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Original Paper

Difference of saccharification yields between organs and growth stages in rice

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Abstract Saccharification is a key step in the efficient production of biofuels and biomaterials from cellulosic biomass. We examined saccharification yields from leaf blades, leaf sheaths and stems at several growth stages in rice. We found that saccharification yields were high before heading and reduced after heading in all three organs examined. Stems showed highest saccharification yields at all growth stages examined, and leaf blades showed lowest saccharification yields. Differences of saccharification yields between rice cultivars were also observed. Our results indicate that saccharification yields are different between rice organs. This suggests that the proportion of organs is one of the determinants of saccharification yields of rice straws, and thus it will be a breeding target for biofuel and biomaterial crops with high saccharification yields. Our results also suggest that the harvesting stage is critical for high saccharification yields.

Key words: Saccharification, straw, rice.

Cellulose, which is a major component of the plant cell wall and the most abundant biomass, is an indispensable and valuable material for production of renewable biofuels and biomaterials, but the physical strength and chemical stability of plant cell walls make it difficult to degrade them and efficiently produce fermentable sugars such as glucose (Hendriks and Zeeman 2009). To overcome this problem, a number of studies have been carried out (Alper and Stephanopoulos 2009; Sainz 2009). These studies focused on pre-treatment of plant cell walls to fractionate its components and remove lignin, and on engineering microorganisms to enhance saccharification and fermentation abilities. In addition, breeding of material plants by mutations or by genetic engineering using cell wall-degrading enzymes has also been carried out (Taylor et al. 2008).

Straws are considered to be useful materials for biofuel and biomaterial production. It was reported that cell wall compositions were different between organs in wheat, switchgrass and *Brachypodium* (Bhandari et al. 2013; Rancour et al. 2012; Zhang et al. 2014). Because cell wall compositions affect saccharification yields, this suggests that saccharification yields are different between organs. If this is the case, the proportion of each organ in straws should affect saccharification yields of entire straws, which consist of leaf blade, leaf sheath and stems. In this case, modification of proportion of organs in straws will be one of the targets for breeding of material plants with high saccharification yields. However, saccharification yields of each of these three organs in straws have yet to be extensively analyzed. In addition, the composition of cell walls was also reported to be different between plant developmental stages in *Brachypodium* (Rancour et al. 2012). This suggests that a harvesting stage also affects saccharification yields. This also needs to be examined.

Several studies have been carried out to enhance saccharification yields from rice straws (Furukawa et al. 2013; Furukawa et al. 2014; Nigorikawa et al. 2012; Sumiyoshi et al. 2013). These studies include expression of enzymes that catalyze cell wall components such as lignin, cellulose or hemicellulose. If saccharification yields are different between organs in straws, changes of proportion of each organ in straws will also contribute to the enhancement of saccharification yields of entire straws. However, no study has yet analyzed saccharification yields of each organ in straws.

Abbreviations: DAH, day after heading.

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In addition, if saccharification yields are different between growth stages of rice, harvesting rice straws at an appropriate stage is also important for efficient saccharification.

In this study, we examined saccharification yields of three organs in rice straws at four growth stages. Our results showed that saccharification yields were greatly different between organs and also between growth stages.

Materials and methods

Plant materials

Oryza sativa cultivars Koshihikari (a japonica type cultivar) and Kasalath (an indica type cultivar) were used. Plants were grown in pots in a green house under natural temperature and light conditions. A heading date of each panicle was labeled. Plants were grown from April to September or October in 2013 and 2014 at the Graduate School of Agricultural Science, Tohoku University, in Sendai, Japan.

Enzymatic saccharification

Rice straws were harvested at an indicated growth stage and dried at 105°C for 1 to 2 h. For a saccharification analysis of entire straws, straws were directly subjected to grinding into powder. For a saccharification analysis of each organ, leaf blades, leaf sheaths and stems were separated and then subjected to grinding into powder. Enzymatic saccharification was carried out as previously described (Furukawa et al. 2014). Dried and grounded samples were fractionated with mesh to collect particles at sizes less than 77 μm in diameter. The samples (15 mg each) were incubated at 50°C for 48 h in a 1 ml reaction mixture containing 100 mM sodium citrate (pH 4.8), 0.03 FPU of Celluclast 1.5L (Sigma-Aldrich) and 0.12 units of Novozyme 188 (Sigma-Aldrich). Reactions without the enzymes were also carried out similarly. Reducing sugars were measured with the DNS method (Sumner 1921). Saccharification yields were calculated by subtracting the value of the reactions without the enzymes from that with the enzymes, and they were shown as a percentage of saccharified biomass for the biomass used for the reaction.

Analysis of components of a straw

Cell wall fraction was prepared by the method described by Ishii et al. (2001). Cell wall components were quantified as described by Sato et al. (2001). In addition, the residual substances composed of lignin (Klason lignin) and ash were heated for 6 h at 750°C to yield ash fraction, and the weight decreased by the volatilization was designated as the Klason lignin fraction.

Measurement of proportion of a straw

Rice straws at heading and at 40 DAH were dried at 105°C for 1 to 2 h and dissected into leaf blades, leaf sheaths and stems. Dry weight of each organ was measured and a percentage of each organ in a tiller was calculated.

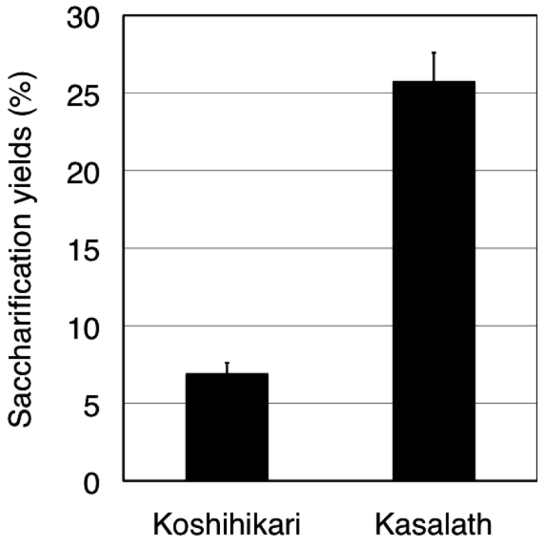

Figure 1. Saccharification yields of rice straws. Rice straws were harvested when the first tiller showed heading. Saccharification yields were shown as percentages of enzymatically saccharified biomass for the biomass used for the reaction. Error bars indicate standard errors.

Results

Saccharification of rice straws

We examined saccharification yields of entire straws of two rice cultivars, Koshihikari (a japonica cultivar) and Kasalath (an indica cultivar). Koshihikari is an elite cultivar widely cultivated in Japan, and Kasalath is often used for a genetic analysis in combination with a japonica cultivar. We harvested rice straws when the first tiller showed heading, and thus the samples were a mixture of tillers at heading and those before heading. Three individual stocks of straws were separately used for the experiments, and the averages were shown. The result showed that saccharification yields were different between these two cultivars (Figure 1). Kasalath showed higher saccharification yields than Koshihikari. This suggests that saccharification yields are different between cultivars at heading. This difference may be caused by compositions of organs in the straws, growth stages of each organ and different saccharification yields of each organ at the same growth stage. To examine these possibilities we analyzed in detail saccharification yields of each organ of Koshihikari and Kasalath at several different growth stages.

Saccharification of leaves at sixth and tenth leaf stages

We examined saccharification yields of young growing rice plants. Leaf blades and leaf sheaths at the sixth and tenth leaf stages were subjected to a saccharification analysis. Three leaves are separately used for the experiments, and the averages were shown. The results showed that in both Koshihikari and Kasalath, tenth leaf blades showed higher saccharification yields than

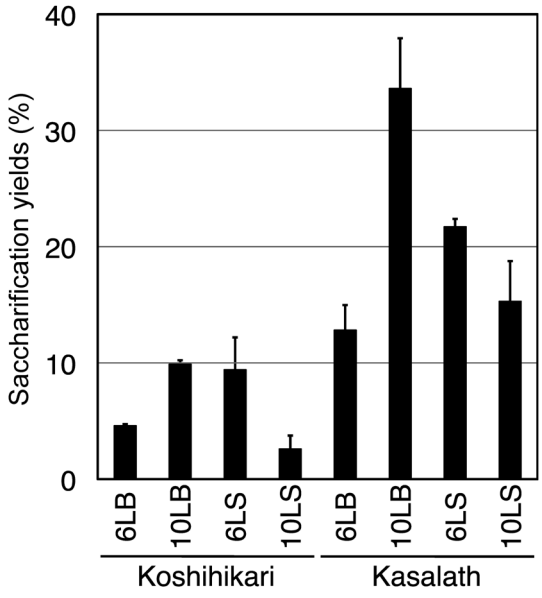

Figure 2. Saccharification yields of leaves at growing stages. Blades and sheaths of the sixth leaves and tenth leaves were subjected to saccharification analysis. Saccharification yields were shown as percentages of enzymatically saccharified biomass for the biomass used for the reaction. Error bars indicate standard errors. 6LB: 6th leaf blade; 10LB: 10th leaf blade; 6LS: 6th leaf sheath; 10LS: 10th leaf sheath.

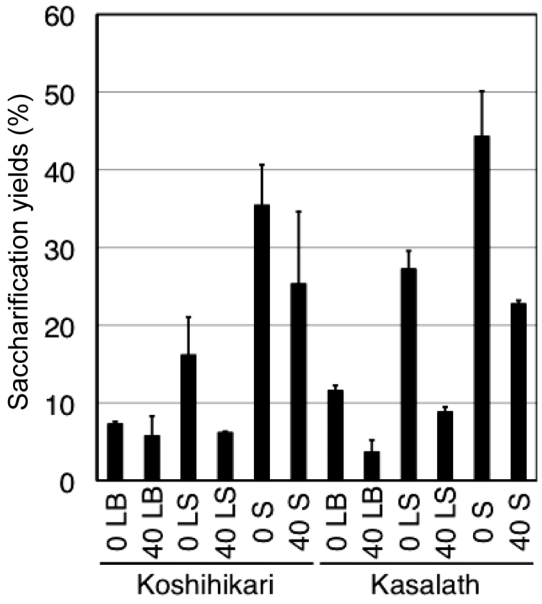

Figure 3. Saccharification yields of rice straws in 2013. Samples were grown in pots in the experimental fields in 2013. Leaf blades, leaf sheaths and stems at heading and 40 DAH were subjected to the saccharification analysis. Saccharification yields were shown as percentages of enzymatically saccharified biomass for the biomass used for the reaction. Error bars indicate standard errors. 0LB: leaf blade at heading; 40LB: leaf blade at 40 DAH; 0LS: leaf sheath at heading; 40LS: leaf sheath at 40 DAH; 0S: stem at heading; 40S: stem at 40 DAH.

the sixth leaf blades, while the tenth leaf sheaths showed lower saccharification yields than the sixth sheaths (Figure 2). Kasalath showed higher saccharification yields than Koshihikari in each sample. This indicates that saccharification yields were different between the leaf blade and leaf sheath, between the two leaf stages, and between the two cultivars.

Saccharification of rice straws at heading

Tillers were harvested at heading and a single tiller was subjected to a saccharification analysis. Leaf blades and leaf sheaths from a flag leaf and its prior leaf were used. The first and second internodes were used as a stem. Experiments were carried out in 2013 and 2014, and similar results were obtained. In each year the experiments were repeated three times using three different tillers. The results showed that a stem showed higher saccharification yields than leaf blades and leaf sheaths in both Koshihikari and Kasalath (Figure 3). Comparing leaf blades and leaf sheaths, leaf sheaths showed higher saccharification yields than leaf blades in both cultivars (Figure 3). This indicates that saccharification yields were different between rice organs at heading. Similar results were obtained in another year (Supplemental data).

Saccharification at 40 DAH

Rice straws were harvested at a grain-harvesting stage, which is around 40 DAH. The samples were similarly collected and separated into leaf blades, leaf sheaths

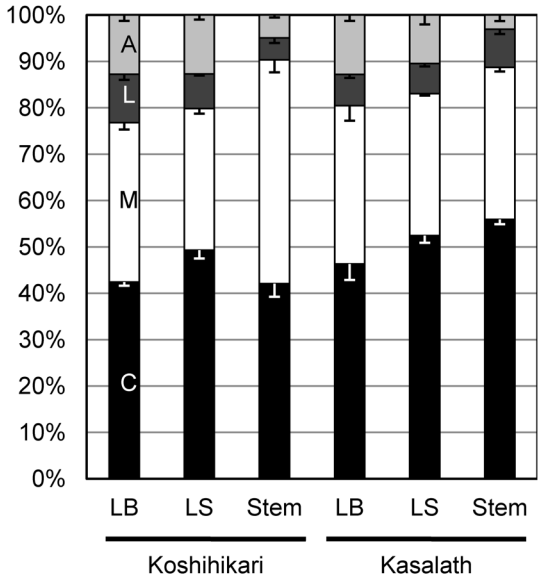

Figure 4. Composition of cell walls of leaf blades, leaf sheaths and stems at heading. A: Ash, L: lignin, M: matrix polysaccharides, C: cellulose, LB: leaf blade, LS: leaf sheath.

and stems. The results indicated that a stem showed much higher saccharification yields than leaf blades and leaf sheaths, and leaf sheaths showed slightly higher saccharification yields than leaf blades in Kasalath, but they were comparable in Koshihikari (Figure 3). This indicates that saccharification yields were different

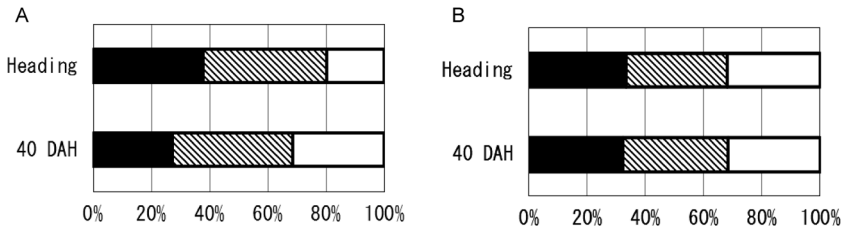

Figure 5. Proportion of organs in rice straws. A: Koshihikari. B: Kasalath. Dry weights of leaf blades, leaf sheaths and stems at heading and at 40 DAH were measured, and their proportion was shown by a percentage. Filled bar: leaf blade; hatched bar: leaf sheath; open bar: stem.

between rice organs at 40 DAH, as was the case at heading.

When saccharification yields at heading and at 40 DAH were compared, higher saccharification yields were observed at heading than those at 40 DAH in all three organs examined in both cultivars (Figure 3). This indicates that saccharification yields from rice straws reduce after heading. Similar results were obtained in another year (Supplemental data).

Cell wall composition of each organ of a straw

Since cell wall composition is an important factor that determines saccharification yields, we examined composition of cell walls of each organ at a heading stage. In Koshihikari, the proportion of lignin was inversely correlated with saccharification yields, and stems, which showed high saccharification yields, had less lignin, and leaf blade, which showed low saccharification yields, had more lignin (Figure 4). In Kasalath, the proportion of cellulose was correlated with saccharification yields, and stems had more cellulose than leaf blades (Figure 4). However, the difference of the proportion of cellulose was not as significant as the difference of saccharification yields between organs (Figures 3 and 4). In both cultivars, stems had less proportion of ash compared to leaf blades and leaf sheaths (Figure 4).

Proportion of organs in a straw

Proportion of leaf blades, leaf sheaths and stems in rice straws is a notable factor that determines saccharification yields of entire rice straws, since each organ showed different saccharification yields. To examine possible effects of proportion of organs on saccharification yields of entire straws, we examined a dry weight ratio of these three organs in rice straws at heading and at 40 DAH. Rice straws were dried, separated into leaf blades, leaf sheaths and stems, and the weight of each organ was measured. The results showed that at heading leaf blades, leaf sheaths and stems occupied 38%, 42% and 20% of straws, respectively, in Koshihikari, and 33%, 35% and 32% of straws, respectively, in Kasalath (Figure 5). This indicates that Koshihikari has a lower proportion of stems (a high saccharification yield organ) than Kasalath, and Koshihikari has more leaf blades (a low saccharification yield organ) than Kasalath. At 40 DAH, leaf blades, leaf sheaths and stems occupied 27%, 41% and 31% of straws, respectively, in Koshihikari, and 32%, 36% and 32% of straws in Kasalath (Figure 5). Since no decrease of stems (a high saccharification yield organ) and no increase of leaf blades (a low saccharification yield organ) were observed after heading, proportion of organs in straw is not associated with reduction of saccharification yields after heading.

Discussion

Rice straws are potential biomass that can be used for production of biofuels and biomaterials, and efficient saccharification of rice straws is key for practical production from rice straws, and thus saccharification yields of rice straws will be a target of breeding by conventional methods and genetic engineering (Furukawa et al. 2013; Furukawa et al. 2014; Nigorikawa et al. 2012; Sumiyoshi et al. 2013). In this study, we examined saccharification yields of rice straws. Since all the experiments were carried out using rice plants grown in pots in our experimental fields, we cannot exclude the possibility that different saccharification yields might be obtained under different growth conditions.

We demonstrated that saccharification yields were different between organs and also between growth stages (Figures 2 and 3). Difference of saccharification yields was also observed between two rice cultivars (Figures 1 to 3). Since stems showed higher saccharification yields than leaves, rice straws with a higher proportion of stems and lower proportion of leaves should show higher saccharification yields. In addition, straws at heading showed higher saccharification yields than those at 40 DAH. This indicates that an approach to avoid the reduction of saccharification yields after heading will be one of the ways to enhance saccharification yields of rice straws. Suppression of senescence after heading without affecting grain maturation might be one possible approach.

It is not clear what determines saccharification yields of rice straws. Lignin is known to inhibit saccharification of lignocellulosic biomass, and plants with less lignin show higher saccharification yields (Chen and Dixon 2007). For example, *Arabidopsis* plants with a mutation in a lignin biosynthesis gene showed higher saccharification

yields (Berthet et al. 2011). Switchgrass genetically manipulated to reduce lignin contents also showed high conversion of biomass (Fu et al. 2011; Shen et al. 2012). However, reduction of lignin contents does not always enhance saccharification yields, probably due to the compensation of cell wall strength in rice (Furukawa et al. 2013). In contrast, overexpression of arabinofuranosidase decreased arabinose contents and increased cellulose contents, and this change of cell wall composition brought about an increase of saccharification yields of rice straws (Sumiyoshi et al. 2013). Constitutive overexpression or senescence-induced expression of cellulase in rice also showed enhanced saccharification yields of straws (Furukawa et al. 2014; Nigorikawa et al. 2012). These results suggest that the composition of cell walls as well as the structure of cell walls may affect saccharification yields of rice straws.

To examine possible effects of cell wall composition on the difference of saccharification yields between organs in rice straws, we examined cell wall composition of each organ of rice straws at heading (Figure 4). We showed that the proportion of lignin was inversely correlated with saccharification yields in each organ in Koshihikari. This suggests that proportion of lignin is one of the factors that determine the differences of saccharification yields between organs in Koshihikari. In Kasalath, however, such correlation between lignin and saccharification yields was not observed. Instead, saccharification yields were correlated with the proportion of cellulose, although the difference of the proportion of cellulose between organs in Kasalath was not as significant as the difference of saccharification yields between organs. Thus, the proportion of cellulose may not be a main factor in creating a difference of saccharification yields between organs in Kasalath, even if it had some effect on saccharification yields. It seems likely that various factors, which include proportion of cell wall components such as lignin and cellulose, are associated with differences of saccharification yields between organs in rice straws.

Cell wall composition was also examined in *Brachypodium distachyon* (Rancour et al. 2012). Differing from rice, this grass showed more lignin in stem than in leaf blades. This suggests that cell wall composition in each organ was different between grass species. For enhancement of saccharification yields from biomass by manipulating cell wall compositions, different targets and approaches appropriate to each organ and species should be applied.

We demonstrated that saccharification yields from entire rice straws were different between the two cultivars, Koshihikari and Kasalath (Figure 1). Our results indicate that, in addition to difference of saccharification yields in each organ of the two cultivars, the proportion of organs in straws is also one of the reasons for the difference of saccharification yields of entire straws of the two cultivars. Stems showed higher saccharification yields than leaf blades and leaf sheaths, and the proportion of stems in straws in Kasalath, which showed high saccharification yields at heading, was more than that in Koshihikari, which showed low saccharification yields (Figures 3 and 5). Breeding rice plants with more biomass in stems at harvest will be an approach to enhance saccharification yields of rice straws.

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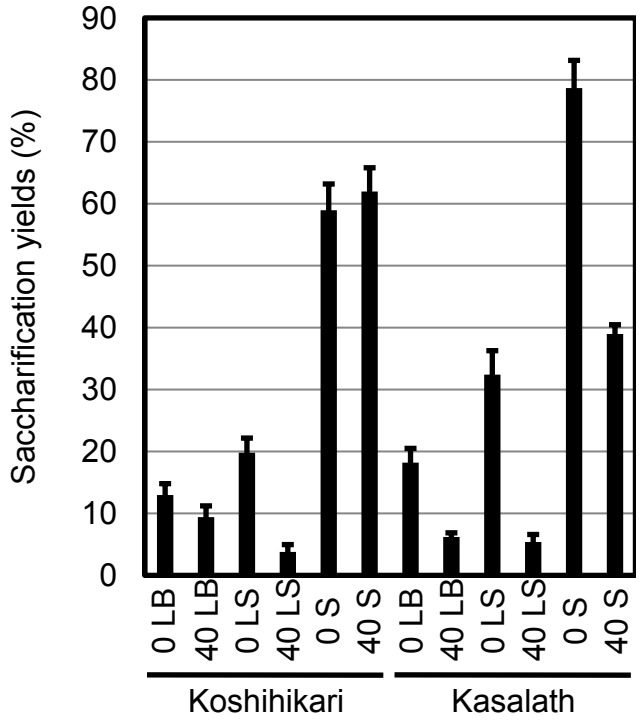

Supplemental data. Saccharification yields of rice straws in 2014.
Samples were grown in pots in the experimental fields in 2014. Leaf blades, leaf sheaths and stems at heading and 40 DAH were subjected to the saccharification analysis. Saccharification yields were shown as percentages of enzymatically saccharified biomass for the biomass used for the reaction. Error bars indicate standard errors. 0 LB: leaf blade at heading; 40 LB: leaf blade at 40 DAH; 0 LS: leaf sheath at heading; 40 LS: leaf sheath at 40 DAH; 0 S: stem at heading; 40 S: stem at 40 DAH.