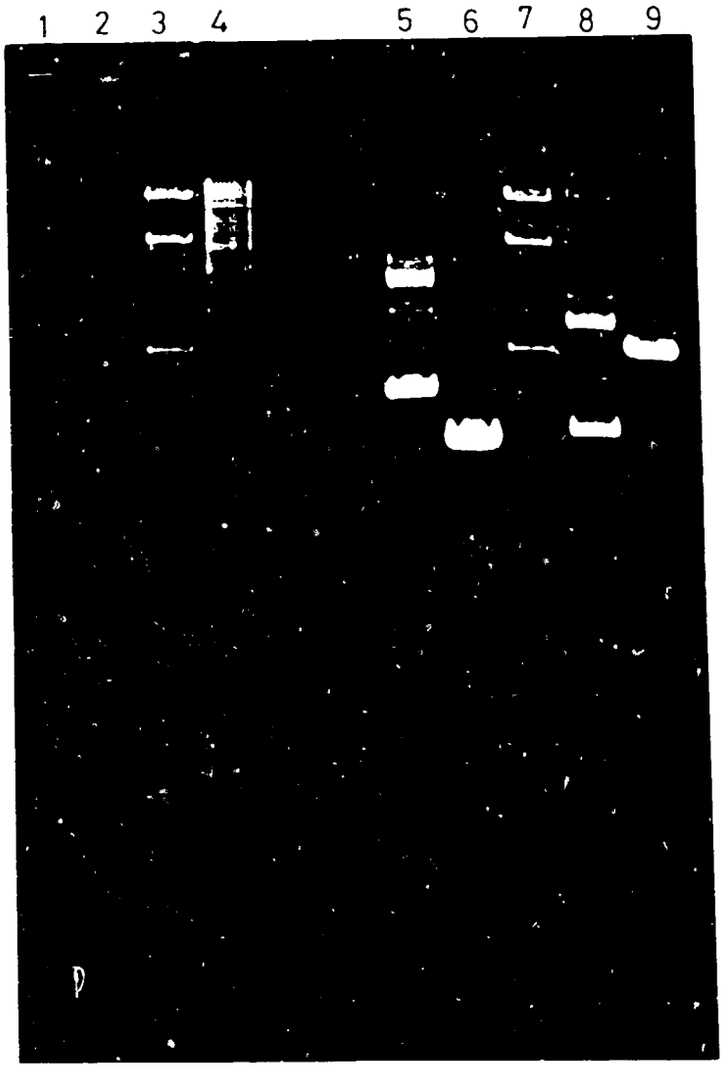

Fig.4. BamHI and Sau3AI restriction digests of Cellulomonas DNA used in the ligation reactions, and the plasmids pBR322 and pUC18, the original preparations and those linearised with BamHI. Lanes from left: 1, uncut DNA; 2, Sau3AI digest of Cellulomonas DNA; 3, ClaI restriction fragments of T_7; 4, BamHI digest of Cellulomonas DNA; 5, original preparation of pUC18; 6, pUC18 linearised with BamHI; 7, ClaI restriction fragments of bacteriophage T_7; 8, original preparation of pBR322; 9, pBR322 linearised with BamHI.

Ligation reactions

Restriction digests of the chromosomal DNA with Sau3AI and BamHI and the plasmids pUC18 and pBR322, linearised with BamHI (Fig.4), were used for the ligation reactions. The DNA restriction digests as well as the linearised plasmids were purified by washing with phenol: chloroform mixture and precipitating with alcohol as described above. Each of the Sau3AI and BamHI partials were used for ligation with the linearised pUC18 in proportions of 1:2, 2:1, 1:1, with total DNA content of 1 µg, 1 U of ten-fold diluted T4 DNA ligase (BRL, Cambridge, UK), 1 µl 10 x T4 DNA ligase buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM dithiothrietol), 1 µl 5mM(10x)ATP and water to make 10 µl in an Eppendorf tube. The reaction mixtures were incubated in a water bath at 12.5°C for 18 hours. 1 mg of the linearised pUC18 and the DNA partials were also religated alone under the above conditions. The ligation mixtures were electrophoresed on 0.8% agarose. As shown in Fig.5 much of both the Sau3AI and BamHI partials of the chromosomal DNA and almost all the linearised plasmid were religated when reacted alone. A proportion of 1:2 between the linearised plasmid and the DNA partials seemed best as the bands corresponding to religated linearised plasmid were weakest, which would ensure a higher proportion of the bacterial cells transformed with recombinant DNA carrying a DNA fragment.

Electrophoretic patterns obtained with ligation mixtures between linearised pBR322 and Sau3AI and BamHI partials (Fig.6) showed quite similar results as in the case of ligations involving pUC18. Ligation reaction was also carried out under the conditions described above initially for three hours, the reaction mixture was then diluted ten-fold with 9 µl 10xligase buffer, 9 µl 10xATP, 1 U of T_4 DNA ligase and water to make 100 µl, and ligation continued overnight at 12.5°C. The ligation mixture thus obtained was tested for transformation of the cells with the plasmid carrying DNA inserts.

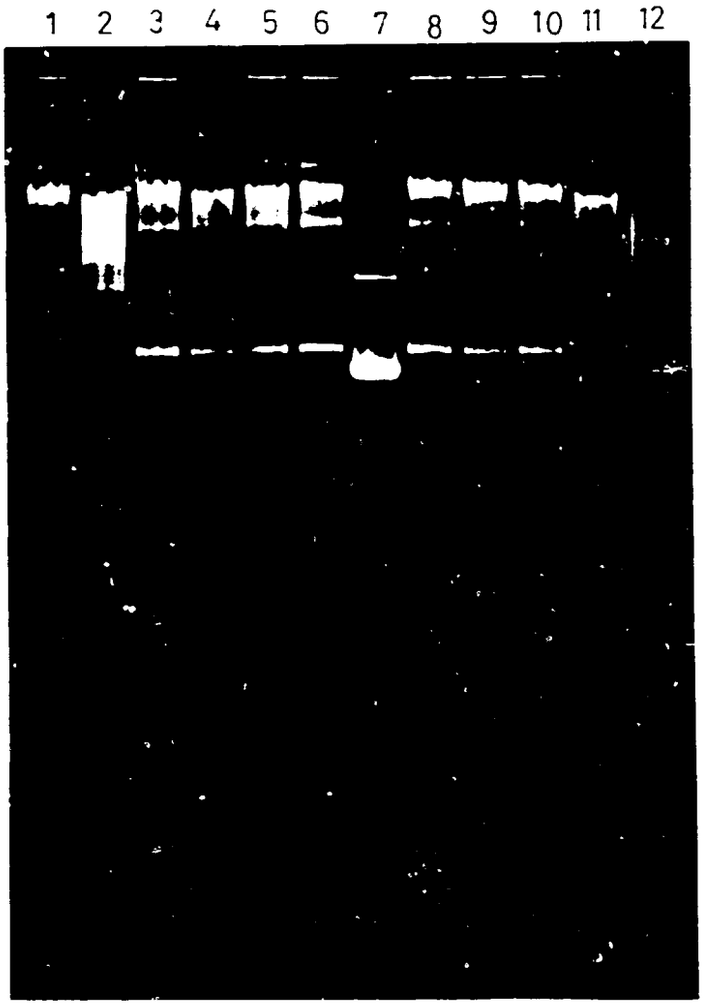

Fig. 5. Ligation mixture of BamHI and Sau3AI partials of Cellulomonas DNA and linearised pUC18. Lanes from left: 1, BamHI partials religated on itself; 2, BamHI partials used in the ligated reactions; 3-5, ligation mixtures of linearised pUC18 and Sau3AI partials in proportions of 2:1, 1:2, and 1:1, respectively; 6, linearised pUC18 religated on itself; 7, pUC18 linearised with BamHI; 8-10, ligation mixtures of linearised pUC18 and BamHI partials in proportions of 2:1, 1:2 and 1:1, respectively; 11, Sau3AI partials religated on itself; 12, Sau3AI partials used in the ligation reactions.

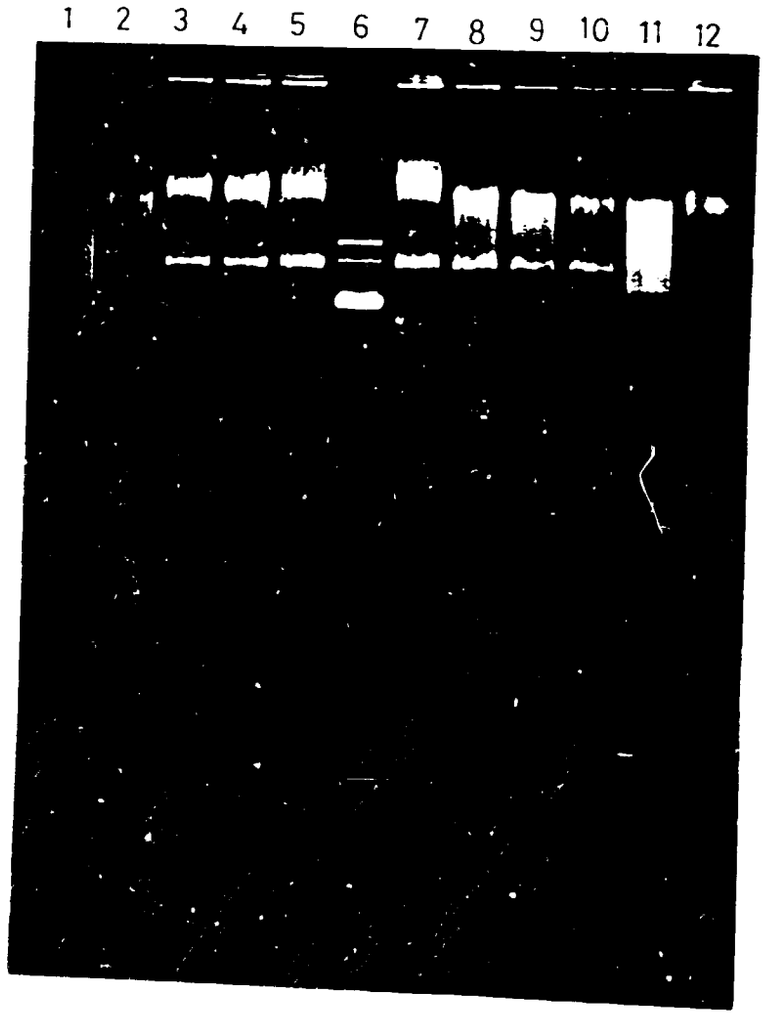

Fig.6. Ligation mixtures of BamHI and Sau3AI partials of Cellulomonas DNA and linearised pBR322.
Lanes from left: 1, Sau3AI partials used in the ligation reactions; 2, Sau3AI partials religated on itself; 3-5, ligation mixtures of linearised pBR322 and BamHI partials in proportion of 2:1, 1:2 and 1:1; 6, pBR322 linearised with BamHI; 7, linearised pBR322 religated on itself; 8-10, ligation mixtures of linearised pBR322 and Sau3AI partials in proportions of 2:1, 1:2 and 1:1; 11, BamHI partials used in the ligation reactions; 12, BamHI partials religated on itself.

Preparation of competent cells

JM107 and L5 strains of E. coli were cultivated overnight at 37° and 30°C respectively in a 10 ml broth contained in a 30 ml test tube with sterile air blowing through it. Inoculated 40 ml L broth contained in a 250 ml flask with 1 ml of this culture and incubated in a shaking water bath at 37° or 30°C until OD_{550} = 0.30, chilled the culture, centrifuged off the cells and washed with 20 ml 10mM NaCl asceptically. Washed the cells again with 20 ml 30mM $CaCl_2$, resuspended in 20 ml 30mM $CaCl_2$ and placed on ice for 20 minutes. After centrifugation the cells were resuspended in 4 ml 30mM $CaCl_2$ containing 15% glycerol and stored in aliquots of 0.2 ml in Eppendorf tubes at -70°C. The competent cells thus prepared were found good with respect to viability and transformability.

Transformations and screening for positive clones

Transformations were done by thawing 0.2 ml of the competent cells on ice and then adding an aliquot of the ligation mixture (total DNA content approx. 0.5 μg) followed by incubation on ice for 1 hour. Then incubated the transformation mixture of L5 cells at 35°C and that of the JM107 at 42°C for 2 mins. After incubating the mixtures at 30°C and 37°C for the L5 and JM107 strains, respectively, for 30 mins. added 1 ml L broth and incubated for another 3 hours at the same temperature. 0.1 ml aliquots of the transformation mixtures were then spread on CMC-agar plates for screening.

JM 107 strain of E. coli was employed to monitor the rate of DNA inserts in the linearised pUC18 in the ligation reactions. The transformed cells were spread on the x-gal plates and incubated overnight at 37°. The proportion of white colonies among the blue ones showed the efficiency of DNA inserts in the recombinant DNA. As shown in Table 1 the rate of DNA inserts in the plasmid was only 7-9% with the BamHI partials while it was over 20% with the Sau3AI partials. However, when the ligation mixture was diluted ten times after an initial reaction for three hours, the rate of DNA inserted into the plasmid increased to approximately 35%. The ligation mixtures obtained thus were used to

transform the E. coli L5 strain.

Table 1. Rate of DNA inserts in the linearised pUC18 as shown by the proportion of white colonies when E. coli JM107 cells, after transformation, were grown on x-gal plates.

No.	Ligation mixture used for transformation	Proportion of white colonies
1.	pUC18:BamHI partials (1:2)	7-8%
2.	pUC18:BamHI partials (1:4)	8-9%
3.	pUC18:Sau3AI partials (1:2)	Approx. 20%
4.	pUC18:Sau3AI partials (1:4)	Approx. 25%
5.	Ligation mixture at '4' above reacted for 3 hours, then diluted ten times and ligation continued overnight	Approx. 35%

The lysogenic strain E. coli L5 was transformed with the ligation mixture 5 given the Table 1 according to the method described above. 0.1 ml aliquots of the transformation mixture were spread on each CMC-agar plates containing ampicillin and grown overnight at 30°C. Replica plates were then prepared asceptically using velvet cloth and both the original as well as the replicas were again incubated overnight. The original plates were then incubated successively at 42°C for 15 mins, 37°C for 3 hours and 50°C for 2 hours before staining these with congo red. Clones including over 12,000 transformed with pUC18 having chromosomal DNA inserts were screened for CMCase activity. Two of these clones were found to have the activity as shown by the appearance of haloes on staining with congo red.

Miniscreen preparation of the plasmid DNA from the colonies on the replica plates corresponding to the ones that gave positive tests for CMCase activity were obtained according to the method described by Rodriguez and Tait(28). E. coli L5 when transformed with this preparation showed CMCase activity. Further experiments on characterisation and expression of these genes are in progress.

DISCUSSION

Cellulomonas biazotea, one of the strains in our collection of cellulolytic organisms, was selected as a source of cellulase genes for cloning purposes in this work. Although cloning of cellulase genes have been studied in a procaryote E. coli, it is planned to undertake such investigations eventually on eucaryotic system like yeast. The chromosomal DNA of C. biazotea prepared seemed a good preparation as shown in Figs. 2-4. This DNA could be restricted efficiently with both Sau3AI and BamHI. Experiments were undertaken on restriction with both these enzymes in order to obtain DNA fragments mostly within the size range of 3-10 kb pairs which could be expected to be a suitable size to obtain the enzyme genes in intact form.

The two plasmids pUC18 and pBR322 were prepared from the respective strains of E. coli by growing the organisms in LB medium containing ampicillin and then amplifying the plasmids in the presence of chloramphenicol. Each of the plasmid thus obtained showed a number of bands due to their different forms which could all be rendered to almost a single band of the linearised molecule on restriction with BamHI as shown in Fig. 4.

Ligation reactions were carried out between Sau3AI and BamHI restriction digests of the chromosamal DNA and the linearised plasmids pUC18 and pBR322, keeping different proportions in each case. A higher proportion of the plasmid seemed to yield a higher percentage of the plasmid with DNA inserts which would be apparent by reading Figs. 5 and 6.

The strain E. coli JM107 and the plasmid pUC18 complement each other with respect to the expression of lac operon. The lac region in the pUC18, which has a BamHI site, when cut with this enzyme and religated with a DNA insert at this site, expression of lac operon and thus of β-galactosidase in E. coli JM107 cannot take place when transformed with such a plasmid. On the other hand linearised pUC18 religated on itself, on transforming E. coli JM107 would allow the expression of β-galactosidase, thus hydrolysing X-gal to the blue produce when grown our agar plates. The number of white colonies amongst the blue would thus show the proportion of cells transformed with pUC18 carrying a

chromosomal DNA insert. Ligation mixtures obtained with different proportions of pUC18 and the DNA partials reacted under different conditions were used to transform E. coli JM107. Since BamHI is a 6-base cutter would yield bigger fragments as compared to those of Sau3AI which is a 4-base cutter. The rate of inserts can therefore be expected to be much lower in the former case. As shown in Table 1 the proportion of white colonies was less than 10% when E. coli JM107 was transformed with ligation mixture when obtained from pUC18 and BamHI partials. In the case of SauAI partials there was a 3-fold increase in the rate of insert. After an initial incubation for 3 hours when reaction mixture was diluted 10-fold and then ligation continued overnight with more T_4 DNA ligase the rate of insert increased significantly. Dilution, after initial annealing would provide a better chance for the recombinant molecule to fold back upon itself and religate.

Recombinant DNA molecules used in transformations of E. coli for obtaining cellulase positive clones were constructed from BamHI linearised pUC18 and Sau3AI partials of the chromosomal DNA. Sau3AI partial digest of the chromosomal DNA could obtain some fragments of suitable size having intact cellulase gene, even if it has restriction sites for this enzyme. Nearly complete digestion of the chromosomal DNA would be required for obtaining segments of suitable size if restriction is done with BamHI. If the cellulase gene has a BamHI site it would not be possible to obtain fragments with intact gene in such a case. Sau3AI partials of DNA can be ligated in the BamHI site of the plasmid since recognition sites of the two enzymes are compatible.

E. coli L5 was used for cloning cellulase genes because being a lysogenic strain the cells can be lysed by activating the bacteriophage at 42°C. This would allow the release of the enzymes which may not be secreted and hence difficult to detect. The ligation mixtures prepared as above were used for transformation with pUC18 having DNA inserts. The transformants were screened for cellulase activity by growing these on CMC-agar plates, incubating these at 50°C and then staining the plates with congo red. Other methods have been used by other workers for detecting cellulase transformants. These include measurement of reducing sugars from CMC (17), and immunological detection methods (18,19). Congo red staining method which has been shown to work successfully in the

cloning of β-glucanase (20), was used in this work due to its ease and simplicity. Two clones expressing CMCase activity were detected during this screening. Further experiments on characterisation and expression of the genes thus cloned are planned.

This work has dealt with cloning of endoglucanase genes of Cellulomonas in E. coli. Apart from further studies on cloning of genes coding for this enzyme, work on those of the other enzymes i.e. exoglucanase, cellobiohydrolase and cellobiase are planned. High production of not only the various enzymes involved in the breakdown of cellulose but also those which could break down the lignocellulosic complexes, the form in which cellulose exists natively, is aimed. Capability to break down hemicelluloses which is also a significant content of the native celluloses would also be needed. To be able to clone and express all these activities at a high level would enable to develop a system in which cellulose breakdown could be achieved efficiently. An organism which could grow at elevated temperature and produce the enzymes that are stable at such temperature would be highly desirable from the application point of view. Developing a eucaryotic organism like a yeast from these points of view could allow not only saccharification of lignocellulosics but also utilise the sugars thus produced for the production of alcohol or protein rich biomass in a single process. These are therefore the ultimate objectives of our research on utilization of cellulosic substances. Further line of work in this respect is being proposed separately.

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