和文要旨

河川底泥成分に吸着したセルロース分解活性

劉　文，豊原治彦（京大院農）

東海、近畿及び九州地方の５河川（筑後川，緑川，浜戸川，淀川，田中川）の河口域湿地帯から採集した底泥のセルラーゼ活性を測定した結果、活性レベルは河川により異なっていた。抗生物質で殺菌処理しても底泥は強いセルラーゼ活性を示したことから，セルラーゼの底泥成分への吸着が推測された。上記５河川の底泥のカビ由来セルラーゼの吸着能には違いがあったが，それは底泥中の植物残渣量の違いによるものと考えられた。本研究は、河川底泥においてセルラーゼが植物残渣等の底泥成分に吸着して分解機能を発現している可能性を示唆した。

キーワード：湿地帯、底泥、セルラーゼ、泥、植物残渣

Sediment-complex-binding cellulose breakdown in wetlands of rivers

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Abstract We have been assessing the activity level of cellulase in wetland sediments to clarify the significance of cellulase for the turnover of plant cellulose in wetlands. In the present study, we investigated the cellulose degrading function of sediment in wetlands to clarify the biochemical breakdown mechanism of cellulose. Specifically, we measured cellulase activities of sediments collected from wetlands of the Chikugo River (Fukuoka Prefecture), Midori River (Kumamoto Prefecture), Hamado River (Kumamoto Prefecture), Yodo River (Osaka Prefecture), and Tanaka River (Mie Prefecture). The results revealed that the activity levels differed significantly among rivers. Additionally, the cellulase activities of the sediment were not completely suppressed in the presence of chloramphenicol. These findings suggested that a portion of the cellulase activities were derived from cellulases binding to the components of sediments. Actually, sediments also showed the ability to bind fungal cellulase. Comparison of the binding ability of clay and plant residues, the main components of sediments, revealed that plant residues had significantly higher abilities to bind cellulase. This finding was supported by the fact that there was a strong correlation between the organic matter content in the sediment and the cellulase binding ability (R = 0.66). Results of the present study show that sediment complexes harboring cellulases might be function as a bioreactor to degrade cellulose in wetlands.

Keywords Bioreactor ·Cellulase ·Clay ·Plant residue ·River ·Sediment ·Wetland

Introduction

Cellulose is the main component of the cell walls of plants. Cellulose that is transported from forests to wetlands by rivers is assumed to be utilized by a variety of organisms as a carbon source [1,2,3,4,5,6]; however, the details of the degradation process of cellulose in wetlands remain unknown.

Cellulose is a high molecular weight polysaccharide comprised of glucose bound by β-1, 4 linkage that is biochemically stable when compared with starch, in which glucose is bound by α-1, 4 linkage and α-1, 6 linkage. Accordingly, a series of enzymes such as endo-β-glucanase, cellobiohydrolase, and β-glucosidase, which are collectively designated as cellulases, are required for the enzymatic breakdown of cellulose [7].

Until recently, it has been assumed that herbivores digest cellulose using cellulases derived from the symbiotic microorganisms [8]. Moreover, cellulases in invertebrates, including insects, were long been assumed to originate from symbiotic microbes before demonstration of the endogenous origin of termite cellulase [9]. During the last decade, cellulase genes have been reported in the aquatic organisms such as crayfish [1], mussel [2], abalone [3], bivalve [4], and sea urchin [5].

In addition to these macrobenthos, other organisms such as meiobenthos, fungi, and bacteria are expected to be involved in the degradation of cellulose in the sediments

of wetlands [6,10,11]. In the present study, we attempted to identify organisms contributing to the degradation of cellulose in sediments. The results revealed that cellulases derived from organisms are bound to the components of sediments such as clay and plant residues. Overall, the results of the present study suggest that sediments in wetlands function as a bioreactor to degrade cellulose.

Materials and Methods

Collection of sediments

Sediments from the Tanaka River (Mie Prefecture) were collected on October 20, 2009 and September 8, 2010. Sediments from the Yodo River (Osaka Prefecture) were collected on September 23, 2009. Sediments from the Chikugo River (Fukuoka Prefecture), Hamado River (Kumamoto Prefecture), and Midori River (Kumamoto Prefecture) were collected on October 29, 2010. We selected one collecting site within 50 m from the river mouth and transported these samples at 4 ℃ back to the laboratory at Kyoto University. Sediment samples were stored at 4 ℃ until analyses. Macrobenthos such as bivalves and crustaceans were removed prior to collection of the sediments. Upon arrival at the laboratory, meiobenthos such as nematodes and oligochaetes were carefully removed using tweezers in conjunction with microscopic observation (OLYMPUS-SZX12, OLYMPUS, Tokyo, Japan). To remove the meiobenthos

completely, the sediments were further filtered through a 63 μm mesh and the pass-through fraction was used for the subsequent experiments.

Measurement of cellulase activity of sediments

The cellulase activities of sediments collected from rivers were estimated by measuring reducing sugar released from carboxymethyl cellulose (CMC, Sigma, St. Louis, MO, USA) according to the method described by Somogyi-Nelson [12]. Briefly, 5.0 g of sediment were mixed with 0.5 ml toluene, 10 ml of 0.2 M sodium acetate buffer (pH 5.9) and 10 ml of 1% CMC solution. The CMC was dissolved in water to give a 1% solution. For the blank, water was used instead of CMC solution. Following incubation at 30 ℃ for 24 h, the reaction mixture was centrifuged for 10 min at 2,500 ×*g*, and 1 ml of the supernatant was then transferred to another tube and combined with 1 ml of Somogyi solution (1.2% $KNaC_4H_4O_6 \cdot 4H_2O$, 2.4% Na_2CO_3, 0.4% $CuSO_4$, 1.6% $NaHCO_3$, 18% Na_2SO_4). The mixture was then incubated at 100 ℃ for 10 min. Next, the samples were cooled with cold water, then 1 ml of Nelson solution (1.2% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15% $Na_2HAsO_4 \cdot 7H_2O$, 4.2% H_2SO_4) and 18 ml of water were added. The absorbance at 600 nm was then measured using a spectrophotometer (UV-mini-1240, Shimadzu, Kyoto, Japan) and the activity was represented as the reducing sugar released by 1 g of sediment for 1 h [12]. Unless otherwise specified, reagents of specific grades were purchased from nacalai tesque (Kyoto, Japan).

Effect of chloramphenicol on cellulase activity of sediments

To determine if cellulase activity detected in the sediments was derived from microorganisms, the effects of antibiotics on the cellulase activity of the sediments were investigated. As a preliminary experiment, the effects of ampicillin, kanamycin, tetracycline, and chloramphenicol on the growth of microorganisms in the sediment were tested. Sediments collected from the Midori River were mixed with 400 mg, 40 mg, or 4 mg of ampicillin, kanamycin, tetracycline, or chloramphenicol per gram of wet sediment, after which they were incubated at 37 ℃ for 24 h. The effects of antibiotics were evaluated by plating the incubated sediments on LB (Luria Bertani medium) plates and incubating for five days. Only chloramphenicol (400 mg and 40 mg per 1g sediment) completely inhibited the growth of the microorganisms including bacteria and fungi. Thus, we selected chloramphenicol to sterilize microorganisms in the sediments.

A solution of 2 ml of chloramphenicol dissolved in 50% ethanol to give 100 mg/ml was added to 5 g of per gram of wet sediment collected from the Tanaka and Midori Rivers, which gave 40 mg chloramphenicol per gram of wet sediment in a final concentration. These samples were then incubated for 24 h at 30 ℃. As a control, 2 ml of 50% ethanol in water was added. After incubation, the sediment was spread on a LB plate to validate the sterilizing effect of chloramphenicol on microorganisms.

Meanwhile the sediment was spread on CMC agar plates containing 1% CMC, 0.15% $Ca(NO_3)_2$, 0.05% $MgSO_4$, 0.05% K_2HPO_4 and 1.5% agar and the samples were then incubated at 30 ℃ for three days to detect the remaining cellulase activity of the

sediment (data not shown). Then, the remaining cellulase activities of the two rivers were quantified by Somogyi Nelson method described above [12].

Evaluation of cellulase binding ability of sediments

Sediments collected from all rivers were autoclaved at 121 ℃ for 15 min to inactivate sediment-bound cellulases after vigorous washing with water. After cooling, the following procedures were conducted in the clean bench. Two grams (dry weight) of autoclaved sediment and 6.25 mg of *Aspergillus niger* cellulase (MP Biomedicals, California, USA) dissolved in 5 ml H_2O were mixed and shaken for 1 h at room temperature to bind the cellulase to the sediments. After centrifugation at 2,500 ×*g* for 10 min, the pellet was vigorously washed twice with excess water to remove the unbound cellulase. The cellulase activities of the sediments were measured as described above to determine cellulase binding ability. The activity was expressed as the reducing sugar released per dry weight of sediments.

Comparison of cellulase binding ability between clay and plant residues

Sediments collected from the Midori River were separated into clay and plant residues using tweezers in conjunction with microscopic observation. To inactivate the originally bound cellulase, separated clay and plant residues were autoclaved for121 ℃ for 15 min. Next, the commercially obtained fungal cellulase and autoclaved clay or plant residues

were mixed for 1 h at room temperature as described above. After washing vigorously with an excess amount of water, the cellulase activities bound to clay and plant residues were measured, respectively. The activity was expressed as the reducing sugar released per dry weight of clay or plant residues.

Correlation between organic matter content and cellulase binding ability

Sediments were heated at 600 ℃ for 3 h using a mantle heater (KCA-10A, Koito, Tokyo, Japan). The organic matter content was determined based on the loss on ignition values. The correlation between the cellulase binding ability determined in Table 3 and the organic matter content was then estimated.

Statistical analyses

All data were statistically analyzed by ANOVA.

Results

Cellulase activity of sediments from five rivers

Table 1

As shown in Table 1, sediments collected from all five rivers exhibited cellulase activities. However, the activity levels differed among rivers. Specifically, sediments

from the Yodo River showed significantly higher activity than those from the Hamado River, Midori River and Tanaka River, while those from the Chikugo River had higher values than those from the Midori River and Tanaka River. Sediments from the Hamado River showed significantly higher activity than those from the Tanaka River. However, the activity levels differed among rivers so far as compared by using the data obtained from the collecting sites of each river.

Effect of chloramphenicol on the cellulase activity of sediment

Table 2

As shown in Table 2, chloramphenicol exhibited no effect on the cellulase activity of sediment from the Midori River, while it showed a partially inhibiting effect on that of the Tanaka River. These findings suggest that part of the cellulase activity of the Tanaka River was derived from microorganisms sensitive to chloramphenicol. It should be stressed that a substantial amount of the activity of sediments from both rivers remained, even in the presence of chloramphenicol, suggesting that these activities were derived from cellulases extracellularly secreted from microorganisms and /or benthic animals.

Binding of fungal cellulase to the sediments

Table 3

The finding above suggested us that cellulases secreted from organisms would directly bind to sediments under natural condition. To validate this, we examined the binding ability of sediments to commercial available fungal cellulase. As shown in Table 3,

sediments collected from all five rivers showed fungal cellulase binding ability (Table 3). The sediments from Tanaka River showed significantly lower binding ability than those from Hamado River and Chikugo River, while those from Yodo River showed lower binding ability than those from Chikugo River.

The finding clearly suggested that sediment has the ability to bind cellulase. Sediments are mainly composed of clay and plant residues. Thus, we subsequently compared the cellulase binding ability between clay and plant residues using sediment collected from the Midori River. The result revealed that 11.7 ± 2.3 μg/g of cellulase was bound to clay, while 419.5 ± 47.1 μg/g of cellulase was bound to plant residues. These results suggest that plant residues have significantly higher ($P < 0.05$) cellulase binding ability than clay.

Correlation between organic matter content and cellulase binding ability

Fig.1

As shown in Fig. 1, the organic matter content varied among rivers. Organic matter in sediment was assumed to primarily consist of plant residues. For example, sediment from the Midori River included a large content of plant resides (14.2%), while that of the Tanaka River included a small amount of plant residues (1.3%). A strong correlation between cellulase binding ability and the organic matter content in the sediments was

also observed (R = 0.66), suggesting that plant residues can function as an efficient binder of cellulase in the sediment.

Discussion

A significant amount of cellulase activities remained in the presence of chloramphenicol (Table 2). When sediments incubated in the presence of chloramphenicol were inoculated onto agar plates containing LB medium without chloramphenicol, no colonies of microorganisms (fungi or bacteria) were observed during incubation for five days (data not shown), suggesting that microorganisms in the sediment were completely attenuated by chloramphenicol. Based on these findings, we assumed that sediment-complex-binding cellulases secreted from microorganisms and/or benthic animals function as a bioreactor independent of organisms under natural conditions.

High cellulase activities of sediments in the Yodo River and Chikugo River were detected, while low activity was observed for the Tanaka River (Table 1). This is because sediments from the Tanaka River showed low cellulase binding ability, possibly due to the low content of plant residues (Table 3 and Fig. 1). The cellulase activity in sediment from the Midori River was significantly lower than that of the Yodo

River (Table 1), although the cellulase binding ability of the sediment from the Midori River was not significantly different from Yodo River (Table 3). As described above, these results suggest that a significant amount of cellulase was secreted extracellularly from microorganisms and/or benthic animals. Accordingly, these findings strongly suggest that the low cellulase activity in sediment of the Midori River could be ascribed to the low level of cellulase supplied by microorganisms and/or benthic animals.

The results shown in Table 3 suggest that river sediments have cellulase binding ability but that the level differed among rivers. As shown in Fig.1, the cellulase binding ability of sediments depends on the content of plant residues. Indeed, there was a strong correlation between the organic matter content in the sediments and cellulase binding abilities (R = 0.66). Organic materials in sediments were considered to be almost entirely a result of plant residues because no weight loss was observed before and after heat treatment of sediments from which the plant residues were removed by tweezers as possible as carefully (data not sown). The difference in the level of cellulase activity of the sediments of various rivers could be ascribed to the content of plant residues as well as the content of cellulase supplied by microorganisms and/or benthic animals.

To confirm that plant residues had a higher binding ability than clay, we separated plant residues and clay from the sediment of the Midori River and compared the cellulase binding abilities. Results indicate that the plant residues exhibited approximately 36 times higher cellulase binding than clay. These findings strongly suggest that plant residue functions as an efficient binder of cellulase in the sediment.

Overall, this is the first study to demonstrate that sediments composed of clay and plant residues bind cellulase and act as a bioreactor independent of organisms. We are now identifying the organisms that secrete cellulases and will report these results in the near future.

Acknowledgement

This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 21380131).

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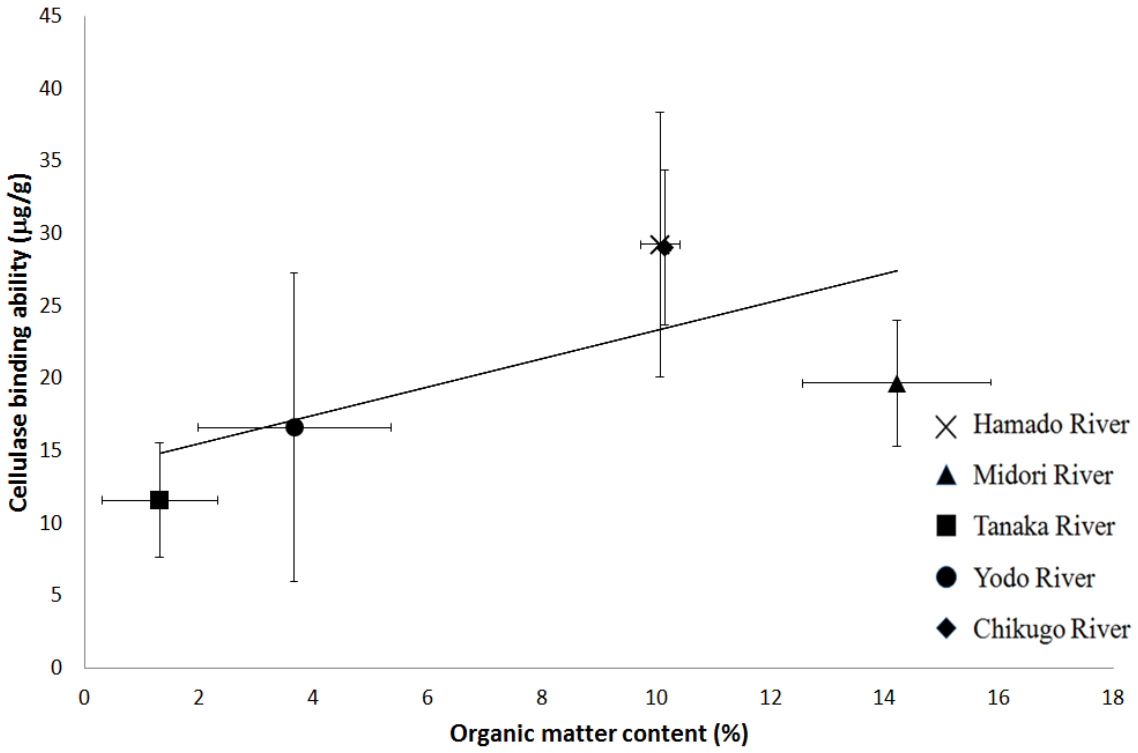
Figure caption

Fig. 1 Correlation between the organic matter content and the cellulase binding ability of the sediments from various rivers. A strong correlation was demonstrated between them ($R = 0.66$).

Figure

Fig. 1


Cellulase binding ability (μg/g)
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
Organic matter content (%)
Hamado River
Midori River
Tanaka River
Yodo River
Chikugo River

Table

Table 1 Cellulase activity of the sediments collected from five rivers

Collecting sites	Chikugo River	Midori River	Hamado River	Yodo River	Tanaka River
Cellulase activity (nmol/gh)	$59.2 \pm 26.0^{a,d}$	$22.5 \pm 3.4^{b,c}$	$33.6 \pm 8.45^{a,c,}$	77.6 ± 7.1^{d}	12.9 ± 8.2^{b}

Different letters indicate a significant difference ($P < 0.05$). Values are means $\pm$ SD (n = 3).

Table 2 Effect of Chloramphenicol on cellulase activities of the sediments from Tanaka River and Midori River

Collecting sites	Tanaka River		Midori River	
	Chloramphenicol		Chloramphenicol	
	+	-	+	-
Cellulase activity (nmol/gh)	27.4 ±1.7[a]	43.1 ±4.7[b]	18.8 ±2.0[a]	28.3 ±7.7[a]

Concentration of chloramphenicol was 40 mg/g sediment. The statistical analyses on both rivers were performed independently. Different letters indicate a significant difference ($P < 0.05$).

Table 3 Binding of fungal cellulase to the autoclaved sediments collected from five rivers

Collecting sites	Chikugo River	Midori River	Hamado River	Yodo River	Tanaka River
Cellulase binding ability (μg/g)	29.0 ±5.4[a]	19.7 ±4.3[b]	29.2 ±9.13[a,b]	16.6 ±10.6[b,c]	11.6 ±4.0[c]

Values are means ±SD (n = 3). Different letters indicate a significant difference (P < 0.05).

Reviewer #2: The reviewer admits that the manuscript has been improved from the original version; however, there are still many points that should be addressed by the authors.

Materials and Methods

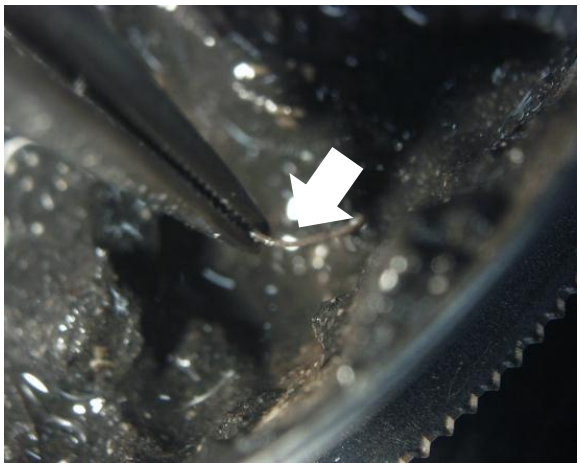
- *L86: $KNaC_4H_4O_6 \cdot Na_2O$ ---- Na_2O may be $4H_2O$.*

  -We have corrected it as the reviewer suggested.

- *L89: $Na_2HAsO_4 \cdot As_2O$ ---- As_2O may be $7H_2O$*

  -We have corrected it as the reviewer suggested.

- *L133-L134 --- The reviewer cannot imagine how the authors distinguished clay and plant residues and how separated them using tweezers? Clay may be too small to pick up with tweezers.*



  -As shown in the photo, clay and plant could be separated easily by tweezers, because the shapes of them completely different. The arrow indicates a plant residue.

- *L136: How many grams of clay and plant residues did the authors use for cellulase-binding assay?*

  -We used approximately 5g (in wet condition) of clay and plant residues for the assay.

Results

- *L165: exhibited no effect on ... from the Midori River, --- The difference in the cellulase activity between plus and minus chloramphenicol for Midori River*

sample, i.e., 18.8 vs 28.3, indicates that the effect of CP is not negligible. The reviewer cannot agree the authors' explanation "exhibited no effect".

-Because of large SD of minus CP (28.3±7.7) we could not recognize a significant difference between the values of plus CP and minus CP in the Midori River samples. Thus, we considered that CP exhibited no effect.

- *L178: The sediment from Tanaka River showed significantly lower… ---- The reviewer does not think that the value for Tanaka River is "significantly" low. The value for Tanaka River is comparable level with that for Yodo River.*

-We would like to show that there is a statistical difference between Tanaka River and Yodo River. We revised as follows to avoid misunderstanding.
Line178-180:"The sediments from Tanaka River showed statistically lower binding ability than those from Hamado River and Chikugo River."

Discussion

- *L203-204: secreted from … and/or benthic animals ---- Do the benthic animals secrete cellulase to the sediment? Generally, the benthic animals are considered to ingest plant tissues and digest them in the digestive tract with cellulase secreted in the tract.*

-We found that cellulases are secreted in feces as active form. We will report this in the next paper.

- *L224: the plant residues were completely removed --- How did the authors confirmed the "complete" removal of plant tissues.*

-It is easy to remove plant residues by tweezers as described above from their shapes. However, as suggested by the referee, it is difficult to declare to be "complete". Thus we revised the sentence as follows in the new manuscript.
L222-225: Organic materials in sediments were considered to be almost entirely a result of plant residues because no weight loss was observed before and after heat treatment of sediments from which the plant residues were removed by tweezers as possible as carefully (data not sown).

- *Fig. 1. The values should be presented as the average values with SDs.*

-According to the suggestion, we added SDs in the figure.

- *Table 1-3. What do "a-d" mean?*

-These alphabet letters show the statistical difference. If the two values are attached by different letters such as “a” and “b”, it means both values are statistically different. This expression is usually used to demonstrate a statistical difference between values. For example, please refer Tables 3-8 in the paper published in Fisheries Science 77, 357-365 (2011).

- *Table 2. The activities for Tanaka River and Midori River in the absence of chloramphenicol, 43.1 and 28.3, are inconsistent with those in Table 1. How can the author explain?*

-We collected sediments from Tanaka River on October 20, 2009 used for the experiment in Table 1, and September 8, 2010 used for the experiment in Table 2. Therefore, the activities in Table 1 (12.9±8.2) and Table 2 (43.1±4.7) are inconsistent. This is possibly because the slight difference in the collecting sites, difference in the month and/or the difference in weather condition between 2009 and 2010.
As for Midori River, we used the same sediments collected on October 29, 2010 . Thus, no statistical difference was observed; 22.5 ±3.4（Table 1）and 28.3 ±7.7 (Table 2).

- *Table 3. The unit "nmol/gh" is not suitable for indication of "Cellulose binding ability".*

-According to the suggestion, we expressed “cellulase binding ability” as the ability to bind the amount of cellulase per one gram weight of dry sediment. To determine the amount of cellulase, we validated the relationship between cellulase activity (absorbance at 600 nm) and amount of fungal cellulase (μg) as shown below. The amount of cellulase binding to sediment was determined according to the formula inserted in the figure. Cellulase binding ability shown in Table 3 is expressed as the value which was determined by dividing the amount of cellulase by the dry weight of sediment.

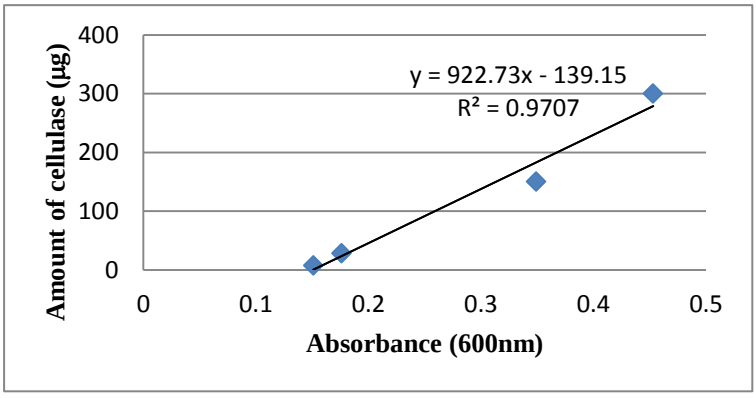


Fig. Relationship between the cellulase activity (absorbance at 600 nm) and amount of fungal cellulase (μg).

By this change, the results of statistical analysis in Table.3 and Fig.1 were altered. In Table.3, altered portions are shown in red letters as described below.

Table 3 Binding of fungal cellulase to the autoclaved sediments collected from five rivers

Collecting sites	Chikugo River	Midori River	Hamado River	Yodo River	Tanaka River
Cellulase binding ability (μg/g)	29.0 ±5.4[a]	19.7 ±4.3[b]	29.2 ±9.13[a,b]	16.6 ±10.6[b,c]	11.6 ±4.0[c]

Values are means ±SD (n = 3). Different letters indicate a significant difference (P < 0.05).

In Fig.1, the slope of line was altered. Sentences in the manuscript corresponded were also altered as described below (altered portions are shown in red letters):

Line 26-27: This finding was supported by the fact that there was a strong correlation between the organic matter content in the sediment and the cellulase binding ability (R = 0.66).

Line 179-181: ... while those from Yodo River showed lower binding ability than those from Chikugo River.

Line 184-185: The result revealed that 11.7 ± 2.3 μg/g of cellulase was bound to clay, while 419.5 ±47.1 μg/g of cellulase was bound to plant residues.

Line 193-195: A strong correlation between cellulase binding ability and the organic matter content in the sediments was also observed (R = 0.66),...

Line 210-213: The cellulase activity in sediment from the Midori River was significantly lower than that of the Yodo River (Table 1), although the cellulase binding ability of the sediment from the Midori River was not significantly different from Yodo River (Table 3).

Line 220-222: Indeed, there was a strong correlation between the organic matter content in the sediments and cellulase binding abilities (R = 0.66).

Line 230-231: Results indicate that the plant residues exhibited approximately 36 times higher cellulase binding than clay.

Line 274-277: Fig. 1 Correlation between the organic matter content and the cellulase binding ability of the sediments from various rivers. A strong correlation was demonstrated between them (R=0.66)