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CELLULOSE BIODEGRADATION STUDIES: APPLICATION OF rDNA AND PROTOPLAST FUSION TECHNIQUES

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Introduction

Cellulose biodegradation refers to the breakdown of cellulose to its component glucose units through the action of enzymes. This process has attracted scientific attention because of the complexity of the enzymes involved and the environmental, as well as economic, significance of the process.

Cellulose, the major structural polysaccharide of plants, is a hydrophilic linear glucose polymer with the anhydroglucose units bonded by B-1.4, glucosidic linkage (Ghose and Mishra, 1984). The number of glucose units may vary from 15 (α-cellulose) to more than 10,000 (α-cellulose) per molecule. The polymer has both crystalline and amorphous regions, the former referring to the portion more resistant to chemical/biochemical attack and the latter, to the portion of the cellulose chain that is prone to easy hydrolysis (Muhlenthaler, 1967).

Cellulose biodegradation is mediated by several enzyme systems. The more studied are the extracellular cellulase systems in fungi that have three components: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and B-glucosidases (EC 3.2.1.21) (Coughlan and Ljungdahl, 1988). The endoglucanases are found to be inactive against crystalline cellulose when acting alone, but hydrolyze amorphous cellulose and soluble derivatives of carboxymethyl cellulose (CM-cellulose). Endoglucanases are also referred to as CM-cellulases. Their attack on amorphous cellulose is characterized by random cleavage of B-glycosidic linkages. By contrast, cellobiohydrolases degrade amorphous cellulose by consecutive removal of cellobiose units from the non-reducing end of the substrate. Endoglucanases and cellobiohydrolases act together to degrade crystalline cellulose and the B-glucosidades complete the hydrolytic process by converting the resultant cellobiose to glucose or removing glucose from the non-reducing end of short cellooligosaccharides. Exoglucohydrolases (EC 3.2.1.74) from *Penicillium funiculosum* and *Talaromyces emersonii* catalyze the removal of glucose residues from the non-reducing end of cellodextrins but do not interact synergistically with endoglucanases in the hydrolysis of cellulose. Oxidative enzymes such as cellobiose oxidase (EC 1.199.18) and cellobiose: quinone oxidoreductase (EC 1.3.5.1) have been found to participate in cellulose degradation. Most, if not all, of the extracellular hydrolytic enzymes of

fungi are glycoproteins. Of the known aerobic cellulolytic bacteria, only three, *Clostridum thermocellum, Thermonospora sp.*, and *Microbispora bispora* possess culture filtrates active against crystalline cellulose. In anaerobic bacteria, an active cellulase complex referred to as cellulosome (Lamed *et al*, 1983) is bound to the cell surface. The bound enzymes are believed to be the more active cellulases in bacteria. However, one can see a more complex structure. The cellulosomes comprise 35 polypeptides ranging from 45kDA to about 200 kDA (Lamed, *et al*, 1983 and Hon-Nam, *et al*, 1986).

This paper describes our research work done at the Natural Sciences Research Institute (NSRI) at the U.P. Diliman campus and at the National Institute of Biotechnology and Applied Microbiology (BIOTECH) at the U.P. Los Baños campus. Research done at the NSRI mainly involves the use of rDNA techniques in studying cellulose biodegradation whereas at the BIOTECH, our work involved the use of protoplast fusion in improving cellulose biodegradation in fungi.

I. *The use of recombinant DNA in cellulose degradation studies*

Recombinant DNA techniques refer to a set of procedures that obtains a piece of DNA from a donor species and inserts this piece to a self-replicating DNA molecule referred to as a vector that has been constructed to easily enter and multiply in a host cell.

At present, a number of laboratories worldwide have employed the recombinant DNA techniques in studying the cellulases of fungi and bacteria. This is based on the underlying concept of molecular biology that the structure determines the function. Primarily, people wanted to examine the structure of the cellulase genes to explain such phenomena as the proper and functional aggregation of a multicomponent complex or the varied activity of the enzyme depending on the source of substrate. In our studies, we wanted to do three things:

One was to clone the cellulase genes from a depressed mutant of *Cellulomonas fimi* and compare it with the genes from the wild type strain. The results would tell us if the genes themselves contain sequences involved in glucose repression and where these sequences would be located.

Two, we wanted to place the cellulase genes in a multicopy plasmid, place it back into the *Cellulomonas* cell to produce a high cellulase producer.

Three, we wanted to identify the exact gene products of the genes we isolated.

We started our work in May, 1986 and with the funds obtained from the U.S. Agency for International Development, we cloned and obtained restriction maps of two cellulase genes, did partial sequencing, identified our constraint in placing the cellulase genes back in the Cellulomonas and are still in the process of identifying the exact gene products of our two genes.

Our procedures involved construction of a *Cellulomonas* DNA library, molecular cloning and subcloning, restriction mapping, Southern blot hybridization, transformation experiments, and enzyme assays. Subcloning, restriction mapping, Southern blot hybridization and partial DNA sequencing were done by one of our

project personnel, Rose Caday, at the laboratory of Professor Seymour Fogel at the University of California, Berkeley, whereas the rest of the work was done at our NSRI laboratory. Details of our procedures were presented in three papers being prepared for publication.

We obtained a DNA library in *Escherichia coli* C600 composed of 3,000 clones using the shotgun approach. Of these, seven clones gave positive reaction when screened for cellulase activity by the Congo red assay. The presence of cellulase activity was further demonstrated by colorimetric activity (Table 1). However, only four clones were further assayed using flourogenic substances and these exhibited substantially increased activities as indicated by bright flourescence under uv light compared with the *E. coli* host. The culture filtrate of the four recombinant clones *E. coli* C602, C609, C610 and C612 were positive for B-glucosidase, cellobiohydrolase and xylanase activities (Table 2) whereas the cell-free extracts were positive only for B-glucosidase (Figure 1). The cell-free extracts of the *E. coli* host also showed high B-glucosidase activity but none of the other enzymes whereas the culture filtrate showed very weak B-glucosidase activity. The donor, *Cellulomonas* strain exhibited high activities for all three enzymes in both its cell-free extracts as well as its culture filtrate. These results indicate that the B-glucosidase, the cellobiohydrolase and the xylanase excreted by the *E. coli* host were of *Cellulomonas* origin. This may also mean that the host produced a lot more B-glucosidase, that some were excreted and the rest were kept inside the cell. This also indicates that both the *Cellulomonas* and the *E. coli* B-glucosidase genes were expressed.

The DNA inserts of recombinant clones, C602 and C610 were subcloned into *E. coli* HB101 for higher plasmid recovery. The recombinant plasmids were extracted and labelled with P32. These were hybridized with the digested *Cellulomonas* genomic DNA. Both inserts hybridized. These were also restricted with a total of 23 restriction enzymes. These two DNA inserts showed strictly different restriction maps indicating different DNA sequences (Figure 2). These genes may

Table 1. Colorimetric assay of cell lysates

Source of Enzymes	*CMCase* *10*	*FPase* *10*	*B-clucosidase* *10*
E. coli C602	7.50	1.16	3.35
E. coli C603	28.06	3.24	6.04
E. coli C606	5.00	2.08	9.73
E. coli C608	41.11	4.86	14.76
E. coli C609	39.44	5.79	14.76
E. coli C610	50.56	11.57	16.10
E. coli C612	2.78	0.92	6.04
E. coli C600	7.78	4.40	5.37

From: Halos, S.C. *et al.* (manuscript in preparation).

Table 2. Cellulase activity of culture filtrate from transformed and control cells

Source of Enzymes	*Substrates*			
	MUG	*MUCb*	*MUxy*	*CMC**
E. coli C602	++	++	++	+
E. coli C603	nt	nt	nt	+
E. coli C606	nt	nt	nt	+
E. coli C608	nt	nt	nt	+
E. coli C609	+	+	+	+
E. coli C610	+	+	+	+
E. coli C612	+	+	+	+
C. coli C600	+/–	+/–	+/–	+
E. coli HB 294	+/–	+/–	+/–	+
C. fimi	+++	+++	+++	+

+++	Strong fluorescence
++	Medium fluorescence
+	Weak fluorescence
+/–	Very weak fluorescence/clearing
*t	Presence of halo
nt	not rested
MUG	4-methylumbelliferyl B-D glucopyranoside (B-glucosidase)
MUCb	4-methylumbelliferyl B-D cellobioyranoside (Cellobiohydrolase)
MUXy	4-methylumbelliferyl B-D xylopyranoside (Xylanase)

From: Halos, S.C. *et al.* (manuscript in preparation)

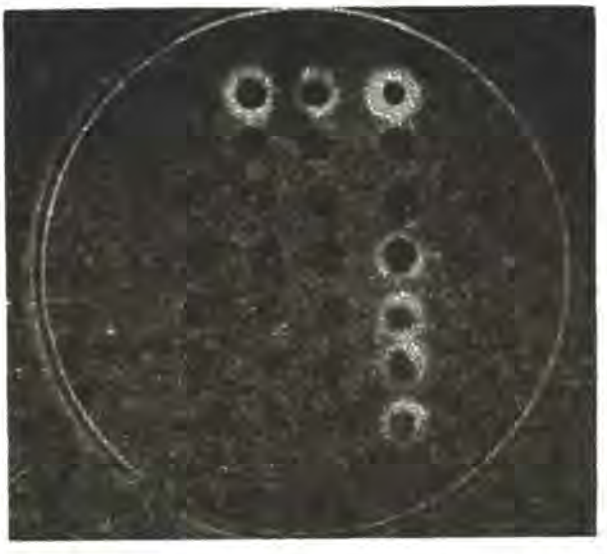

Figure 1. Fluorescence of crude extracts using 4 methylumbeliferyl B-D glycosides. Column 1, MUG; Column 2, MUCb; Column 3, MUXy; Column 4 Mixed substrates, no enzyme. Row 1, C602; Row 2, C609; Row 3, C610; Row 4, C612, Row 5, HB294; Row 6, C600, Row 7, *Cellulomonas fimi.* (Halos, C.S., J. Claudio, D. Sanchez, Manuscript in preparation).

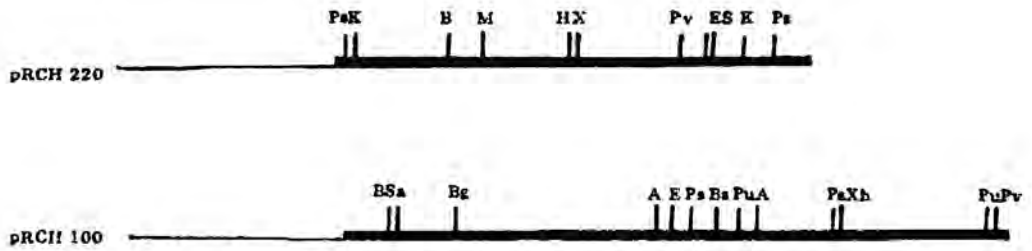

Figure 2. Restriction maps of cloned DNA fragments from depressed mutant of *Cellulomonas fimi* expressing cellulase activity. Ps, PstI; K, KpnI; B, BamHI; M, MluI; H, HindIII; X, XbaI; Pv, PvuI; E, EcoRI; S, SmaI; Sa, SalI; Bg, BglI; A, AvaI; Bs, BstxI; Pu, PvuII; Xh, XhoI. Pu sites were not clearly established from restriction map. (Halos, S.C. & R.A. Caday, manuscript in preparation).

represent the two separately migrating cellobiohydrolases that we have extracted from *Cellulomonas*. However, these restriction maps differed from restriction maps obtained for cellulase genes of various microorganisms, including the wild type of *Cellulomonas fimi* (Figures 3-7). It is possible that we have cloned other cellulase genes and that there could be at least four cellulase genes in *Cellulomonas fimi*. There were ten different DNA fragments expressing cellulolytic activities cloned for *C. thermocellum* and six in *Microbispora bispora*.

The cloned genes, including those obtained from other microorganisms and inserted in pBR322 or its derivatives, were used to transform the *Cellulomonas fimi* mutant. Although we were able to obtain transformation, the cells apparently could not maintain the plasmid. We have observed, however, that *Cellulomonas* has a plasmid which we can use to construct a cloning vector in the future.

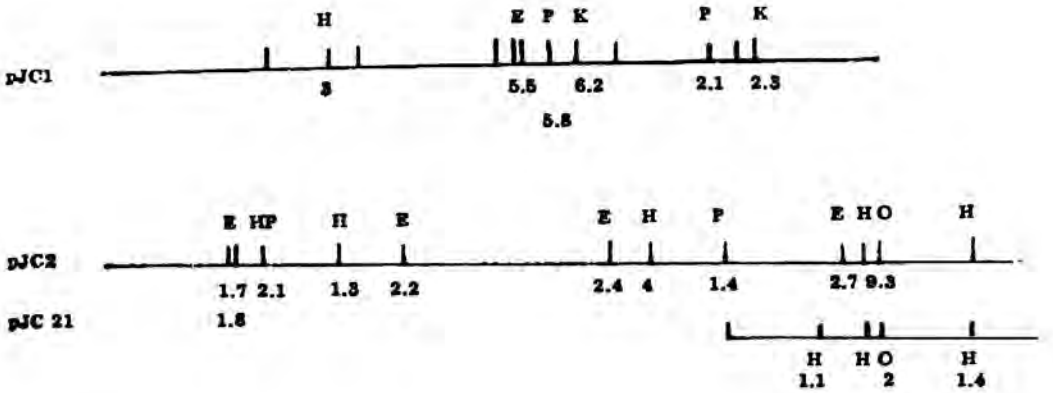

Figure 3. Restriction maps of cellulase genes from *Cellulomonas* sp. H, HindIII; E, EcoRI; K, KpnI, P, PvuII; B, BamHI. (Lohwongwatana, unpublished)

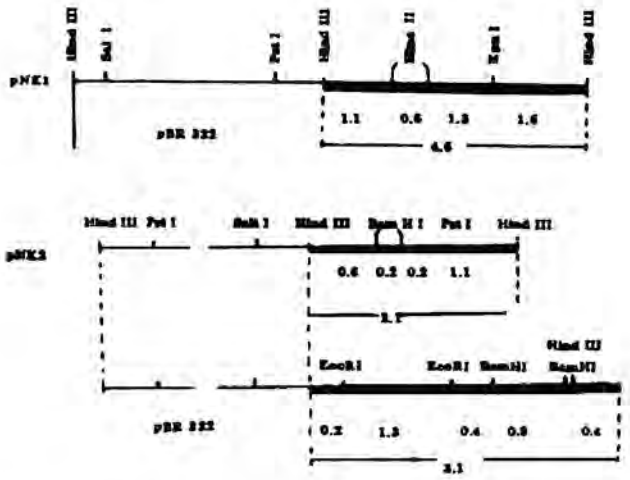

Figure 4. Restriction maps of cloned DNA fragments from *Bacillus* sp. expressing cellulase activity. (Horikoshi and Fukumori, 1988)

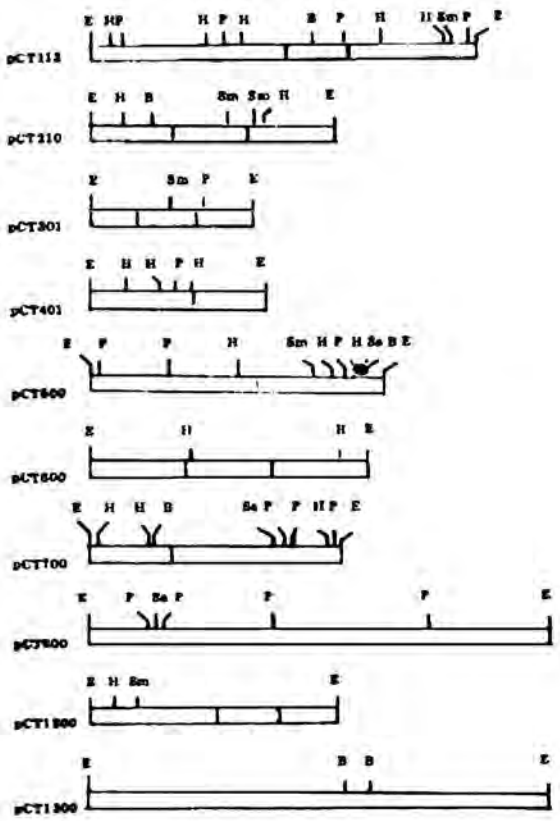

Figure 5. Restriction map of DNA from *Clostridum thermocellum* encoding CMC or MUCb-hydrolyzing activity. B, BamHI; E, EcoRI; H, HindIII; P, PstI; Sa, SalI, Sm, SmaI. pCT800 and pCT1300 contain 6 and 9 HindIII sites, respectively. (Beguin *et al.*, 1988)

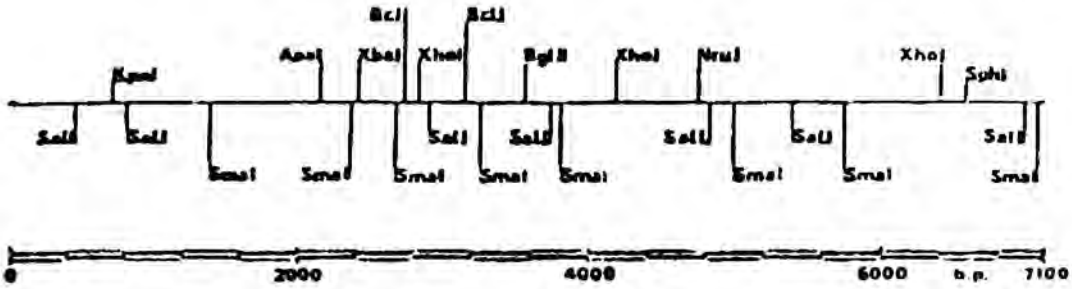

Figure 6. Restriction map of cloned DNA fragment from *Streptomyces* expressing cellulase activity. (Coppolecchia *et al.*, 1987)

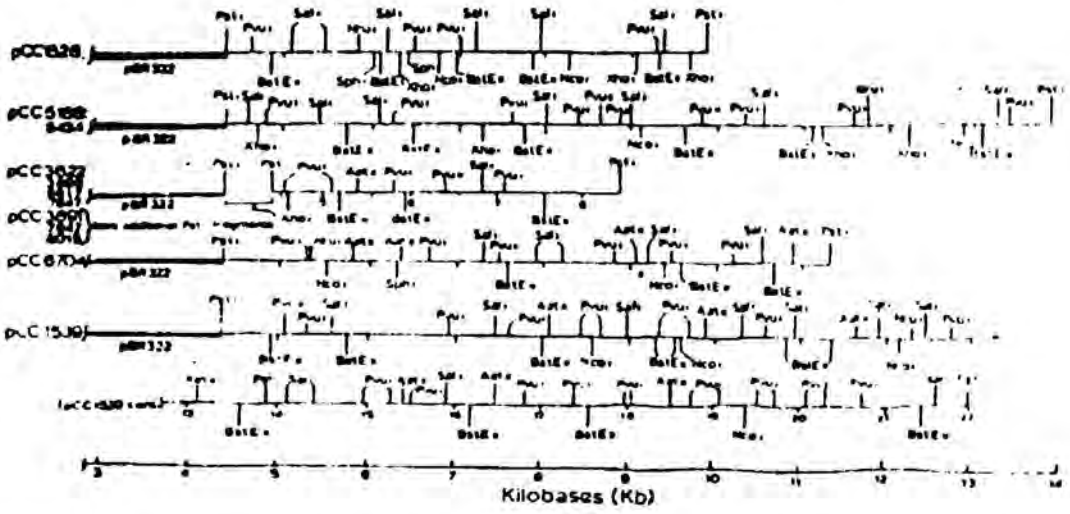

Figure 7. Restriction maps of cloned *Microbispora bispora* DNA fragments expressing active CMCase in *E. coli*. (Yablonsky *et al.*, 1988)

II. *The use of protoplast fusion in cellulose biodegradation studies.*

Protoplast fusion refers to the creation of one cell out of two cells using an agent that removes the cell wall of bacteria, plants or fungi and an agent that makes these membrane-bound cells stick together and adjoin their membranes. With animal cells, only the use of a sticking agent is required. Protoplast fusion allows for the production of hybrids between totally unrelated species provided that viable fusants are obtained. In our studies, we are exploring the use of protoplast fusion in producing new fungal hybrids with improved cellulolytic activity.

Of the various fungal species producing cellulases, the *Trichoderma* and the *Penicillium* species are the ones currently used in the limited commercial production of cellulases. The advantage of using fungal cellulases lies in their relative stability to extraction procedures. However, the search for better sources of cellulases continues since no strain that can be used economically to produce the enzymes has yet been developed or isolated.

In our earliest project of screening for high cellulase producers, we identified, one isolate of *Penicillium funiculosum* Thom No. 171 that was as good as our reference strain, *Trichoderma reesei* RutC30, a strain developed for high cellulolytic activity by researchers of Rutgers University in New Jersey. In addition, *P. funiculosum* No. 171 produced higher levels of B-glucosidase activity than T. Reesei Rut C300 (Cruz, W.T., 1986). This prompted us to explore the possibility of using protoplast fusion to develop intergeneric hybrids of *Trichoderma X Penicillium* that would produce a hybrid cellulase complex incorporating the higher cellulase activities of both parents.

Our study involved establishing the procedures for protoplast isolation and renegeration for *P. funiculosum*, and *T. reesei* RutC30 using resources available to us, identification of suitable selection markers, characterization of fusants and assaying for their cellulolytic activities. Details of procedures and results are presented in two papers submitted for publication.

Of six enzyme preparations tested for protoplast production, a more efficient one was the combination of Novozyme 234 (Novo Industries, Inc.) and Zymolyaze 20T which released 3.6 X 10^7 for *T. reesei* and 1.4 X 10^7 for *P. funiculosum* per mg mycelia (Table 3 and Figure 8). This enzymes combination released protoplast in direct proportion to the amount of mycelia when exposed to the same concentration of enzyme (2.5 mg/ml Novozyme 234 and 5 mg/ml Zymolyase 20T) typical of an enzymatic reaction. *T. reesei* was more susceptible to these enzymes in that protoplasts were first produced from *T. reesei* 60 minutes following treatment of mycelium compared with *P. funiculosum* that started releasing

Table 3. Comparison of lytic enzyme preparations for the release of Protoplasts from *Trichoderma reesei RUT C-30* and Penicillum funiculosum *Thom* MG 171

Enzyme System	*Protoplast yield Trichoderma reesei RUT C-30*	*Penicillum funiculosum Thom MG 171*
Celluclost	–	– –
Cellulase (laboratory prep.)	3.7 X 10^6	2 X 10^6
Novozyme 234	2.9 X 10^5	1.7 X 10^6
Zymolase 20T	0.5 X 10^6	0.28 X 10^6
Celluclast + Zymolace 20T	0.1 X 10^6	0.016 X 10^6
Novozym 234 X zymolase 20T	3.6 X 10^7	1.4 X 10^7

From: Pham, L. & S.C. Halos, 1988. manuscript for publication.

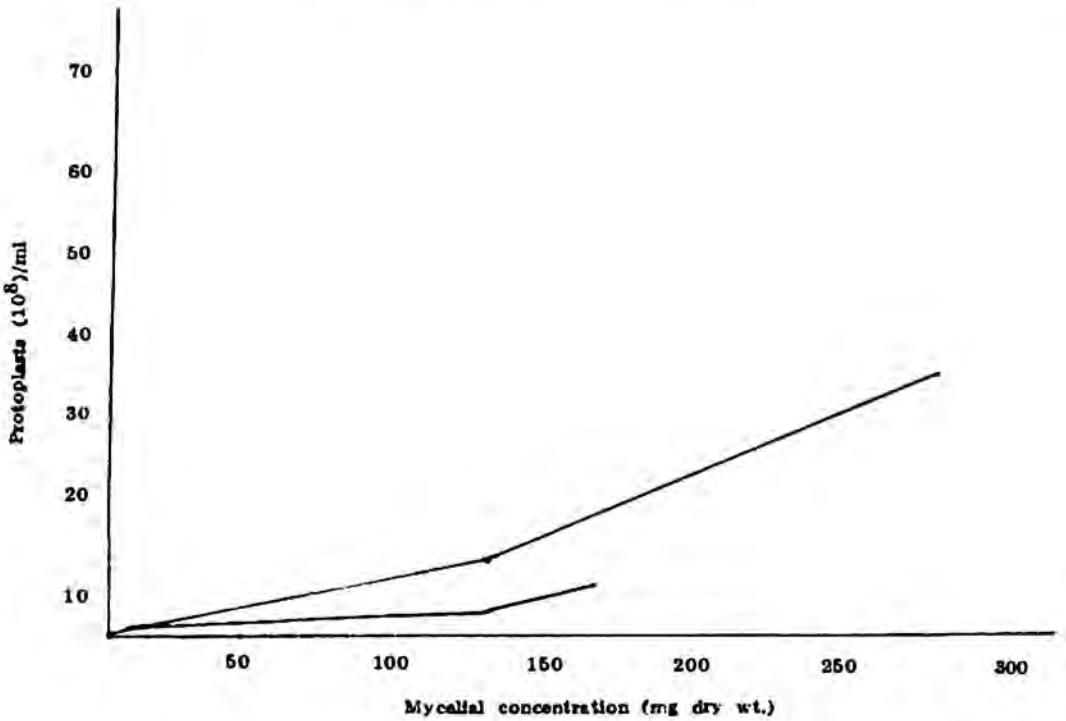

Figure 8. The effect of mycelial oncentration on protoplast formation of *Trichoderma reesei RUT C-30* and *Penicillium funiculosum Thom MG 171*. (Pham, L. & S.C. Halos, 1988. manuscript for publication).

protoplast 90 minutes after treatment. Furthermore, the highest yield of protoplasts was obtained with *Trichoderma* 2 hours after enzyme exposure, whereas with *P. funiculosum* it was 4 hours after. Also, *T. reesei* regenerated more at 92% than *P. funiculosum* which gave a 31.17% regeneration frequency. Regeneration occurred 3 hours after transfer to Winge medium.

Since we had no intention of altering the genetic makeup of our parental strains, we sought for innate properties of these strains to use as selection markers. We screened their resistance to different metal ions (Cu, Na, Co, and Hg), fungicides (Captan and Benlate) and antibiotic (nystatin) at different concentrations. We were able to identify complementing markers Co^R and Hg^S for *T. reesei* and Co^S and Hg^R for *P. funiculosum* (Table 4) as the primary selection markers (Figure 9). Fusants were then selected as Co^R and Hg^R colonies (Figure 10).

Fusants derived were viable and exhibited different morphologies (Table 5) which combine the properties of both parents. Fusants did not exhibit uniform characteristics which could be due to observations that mycelial fungi are multinucleated. Fusants might have represented different combinations of the 3 nuclei that are often found in one mycelial cell.

Table 4. The effect of Metal Ions on Protoplast Regeneration of *Trichoderma reesei* RUT C-30 and *Penicillium funiculosum* Thom MG 171

Metal Ions	*Trichoderma reesei RUT C-30*	*Penicillium funicolusom Thom 171*
Cu (10 ppm)	+	+
Na (10 ppm)	+	
Cu (10 ppm)	+	+
Na (10 ppm)	+	+
Co (10 ppm)	+	–
Hg (1 ppm)	–	+

+ = regenerated
– = did not regenerate

From: Pham, I. & S.C. Halos, 1988. manuscript for publication)

Protoplast from *Penicillium funiculosum.*

Regenerating protoplast from *P. funiculosum.*

Figure 9. Regeneration of *Penicillium funiculosum* protoplast. (Pham, L. & S.C. Halos, 1988. manuscript for publication).

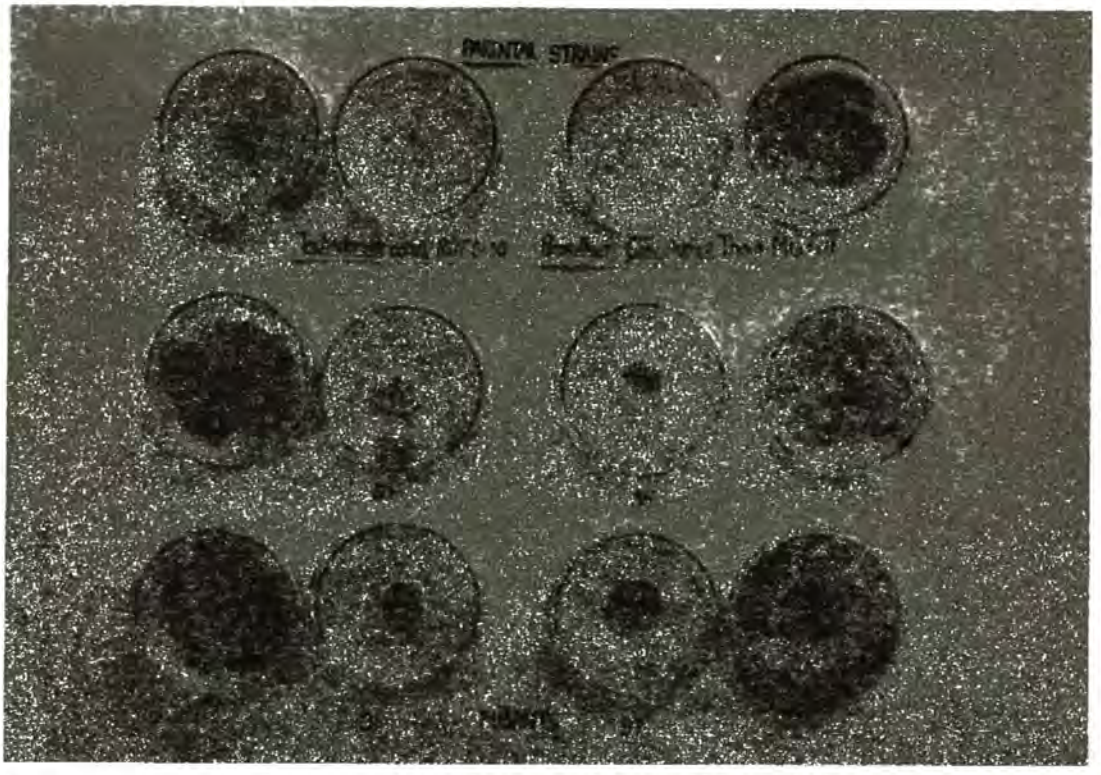

Figure 10. Parental Strains and Fusants derived from *Trichoderma reesei* RutC30 and *Penicillium funiculosum* Thom MG 171. (Pham, L. & S.C. Halos, 1988. manuscript for publication).
extreme left – without Co^{+}, Hg^{++}
center – with Co^{+}, Hg^{++}
extreme right – without Co^{+}, Hg^{++}

Table 5. Cultural characteristics of fusant strains following protoplast fusion compared tn parental strains

FUSANT NU.	*COLONY COLOR*	*COLONY TEXTURE*	*SPORU-LATION*	*PIGMENTATION*
14	white	cottony	none	red
20	graying green	cottony	fourth day	dark brown
24	light blue to moss green	velvetty	second day	sulfur yellow
34	olive green	cottony	fourth day	red to brick red
35	olive green	cottony	fourth day	red to brick clay
36	blue to moss green	velvetty	second day	sulfur yellow
37	blue to moss green	velvetty	second day	sulfur yellow

FUSANT NU.	*COLONY COLOR*	*COLONY TEXTURE*	*SPORU-LATION*	*PIGMENTATION*
46	moss green	velvetty	–	dark brown
47	yellow green	velvetty	–	dark brown
62	olive green	velvetty	–	dark red
66	moss green	irregular	–	dark red
71	moss green	velvetty	–	brown
74	moss green	velvetty	–	brown
76	olive green	velvetty	–	dark brown
77	moss green	velvetty	–	dark brown
PARENTAL STRAINS				
T. reesei RUT C-30	yellow green to olive green	cottony	fourth day	sulfur yellow
P. funiculum Thom MG-171	moss agreen	velvetty	fourthday	red

From: Pham, L. & S.C. Halos, 1988. manuscript for publication.

Table 6. Enzyme activities of fusants and parental strains

FUSANT NO.	*PROTEIN Mg/ml*	*CMCASE IU/ml/min*	*FPASE IU/m/min*	*B-GLUCOSIDASE IU/10 min.*
14	0.38	.157	.007	.101
20	0.68	.055	.051	.037
24	1.09	.565	.154	.126
34	1.52	1.52	1.31	7.79
35	1.55	4.65	1.19	4.267
36	0.95	3.90	0.697	1.877
37	1.93	3.90	1.04	1.319
46	1.14	5.46	.36	–
47	.553	2.17	.36	–
66	1.25	5.46	.332	–
67	1.38	5.05	.408	–
71	1.59	4.83	.344	–
74	1.86	5.23	.398	–
76	1.25	4.42	.352	–
77	1.45	5.62	.359	–
78	1.92	3.56	.334	–
PARENTAL STRAINS				
T. reesei RUT C-30	2.38	7.15	2.49	.178
P. funiculosum TThom MG-171	1.25	4.55	1.40	9.89

From: Pham, L. & S.C. Halos, 1988. manuscript for publication.

The cellulolytic activity of various fusants were in general lower than their parental strains (Table 6) Pham and Halos, 1988). However, preliminary results with the second generation or the progeny of two fusants indicate that these have higher CM-cellulase and FP-cellulase activities than the parental strains (Tables 7-8) (de los Reyes, M. 1988).

Table 7. Average CMcase activity (IU/ml), glucose production (mg/ml) and specific activity (IU/mg) in rice straw of the cellulase enzyme from the fusants and parental strains

Isolate Name/Number	*CMcase act. (IU/ml)*	*Spec. act. (IU/mg)*	*Glucose yield (mg/ml)*
Trichoderma reesei Rut C30	*0.0759*[B]	0.4258[B]	0.1366
P. funiculosum MG 171	0.0557[B]	0.6724[B]	0.1002
B18	0.3454[A]	0.8665[A]	0.6217
B12	0.2857[A]	0.7704[A]	0.5143
B13	0.3639[A]	0.3808[A]	0.6551
B14	0.3605[A]	0.7454[A]	0.6490

From: de los Reyes, C.C., 1988

Table 8. Average FPase activity (IU/ml), glucose production (mg/ml) and specific activity (IU/mg) in rice straw of the cellulase enzyme from the fusants and parental strains

Isolate Name/Number	*FPase act. (IU/ml)*	*Spec. act. (IU/mg)*	*Glucose yield (mg/ml)*
Trichoderma reesei Rut C30	0.0073[B]	0.0431[A]	0.0787
P. funiculosum MG 171	ND'[B]	ND'[A]	ND'
B18	0.0793[A]	0.1581[A]	0.8562
B12	0.0260[A]	0.0714[A]	0.2809
B13	0.0733[A]	0.0767[A]	0.7912
B14	0.0963[A]	0.1995[A]	1.0402

[1] ND, undetectable

'values within a column having similar letters indicate that they are not significantly different according to DMRT result

From: de los Reyes, C.C., 1988

Conclusion

We are just starting to explore the use of the techniques of the new genetics in cellulose biodegradation. In the use of protoplast fusion in improving the cellulose biodegradation ability of fungal strains, it appears that there is more promise

among the second generation fusants of *T. reesei* Rut C30 and *P. funiculosom* No. 171, whereby four second generation fusants exhibited higher cellulolytic activities than their parental strains. In using rDNA techniques, we are confirming results we obtained with other procedures on the presence of at least two genes for cellulases. These genes exhibit different sequences and their protein products exhibited different mobilities upon electrophoresis. Originally, we proposed to place one of these cloned genes back into the *Cellulomonas* cell; however, the *Cellulomonas* cannot retain the pBR322 plasmid. Hence, we are currently studying the *Cellulomonas* plasmid as a possible vector for the cloned cellulase gene.

Acknowledgments

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Literature Cited:

1. Beguin, P. *et al.* The cell (Cellulose Degradation) Genes of *Clostridium thermocellum*. p. 267-282. In Biochemistry and Genetics Cellulose Degradation. eds: Aubert, J.P. *et. al.*, London: Harcourt Brace Jovanovich, Publishes, c 1988.
2. Coppolecchia, M.R. *et al.* 1987. Cloning in *E. coli* of a *Streptomyces* Cellulase Gene. Biotech. Letters. 9(7): 495-500.
3. Coughlan, M.P. and L.G. Ljungdahl. Comparative Biochemmistry of Fungal and Bacterial Cellulolytic Enzyme Systems. p. 11-30. In: Biochemistry and Genetics of Cellulose Degradation. eds. Aubert, J.P. et al. London: Harcourt Brace Jovanovich, Publishers. c 1988.
4. Cruz, W.T. 1986. Isolation, Screening, Characterization, and Identification of Local Lellulase-Producing Fungi and Optimization of Enzyme Production. Master's Thesis. University of the Philippines, Los Baños.
5. de Los Reyes, C.C. 1988. Comparative Cellulase Activities of Fusants of *Trichoderma reesei* Rut C30 x *Penicillium funiculosum* MG171 and their Parental Strains. Undergraduate Thesis. University of the Philippines, Los Baños.
6. Ghose, T.K. and S. Mishra. 1984. Bioconversion of Lignocellulose to Ethanol p. 123-147. In: Proc. Role of Biochemistry in Food and Energy Production Symposium. Manila Philippines.
7. Halos, S.C., J.O. Claudio and D.R. Sanchez. Manuscript in preparation.
8. Halos, S.C. and R.A. Caday. Manuscript in preparation.
9. Hon-Nami, K. *et al.*, 1986. As cited by Coughlan, M.P. and L.G. Ljungdahl. Comparative Biochemistry of Fungal and Bacterial Cellulolytic Enzyme Systems. p. 11-30. In: Biochemistry and Genetics of Cellulose Degradation. eds: Aubert, J.P. *et. al.*, London: Harcourt Brace Jovanovich, Publishers. c. 1988.
10. Horikoshi, K. and F. Fukumori. Modification and Expression of Alkaline Cellulase Genes of Alkalophilic *Bacillus* strains. In: Biochemistry and Genetics of Cellulose Degradation. eds: Aubert, J.P. *et al.* London: Harcourt Brace Jovanovich, Publishers. c. 1988.
11. Lamed, R. *et al.* 1983. as cited by Coughlan, M.P. and L.G. Ljungdahl. Comparative Biochemistry of Fungal and Bacterial CEllulolytic Enzyme Systems. p. 11-30. In: Bio-

chemistry and Genetics of Cellulose Degradation. eds: Aubert, J.P. *et al.*, London: Harcourt Brace Jovanovich, Publishers. c 1988.

12. Lohwongwatana, J. *et al.*, Cloning of B-glucosidase gene from *Cellulomonas sp.* (unpublished).
13. Muhlenthaler, R. 1967. Ultrastructure and Formation of Plant Cell Walls. Ann. Rev. Plant 18:1-24.
14. Pham, L.J. and S.C. Halos. 1988. Manuscript (for publication).
15. Yablonsky, M. D. *et al.* Characterization and cloning of the Cellulase Complex of *Microbispora bispora.* In: Biochemistry and Genetics of Cellulose Degradation. eds: Auber, J.P. *et al.* London: Harcourt Brace Jovanovich, Publishers. c 1988.