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Anaerobic Bacterial Degradation for the Effective Utilization of Biomass

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Abstract Biomass is originally photosynthesized from inorgainic compounds such as CO_2, minerals, water and solar energy. Recent studies have shown that anaerobic bacteria have the ability to convert recalcitrant biomass such as cellullosic or chitinoic materials to useful compounds. The biomass containing agricultural waste, unutilized wood and other garbage is expected to utilize as feed, food and fuel by microbial degradation and other metabolic functions. In this study we isolated several anaerobic, cellulolytic and chitinolytic bacteria from rumen fluid, compost and soil to study their related enzymes and genes. The anaerobic and cellulolytic bacteria, *Clostridium thermocellum, Clostridium stercorarium*, and *Clostridium josui*, were isolated from compost and the chitinolytic *Clostridium paraputrificum* from beach soil and *Ruminococcus albus* was isolated from cow rumen. After isolation, novel cellulase and xylanase genes from these anaerobes were cloned and expressed in *Escherichia coli*. The properties of the cloned enzymes showed that some of them were the components of the enzyme (cellulase) complex, *i.e.*, cellulosome, which is known to form complexes by binding cohesin domains on the cellulase integrating protein (Cip: or core protein) and dockerin domains on the enzymes. Several dockerin and cohesin polypeptides were independently produced by *E. coli* and their binding properties were specified with BIAcore by measuring surface plasmon resonance. Three pairs of cohesin-dockerin with differing binding specificities were selected. Two of their genes encoding their respective cohesin polypeptides were combined to one gene and expressed in *E. coli* as a chimeric core protein, on which two dockerin-dehydrogenase chimeras, the dockerin-formaldehyde dehydrogenase and the dockerin-NADH dehydrogenase are planning to bind for catalyzing CO_2 reduction to formic acid by feeding NADH. This reaction may represent a novel strategy for the reduction of the green house gases. Enzymes from the anaerobes were also expressed in tobacco and rice plants. The activity of a xylanase from *C. stercorarium* was detected in leaves, stems, and rice grain under the control of CaMV35S promoter. The digestibility of transgenic rice leaves in goat rumen was slightly accelerated. *C. paraputrificum* was found to solubilize shrimp shells and chitin to generate hydrogen gas. Hydrogen productivity (1.7 mol H_2/mol glucose) of the organism was improved up to 1.8 times by additional expression of the own hydrogenase gene in *C. paraputrficum* using a modified vector of *Clostridium perfringens*. The hydrogen producing microflora from soil, garbage and dried pelletted garbage, known as refuse derived fuel (RDF), were also found to be effective in converting biomass waste to hydrogen gas.

Keywords: anaerobic bacteria, biomass utilization, cellulase, cellulosome, garbage, hydrogen gas, waste

INTRODUCTION

Biomass is the biologically photosynthesized organic materials from carbon dioxide, minerals, water and solar energy and is continuously produced by plants and microorganisms in any places on the earth surface. Starchy components in the biomass are easily digested by human enzymes, but cellulosic materials, such as grass and woods, are not easily digested by human enzymes. Therefore, human cannot degrade such recalcitrant biomass but some microorganisms can do by their enzymes, cellulases, xylanases and chitinases. Utilization of the microbial functions may one day convert the recalcitrant biomass into food for human consumption. The rapid increase in human population as well as global warming of the earth may lead to shortages in food and energy resources. Thus, there is a great need for the development of techniques for the effective utilization of biomass. Moreover, the massive amount of biomass waste produced by human activity will likely be detrimental to our civilization necessitating a counter measures to protect and re-establish the environment. It is in this context, we studied degradation

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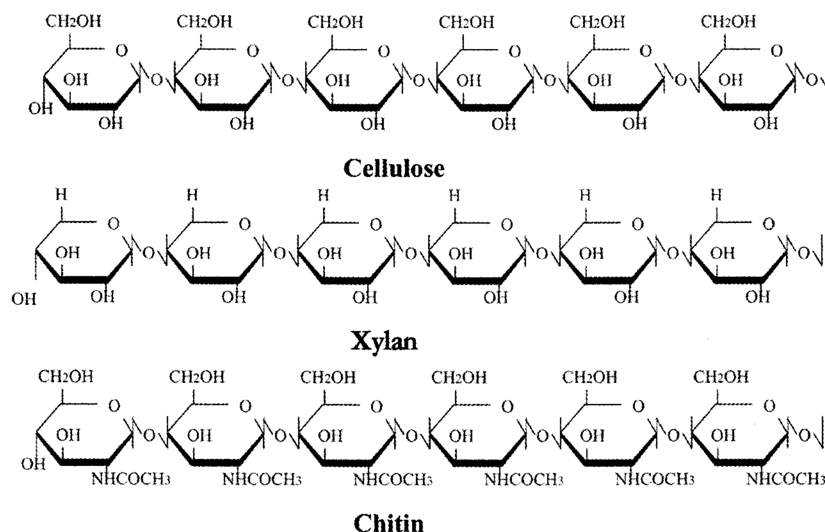

Fig. 1. Main components of unutilized biomass.

and solubilization of unutilized recalcitrant biomass, especially cellulosic biomass, by microorganisms and their enzymes for useful materials. The characterization of their cellulolytic enzymes was accomplished based on genetic analysis, and the functions of new enzymes and domains were found.

Based on these results, the specific binding functions between the binding sites, cohesins in a CIP and dockreins in enzyme molecules, of the cellulosome, a cellulase complex found in anaerobic bacteria, was elucidated by measuring the interaction between these binding proteins. Based on the cohesin-dockerin binding specificities, we are attempting to bind enzymes on the chimeric CIP to construct an artificial enzyme complex, like a CO_2 fixing system. Furthermore, the xylanase gene from *Clostridium thermocellum* was integrated and expressed in the rice plant. The transgenic plant leaves were accelerated digestion by rumen bacteria in the goat rumen. A chitin degrading anaerobic bacterium, *C. paraputrificum* was also isolated from beach soil, and transformed to enforce the activities of its own chitinase and hydrogenase for increasing productivities of hydrogen gas from chitin-containing biomass waste.

Analyses of the Cellulase Genes in Anaerobic Bacteria and Their Enzymatic Functions

Cellulosic, xylanous, and chitinous compounds take time to be hydrolyzed enzymatically and thus remain as a large component of unutilized biomass. These compounds including cellulose, xylan and chitin are respectively consisted from monosaccharide, glucose, xylose and N-acetylglucosamine, linked by β-1,4-glycoside linkages (Fig. 1). Therefore, their solubilization and monomerization would give us vast quantities of each individual compound. These mono-sugars are important nutrients, not only for human consumption, but also for other organisms. In general, β-1,4-glycosyl linkages are difficult to be digested by mammalian systems due to the lack of enzymes cleaving β-1,4-glycoside bonds. Certain bacteria, which produce enzymes that can cleave β-glycosyl linkages, have been found in rumen, in compost and in soil. Therefore, the study of these anaerobic bacteria and their enzymes are of fundamental importance for the development and the application of recalcitrant biomass utilization.

We investigated the activities of *Ruminococcus albus,* a major anaerobic cellulolytic microorganism located in the rumen in cattle and goat rumen. Establishment of the anaerobic cultivation conditions of *R. albus* were created in a jar-fermentor use [1-3] to harvest a sufficient volume of bacterial cells and culture supernatant containing extra-cellular enzymes. From these culture preparations, we purified β-glucosidase [4] and endoglucanase, EgI [5]. The *egI* gene encoding the endoglucanase I from *R. albus* was then cloned and analyzed, revealing that the enzyme consisted only of the catalytic domain of family 5 [6,7]. The *egI* gene was truncated by DNAase randomly and resultant genes were expressed in *E. coli.* Among the truncated enzymes, the enzyme with 15 amino acids deleted from the N-terminus following the signal peptide of 43 amino acids, expressed the highest specific activity [8].

In addition to EgI, EgII and EgIII were isolated and purified from the culture supernatant of *R. albus*. Each of the enzymes was distinguished immunologically and was found to work together in a synergistic fashion [9]. Although the genes encoding these endoglucanase-II (*egII*) and -III (*egIII*) were not yet cloned from *R. albus, eg-IV, -V, -VI* and *-VII* have already been cloned, in which EgIV also consists only of a catalytic domain [10]. The catalytic domain of EgIV was chimeralized at the N-terminus by connecting the cellulose-binding domain (CBD) of a *C. stercorarium* xylanase gene to construct EgIV-CBD. The chimeric enzyme molecule newly obtained cellulose-binding properties due to the integration of CBD in to the chimeric molecule. This allowed the chimera mole-

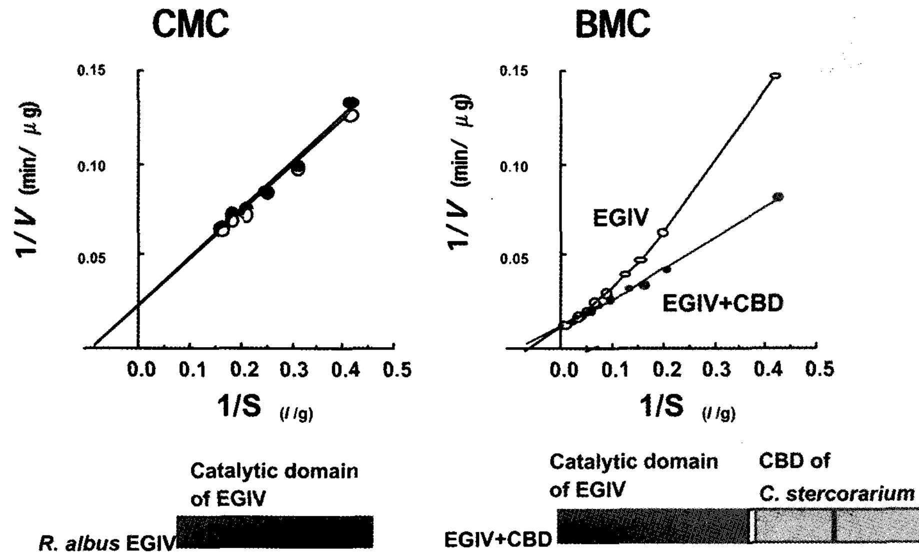

Fig. 2. The fortification of *R. albus* cellulase activity by adding CBD of *C. thermocellum* to the EGIVcatalytic domain. CMC: Carboxymethyl cellulose as substrate. BMC: Ball-milled cellulose as substrate.

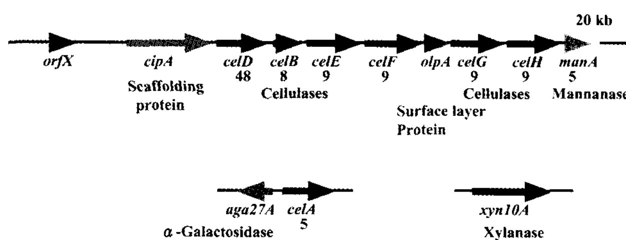

Fig. 3. Cellulase gene cluster in *C. josui.*

cule to elevate cellulose-degrading activity due to the concentration of catalytic domain on cellulosic substrate by adsorption (Fig. 2) [11]. This CBD property to bind cellulose was also applied to purify the other enzymes by genetically chimeralizing the enzymes with CBD. Then the chimeralized enzyme only gained cellulose-binding properties when the gene was expressed in *E. coli*. Therefore, the chimeralized enzyme only adsorbed to the cellulose in the crude extract of the transformant by mixing cellulose to the crude enzyme preparation for adsorption. The cellulose-enzyme complex was then washed well to remove contaminated proteins followed by elution of the enzyme with glucose [12]. The results suggested that each domain fully expressed its function in a chimerized enzyme molecule.

A study by Jiraporn *et al.*, isolated a novel strain of clostridia from Thai compost, which was characterized and named as *Clostridium josui* [13]. Several genes of the bacteria were cloned and found to encode a scaffolding protein and enzymes, such as cellulases and mannanase. They were found to be lined up in order on a DNA fragment from the N-terminus to make a cluster while under the control of a promoter on the genome, indicating that these cellulosome (cellulase complex) members might be expressed sequentially on the same DNA fragment (Fig. 3) [14-17].

The structure of *C. stercorarium* xylanase A has been reported to have a CBD bound to cellulose with two repeated sequences classified to family 6 as well as a catalytic domain [18-20]. The function of CBD allows xylanase, similar to cellulase, to adsorb on cellulose for accelerating the hydrolysis of xylan around the cellulose fiber [18-20].

Overall, more than 20 genes including those for cellulase and xylanase have been cloned and characterized from cellulolytic anaerobes such as *C. thermocellum* [21] and *C. stercorarium* [22,23]. All of these work were done by our group. These findings reveal the cellulose binding properties of these bacteria, which allow enzymes adsorption on substrate cellulose, or in some cases, the binding of the bacterial cell itself onto the cellulose. In the latter case, solubilized compounds from plant fibers, such as glucose, xylose or their oligomers, are able to diffuse immediately to the bacterial cells given the short distance. This substrate supply system to the bacterial cells is very effective and valuable for the transfer of energy compounds to cellulolytic anaerobic bacteria in the rumen, where many different rumen anaerobes compete for nutrients.

Mechanism for Cellulosome Formation and Construction of Artificial Enzyme Complex

The cellulase, xylanase [24,25] and β-galactosidase [26] genes from *C. josui* and *C. thermocellum* were cloned in *E. coli*. All these enzyme genes had sequences homologous to a binding domain, which forms dockerin. The binding counterpart of dockerin, the so-called cohesion sequences were found to form a scaffolding protein upstream of these enzyme genes on the same DNA frag-

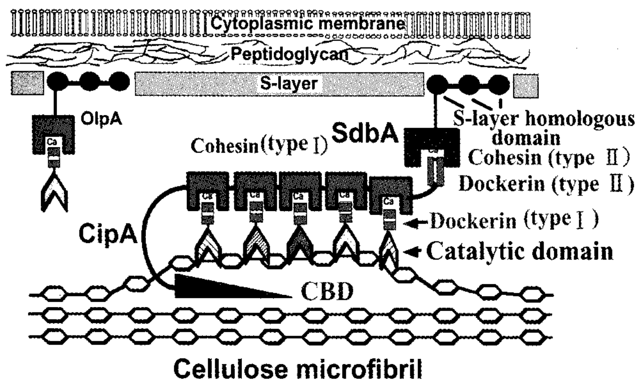

Fig. 4. A model of *C. thermocellum* cellulosome. CipA: cellulase integrating protein A (scaffolding protein A). S-layer: surface layer. SdbA: surface layer dockerin binding protein A.

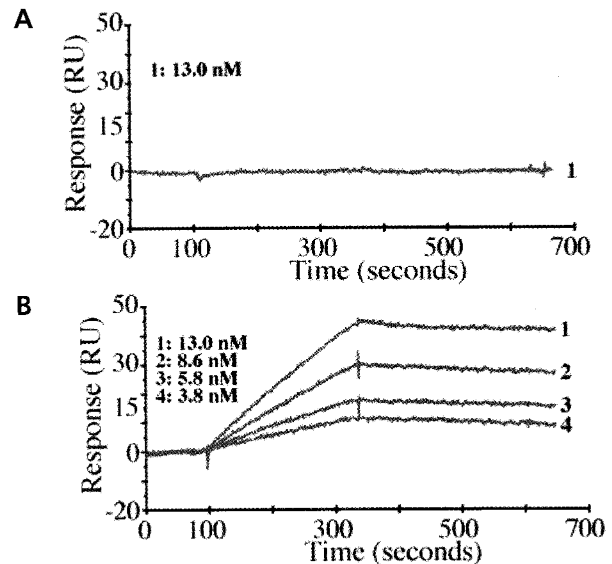

Fig. 5. Sensorgrams of cohesin/dockerin interaction measured by BIAcore. A: Sensorgram of the *C. thermocellum* cohesion (type I) and *C. josui* Aga dokerin (type I). B: Sensorgrams of the *C. josui* cohesin (type I) and the *C. josui* Aga dokerin (type I). 1~4: Concentrations of *C. josui* Aga dokerin (type I).

ment. A promoter was also identified that was upstream of the scaffolding protein, indicating that these genes are expressed under the control of the promoter. This cluster of genes forms a cellulosome proposed by Bayer *et al.* in reference [30]. Cohesins, binding counterparts of dockerin, are found on the scaffolding protein as repeated sequences of around 60 amino acids (6 repeated cohesins on the *C. josui* scaffolding protein and 9 repeated cohesins on the *C. thermocellum* scaffolding protein). The scaffolding protein of *C. thermocellum* was found upstream of the cellulosome gene cluster.

A gene coding a surface layer homologous (SLH) protein is known to bind cellulosome *via* SLH cohesin (type II cohsin) and type II dockerin on the scaffolding protein of the cellulosome as shown in Fig. 4. In the case of the *C. thermocellum* scaffolding protein, there were 9 repeated cohesin sequences, while *C. josui* contained 6 repeated cohesin sequences. These cohesins were classified as type I cohesins, on which a cognate type I dockerin bound each catalytic domain, such as cellulase and xylanase, to form the cellulosome (Fig. 4).

Thus, the scaffolding protein in these anaerobes seem to be a long protein molecule containing a string of 6-9 cohesins, along which each enzyme molecule is bound to form the cellulosome. The cellulosome may function to bind bacterial cells and substrate cellulose as described above.

The cellulosome system was also found in *R. albus* by detecting molecular enzyme complexes by acrylamide gel electrophoresis SDS-PAGE. One of cellulolytic enzymes, *R. albus egV* [27], was found to contain a dockerin sequence and 40 kD cohesin-containing protein (cellulase-integrating protein: CIP or scaffolding protein) was found in an *R. albus* cellulosomal protein preparation with a 40 kD molecular weight [28]. The molecular weight of the *R. albus* scaffolding protein was lower than that of *C. thermocellum* and *C. stercorarium*, indicating that the *R. albus* cellulosome might be smaller than that of clostridia. The formation of cellulosomes in *R. albus* cells was also observed by the scanning electron microphotograms [29].

The binding specificities between cohesins and dockerins from *C. josui* and *C. thermocellum* were determined by measuring the surface resonance response with a BIAcore 2000 (BIAcore AB, Uppsala, Sweden). We used PCR to prepare dockerin and cohesin from the respective genes encoding catalytic molecules and scaffolding proteins [30]. Cohesin molecules, consisting of approximately 200 amino acids, were immobilized on the sensor disk of BIAcore, on which dockerin molecules (about 60 amino acids) were fed as a solution to detect interaction between them. Surface resonance units between *C. thermocellum* coheshin and *C. josui* dockerin did not increase indicating that cohesin-dockerin binding was not detected on the sensor (Fig. 5A), In the case of cognate coheshin-dockerin pairs of *C. thermocellum,* surface resonance units had increased according to increases in protein concentration (Fig. 5B). In this experiment, ten different cohesin peptides and ten different dockerin peptides were prepared and their binding properties measured by calculating association/dissociation constants obtained from the sensor gram ($k$on/$k$off) as listed in Table 1. Based on the association-dissociation constants (KD) between cohesin and dockerin molecules, it was concluded that cognate cohesins in the scaffolding proteins (type I cohesin) and dockerins in the cognate enzymes (type I dockerin) bound each other; however, noncognate cohesin did not bind to noncognate dockerin. In other words, type I cohesin and type I dockerin from *C. thermocellum* can bind each other. Type I cohesin from *C. thermocellum* cannot bind to type I dockerin from *C. josui* and vise versa. Moreover, type II cohesin on the SLH protein of *C. thermocellum* can bind only to type II dockerin located on a scaffolding protein of *C. thermocellum,*

Table 1. Coehsin/Dockerin binding analysis by BIAcore

Cohesin \ Dpckerin		*C. josui* Cj-Aga27Doc		
		$K_{on}(s^{-1}M^{-1})$	$K_{off}(s^{-1})$	$K_D(M)$
C. thermocellum	Coh1-Ct	No Binding		
	Coh2-Ct	"		
	Coh3-Ct	"		
	Coh4-Ct	"		
	Coh7-Ct	"		
C. josui	Coh1-Cj	1.8×10^5	3.4×10^{-4}	$1.9 \times 10^{-9}(7.9)$
	Coh2-Cj	6.9×10^5	1.7×10^{-4}	$2.4 \times 10^{-10}(1.0)$
	Coh5-Cj	3.9×10^5	2.5×10^{-4}	$6.3 \times 10^{-10}(2.6)$
	Coh6-Cj	2.1×10^5	1.3×10^{-4}	$6.5 \times 10^{-10}(2.7)$

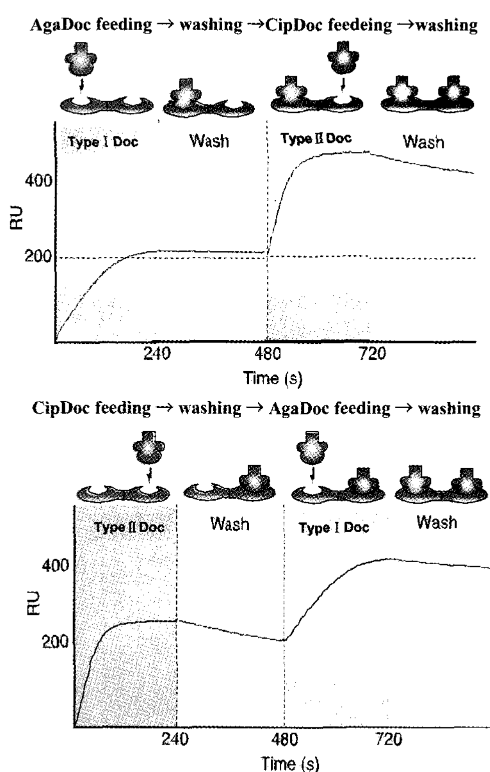

Fig. 6. Binding specificities between chimeric scaffolding proteins (consisting type I cohesin and type II cohesin) and dockerins from *C. josui* Aga dockerin (type I) and *C. thermocellum* CipA dockerin (type II). RU: resonance unit.

but cannot bind to the dockerin in cognate catalytic molecules (type I dockerin of *C. themocellum*), even though they originated from the same cognate organisms. Type I dockerins of both *C. thermocellum* and *C. josui* were found to be bound to cohesins in the respective cognate scaffolding proteins (type I). Based on these binding properties between cohesin and dockerin, 3 pairs of cohesin/dockerin with specific binding properties were selected.

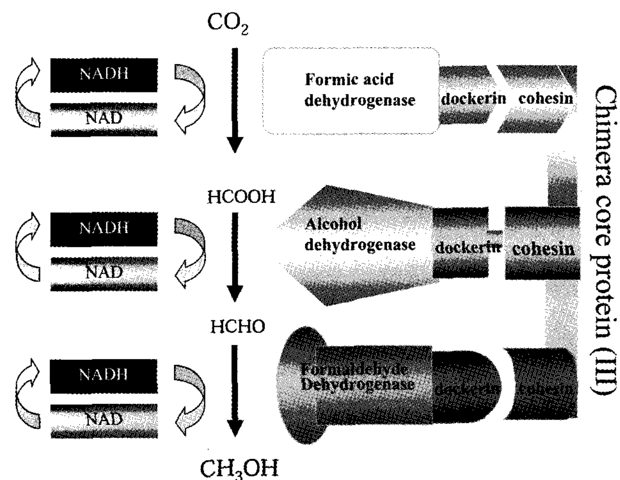

Fig. 7. Hypothetical construction of an artificial enzyme complex for converting CO_2 to methanol.

For further study of the binding properties of chimeric cohesin, two genes encoding *C. thermocellum* type I cohesin and *C. thermocellum* type II cohesin were connected in this order. The chimeric cohesin gene was expressed in *E. coli* and the protein produced was a chimeric scaffolding protein (CSP2). CSP2 was immobilized on the sensor tip of BIAcore to which the *C. themocellum* alpha (α) galactsidase A (AgaA) dockerin (type I) solution was fed. The binding of AgaA dockerin to CSP2 was monitored by increase in resonance units as shown in Fig. 6. Thereafter, type II dockerin solution was fed. The binding curves obtained were similar to those obtained in a previous study of dockerin (type I), which indicated that AgaA dockerin binds to type I cohesin while type II dockerin binds to type II cohesin (Fig. 6). When the feeding order of each dockerin solution was reversed, a similar resonance curve was obtained, indicating that both dockerins were selectively bound to their counterparts with equal molecular numbers as indicated by equal amount of increase in resonance units, *i.e.* binding molecular ratio of dockerin(type I), dockerin(type II), and chimeric cohesin (CSP2) was 1:1:1.

For future studies, we would like to arrange the dockerin-enzyme chimeras on CSP2 (chimeric scaffolding protein) to construct artificial enzyme complexes. The construction of an artificial enzyme containing dockerin-formic acid dehydrogenase as well as dockerin-NADH dehydrogenase on CSP2 may provide a novel method to reduce CO_2 to methanol (Fig. 7).

Expression of Anaerobic Bacterial Enzymes in the Rice Plant and Digestibility of the Transgenic Plant

Rice straw, a cellulosic biomass with high silica content, is not easily digested by rumen bacteria or solubilized by enzymes. Thus, rice straw cannot be effectively utilized as feed or food. We sought to improve digestibility of rice straw by cellulolytic anaerobes by expressing xylanase and cellulase genes. We used a tobacco BY2 host-vector sys-

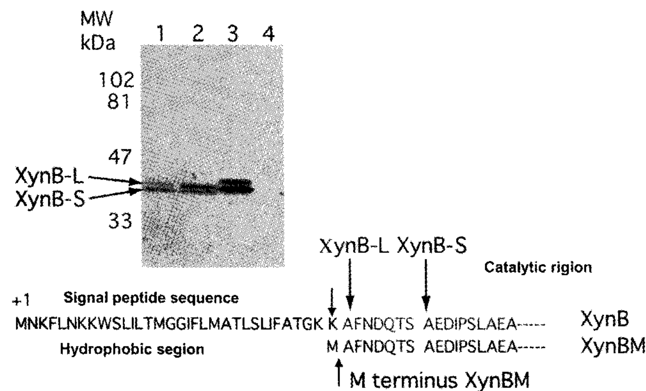

Fig. 8. Processing of *C. stercorarium* XynB in tobacco suspension cells.

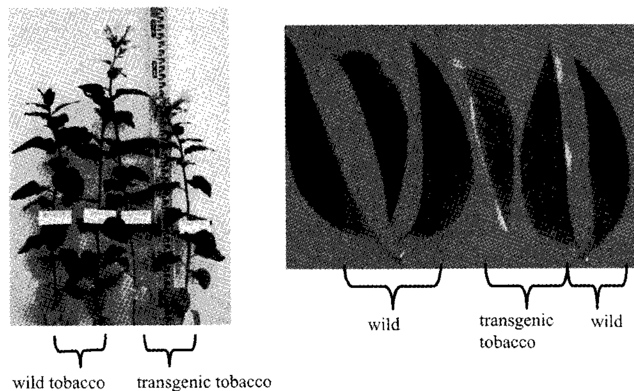

Fig. 9. Transgenic tobacco plants expressing *R. albus* t-Egl. (right: growth, legt: activity).

tem [31] to integrate *R. albus t-egl,* an *R. albus* endoglucanase gene which was truncated at the signal peptide sequence of 43 amino acids and the N-terminal sequence of 15 amino acids, t-EgI [32,33]. The activity of the t-EgI was several times higher than the full-size enzyme EgI. The *t-egl* gene was integrated into the binary vector under the control of the cauliflower mosaic virus 35S promoter (CaMV35S) and expressed in a tobacco BY2 cell suspension [31]. Almost 95% endoglucanase activity was detected in the cytoplasm of the transgenic cells without apparent delay or depression in their growth. Other genes of *C. thermocellum xynA1* (catalytic domain of xylanase A), *xynA2* (catalytic domain, and cellulose binding domain of xylanase A) [34], and *C. stercorarium xynA* [35] containing CBD which adsorbs to both cellulose and oatspelt xylan, and *C. stercorarium xynB* [22,36, 37] were also expressed in BY2 [38]. Transgenic BY2 produced XynB, which was found to degrade barley straw. A XynB protein band resulting from SDS electrophoresis was extracted from the gel and its N-terminal amino acid sequence was determined as shown in Fig. 8, indicating that 33 amino acids at N-terminal of the full sequence functioned as a signal peptide, even in the tobacco BY2 cell suspension.

The *R. albus t-egl* gene was integrated into a tobacco callus by an *Agrobacterium* infection method and expressed under the control of the CaMV35S promoter [39]. Then, transgenic calli were selected against antibiotics (hygromycin and kanamycin), and differentiated to transgenic tobacco plants. The activity of t-EgI was detected in the cytoplasm, which was released from cut sites of the transgenic tobacco leaves. The t-EgI protein content in the cytoplasm was determined to be 0.1~0.5% of the total soluble proteins in the leaf. The apparent growth rate of the transgenic tobacco plants was similar to that of wild type plants (Fig. 9). When transgenic leaves were ground and incubated at 37°C, soluble sugar content was substantially higher than that of wild type tobacco, indicating that t-EgI degraded cellulosic materials in leaves. By using this method, enzymes such as cellulases and xylanases can be produced in tobacco plant cells during its growth under the photosynthesis. This method pro-

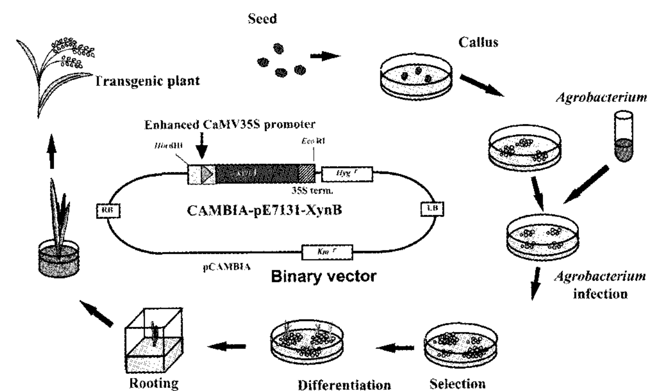

Fig. 10. The *Agrobacterium* transformation procedure of rice plant by using binary vector harboring *C. stericorasum* XynA.

vides a low cost technique to produce bacterial enzymes in plant.

However, because tobacco is not acceptable feed for ruminants, we attempted to apply the transgenic technique to rice plants for the production of xylanase. Details of the procedure to integrate the bacterial xylanase genes are shown in Fig. 10. Hygromycin, an antibiotic, was used as a selection pressure of transgenic rice callus. In the first part of the experiment the *C. thermocellum xynA* gene, without the signal peptide region, was integrated into the rice callus [40]. The resulting transgenic rice plants revealed XynA activity in leaves, stems and grains. Growth rates of the transgenic rice plants and their outward appearance were very similar to wild type plants, indicating that normal growth of the transgenic rice plants was observed. The cell free extracts of the transgenic rice leaves depicted high xylanase A active even after incubation at 60°C for 24 h.

C. stercorarium xynB was also successfully expressed in rice plants by the same procedure as described above. When the gene, that was modified by removing the signal peptide region from *xynB* (denoted as *xynBM),* was expressed in the rice plant, the resulting enzyme XynBM was found to have accumulated in plant cells, thus allow-

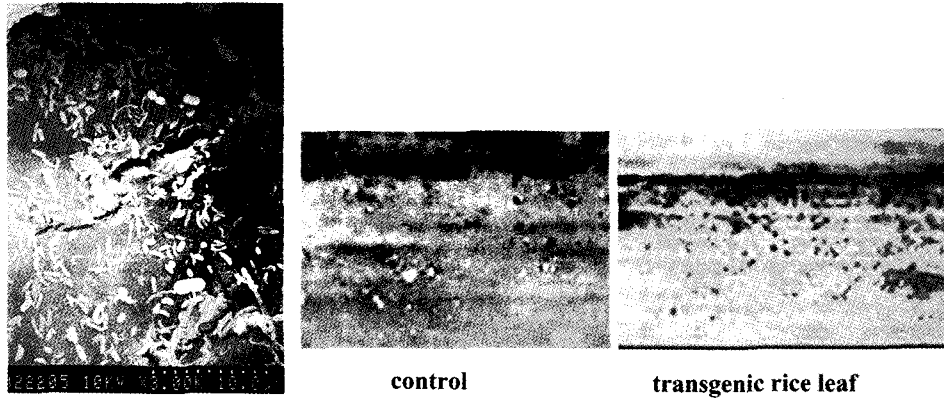

Fig. 11. Rumen microorganisms absorbed on the transgenic rice leaves in goat rumen.

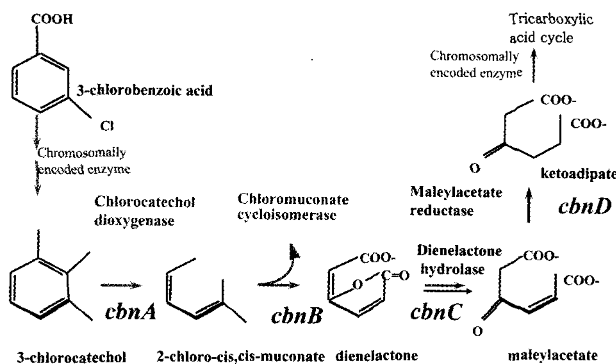

Fig. 12. Chlorocatechol–degrading pathway of *Ralstonia eutropha* NH9.

ing for the normal growth of transgenic rice plant and matured. XynBM activity was detected in leaves, stems and grains.

In contrast, when *xynB* with the signal peptide region was expressed, the growth of the transgenic rice plants were remarkably slower than that of wild type plants and they died about 2 weeks after differentiation of the callus (unpublished findings). Since the signal sequence of XynB functioned in tobacco plants, it also might work to secrete XynB from the rice plant cells. This secretion allowed for enzyme attack of the cell walls of rice plants and then caused the plant to die due to inhibition of the normal cell wall synthesis.

These results indicate that the bacterial cellulolytic and xylanolytic genes can be expressed in rice plants and accumulated in their cells if their signal peptide sequences are removed from the genes. Therefore, when the transgenic rice straw is given as animal feed, xylanase B might be released from the disrupted cells after chewing the straw, which enhance degradation and solublization of rice straw, functioning similarly to the enzyme added to the grass silage to enhance partial digestion of grass.

In another experiment, leaves of the transgenic rice straw were dipped into rumen solution for 2 days in the goat rumen through a fistula. Many more rumen microbial cells were adsorbed on the transgenic leaves compared to wild type (Fig. 11). The adsorption of bacterial cells on the leaves may trigger the initiation of degradation of recalcitrant biomass. Enhancement of cell adsorption of rumen bacteria on the transgenic rice leaves seemed to be induced by expressing bacterial XynB. This may lead to the acceleration of bacterial and enzymatic digestion of cellulosic biomass. To confirm this observation, we determined the weight of the transgenic leaves dipped in the rumen solution through the fistula and observed that the transgenic leaves were much lighter than wild type leaves, suggesting that the XynB-transformed rice plant had accelerated bacterial digestibility in the rumen.

Other transgenic rice plants were bred to add a pesticide-degrading ability to remove residual pesticides in soil by bacterial enzyme genes [41]. A partially degraded pesticide component, 3-chlorobenzoic acid is known to be sequentially degraded to adipic acid by 4 enzymes, *cbnA, cbnB, cbnC,* and *cbnD,* found in *Ralstonia eutropha* (Fig. 12). In first step of the degradation, the *cbnA* encoding the chlorocatechol dioxygenase (CbnA) was integrated into the binary vector for plant transformation under the CaMV35S promoter [41]. Thereafter, the recombined vector was used to transform rice plant. The expression of CbnA protein in the transgenic rice plant was detected by using CbnA antibodies in leaves, stems, grains and roots. The cell free extract of the transgenic leaves revealed activity which converted 3-chlorocatechol to 2-chloromuconate. The expression of CbnA negligibly affected growth of transgenic rice plants.

The two genes, *cbnA* and *cbnB,* of *R. eutropha* were expressed in rice plants. CbnA degraded 3-chlorocatechol to 2-chloromuconate and then CbnB degraded 2-chloromuconate to dienlactone (unpublished findings). For further degradation, we would like to express CbnC and

Form	rods(long)
Cell size	1 by 5 to 10mm
Gram stain	positiveat youngage
Motility	twist
Spore	+(plectridiumform)
Flagellum	peritrichous
GC content	27(mol%)
Relation to oxygen	anaerobe
Nitrarte reduction	-
Starch hydrolysis	+
Esculin hydrolysis	+
Milk coagulation	-
Gelatin hydrolysis	-
Indole formation	-
Catalase	-
Lecitimase	-
Urease	-
Hemolysis	-

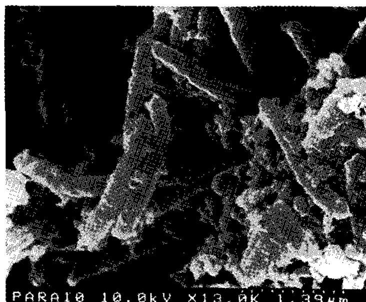

Fig. 13. Characteristics of *Clostridium paraputrificum* and its scanning electronmicrograph.

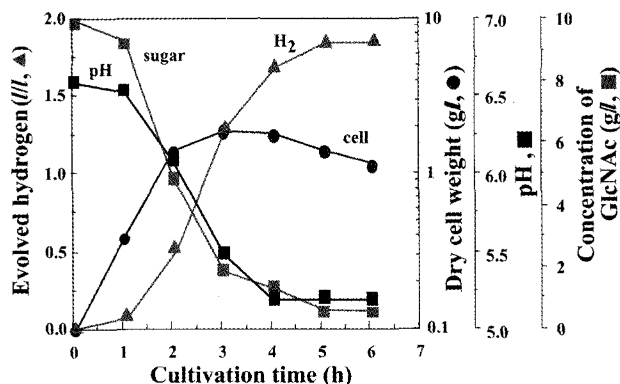

Fig. 14. Time course of the *C. paraputrificum* M-21 cultivation under optimal conditions (Initial medium pH 6.5, 45℃, agitation speed of 250 rpm and working volume, 500 mL).

CbnD in the same rice plant. In addition, we are searching for the promoter sequence, which allows for their expression at the plant root tissue, since residual pesticides are mainly located in the soil.

In conclusion, tobacco and rice plants may be effective plants for the production and accumulation of bacterial enzymes with lower production cost than that of bacterial use, since cultivation of plants by using solar energy, CO_2 and inorganic nutrients is simpler than that of bacteria which inquires complicated organic components as nutrients in the soil of wide field to maintain the recombinant bacterial cells with high population. The results described here may represent novel methodologies for the effective utilization of recalcitrant biomass and plant function by expressing bacterial genes.

Production of Hydrogen Gas from Recalcitrant Unutilized Biomass by Anaerobic Chitin-degrading Bacteria

An anaerobic bacteria, which mainly digests shrimp shell and chitin, was isolated from beach soil at the Mie University campus and was identified as *Clostridium paraputrificum* (Fig. 13) [42]. The organism consume N-acetyl glucosamine, a monosugar of chitin, as the main carbon source within several hours. The rate of bacterial growth doubled every 30 min. The organism produced around 2 L of hydrogen gas and one liter of CO_2 from N-acetyl glucosamine (10 g/L medium) at 37°C (Fig. 14), showing that the organism produced 1.7 mol H_2 gas/mol N-acetyl glucosamine [43]. The productivity of hydrogen was close to the reported values in other papers (2 mol H_2/mol glucose) [43].

We had cloned and characterized certain genes from our isolate, including *chiA* which encodes a chitin-degrading enzyme [44,45]. ChiA degraded chitin, colloidal chitin, glycol chitin, and 4-methyl unberiferil diacetyl chitobiose. Chitooligomers such as chitotetraose, chitopentaose and chitohexaose were mainly degraded to chitobiose. ChiA adsorbed to chitin as well as cellulase to cellulose. Another chitinase gene *chiB* [46], found downstream of *chiA*, had its promoter sequence on the structure gene of *chiA*, suggesting that these genes existed in a compact form. Since both ChiA and ChiB produced by respective *E. coli* transformants consisted of an 87 kD catalytic domain and a cadherin like chitin-binding domain, they can adsorb to chitin for degradation.

The β-N-acetylglucosaminidase gene from *C. paraputrificum* was cloned [47]. This enzyme belonged to family 3 with a molecular weight of 46 kD and degraded chitooligomers such as chito-hexaose, -pentaose, -tetraose, -triose, and -diose to N-acetylglucosamine. The chitobiose degradation rate was the highest in these substrates. Another β-N-acetylglucosaminidase belonging to family 84 with a molecular weight of 174 kD was also cloned from the organism [48]. The enzyme revealed high homology to a hyaluronidase from *C. perfringens*, but did not hydrolyzed hyaluronic acid. In addition, this enzyme was induced by the substrate chitin. Therefore, the enzyme with high molecular weight was classified as novel N-acetyl glucosaminidase.

C. paraputrificum chitinases, therefore, degraded chitin to chitooligomers and N-acetyl glucosaminidases to N-acetylglucosamine, which might be thereafter taken in to the bacterial cells as a carbon and energy source. Due to metabolism of the compound, the organism generated significant volumes of hydrogen and CO_2 in a ratio of 2:1. If hydrogen can be produced in sufficient quantity from the degradation of unutilized agricultural bioresources and fishery refuse by microorganisms, we may obtain the cleanest source of biofuel and reduce the environmental load by microbial degradation of waste.

Based on these results, we attempted to increase hydrogen productivity from chitinous substrates using our isolates of *C. paraputrificum*. For this purpose, we genetically modified *C. paraputrificum* to improve its hydrogen production ability. The first part of the experiment involved the development of a new host-vector system for *C. paraputrificum* [49]. A host-vector system of *C. perfringens*, a pathogenic anaerobe, was kindly provided by Dr. J. Rood, Professor of Monarsh Unicersity, Australia. Fortunately, the vector for *C. perfringens* functioned in our strain of *C. paraputrificum* as well. Chromosomal DNA was then prepared from our strain, and hydrogenase gene

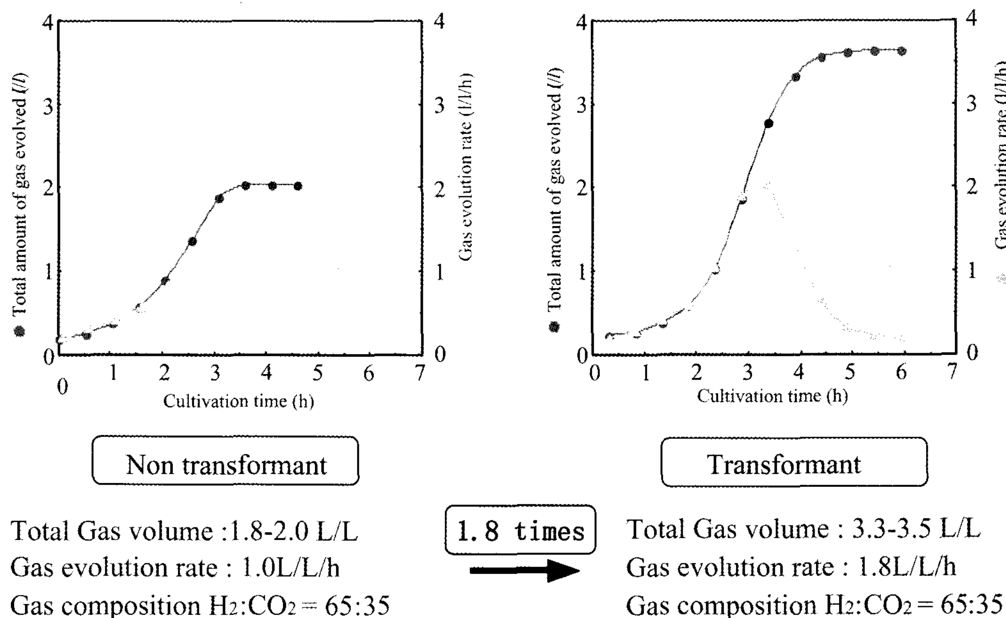

Fig. 15. Increase in hydrogen production by additional expression of a hydrogenase gene in *C. paraputrificum*.

was cloned by PCR using primers containing the conserved sequences in homologous hydrogenase genes from other microorganisms. The DNA sequence of the cloned gene was determined by using a DNA sequencer (Licor 4000L, Lincoln, Nebraska, USA). The gene was integrated into the *C. perfringens* vector and expressed in our strain by electroporation. The transformant bred by the above procedure resulted in an enhanced hydrogen productivity 1.8-fold higher than our wild type strain (Fig. 15) (unpublished findings). Unfortunately, the transformant with the high hydrogen-producing ability depicted low activity for the hydrolysis of high molecular weight substrates, such as chitin. To compensate for this limitation of the transformant, we bred another transformant of *C. paraputrificum* with the enhanced chitinase genes, *chiA* and *chiB*, which increased the chitin degrading ability.

We are still in the process of co-cultivating these transformants to improve hydrogen yield from unutilized biomass and waste.

Microflora with significant hydrogen production was found from refuse-derived fuel (RDF) [50]. RDF is a new fuel, manufactured from municipal waste, which is dried and pelletized to be 1 cm in diameter and several cm in length. RDF is to be burned for heat recovery from waste. Adequate waste disposal is difficult for local rural governments in Japan except this way. However, caution is needed in the use of RDF. Long-term storage (6 months or more) of RDF resulted in explosion of the storage tank. In our experiments, RDF dipped in water at 37°C began to generate flammable gas composed of H_2:CO_2 at a 2:1 ratio (Fig. 16). Total DNA collected from RDF was used to clone 16SrDNAs by PCR. 16SrDNAs were separated in SDS-PAGE and each of them was sequenced to identify bacterial species. All the species in RDF identified had the ability to produce inflammable gas. 50 mL of the gas rotated a small propeller attached to a fuel cell for about one minute. The gas consisted of hydrogen and CO_2 in a 2:1 ratio, similar to that generated by *C. paraputrificum*.

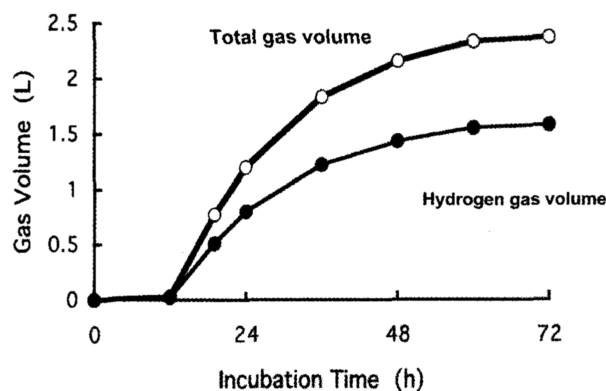

Fig. 16. Hydrogen gas production from RDF soaked in water at 37°C.

Hydrogen producing microflora was also isolated from a compost of rice field soil, rice straw, and pruned tree branches. The microflora isolated from this compost demonstrated cellulolytic activity. Another microflora having starch-degrading activity was also isolated and identified as the hydrogen-producing microflora from garbage and waste drained from nearby restaurants. The hydrogen-producing ability of these microflora could be improved by adding cellulosic and/or starchy materials repeatedly as carbon and energy sources.

We had designed and constructed a biogas fermentor (Fig. 17) which consisted of two vessels, one for hydrogen-producing microflora and another for methane-

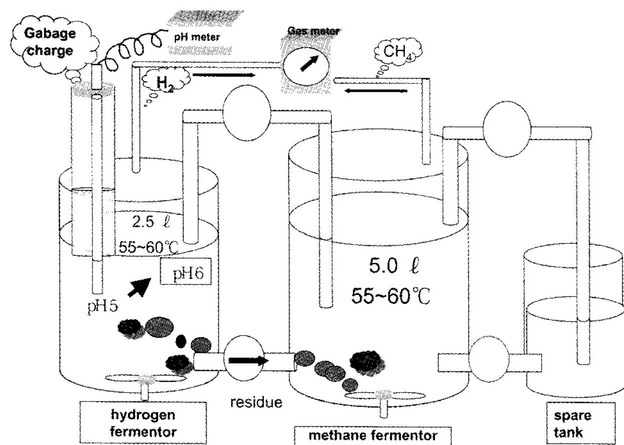

Fig. 17. Two step fermentation of refuse for the production of hydrogen and methane.

producing microflora. In the hydrogen fermentor, hydrogen production was observed within a day but solubilization of solid materials was slower than that occurring in a methane fermentor. Therefore, a combination of the two-step fermentation is now the focus of the NEDO (New Energy Development Organization) project to reduce the bulk of biomass waste and convert it to energy compounds. The pilot study currently taking place at the National Institute of Advanced Industrial Science and Technology (AIST) in Tsukaba, where our microflora are evaluated for hydrogen- and methane-producing capability.

CONCLUSION

The accumulation of unutilized cellulosic biomass is largely a result of modern industrialized life which is characterized by large population increases as well as higher per capita energy and resource consumption. To develop novel approaches to address this problem, we focused attention on enzymatic solubilization or microbial degradation of recalcitrant biomass for the conversion to useful material for human needs or animal consumption. To accomplish these objectives, we isolated and characterized several cellulolytic anaerobes, in which we cloned more than 20 genes encoding the fibrous biomass-degrading enzymes such as cellulase, xylanase and other non-catalytic proteins. Certain enzymes formed part of the cellulosome, a cellulase complex, which organizes synergistic enzyme action for fibrous biomass degradation. The major binding sites for the complex formation are cohesins in scaffolding proteins and dockerins in catalytic proteins. Currently, we are attempting to apply their binding properties for the formation of artificial enzymes, such as dehydrogenase complex for sequential reduction of CO_2 to methanol.

Certain genes such as xylanase A from *C. thermocellum* and xylanasse B from *C. stercorrarium* were expressed in rice plants to accelerate the digestibility of rice straw as animal feed. Another kind of bacterial gene, *cbnA* and *cbnB* from *R. eutropha* were expressed for decomposing residual pesticides in soil.

The chitinolytic anaerobe, *C. paraputrificum* generated hydrogen gas when it was grown on chitinous waste such as lobster and shrimp shells.

The results obtained indicate the important role in microbial conversion of unutilized and/or wasted biomass to valuable materials may play in the near future. Microbial degradation of biomass may contribute to reduce environmental pollution and burden resulting from excessive waste production by humans.

Biomass is bulky organic material that is found throughout the world so would be ideal as a source of fuel. However, its energy density is much lower than fossil fuel. Research into the degradation and possible utilization of biomass is urgently needed for the development of a sustainable society. The studies described here may provide new insights for the development of approaches to preserve the environment.

Acknowledgments K. Ohmiya started to study "Microbial Conversion of Cellulosic Biomass to Valuable Materials" more than 25 years ago when he was a research associate at the Nagoya University, and a member of the cooperative research project between Japan and Thailand, organized by International Center of Biotechnology, Osaka University under the support of The Japan Society for the Promotion of Science. During this period, many Japanese as well as Southeast Asian undergraduate students, postgraduate students, postdoctoral fellows and scientists participated in this study. As the result of their dedicated contribution, this study was awarded by The Society for Biotechnology as "Award for Biotechnology and Bioengineering 2004", He sincerely appreciates from the bottom of his heart all the people who dedicated their valuable time to this study. He also deeply appreciates all the scientists from Japanese universities and The Society for Biotechnology who supported and evaluated our work.

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