PN-ABS-269
6.163
logged

FINAL REPORT

Covering Period: Dec. 1, 1988 to June 30, 1992

Submitted to the Office of the Science Advisor U.S. Agency for International Development

Bioconversion of Lignocellulosic (LC) Biomass Produced on Saline Lands by *Cellulomonas*.

Principal Investigator: **Dr. M. Ibrahim Rajoka**

Grantee Institution: Nuclear Institute for Agriculture and Biology, P.O. Box 128, Faisalabad. PAKISTAN.

Collaborator: **Dr. Carl A. Batt**

Institution: Department of Food Science, Stocking Hall, Cornell University, Ithaca NY, 14853. U.S.A.

Project Number: **PSTC 6.163**

Grant Number: **936-5542-G-00-8942-00**

A.I.D. Grant Project Officer: Dr. Ronald S. Senykoff

Project Duration: Dec. 1, 1988 to June 30, 1992

OCT 15 1993

TABLE OF CONTENTS

Executive Summary	:	1
Objectives	:	2
Methods	:	3
Results	:	5
Impact, Relevance and Technology Transfer	:	13
Project Activities / Outputs	:	13
Project Productivity	:	14
Future Work	:	14
References	:	15

EXECUTIVE SUMMARY

The proposed research was aimed at (1) Mutant selection of *Cellulomonas biazotea* for production of cellulases and hemicellulases to permit rapid cellulolysis.(2) Fractionation of enzymes from the crude extracts,purification and characterization of cellulases(3) Improvement and simplication of saccharification process using recombinant DNA techniques.

Two hypersecreting cellulolytic mutants of *C.biazotea* NIAB 442 were isolated after gamma ray-induced mutagenesis. Subsequent chemical mutagenesis yielded a variety of further mutants. The mutants showed improved saccharification of *Leptochloa fusca* (L) Kunth (Kallar grass).

Endoglucanase (CMCase) and B-glucosidase components of *C.biazotea* were differentiated and characterized by non-denaturing PAGE using zymographic techniques.A quick and efficient method was developed for staining protein gels.

Chromosomal DNA from *C.biazotea* was used for isolation and cloning of cellulase genes. For this purpose plasmid pUC18 was used for cloning fragments in the range of 1-9 Kb in its *BamHI,SauIII AI* or *SacI* site. Endo-glucanase and B-glucosidase genes were cloned in *Escherichia coli* HB101.After transformation of *E.coli*,B-glucosidase and endo-glucanase positive clones were detected *in situ*. The recombinants were characterized in plate tests as well as in aqueous cultures. A *Sma1* genomic library from *C.biazotea* was constructed in *E.coli* HB101 using shuttle vector pBLU- D.The recombinant plasmids were transformed to competent cells of a Cir° yeast. *In vivo* studies indicated that the genes were fully expressed in yeast as well. Similarly genomic library was constructed in *SmaI* site of plasmid YEP24.Three clones able to hydrolyse CMC have been isolated.Thus some of the objectives outlined have been completed.The facilities developed are being used by three groups of NIBGE to complete ongoing projects.

OBJECTIVES

Pakistan meets 65% of its very low energy consumption through indigenous natural gas, petroleum oil, coal and non-conventional sources; 35% of the consumptions particularly liquid energy needs, are met through imports consuming more than 32% of all foreign earnings. The per capita consumption of energy in Paki stan is about half of that of the other developing countries. This situation warrants exploitation of alternate sources of energy and unconventional proteinous materials for conservation of energy.

Of the many forms of energy, such as hydro, fossil fuels, nuclear etc, renewable such as solar, wind, recycled wastes, biomass etc. offer feasible additional sources of commercial energy. The most abundant use of solar energy is through plants which convert it into chemical energy through the process of photosynthesis; fossil fuels are also a result of the same process. Production of energy through biomass is a very attractive proposition, particularly for a country whose economy is based on agriculture. When oil and gas reserves start to run out, LC biomass could come to rescue and provide bioethanol, to supplement and eventually replace those from oil and natural gas.

Biotechnological processes can play a role in producing biofuels, (Gibbons and Westly, 1986), improve the quality of animal feed by enhancing protein value (Gibbons and Westly 1987). Microbes are amenable to changes and can be manipulated to hyper-produce the desired end product.

The hydrolysis of cellulose in LC biomass to glucose is an important unit operation in the production of fuels and chemicals from LC biomass.To achieve complete hydrolysis of cellulosic substrates, the different enzyme components of the cellulase complex must be present in the right proportion. This requirement for the synergistic action of different endo-and exo-B-glucanases (EC 3.2.1.4 and 3.2.1.91) and B-glucosidase (EC 3.2.1.21) for complete hydrolysis has been reported (Khan et al, 1985; Mandels, 1982,1985; Szczodrak, 1988). The complexity of the enzyme system is partly genetic in origin , with a number of the enzymes derer mined by different genes. Many fungi (Goyal et al,1991;Marsden and Gray,1986) and bacteria (Gilkes et al ,1991 ; Malek et al, 1988; Nakamura and Kitamura,1982 ,Rajoka and Malik, 1986; Rickard et a, 1981; Sami et al, 1988; Waldron et al, 1986) produce cellulase enzymes. Bacterial strain, *C.biazotea* secretes potent cellulases (Rajoka and Malik; 1986) and may serve as a better donor for cellulases. Many attemps have been made towards strain development of *Trichoderma* spp and *Cellulomonas* spp. (Mountenecourt and Eveleigh ,1979;Mountenecourt and Eveleigh,1977; Choi et al,1978; Haggett et al,1978).However ,synthesis of the cellulase enzymes by the wild strain is subject to a number of biochemical and genetic controls and cause low yield. Previous attemps to mutate strains of *Cellumonas* have not given stipulated improvement in enzyme production ability by the use of classical genetic techniques. Using UV or chemical mutagenesis,2-3 fold improvement in production of of endo-glucanase and cellobiohydrolase has been possible in fungi by induced mutagenesis (Macris,1984). Mountenecourt and Eveleigh(1979) have reported *T.reesei* strain using UV and chemical mutagenesis and have re ported many fold improvement in the cellulase complex of the organism. In a new strtegy, use of gamma rays induced mutations coupled with chemical mutagenesis may generate a new strain giving high cellulolytic activities.

Cloning of bacterial genes in yeast is simple and straight forward as no introns are involved (Penttila; 1989). Most of the cellulase genes cloned so far have been cloned in *E.coli*. Efforts will be made to clone and express each individual cellulase gene. Our ultimate aim is to express these genes in a single osganism of choice for concerted production of cellulases and produce bioethanol most probably in a single step. The yeast *S.cereviceae* is used in many biotechnical processes where plant raw material is used but it does not secrete cellulases.Using gene technology, it should be possible to construct cellulolytic yeast which can grow on lignocellulosic wastes for fuel ethanol production.

Currently cellulase genes from a wide variety of micro-organisms have been cloned (Teeri et al 1983; Johnson et al 1986; Knowles et al, 1987; Skipper etal, 1991; Penttila et al, 1989; Hu and Wilson, 1988;. Endo-B-glucanases from bacteria, e.g., from *Bacillus subtilis*(Cantwell et al, 1986), *Clostridium thermocellum* (Sacco et al,1984), *C.fimi* (Curry et al, 1988; Skipper et al 1985; Wong et al, 1988) *Ruminococcas albus* (Honda et al, 1988) have been cloned in *Saccharomyces cerevisiae*; in some cases, low expression of genes has been observed. This report describes cloning of all genes of the cellulase complex to develope cellu lolytic yeast for fermentation of LC substrates.

The successful process requires, amongst other things, a clear understanding of the molecular biology of the expression and secretion of heterologous proteins by yeast.Without prior knowledge of these aspects,further development is very diffi cult. For this reason, expression of cellulase genes has first been observed in *E.coli* and then in yeast.

MATERIAL AND METHODS

Mutagenesis.

Organisms: *C. biazotea* NIAB 442 and its mutant derivatives were used throughout these studies. The strains were maintained on Dubos salts-CMC (0.7 % carboxymethyl cellulose - Na salt, low viscosity) slants as described earlier (Rajoka and Malik, 1984, 1986).

Growth media: *C. biazotea* was grown in a cellobiose salt medium supplemented with 0.2 % yeast extract (CSYE medium). The pH of the medium was adjusted to 7.3 with HC1. In other growth studies, the cellobiose was replaced by other carbon sources, mentioned in the text and tables.

Substrate preparation: Alkali treated LC biomass was prepared as described earlier (Rajoka and Malik, 1984).

Isolation of Mutants: Actively growing *C. biazotea* cells (2 x 10^9 cells/ml) were exposed to a range of doses of gamma radiation in a Co-60 gamma cell. To prepare a cell survival curve following irradiation, the cells were seriallly diluted in biological saline supplemented with 0.1 % yeast extract, plated on nutrient agar plates and colonies counted after 72 hr. The survivors from 30, 40, 50 Krad were allowed to express in CSTE medium and expressed cells were treated as follows: To isolate (a) Str resistant (Str) mutants, cells were plated on a medium containing 40 ug/ml streptomycin (Str).(bTo isolate Rf' mutants 20 ug/ml rifampicin (Rf) was

used .The selected cells were serially diluted and plated on esculin selection plates to select for beta-glucosidase mutants. (Choi et al., 1978; Montenecourt and Eveleigh, 1977), containing either (a) Str or (b) Rf. The colonies were counted and re-plated to prepare master plates containing approximately 600 colonies/plate. Mutants showing hyperproduction of beta-glucosidase were selected.

The expressed cells at the absorbance of 0.6, were plated on CMC, CMC + glucose, Sigmacel 100, or Sigmacel 100 + glucose. The colonies were used to prepare master plates and assayed for hyperproduction of endoglucanase by the size of the clearing zone evidenced by the congo red staining technique (Theather and wood, 1982). The master plates with Sigmacel, were used for selecting hyper-producing mutants that potentially produced exo-glucanase (Sigmacelase) as evidenced by greater size of the clearing zone.
Chemical mutagenesis: The hypersecretive mutants were subsequently mutated with NMU (140 ug/ml) (EMS 160 ug/ml) or MNNG (200 ug/ml) for about 1 hr yielding a 90 % kill. Fig. 1 illustrates the geneology of the mutant selections.

Selection of constitutive mutants for hyperproduction of beta-glucosidase: The mutated and expressed cells were plated on 1 % glucose-agar medium, and subsequently the colonies were then overlayed with esculin medium in 0.5 % agar. The colonies showing earlier and larger zones of blackening were picked, and then re-screened in plate tests to confirm hyper-glucosidase production. The selected strains were stored at 4°C for further studies.

Fermentation Studies: The parent, *C. biazotea* and its mutant derivatives were grown in liquid salt medium containing the growth substrate. The cells were processed, as described previously (Rickard et al., 1981), to gain extracellular and cellular fractions. The latter were washed, whole cell sonicates. Exo-glucanase, endo-glucanase, beta-glucosidase and beta-xylosidase were determined (Rajoka and Malik, 1986).

Saccharification Studies: Portions of 0.5 g of Kallar grass straw were dispensed into 50 ml conical flasks to which 3 ml of phosphate buffer 0.05 M pH 7.0 were added. They were incubated with 7 ml of crude enzyme preparation obtained after growth of the test organisms on 1 % Kallar grass straw. Studies were at 40°C in shaking water bath incubator, and duplicate flasks were periodically removed to measure reducing sugars using the dinitrosalicylic acid method.

Characterization of Cellulases

Characterization of cellulases was done after Ashfaq et al (1991)

Cloning of cellulase genes and characterization of gene products:

Cloning in E.coli: For cloning cellulase genes from *C.biazotea* into *E.coli*,the protocols adopted by Bashir et al (1991) were followed.

Cloning in Yeast: These cloning experiments were performed as described by Rajoka et al (1992) and Parvez et al (1993).

RESULTS AND DISCUSSION

Selection of Mutants: The survivors following radiation at 30, 40 and 50 Krad were allowed to express and were processed as described in Materials and Methods for isolation of Str and Rf' mutants. 600 colonies were plated for selection of mutants with the ability to hyper-produce endo-glucanase studies as evidenced via the congo red plate selection procedure (Teather ad wood 1982).

Four Str' mutants and 3 RF' mutants were selected (Figure 1) Str' mutants were also evaluated for hyper-production of beta-glucosidase . 51 Str' and 7 Rf' were selected as hyper-beta-glucosidase producers. Fig. 2 and 3 illustrate the glycosidase activities of the parent and its mutant derivatives after growth in salicin (0.25 % W/V). Substantial improvement in biosynthesis of beta-glucosidase was found especially in 51 Str' and 7 Rf' (3 fold increase). The effect of Kallar grass on the production of beta-glucosidase was also studied in all the strains. 51 Str' and 7 Rf' mutants along with the parent were grown on Kallar grass straw-liquid culture to observe production of the above mentioned latter two glycosidases. Figures 4 and 5 illustrate the time course production of beta-glucosidase and beta xylosidase. All studies were carried out at 30° with an initial pH of 7.3. The cells were removed by centrifugation, resuspended in buffer and sonicated. The whole cell preparations were assayed. It was observed that 2.2 fold yield in the production of these activities occurred (Figs. 4 and 5).

Chemical mutagenesis: Mutant M-NMUI was selected using NMU, mutant AVIS and copper resistant mutant M-CU1 were selected using EMS, and BG-4 with MNNG from 600 various, based on producing larger zones of blackening in combined esculin-deoxyglucose medium. These four mutants were grown in Dubos liquid medium containing 1% pretreated Kallar grass straw, and periodically assays were carried out for intracellular (total cell homogenates) and extracellular beta-glucosidase, beta-xylosidase, CMCase activities (Table 1). The former two activities were produced mainly associated with the cell while the CMCase was extracellular. There was no change in the biosynthesis of Sigmacelase and xylanase. Mutant AVIS (a penicillin resistant mutant (11.25x) and BG-4 improved with respect to beta-glucosidase, beta-xylosidase and CMCase production over the wild parent and mutant parent 51 Str'.

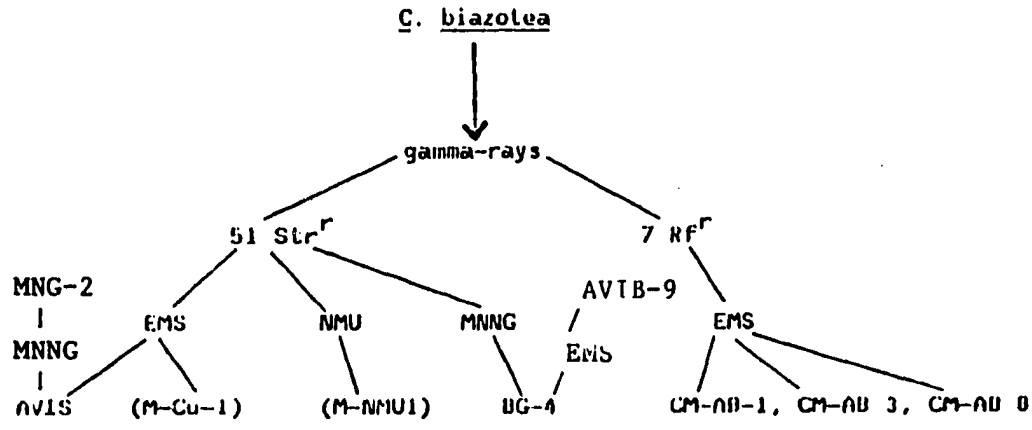

Fig. 1 Geneology of Cellulomonas biazotea mutants

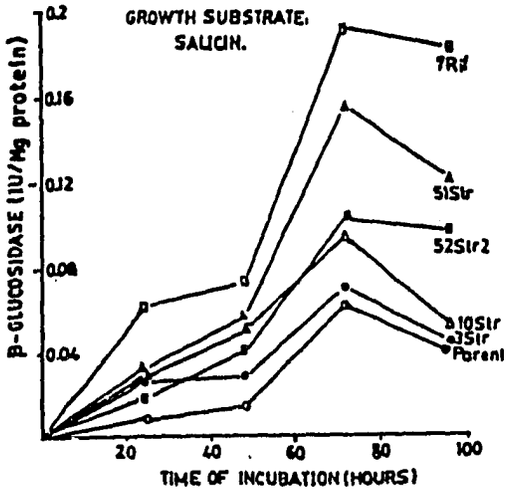

Fig. 2 Production of β-glucosidase from salicin using C. biazotea and its mutant derivatives.

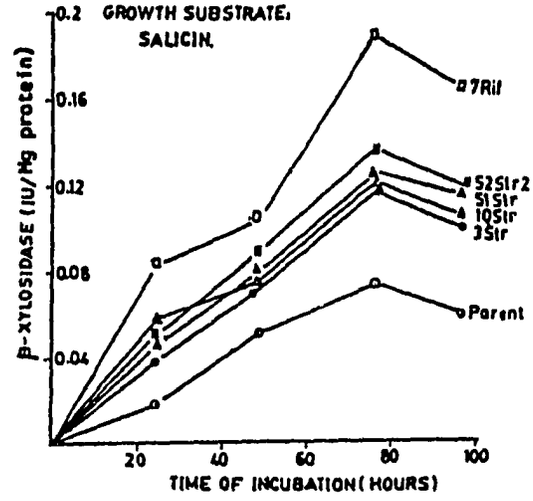

Fig. 3 Production of β-xylosidase from salicin using C. biazotea and its mutant derivatives.

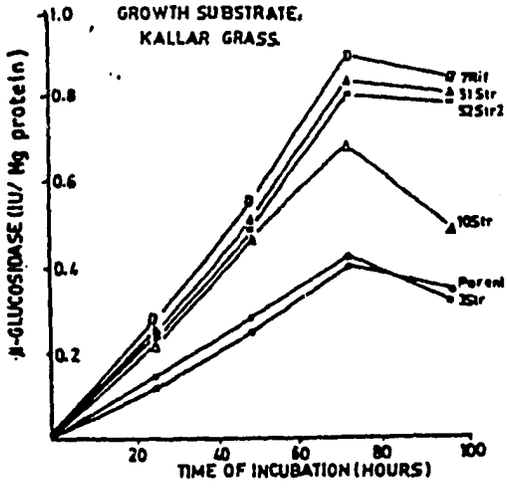

Fig. 4 Production of β-glucosidase from kallar grass using C. biazotea and its mutant derivatives.

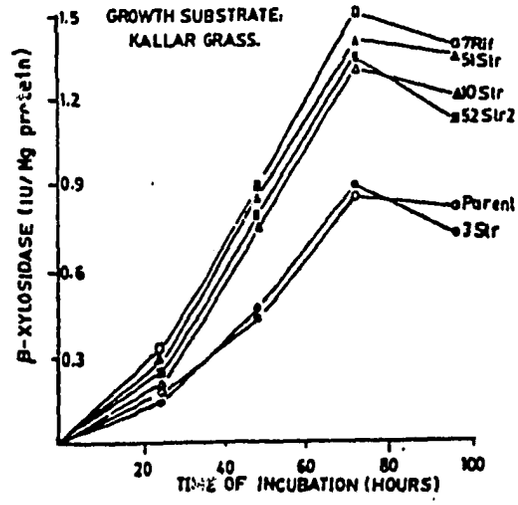

Fig. 5 Production of β-xylosidase from kallar grass using C. biazotea and its mutant derivatives.

Table 1: Time course study to produce CMCase, ß-glucosidase and ß-xylosidase after growth on 1% Kallar grass straw.

	CMC*ase (IU/mg protein) at day					B-glucosidase**(IU/mg protein)at day					B-Xylosidase** at(IU/mg protein at day				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Wild	0.43	2.10	3.5	2.96	2.3	0.17	0.18	0.40	0.20	0.2	0.5	0.69	0.71	0.59	0.50
M-51 Str'	0.98	3.82	4.3	4.13	3.2	0.32	0.42	0.95	0.85	0.78	0.05	1.25	1.43	1.39	1.25
M- 7 Rf'	0.5	2.45	3.65	3.5	3.0	0.28	0.51	0.98	0.69	0.68	0.96	1.10	1.53	1.79	1.26
M-AVIS	1.50	3.94	6.2	5.0	4.3	0.34	0.64	0.15	1.0	0.90	1 25	1.75	2.0	1.7	1.50
M-CU-1	0.7	3.85	5.6	4.3	3.4	0.34	0.45	0.95	1.02	1.20	0.75	1.3	1.5	1.51	1.60
M-NMU-1	0.78	3.4	5.4	5.0	4.5	0.40	0.50	1.2	1.1	0.90	1.10	1.75	1.9	1.45	1.30
CM-AB-3	1.20	4.0	5.0	5.2	6.0	0.50	0.62	1.30	1.5	1.52	1.45	1.90	2.1	1.8	2.1
M-BG-4	0.97	3.5	4.40	4.3	3.3	0.52	0.82	2.1	1.60	1.50	1.5	2.0	2.1	2.0	1.9

* CMCase was mainly extracellular.
** ß-glucosidase and ß-xylosidase were cell associated.

Table 2: Constitutive production of glycosidases after growth on 1 % glucose.

	Beta-glucosidase(IU/mg protein) at day			Beta-Xylosidase(IU/mg protein)at day		
	1	2	3	1	2	3
Wild parent	0.07	0.07	0.06	0.00	0.00	0.03
Mutant parent 7Rf'	0.07	0.04	0.05	0.00	0.00	0.03
CM-AB-1	1.15	1.28	1.68	0.23	0.21	0.19
CM-AB-3	1.56	1.68	1.84	0.23	0.16	0.15
CM-AB-8	0.87	1.53	1.76	0.05	0.05	0.035

The test organism were grown on 1 % glucose in shake flasks cultures. The cells were harvested and the total cells assayed for glycosidase activities.

Table 3: Comparison of enzymes of *C. blazotea* and mutant derivatives in the saccharification of *Kallar grass.

	5 h		20 h		25 h	
Wild parent	10.0	(27)	20.8	(56)	20.8	(56)
Mutant parent 51 Str'	11.3	(30)	24.0	(64)	26.4	(70)
Mutant parent 7 Rf'	11.3	(30)	23.8	(64)	26.0	(70)
AVIS	11.8	(32)	26.2	(70)	28.4	(76)
AB-3	11.4	(30)	24.2	(65)	26.4	(70)
BG-G	12.5	(33)	26.2	(70)	27.0	(72)

* Kallar grass (5%) [75% sugar]
Sugar measured as mg/ml
5 h, 20 h, 25 h, - Incubation time
Values in parenthesis indicate % saccharification.

Constitutive mutants: Actively growing cells of 7 Rf' were mutated with EMS and expressed cells were plated on Dubos Salts-glucose medium. The colonies were overlayed with esculin-ferric ammonium citrate agar medium. The colonies showing faster and larger blackening zones were picked, and tested in plate tests to confirm constitutive beta-glucosidase production. Three mutants (CM-AB-I,CM-AB-3 and CM-AB-8) proved stable and were grown at different glucose concentrations (Table2) and levels of cell associated beta-glucosidase production were assayed to find the optimum substrate concentration for production. 1 % glucose was optimal for CM-AB-3, The best strain.The enhanced yields of beta-glucosidase and beta-xylosidase by the CM-AB mutant series is quite evident(Table 1) Yields (1.8 U/mg) are a 4-5 fold improvement over the best mutant of *Cellulomonas* selected by Haggett et al. (1978).

The best mutants were further mutated with EMS or MNG to select mutants which could produce ß-glucosidase and CMCase in the presence of increasing concentration of DOG(1.5,1.75 and 2%).Mutant MNG-2 ad AVI-9 were selected as the best mutants.These mutants alongwith other mutants and wild parent were characterized for production of CMCase and B-glucosidase in the presence of increasing concentration of glucose or glycerol in plate tests.B-glucosidase was not inhibited in the presence of10 or 15% concentration of glucose in mutants while 10% glucose did not significantly repess CMCase biosynthesis in mutats while 15% glucose inhibited 65 % activity of parent.

Liqid culture screening for production of cellulases and xylanases from 14 differen indicucers rvealed that the parent and the mutant strain grew rapidy in liquid media and produced cellulases. Filter paper, cotton wool or sigma cell 100 resulted in increased yields of FPase followed by kallar grass raised on saline lands while high xylanase activities were detected when kallar grass was used as inducer. The greatest enzyme production occurred at 30C, pH 7.3 after 3days of incubation both from realistic cellulosic substrates viz, filter paper, x-cellulose, or sigma cell 100 and LC substrates. AVI-9 and MNG-2 were selected as the best mutants with respect to production of these enzymes in the presence of 10% glucose.Aqueous culture studies indicated that there was marginal improvement in cellulases and hemicellulase production in new mutants.

Saccharification studies: Strains were grown on Kallar grass-liquid medium for 3 days and the whole cultures assayed for enzymes of the cellulase complex. Enzyme preparation were diluted in 0.5 M citrate buffer to equivalent levels of CMCase and then Kallar grass saccharified with these enzyme preparations and reducing sugars produced were measured (Table 3). Although equal amounts of CMCase were used, the total cellulase activity varied between preparation. some mutants (AVIS &BG 4) showed up to 1.4 fold improvement over wild parent in the saccharification of Kallar grass.

Gamma ray-induced mutation has yielded some mutants which hyperproduce endo-glucanase and beta-glucosidase. One significant anomaly was that though the plate screening for beta-glucosidase was based on the size of the blackening zone around the colony, the mutants in liquid culture only produced a cell-associated enzyme. Hyper-enzyme production was also obtained when these mutants were grown on salicin and Kallar grass straw. These strains were chemically mutated yielding some useful inducible and constitutive mutants. Some of the mutants showed improved cellulolysis of Kallar grass which had been produced on saline lands in Pakistan.

Characterization of Cellulases:

Ashfaq et al (1991) indicated that endoglucanase(CMCase) and B-glucosidase components of C.biazotea NIAB 442 were differentiated and characterized by non-denaturing PAGE using zymographic techniques. Activity stains showed three electrophoretically distinct CMCase isozymes, while B-glucosidase was found to be in the range of 90-110 kdal. Electrophoretic elution of B-glucosidase fragment on SDS-PAGE gave bands in the range of 20-60 kdal.The study of partial proteolysis of CMCases by trypsin showed no significant change in its activity.This suggests that the structural configuration of the enzymes possibly protects them against the proteolytic action of trypsin.A quick and efficient method was developed for staining protein gels(Siddiqui and Rashid,1991).

Cloning of Cellulase Genes:

Bashir et al(1991) have described that a total of 6781 recombinants were screened by replica plating on four different media having x-gal, Esculin, CMC and Sigma cell 100, in each medium along with 50 ug/ml ampicilin to identify blue/white colonies, B-glucosidase, CMC-ase and FP-ase positive colonies respectively. After 48 hour incubation, one of the recombinant was found positive for B-glucosidase production on medium having Esculin . Congo red staining of replica plates having 0.5% CMC after 72 hours incubation yielded one colony positive for CMC-ase. Chromosomal DNA fragments cut with *SaCI* in the range 1-7 Kb were ligated in *SaCI* linearized pUC18 in its polycloning site (Bashir et al,1990). The ligation mixture was transformed to competent cells of *E. coli* HB101 and the recombinants were selected on Esculin-Dubos salt-yeast extract Amplicin-IPTG-medium to isolate B-glucosidase (BG) recominants and CMC selection medium to select endo-gluca nase (EG) recombinants. out of 6891 recombinants, 7 BG+, and 6EG positive recombinants were selected. BG recombinants were characterized for inductive as well constitutive production of ß-glucosidase in plate tests (Table 4). The recombinants produced two fold more ß-glucosidase than that produced by the donor. The recombinants were fast growing organism on Dubos salt medium than the donor. The recombinant carrying eg genes were also characterized in Plate tests (Table 5). The recombinants were further characterized after growth in CMC-Dubos salt-Amp liquid medium.

Results of cellulase production from recombinants grown in CMC medium have been presented in Table 6. Initial liquid culture screening have indicated that 8 recombinants produce CMCase while 6 have shown B-glucosidase production in Dubos-CMC and Dubos-Cellobiose medium respectively. Zymographic Studies have indi cated that both CMCase and B-glucosidase produced by recombinants show similar electrophoretic mobility as that depicted by the donor . Recombinant plasmids were subjected to restriction wih *SacI*.The size of the recombinant plasmid varied from 1.94-3.72 . The recombinant plasmids were treated with *EcoRI* . Plasmid Carrying 3.72 kb insert was subjected to erase a base technique using *EXo III* and Bal 31.

Various transformations were made in *E. coli* HB101 to know the size of insert encoding for ß-glucosidase.These studies indicat ed that the size of plasmid coding for B-glucosidase in this plasmid was 3.2 kb. The enzyme properties of the cellulases produced by the recombinants were similar to those shown by the donor.

Table 4: Production of ß-glucosidase by transformants harbouring recombinant plasmids measured in plate tests after 24 hours of growth.

Recombinant plasmid in HB101	Inductive*	Constitutive**
	measured as zone of blackening (mm)	
pUC 18 (Control)	0	0
pRM 1-1	15	20
pRM 4-1	26	28
pRM 8-1	10	10
pRM 13-1	15	15
pRM 14-1	12	10
pRM 18-1	18	ND
pRM 11-1	21	24
pRM 54	40	39

* The cells were grown on 1% glucose solid medium for 72 hours. The growth was overlayed with esculin (0.2%) dissolved in phosphate buffer of pH 7. The zone of blackening was measured in millimeters.

** The cells were grown on 0.2% esculin medium using stab tech nique. The zone of blackening measured in millimeters was taken as indicator of esculinase (ß-glucosidase) production after Montenecourt and Eveleigh (1977). *C. biazotea* under simi lar conditions produced 10 mm zone of blackening.

Table 5 : Endo-glucosidase (CMCase) production by some of the recombinants measured as zone of hydrolysis.

E. coli HB101 with recombinant plasmid	Zone of hydrolysis (mm)
pUC18 (Control)	0
pRM 2-1	15
pRM 3-1	18
pRM 7-1	14
pRM 12-1	18
pRM 13-1	14
pRM 15-1	14
pRM 54	5

* 10 ul of cells of equal optical density were stabbed in CMC- Dubos-Amp, solid medium. The cells were allowed to grow for 24 hours. The enzyme released was measured as zone of hydrolysis (measured in millimeters) after Teather and Wood (1982). The donor (*C. biazotea*) under similar conditions gave a 10 mm zone of hydrolysis.

Table 6: Production of cellobiohydrolase (FPase) , CMCase and salicinase from the recombinants after 20 hours growth in Dubos medium a,b of pH 6.8.

E. coli HB101 with recombinant plasmid	(a) FPase	(a) CMCase	(b) Salicinase	(b) ß-glucosidase
	----------(u mole/ml. h----------			
pUC 18 (Control)	0.0	0.0	0.0	0.0
pRM 1-1	0.0	0.0	0.0	1.3
2-1	4.2	5.6	0.0	T
3-1	4.8	0.6	0.1	0.5
4-1	0.0	0.0	1.7	2.1
7-1	0.0	3.4	0.2	T
12-1	0.0	2.3	0.0	0.0
13-1	0.5	5.0	T	0.2
15-1	1.0	1.5	0.0	0.0
C 12	4.0	12.0	0.0	0.0
C 22	2.4	11.2	0.0	0.0
C 28	2.3	6.32	0.0	0.0

T = Traces

All cultures were grown at 30°C at 100 rpm shaking speed in orbital shaker.

a) All grown on CMC -SYE medium

b) All grown on CSYE medium

The molecular cloning of cellulase genes has been currently reviewed (Gilkes et al,1991;Knowles etal,1987). Endo-glucanase genes from *C. uda* (Nakamura etal 1986 a), *C. flavigena* (Silva et al,1988), *C. fimi* (Gilkes et al ,1984) and B-glucosidase genes from *C. uda* (Nakamura et al,1986 b) have been cloned and expressed in *E. coli.* Bashir et al (1990) cloned B-glucosidase gene from *C. biazotea* in *E. coli* JM 109 but the recombinants grew poorly on cellobiose-LB or CSYE medium and supported the work of Nakamura et al (Nakamura et al,1986 b). All recombinants re ported here grew very fast and profusely (to an OD of 3.0) *in* CSYE, CMC-SYE or CMC-LB medium supplemented each with Amp. The activity produced in the plate tests was very high but in liquid medium, the activity was low (about one tenth of that produced by the donor) which indicates that *C. biazotea* cellulase genes are expressed at low level in *E. coli* but this work supports the work of Ghangas and Wilson (1987) and Lejeune et al (1986).Although *E. coli* is a useful host for primary cloning of cellulase genes, it has the limitation for the utilization of the gene products. It does not actively secrete enzymes, nor does it make useful metabolic products in sufficient quantity. Presently we are trying to clone cellulase genes in *S. cerevisiae* which is widely used biotechnically in brewing, baking and ethanol production processes.

Cloning of ß-glucosidase genesin yeast: A total of 300 Amp' clones were obtained. Three clones showed detectable ß-glucosidase activity. The recombinant plasmids isolated from these clones were named pRPI, pRP2 & pRP3. Thier expression and characterization has been presented by Rajoka et al (1992).

Cloning of endoglucanase genes in yeast: Genomic library for endoglucanase was also constructed in *Smal* site of plasmid YEP24 (Parvez et al,1993).Tree clones able to hydrolyse CMC have been isolated.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

These findings will improve the working capability of the research workers of the department. Project provided sufficient equipment and chemicals for carrying out the ongoing projects of the individuals in the laboratory and scientists of two other groups of the institute. Some Ph.D. and M.Sc. university students used the equipment for completing their theses. The results will be initially used by the institute for development of all techniques of molecular biology in the institute and collaborating universities. The equipment purchased under the project is being used by three groups of the institute as well as by research students of the universities for completing ongoing projects. These may kindly be left here in the developing country to improve the scientific capability of the researchers.

PROJECT ACTIVITIES/OUTPUTS

Participation in National and International Conferences/Symposium:

1. PSTC grantees meeting on "Biomass Production and Conversion" Dec. 13-15, 1989, Serena Hotel Faisalabad, Pakistan.

2. International Symposium on Biotechnology for Energy Dec. 16-21, 1989, Serena Hotel, Faisalabad, Pakistan.

3. Participated in "Twelfth Symposium on Biotechnology for Fuels and Chemicals" Gatlinburg, TN, USA May 7-11 , 1990.

4. 3rd Conference of Chemical Society of Pakistan, Univ. of D.I. Khan, D.I. Khan Dec., 1991.

5. Science International conference May 16-22, 1992, Khanaspur, Pakistan.

6. Second International Symposium on Genetic Engineering and Biotechnology, Jan. 6-9, 1993, Karachi, Pakistan.

7. 7 months training in "Molecular Biology of Biomass Conversion" from Department of Food Science, Cornell University, Ithaca, NY June 6, 1990 - January 1, 1991.

List of Publications\Presentations

1. Ashfaq R, A Bashir, MI Rajoka and KA Malik (1989). Structural activity of cellulose degrading protein from *Cellulomonas biazotea* NIAB 442 abstracted in Second Intern. Symp. on Protein Structure function Karachi Dec. 11-14, 1989.
2. Rajoka MI, A Bashir, SR Ashfaq and KA Malik (1989). Bioconversion of lignocellulosic (LC) biomass produced on saline lands by *Cellulomonas*: Cloning of genes for cellulase degradation PSTC grantees meeting on Biomass Production and Conversion Dec. 13-15, 1989 Hotel Serena Faisalabad, Pakistan.
3. Bashir A, SR Ashfaq, MI Rajoka and KA Malik (1990). Cloning of cellulase genes for the utilization of agricultural wastes. Abstracted in Proceedings National/International Telecommunication Symposium on Plant Biotechnology Aug. 16-19, 1991.
4. Ashfaq SR, A Bashir, MI Rajoka and KA Malik (1991). Rapid zymographic technique for the localization of cellulases on non-denaturing polyacrylamide gel. Proc. Int. Symp. on Biotechnoloy for Energy,Dec.16-21,1989.Faisalabad, Pakistan. Eds.K.A.Malik, S.H.M.Naqvi and M.I.H.Aleem.Published by NIAB/NIBGE.Faisalabad,Pakistan. p.77-84.
5. Bashir A, SR Ashfaq, MI Rajoka and KA Malik (1991). Cloning of cellulase genes using pUC18 and lamba 2001 vectors Ibid. p.55-64.
6. Rajoka MI, A Bashir, R Ashfaq, KA Malik & CA Batt (1992). Cloning of genes for endo-glucanase and B-glucosidase in *Escherichia coli* HB101 and characterization of recombinants for enzyme production. In Proc. third Natl. Conf. Chem., 1991 (in press).
7. Rajoka MI, S Parvez and KA Malik (1992). Cloning of structural genes for ß-glucosidase from *Cellulomonas biazotea* into *E.coli* and *Saccharomyces cerevisiae* using shuttle vector pBlu-D. Biotechnol. Letts. 14(11): 1001-1006.
8. Parvez S, MI Rajoka and KA Malik (1993). Utilization of agricutural wastes: Cloning of endoglucanase genes from *Cellulomonas biazotea* into *E.coli* and *Saccharomyces cerevisiae* using shuttle vector YEP24.Proceedings Second Inernational symposium on Genetic Engineering and Biotechnology, Karachi, Pakistan Jan.1993(in press).

PROJECT PRODUCTIVITY

All goals proposed have not been achieved because of technical difficulties faced during the proposed studies. Many difficulties were faced in isolating desired genes and their expression in foreign hosts. Isolated genes could not be expressed back in the donor. Similarly many efforts to clone exo-glucanase gene in any host have not been successful. Studies are still under progress to achieve the proposed objectives as these are some of the long term objectives of our Biofuel group of the institute.

FUTURE WORK

Project has led to development of sub-projects like i) Efficient and quick method to stain PAGE gels, ii) Development of simple and cheap electrophoretic apparatus for purification of proteins, iii) Inclusion of X-factor for improved secretion of gene product by yeast, iv) Cloning of exo-glucanase gene in yeast to allow rapid saccharification and fermentation to ethanol.

REFERENCES

Ashfaq SR, A Bashir, MI Rajoka and KA Malik (1991). Rapid zymographic technique for the localization of cellulases on non-denaturing polyacrylamide gel.Proc.Int.Symp.on Biotechnoloy for Energy,Dec.16-21,1989.Faisalabad, Pakistan. Eds.K.A.Malik, S.H.M.Naqvi and M.I.H.Aleem.Published by NIAB/NIBGE Faisalabad,Pakistan. p.77-84.

Bashir A, SR Ashfaq, MI Rajoka and KA Malik (1991). Cloning of cellulase genes using pUC18 and lamba 2001 vectors Ibid. p.55-64.

Birnboim, H.C. and Dolly, J. (1983). A rapid alkaline extraction procedure for screening recombinant plasmid DNA Nucl. Acids Res. 7: 1513-1523.

Cantwell, B.A., Sharp, P.M., Gormley, E. and McConnel, D.J. (1988). Molecular cloning of *Bacillus* ß-glucanase. In Biochemistry and Genetics of cellulose degradation, FEMS Symposium 43 ed. J.-P. Aubert, P. Beguin & J. Millet. Academic press, London, pp: 181-201.

Choi, W.Y., Haggett, K.D. and Dunn, N.W. (1978). Isolation of a cotton wool degrading strain of *Cellulomonas*: Mutants with altered ability to degrade cotton wool. Aust J Biol Sci. 31: 553-64.

Curry, C., Gilkes, N., O'Neill, G., Miller, R.C. Jr. and Skipper N. (1988). Expression and Secretion of a *Cellulomonas fimi* exo-glucanase gene in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 54: 476-84.

Ghangas, G and DB Wilson (1987). Expression of a *Thermonospora fusca* cellulase gene in *Spreptomyces lividans* and *Bacillus subtilis* Appl. Environ Microbial 53:1470-1475.

Gibbons, W.R. and Westly, C.A. (1986). Effect of pulp pH on solid phase fermentation of fodder beets for fuel ethanol production Biotechnol Lett 8: 657.

Gibbons, W.R. and Westly, C.A. (1987). Effect of fodder beet cube size on ethanol production via diffusion fermentation Biotechnol Lett 9: 135-138.

Gilkes, N.R., Kilburn, D.G., Miller, R.C. and Warren, R.A.J. (1984). A mutant of *Escherchia coli* that leaks cellulase activity encoded by cloned cellulase genes from *Cellulomonas fimi* Bio/Technol 3: 259-265.

Gilkes, N.R., Kilbern, D.G., Miller, R.C. Jr. & Warren, R.A.J. (1991). Bacterial cellulases. Bioresource Technology 36: 21-35.

Goyal, A., Ghosh, B. and Eveleigh D.E. (1991). Characteristics of fungal cellulases. Bioresource Technology 36: 37-50.

Haggett, K.D., Choi, W.Y. and Dunn, N.W. (1978). Mutants of *Cellulomonas* which produce increased levels of B-glucosidase. Eur. J. Appl. Microbiol. Biotechnol. 6: 189-191.

Honda, H., Saito, T., Lijima, S. and Kobayashi, T. (1988). Molecular cloning and expression of B-glucosidase from *Ruminococcus albus* in E.coli. Enzyme Microb. Technol. 10: 559-562.

Johnson, J.A., Wong, W.K.R. and Beatty, J.T (1986). Expression of cellu lase gene in *Rhodobacter capsulatus* by use of plasmid expression vectors. J. Bacterial. 167: 604-610.

Khan, A.W., Chan, A. and Barid, S. (1985). Use of charcoal for product inhibition in enzymic hydrolysis of cellulose. Biotechnol. Lett. 7: 447-450.

Knowles, J., Lehtovaara, P. and Teeri, T. (1987). Cellulase families and their genes. Trends Biotechnol., 5: 255-261.

Lejeune, A., Colson, C., & Eveleigh D.E. (1986). Cloning of an endo-glucanase gene from *Pseudomonas flurescens* var. *Cellulosa* into *Escherichia coli* and *Pseudomonas flurescens.* J. Ind. Microbiol 1: 79-86

Macris, B.J. (1984) Enhanced cellulase and B-glucosidase production by a mutant of *Alternaria alternata*. Biotecnol. Bioeng. 26: 194-196.

Malek, M.A., Chowdhury, N.A., Yousuf, Q.M. and Choudhury, N. (1988). Bacterial cellulases and saccharification of lignocellulosic materials. Enzyme Microbiol. Technol. 10: 750-753.

Mandels, M. (1985). Application of cellulases. Biochem Soc Trans 13: 414-416.

Montenecourt, B.S. and Eveleigh, D.E. (1977). Semiquantitative plate assay for determining cellulase production by *Trichoderma viride* Appl Environ Microbiol 33: 178-183.

Montenecourt, B.S. and Eveleigh, D.E. (1979). Selective screening methods for the isolation of high yielding cellulase mutants of *T. reesei* In: Brown RD Jr, L Jurasek (Eds) Hydrolysis of cellulose: Mechanisms of enzymic and acid catalysis Ad. 289.

Marsden, W.L., Gray, P.P. (1986). Enzymatic hydrolysis of cellulose in lignocellulosic material. CRC Critical Reviews in Biotechnology 3: 235-276.

Nakamura, K., Misawa, N. and Kitamura, K. (1986a) Cellulase genes of *Cellulomonas CB4.* Cloning and expression of a CM-cellulose hydrolyzing enzyme (endoglucanase) gene in *E.coli.* Journal of Biotechnology 3: 239-246.

Nakamura, K., Misawa, N and Kitamura, K. (1986b). Cellulase genes of *Cellulomonas uda* CB4. 1 Cloning of and expression of B- glucosidase genes in *Escherichia coli* J. Biotechnology 3: 239-246.

Nakamura, K. and Kitamura, K. (1982). Isolation and identification of crystalline cellulose hydrolysing bacterium and its enzymatic properties. J. Ferment. Technol. 60; 343-348.

Parvez S, MI Rajoka and KA Malik (1993). Utilization of agricutural wastes: Cloning of endoglucanase genes from *Cellulomonas biazotea* into *E.coli* and *Saccharomyces cerevisiae* using shuttle vector YEP24.Proceedings Second Inernational symposium on Genetic Engineering and Biotechnology, Karachi, Pakistan Jan.1993 (in press).

Penttila, M., Lehtovara, P. and Knowles, J. (1989). Cellulolytic yeast strains and their application. In Yeast Genetic Engineering. Eds. Barr et al Pubs. Butterworths, Singapore.pp. 247-268.

Rajoka, M. I., Parvez, S. and Malik, K.A. (1992). Cloning of structural gene for B-glucosidase from cellulomonas biazotea into *E. coli* and *S.cerevisiae* using shuttle vector pBLU-D.Biotech.letts.14:1001-1006.

Rajoka, M.I. and Malik, K.A. (1986). Comparsion of different strain of *Cellulomonas* for production of celluloyltic and xylanlytic enzyme from biomass produced on saline lands. Biotechnol. Letts. 8 (10): 753-756.

Rajoka, M.I. and Malik, K.A. (1984). Cellulase and hemicellulase production by *Cellulomonas flavigena* NIAB 441. Biotechnol Lett 6: 597-601.

Rajoka MI, S Parvez and KA Malik (1992). Cloning of structural genes for B-glucosidase from *Cellulomonas biazotea* into *E.coli* and *Saccharomyces cerevisiae* using shuttle vector pBlu-D. Biotechnol. Letts. 14(11): 1001-1006.

Rickard, P.A.D., Rajoka, M.I. and Ide, J.A. (1981). Glycosidases of *Cellulomonas* Biotechnol Lett 3(9) 487-492.

Sacco, M., Millet, J., Aubert, J.P. (1984). Ann. Inst. Pasteur (Microbiol.)135 A:485-8.

Sami, A.J., Akhtar, W., Malik, N.N. and Naz, B.A. (1988) Production of free and substrate bound cellulases of *Cellulomonas flavigena* Enzyme Microb. Technol 10: 626-631.

Siddiqui, K.S. & Ali, R. (1991). A sensitive and simplified method for staining sodium dodecyl sulphate protein gels. Technique 3: 187-190.

Silva, A., Benitez, J. and Hollenberg, C, P. (1991). Endoglucanase A gene fusion vectors for monitoring protein scretion and glycosylation in yeast.Anal. Biochem. 197:290-295.

Skipper, N., Sutherland, M., Davies, R.W., Kilburn, D., Miller, R.C. Jr., Warren, A. and Wong, R. (1985). Scretion of a bacterial cellulase by yeast. Science. 230:958-960.

Taylor, K.A., Crosby, B., McGavin, M., Forsberg, C.W. and Thomas D.Y. (1987). Characteristics of the endo-glucanase encoded by a cel gene from *Bacteriodes succinogenes* expressed in *Escherichia coli*. Appl. Environ. Microbiol. 53: 41-46.

Teather, R.M. and Wood, P.J. (1982). Use of congored polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from bovine rumen. Apl. Environ. Microbiol. 43: 777-780.

Teeri, T., Salovuori, I and Knowles, J. (1983). The moleculatr cloning of the major cellulase gene from *Trichoderma reesei*. Bio/technology 1: 696-699.

Waldron, C.R., Becker-Vallone, C.A. and Evaleigh D.E. (1986). Isolation and characterization of a cellulolytic actinomycete *Microbispora bispora* Appl. Microbiol. Biotechnol. 24: 477-86.

Wong, W.K.R., Curry, C., Parekh, R.S., Parekh, S.R., Wayman, M., Davies, R.W., Kilburn, D.G. and Skipper, N., (1988). Wood hydrolysis by *Cellulomonas fimi* endo-glucanase and exo-glucanase co-expressed as secreted enzymes in *Saccharomyuces cerevisiae*. Biotechnology, 6:7317-19.