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FINAL REPORT C8-027

GRANT No. DPE 5544-G-SS-8061-00

DEGRADATION OF PLANT CELL WALLS BY CELLULOSOMES OF ANAEROBIC BACTERIA

ICAITI
GUATEMALA, SEPTEMBER, 1992

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Guatemala, september 11, 1992

Doctor
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Dear Dr. Rechcigl:

Enclosed please find the final report of the project "*DEGRADATION OF PLANT CELL WALLS BY CELLULOSOMES OF ANAEROBIC BACTERIA*", number: C8-027; grant number: DPE 5544-G-SS-8061-00. This report covers the grant period of september 1989 to the 31st. of august, 1992. Research activities concluded on august 31, 1992.

Looking forward to your comments, and for future collaboration with your program, I remain,

Sincerely yours,

W. Ludwig Ingram, Jr.
Director

F I N A L R E P O R T

Submitted to the Office of Research

Bureau for Research and Development

U. S. Agency for International Development

DEGRADATION OF PLANT CELL WALLS BY CELLULOSOMES OF ANAEROBIC BACTERIA

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Investigator: Fabiola Prado de Micheo
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Principal Investigator: Raphael Lamed
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Project Number: C8-027
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Project Duration: September 1988 - August 1992

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1. EXECUTIVE SUMMARY

The general objetive of this research was to study the degradation of lignocellulosic material by complex enzymatic structures, which have been named "cellulosomes", of *Clostridium thermocellum* (YS strain). The scope of work was later enlarged to include other bacteria.

The first line of reseach included work associated with the characteristics of the cellulosome from the model bacteria.

The cellulosome from *C. thermocellum* lacks the betaglucosidase to complete the transformation of cellulose to glucose. Addition of enzyme is needed to reduce inhibitory effects by cellobiose.

Xylanase activity was found in the cellulosome and in non cellulosome protein fractions. The first may coincide with cellulosomal subunits also exhibiting endo- or exo-glucanase activity. The second has only xylanase activity.

A cellobiohydrolase in the truncated Mr 68000 fragment of the cellulosome was isolated and characterized, as having high ratio of exo- to endo-glucanase activities, as being inhibited by cellobiose and by its unestability at high tempertures in absence of calcium or other divalent ions.

A new procedure for the cellulosome has been developed, which provides 5-fold higher yields of the cellulosome while enhancing the solubilizing activity.

The second research line included work with bacteria besides the reference stain YS of *C. thermocellum*.

The cellulase system of four anaerobic cellulolytic bacteria was characterized for endoglucanase activity following sequential purification steps. The results suggests the existence of cell-associated cellulolytic enzymatic complexes, as in the reference strain, in two of the bacteria studied. One of them also showed non-associated enzyme activity.

Cellulosome-like entities have been discovered and describes in *Bacteroides cellulosolvens*. They also present xylanases.

The third line of reseach addressed applications of the cellulosomes to agroindustrial wastes.

Cellulosomes and beta-glucosidase mistures were used to treat six agroindustrial lignocellulosic residues. Several of these substrates seeem adequate as carriers because of the high adsorption rate. The coversion to reducing sugars was similar in several substrates, but the production of glucose was higher in one: banana stem fibers.

The degradation of banana stem fibers was further compared with that of microcrystaline cellulose, using the same enzymatic mixture described above. The addition of the beta-glucosidase inreased the reaction rate but the effect of other compounds in the banana fibers reduced the conversion of the substrate to glucose. The hydrolysis can be described using a Langmuir-type model.

2.. RESEARCH OBJECTIVES

2.1 General objective

To study the degradation of plant cell walls by "cellulosomes" of anaerobic bacteria.

2.2 Specific objectives

To characterize the enzyme complexes ("cellulosomes") produced by *Clostridium thermocellum*, and other anaerobic bacteria.

To study the absorption of cellulosomes to plant cells and the resulting degradation of the cellulosic material.

To enhance the cellulosomes by adding the necessary enzymes once the enzymatic defficiency for plant cell wall is identified.

2.3 Importance

Most developing countries are located in tropical regions of the world, where the lignocellulosic material is abundant. These countries, in general, are interested in a). using biomass residues in a more productive way and b). increasing the yields of food crop plants. The research proposal has a direct impact specially on the adequate utilization of lignocellulosic agroindustrial residues through the conversion of cellulose to sugars.

For effective degradation of hollocelulose to occur, a complete enzymatic system is needed, a complex capable of degrading native cellulose and possibly other structural polysaccharides forming the plant cell walls.

There was experimental evidence that some anaerobic bacteria have a surface-associated complex enzymatic structure ("cellulosome") which is capable of degrading pure intact cellulose. The cellulosome of the thermophillic anaerobe *Clostridium thermocellum* had been characterized by Lamed and Bayer (1986).

The first step for the enzymatic hydrolysis of a given lignocellulosic raw material is the effective adsorption of the enzymatic complex to the surface of the substrate: Several works had treated the adsorption problem of cellulases (Binder and Ghose, 1978; Ghose and

Bisaria, 1979; Maloney and Coughlan, 1983; Ooshima et al., 1983; Pietersen et al., 1977; Ryu et al, 1982, 1984; Stuart and Ristroph, 1985) and the relationship between the adsorption of cellulases and the rate of hydrolysis (Tanaka et al, 1986) It was found that the adsorption behaviour of a purified cellulase was different from that of crude cellulases.

Most of the previous work had been done using fungal cellulases and pure cellulose as substrate. The behaviour of the cellulosomes of anaerobic bacteria on lignocellulosic raw materials had not been explored.

The cellulosome from *Clostridum thermocellum* lacks the betaglucosidase to complete the transformation of cellulose to glucose. A modified complex was proposed.

A collaborative effort was established between the Central American (ICAITI) and Israeli (Tel Aviv University, Weismann Institute of Science) groups to study some applied aspects of the cellulosome and enhanced cellulosome from *Clostridium thermocellum* (and eventually from other anaerobic bacteria).

3. METHODS AND RESULTS

Three lines of research were supported by this project.

The first covered characterization of the enzymatic complexes of *Clostridium thermocellum* (strain YS) and improved methodology for the high yield purification of these "cellulosomes". Publications 1-4 describe the work and the abstracts are included here. The full articles appear in the ADDENDUM.

3.1 Lamed, R., Kenig, R., Morag, E., Calzada, J. F., Micheo, F., Bayer, E. A. 1991. **EFFICIENT CELLULOSE SOLUBILIZATION BY A COMBINED CELLULOSOME-BETAGLUCOSIDASE SYSTEM.** Appl. Biochem. Biotechnol. 27, 173-183

The cellulosome, the multienzyme complex of the cellulase system of *Clostridium thermocellum* that mediates the solubilization of insoluble cellulose, is strongly inhibited by the major end product, cellobiose. By combining a purified betaglucosidase from *Aspergillus niger* with the cellulosome, accumulated cellobiose was hydrolyzed, thereby resulting in a dramatic enhancement (up to 10-fold) of cellulose degradation. The obseved enhancement was expressed both in the rate and degree of solubilization of microcrystaline cellulose, compared with that observed for the unsupplemented cellulosome. Near-complete conversion of cellulose to glucose could be obtained from dense substrate suspensions (up to at least 200 g/l).

3.2 Morag, E., Bayer, E. A., Lamed, R. 1990. **Relationship of cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum*.** J. Bacteriol. 172, 6098-6105

Xylanase activity of *Clostridium thermocellum*, an anaerobic thermophilic cellulolytic bacterium, was chartacterized. The activity was localized both in the cellulosome (the principal multienzyme, cellulose-solubilizing protein complex) and in noncellulosomal fractions. Each of these fractions contained at least four major polypeptide bands which contributed to the

xylanolytic activity. In both cases, pH and temperature optima, product pattern as well as other features of the xylanase axtivity were almost identical. The main difference was in the average molecular weights of the respective polypeptides which appeared responsible for the activity. In the noncellulosomal fraction, xylanases of Mr ranging from 30,000 to 65,000 were detected. Distinct from these were the cellulosomal xylanases which exhibited much larger Mr values (up to 170,000). The cellulosome-associated xylanases corresponded to known cellulosomal subunits, some of which also exhibited endogluconase activity, other coincided with subunits which expressed cellobiohydrolase-like (exoglucanase) activity. In contrast, the noncellulosomal xylanases hydrolized xylan exclusively. B-Glucosidase and B-xylosidase activities were shown to be the action of differente enzymes; both were associated exclusively with the cell and were not components of the cellulosome. Despite the lack of growth on and utilization of xylan or its degradation products, *C. thermocellum* produces a highly developed xylanolytic apparatus which is interlinked with its cellulase system.

3.3 Morag, E.; Halevy, I.; Bayer, E.; Lamed, R.; 1991. **Isolation and properties of a major cellobiohydrolase from the cellulosome of *Clostridium thermocellum*.** J. Bacteriol. 173, 4155-4162.

In the anaerobic, thermophilic, cellulolytic bacterium, *Clostridium thermocellum*, efficient solubilization of the insoluble cellulose substrate is largely accomplished through the action of a cellulose-binding, multi-enzyme complex, the cellulosome. A major cellobiohydrolase activity from the cellulosome has been traced to its Mr 75,000 S8 sybunit, and an active fragment of this subunit was prepared by a novel procedure involving limited proteolytic cleavage. The truncated Mr 68,000 fragment, termed S8-tr, was purified by gel filtration and high performance ion exchange chromatography. The purified proteind adsorbed weakly to amorphous cellulose, and its enzymatic action yielded cellobiose as the major end product from both amorphous and crystalline cellulose preparations. The high ratio of exo- to endo-B-glucanase activities was supported by viscosimetric measurements. Using model substrates, the smallest cellodextrin to be degraded was cellotetraose, but cellopentaose was degraded at a much greater rate. Cellobiose dramatically inhibited the cellulotyc activities. In the absence of calcium or other bivalent

metal ions, the truncated cellobiohydrolase was relatively unstable at temperatures above 50°C. Cysteine further enhanced the stabilizing effect of calcium. This is the first report of the defined cellobiohydrolase in *C. thermocellum*. Its association with the cellulosome and the correspondence of several of their major distinctive properties suggest that this cellobiohydrolase plays a key role in the solubilization of cellulose by the intact cellulosomal complex.

3.4 Morag, E., Bayer, E. A. and Lamed, R. 1992. **Affinity Digestion for the near-total recovery of purified cellulosome from *Clostridium thermocellum*.** Enz. Microb. Technol. In Press

The affinity properties displayed by the cellulosome, the multienzyme complex from *Clostridium thermocellum*, for its insoluble polymeric substrate, cellulose, have previously been employed for its purification by affinity chromatography. In the present communication, a new purification procedure is described which provides 5-fold higher yields (>90%) of the cellulosome with enhanced solubilizing activity (approximately 1.5 fold). The new method is based on the efficient adsorption of the cellulosome onto phosphoric acid-treated substrate (amorphous cellulose) and the subsequent digestion of the carrier/substrate by the adsorbed enzyme complex. The term "affinity digestion" is proposed for such systems in which the affinity matrix is degraded totally by the adsorbed enzyme(s), thus facilitating its recovery.

The second research line included work with other bacteria that seem to present cellolosome-like structures and the pertinent publications are 5 and 6, whose abstracts follow this paragraph. Full publications in the ADDENDUM.

3.5 Micheo, F., Morales, C., Calzada, J. F. 1992. **Characterization of Endoglucanase Activity during Purification of Five Cellulolytic Anaerobic Bacteria.** Submitted for publication in World Journal of Microbiology and Biotechnology.

The cellulase system of four anaerobic cellulolytic bacteria were characterized for endoglucanase activity

following sequential purification steps. Three strains of *Clostridium thermocellum* (DSM 1237, DSM 2360 and ATCC 31549) and one unclasified mesophilic bacterium (JCAITI M5) were tested against *Clostridium thermocellum YS*, the model organism previously studied for cellulosome structures. Sequential purification included: centrifugation, affinity chromatography on microcrystalline cellulose, gel chromatography. Polyacrylamide gel electrophoresis was used for the ccharacterization. Endoglucanase activity was also studied in sonicated extracts from the biomass pellets. Strain DSM 2360 showed the highest endoglucanase specific activity in all cases. Adsorption to cellulose was only partial in all experiments. Gel filtration showed that protein groups appear in different molecular weight ranges in the different strains; the specific activities were associated with them. Electrophoretic patterns exhibited polypeptide distribution with some similarities with the reference strain; only DSM 2360 presented all of them. The supernatants from sonicated biomass pellets of DSM 2360 and ATCC 31549 showed endoglucanase activity, suggesting the existence of cell-associated cellulolytic enzymes, as in the reference YS strain.

3.6 Lamed, R., Morag, E., Mor-Yosef, O., Bayer, E. A. 1992. **Cellulosome-like entities in *Bacteroides cellulosolvens*.** Curr. Microbiol. In Press.

Cellulosome-like complexes were identified in the broth and sonic extracts of cellobiose- and cellulose-grown cells of *Bacteroides cellulosolvens*. The extracellular fractions contained 3 to 4 major polypeptides and several minor polypeptides bands which were localized in two major gel filtration peaks indicating average molecular wights of about 700 kDa and >10 MDa. A relatively large molecular weight component (Mr 230 kDa) was found to contain carbohydrate, but no apparent enzynmatic activity of its own could be detected. The cell sonicate displayed a more complicated polypeptide profile, and glycosylated polypeptides were larger (ca. 310 and 290 kDa) that that of the extracellular fraction. The 230 kDa extracellular component interacted strongly with the GSI isolectin from *Griffonia simplicifolia*, exhibited immunochemical cross-reactivity with the S1 subunit of the cellulosome from Clostridium thermocellum, and displayed anomalous pH- and salt- dependent migratory behavior in SDS-PAGE. Taken together, this evidence strongly suggests a structural similarity between the glycoconjugates of these two distinct cellulolytic bacteria. A major 84-kDa

polypeptide was identified as a xylanase, and a 50-kDa polypeptide displayed endogluconase activity. Additional biochemical and cytochemical evidence indicated that cellulosome-like cellulolytic complexes are associated with the cell surface in this bacterium.

The third line of research included the utilization of cellulosomes from Clostridium thermocellum in the degradation of lignocellulosic raw materials, compared with the pure cellulose used before. Publications 7 and 8 present the work. Abstracts follows and full publications are in the Appendix.

3.7 Micheo, F., Calzada, J. F., Lamed, R., Bayer, E. A. 1992. **Degradation of Lignocellulosic raw materials by cellulosomes from *Clostridium*.** Submitted for publication in Bioresource Technology.

Lignocellulosic agroindustrial wastes may require selective degradation for efficient biological utilization. The adsorption of the enzymatic complexes "cellulosomes" from *Clostridium thermocellum* YS to the solid substrate and the subsequent degradation have been explored for six agroindustrial wastes. These substrates include: untreated sugar cane bagasse, Organosolv treated sugar cane bagasse, untreated sugar cane pith, organosolv treated sugar cane pith, oil palm bagasse, lemon tea bagasse, banana fruit stem and microcrystalline cellulose. In all cases, the conversion was much lower in the waste substrates than in cellulose. Other components of the lignocellulosic material seem to interfere. Several of the wastes seem adequate as carriers because of high adsorption rate (79-96%). The conversion to reducing sugars, with the addition of beta-glucosidase was similar in various substrates. The production of glucose was higher in the banana stem fibers (7 umoles/ml at 30 hours) than in any other waste.

3.8 Alvarado, J. H., Micheo, F., Calzada, J. F. 1992. **BANANA STEM FIBERS AS SUBSTRATE FOR THE ADSORPTION AND HYDROLYSIS BY CELLULOSOMES FROM *Clostridium thermocellum*.** Submitted for publication in Bioresource Technology.

The degradation of banana stem fibers was compared with that of the microcrystaline cellulose using

cellulosomes from *Clostridium thermocellum YS*. Reaction profiles of the adsorption and hydrolysis were determined for both cases, using cellulosome alone and in mixtures with beta-glucosidase. Batch fermentations were carried out for 72 hours, using cellulose concentrations of 1.5%, 2% and 2.5% (W/V). The adsorption of cellulosome was higher on the cellulose (70% in two minutes, 90% in one hour) than on the banana stem fibers (52% and 60%, respectively). The product formation was also followed in the experiments, with approximately 10% of the total degradation occurring in the first hour in both substrates, when only cellulosome was used. The addition of the beta-glucosidase increased the reaction rate but the effect of other compounds in the banana fibers reduced the conversion in this substrate (24-28% of that for cellulose). The hydrolysis can be described using a Langmuir-type model.

4. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The importance of the biomass resources in the tropical countries without pretroleum. Practically all of them are interested in:

a. Using biomass residues in a more productive way.

b. Increasing the yields of valuable crop plants.

The research has a direct impact on the first aspect and an effect on the second. The reorientation of the research during the lifespan of the project focused it, to more applied aspects of the technology, specially those dealing with the utilization of agroindustrial residues containing cellulose. On the other hand, the search for better microbial and enzymatic systems to hydrolyse plant cell walls, originally envisioned in the research proposal, has been advanced, to be further developed in the areas of vector preparation for biological nitrogen fixation associations.

An important result fron the project is the transfer of the technology for the production of cellulosome from the Tel Aviv University ot ICAITI. It is now the task of the latter to disseminate this knowledege to the Universities and Research Institutes of Central America. The search for funds to carry on applied joint research with therm on this topic has already been initiated.

The collaborative effort must be mentioned. A permanent communication between the two research teams has been established as a result of this project and new possiblities of join research activities are also been explored.

Students from a local university (Biological Chemistry and Chemical Engineering Departments, San Carlos University, Guatemala) have participated in the research activities, preparing their thesis on topics included in the project. One of them presented the results in the 4th National Symnposium on Biotechnology (Guatemala), after a selection procedure by reviewers.

5. PROJECT ACTIVITIES

5.1 Coordination Meetings

The first coordination meeting of this project was carried out in five sessions in March 1989 in Tel Aviv, Israel. Rapahel Lamed, Edward Bayer, Fabiola de Micheo and José Francisco Calzada attended this meeting.

The second coordination meeting was held in Guatemala City, in August 1990 and it was attended by Raphael Lamed, Edward Bayer, Fabiola de Micheo, Carlos Morales and José Francisco Calzada.

A third meeting had been planned for the last quarter of 1991 in Tel Aviv, but last minute problems caused its cancellation.

Communication was maintained between the two groups through regular and electronic mail.

5.2 Training Stays in Israel

The first training stay (Fabiola de Micheo) was in March-April 1989 and included several research activities, as follows:

5.2.1 Separation an purification of the cellulosome.

5.2.2 Determination of Congo Red-Polysaccharide Interactions in the supernatant, 24 hours cell growth of *Clostridium thermocellum.*

5.2.3 Detection of protein in the supernatant, 24 hours cell growth of *Clostridium thermocellum,* by the reaction of lectin bound to peroxidase, on nitrocellulose paper.

5.2.4 Degradation of microcrustalline cellulose (Avicel) by: 1. Cellulosome; 2. Cellulosome and B-Glucosidase (Novozym).

5.2.5 Degradation of alfalfa cell walls (untreated, by: 1. Cellulosome; 2. Cellulosome with B-Glucosidase.

5.2.6 Determination of cellulosome adsorbed by alfalfa cell walls.

The second training stay was in January 1991, also by Fabiola de Micheo. Activities included:

5.2.7 Simultaneous adsorption and hydrolysis of microcrystaline cellulose by cellulosomes and beta-glucosidase.

5.2.8 Purification of cellulosomes using amorphous cellulose and a dialisis procedure.

5.3 Training Stays at ICAITI

Two students from San Carlos University (Guatemala) partook in the activities of the research project. Their particular research originated graduation thesis in the Schools of Chemical Engineering and Biology. The students were Carlos Morales and Jaime H. Alvarado.

Carlos Morales stayed for six months, working in the characterization of the cellulosomes in some of the purification stages.

Jaime Haroldo Alvarado stayed for nine months, with work on the degradation of banana stem fibers by cellulosomes.

5.4 Technical Meetings attended:

Jaime H. Alvarado presented an oral paper in the 2d. National Biotechnology Symposium (Guatemala, July 10, 1992).

José F. Calzada presented one poster paper associated with this research and two related to other projects in the 9th International Symposium on Biotechnology (Crystal City, Virginia, August 16-21).

5.4 Publications

5.4.1 Lamed, R., Kenig, R., Morag, E., Calzada, J. F., Micheo, F., Bayer, E. A. 1991. **Efficient cellulose solubilization by a combined cellulosome-betaglucosidase system.** Appl. Biochem. Biotechnol. 27, 173-183.

5.4.2 Morag, E., Bayer, E. A., Lamed, R. 1990. **Relationship of cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum*.** J. Bacteriol. 172, 6096-6105.

5.4.3 Morag, E.; Halevy, I.; Bayer, E.; Lamed, R.; 1991. **Isolation and properties of a major cellobiohydrolase from the cellulosome of *Clostridium thermocellum*.** J. Bacteriol. 173, 4155-4162.

5.4.4 Morag, E., Bayer, E. A. and Lamed, R. 1992. **Affinity Digestion for the near-total recovery of purified cellulosome from *Clostridium thermocellum*.** Enz. Microb. Technol. In Press.

5.4.5 Micheo, F., Morales, C., Calzada, J. F. 1992. **Characterization of Endoglucanase Activity during Purification of Five Cellulolytic Anaerobic Bacteria.** Submitted for publication in World Journal of Microbiology and Biotechnology.

5.4.6 Lamed, R., Morag, E., Mor-Yosef, O., Bayer, E. A. 1992. **Cellulosome-like entities in *Bacteroides cellulosolvens*.** Curr. Microbiol. In Press.

5.4.7 Micheo, F., Calzada, J. F., Lamed, R., Bayer, E. A. 1992. **Degradation of Lignocellulosic raw materials by cellulosomes from *Clostridium*.** Submitted for publication in Bioresource Technology.

5.4.8 Alvarado, J. H., Micheo, F., Calzada, J. F. 1992. **BANANA STEM FIBERS AS SUBSTRATE FOR THE ADSORPTION AND HYDROLYSIS BY CELLULOSOMES FROM *Clostridium thermocellum*.** Submitted for publication in Bioresource Technology.

6. PROJECT PRODUCTIVITY

The project accomplished most of the proposed goals of the original proposal and reached others not included in it.

Difficulties in the maintenance of an homogeneous suspension of free cells suggested the convenience of shifting to lignocellulosic wastes, area not covered in the original technical work plan. The third research line originated from this change.

The concept of "supercellulosome" was substituted for the addition of the lacking enzymes to the cellulosome-substrate systems, after taking into consideration the potential practical application. Some research was continued, however, in the original line also.

The utilization of different strains of bacteria, other than Clostridiun thermocellum, was a consequence of the findings during the research, enriching the original proposed work.

7. FUTURE WORK

Research work can be envisioned on the characterization of the protein fractions of the bacteria (other than *Clostridium thermocellum*) that have shown cellulolytic activity.

The inhibitory effect of the galacturonic acid, resulting from the degradation of pectin, on some of the reported enzymatic activities should also be explored, specially if pectin-rich agroindustrial wastes are considered for degradation by cellulosome-like entities. Pectinolytic bacteria such as *Erwinia* could also be associated in this research.

Utilization of agroindustrial waste materials as raw materials for the production of multienzyme complexes is an interesting proposition, still lacking further research to prove feasible in economic terms. Overcoming the inhibitory effects of intermediate or end products is an area requiring more research.

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