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Bamboo saccharification through cellulose solvent-based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings

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ABSTRACT

The modified cellulose solvent- (concentrated phosphoric acid) and organic solvent- (95% ethanol) based lignocellulose fractionation (COSLIF) was applied to a naturally-dry moso bamboo sample. The biomass dissolution conditions were 50 °C, 1 atm for 60 min. Glucan digestibility was 88.2% at an ultra-low cellulase loading of one filter paper unit per gram of glucan. The overall glucose and xylose yields were 86.0% and 82.6%, respectively. COSLIF efficiently destructed bamboo's fibril structure, resulting in a ~33-fold increase in cellulose accessibility to cellulase (CAC) from 0.27 to 9.14 m^2 per gram of biomass. Cost analysis indicated that a 15-fold decrease in use of costly cellulase would be of importance to decrease overall costs of biomass saccharification when cellulase costs are higher than $0.15 per gallon of cellulosic ethanol.

1. Introduction

The production of second generation biofuels (e.g., cellulosic ethanol) or third generation biofuels (e.g., hydrogen) from renewable lignocellulosic biomass will result in a new industrial revolution from a fossil fuel-based economy to a sustainable carbohydrate economy (Lynd et al., 2008; Zhang, 2008, 2009). Cost-effective production of fermentable sugars from recalcitrant biomass remains the largest obstacle to emerging cellulosic ethanol biorefineries (Lynd et al., 2008; Wyman, 2007; Zhang, 2008). Significant advances in reduction of 20- to 30-fold of enzyme costs have been made through enzyme production process optimization and cellulase engineering (Himmel et al., 2007; Zhang et al., 2006b), but cellulase, whose costs may range from ~30 cents to more than 100 cents per gallon of cellulosic ethanol at a typical cellulase loading of 15 filter paper units per gram of glucan, is still far more expensive than that of starch-hydrolyzing enzymes for corn kernel-based ethanol biorefineries (e.g., ~5 cents per gallon of starch ethanol).

Bamboos are giant woody, tree-like, perennial evergreen C_4 grasses with more than 70 genera and about 1000 species. Bamboos grow naturally in tropical, subtropical, and temperate regions around the world (Gratani et al., 2008). Bamboos are of economic and high cultural significance in East Asia and South East Asia. Since they are both lightweight and exceptionally durable, the treated bamboos are used extensively as building materials for houses, construction scaffolding, flooring, bridges, etc. Also, they are extensively used to make furniture, chopsticks, food steamers, paper pulp, etc., and are grown as ornamental plants. *Phyllostachys pubescens* (moso bamboo), one of the most popular bamboos, can grow to heights of over 20 m with a diameter of nearly 18 cm. Moso bamboo flourishes in moist, well drained, and fertilized soils with pH from 4.5 to 7.0 and annual precipitation between 800 and 1800 mm. Marginal lands, such as mountain valley, foot of mountain, and gentle slope, are suitable for moso bamboo growth.

Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) has been developed to separate lignocellulose components using a cellulose solvent (concentrated phosphoric acid), an organic solvent (acetone), and water (Moxley et al.,

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2008; Zhang et al., 2007). High glucan enzymatic digestibility of the COSLIF-pretreated biomass is mainly attributed to large cellulose accessibility to enzymes (Zhu et al., 2009).

In this study, we investigated the feasibility of bamboo saccharification by the modified COSLIF followed by enzymatic hydrolysis at ultra-low cellulase loadings.

2. Methods

2.1. Chemicals and materials

All chemicals were reagent grade and purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise noted. Phosphoric acid (85%) and ethanol (95%) were purchased from Fisher Scientific (Houston, TX). The *Trichoderma* cellulase (Novozyme® 50013) and beta-glucosidase (Novozyme® 50010) were gifts from Novozymes North American (Franklinton, NC). They had activities of 84 filter paper units (FPUs) per mL and 270 beta-glucosidase units per mL, respectively. The bamboo used in this study was moso bamboo grown in Taiwan. The full-size culm with around a half- to one-year age was harvested in April 2008, and then dried naturally from April to August 2008. The naturally-dried material was milled to small particles by a Pallmann counter rotating knife ring flaker (Clifton, NJ) to the nominal sizes of <40 and >60 mesh.

2.2. Modified COSLIF procedure

The modified COSLIF pretreatment for bamboo was conducted using 95% (v/v) ethanol as an organic solvent, as described elsewhere (Sathitsuksanoh et al., 2009).

2.3. Carbohydrate and lignin assays

The structural carbohydrate composition of the completely dry biomass was determined using a modified quantitative saccharification (QS) (Moxley and Zhang, 2007). Monomeric sugars were measured by a Shimadzu HPLC with a Bio-Rad Aminex HPX-87P column (Moxley et al., 2008). Lignin and ash were measured according to the standard NREL biomass protocol (Sluiter et al., 2006).

2.4. Enzymatic hydrolysis

The pretreated bamboo samples were diluted to 10 g of glucan per liter in a 50 mM sodium citrate buffer (pH 4.8), as described elsewhere (Moxley et al., 2008; Sathitsuksanoh et al., 2009; Zhu et al., 2009). The enzymatic glucan digestibility, overall glucose and xylose yields during the modified COSLIF pretreatment and enzymatic cellulose hydrolysis were calculated, as described elsewhere (Sathitsuksanoh et al., 2009; Zhang et al., 2009).

2.5. Substrate accessibility assays

The total substrate accessibility to cellulase (TSAC) was determined based on the maximum adsorption capacity of the TGC protein containing a green fluorescence protein and a cellulose-binding module (Hong et al., 2007; Zhu et al., 2009). The recombinant TGC fusion protein was produced in *Escherichia coli* BL21 (pNT02) (Hong et al., 2007) and purified by affinity adsorption on regenerated amorphous cellulose (Zhang et al., 2006a,b) followed by modest desorption using ethylene glycol (Hong et al., 2008). Cellulose accessibility to cellulase (CAC, m^2/g biomass) was measured based on the maximum TGC adsorption capacity after BSA blocking (Zhu et al., 2009).

3. Results and discussion

The functionally-based model for enzymatic cellulose hydrolysis suggests that increasing substrate accessibility is more important than decreasing the degree of polymerization of cellulose for biomass pretreatment (Zhang and Lynd, 2006). The most efficient way for increasing cellulose accessibility is to dissolve cellulose fibers and regenerate them as amorphous form (Kuo and Lee, 2009; Zhang et al., 2006a,b). The COSLIF V1.0 utilizes a highly-volatile organic solvent (acetone) between a cellulose solvent (concentrated phosphoric acid) and water (Moxley et al., 2008; Zhang et al., 2007). Low boiling-point acetone can be recycled easily by simple flashing, but it must be recycled with very high yields (e.g., >99.99%). Any loss in acetone would negatively impact the economics of COSLIF implementation and cause environmental pollution. Since it was found that removal of lignin from the COSLIF-pretreated biomass was not as important as increasing cellulose accessibility to cellulase (Zhu et al., 2009), here we used ethanol as the organic solvent for the modified COSLIF V2.0. The use of ethanol as an organic solvent would bring several benefits: (1) lower recycling efficiencies of ethanol during the pretreatment (e.g., 98–99%) compared to those of acetone, because the remaining ethanol in the hydrolysate and cellulose phase can be recycled after the subsequent ethanol fermentation, (2) ethanol is more chemically stable than acetone for sequential solid/liquid separation, and (3) ethanol is less corrosive to membrane-based separation. Furthermore, the replacement of acetone by ethanol allows a decrease in organic solvent utilization from 100 to 60 volumes. Since bamboo is a wood-like grass, similar to common reed and poplar, the modified COSLIF pretreatment conditions were determined to be 8 mL of 85% phosphoric acid for 1.06 g of bamboo with a moisture content of ~6% at 50 °C and 1 atm for 60 min.

The COSLIF-pretreated bamboo samples were hydrolyzed by a commercial *Trichoderma* cellulase at the enzyme loadings of 1, 2, 5, and 15 filter paper units and 10 units of beta-glucosidase at 50 °C (Fig. 1A). At 15 FPUs per gram of glucan, the sample was hydrolyzed fast for the first 12 h; the glucan digestibility was 89.9% at hour 12 and rose slightly to 94.9% at hour 72. Decreasing cellulase loadings from 5 to 2 to 1 FPU per gram of glucan significantly decreased initial hydrolysis rates but slightly decreased final glucan digestibilities from 93.3% to 89.8% to 88.2%, respectively. Glucan digestibilities at different enzyme loadings were much greater for 12-h or 24-h hydrolysis than for 72-h hydrolysis (Fig. 1B). The results clearly suggested efficient enzymatic hydrolysis at an ultra-low cellulase loading for 72 h.

Fig. 2 presents the mass balance for modified COSLIF pretreatment and enzymatic hydrolysis based on 100 g of dry biomass. After modified COSLIF pretreatment, 2.1 g of soluble glucose equivalent and 10.9 g of soluble xylose equivalent were removed before enzymatic hydrolysis. The reactive cellulosic material was hydrolyzed at one FPU per gram of glucan, releasing 39.1 g of soluble glucose and 3.0 g of xylose equivalent. The overall glucose and xylose yields were 86.0% and 82.6%, respectively. By contrast, the overall glucose and xylose yields were 92.8% and 85.0%, respectively, when 15 filter paper units per gram of glucan were used.

The high glucan digestibility was obtained for the COSLIF-pretreated bamboo sample even when cellulase loading was decreased by 15-fold from a typical 15 FPUs per gram of glucan. This result was mainly attributed to drastic changes in surface morphology of intact and COSLIF-treated bamboo samples. The intact plant cell wall structure of bamboo had its microfibril structure; while a COSLIF-treated lignocellulose sample showed no obvious fibrous structure (data not shown), as shown elsewhere for other biomass samples (Moxley et al., 2008; Zhu et al., 2009). In addition to qualitative images, we further measured the substrate accessibility before and after the modified pretreatment.

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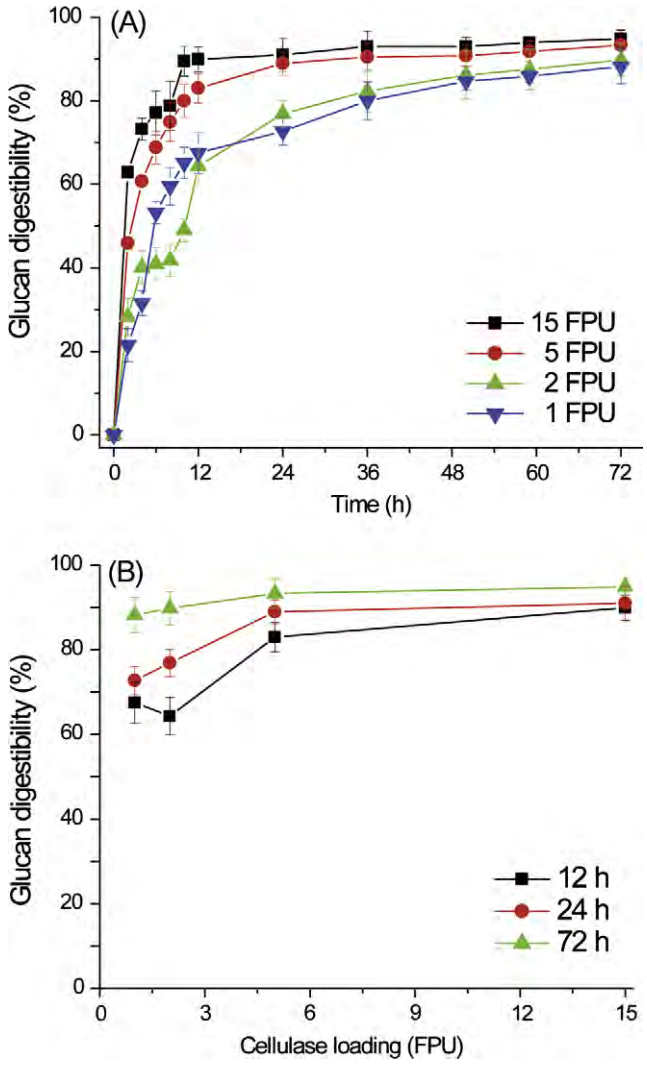

Fig. 1. Enzymatic cellulose hydrolysis profiles for the COSLIF-pretreated bamboo at different enzyme loadings (A, 1, 2, 5, and 15 FPUs of cellulase as well as 10 units of beta-glucosidase) and glucan digestibilities in terms of cellulase loadings for different hydrolysis time lengths (B).

The total substrate accessibility to cellulase (TSAC) increased from 0.45 ± 0.04 to 9.68 ± 0.76 m^2 per gram of COSLIF-pretreated biomass. In order to eliminate interference from the remaining lignin and other non-cellulose components, cellulose accessibility to cellulase (CAC) was measured based on the adsorption of TGC after blocking with BSA. The CAC values of the intact bamboo and pretreated bamboo were 0.27 ± 0.03 and 9.14 ± 0.64 m^2 per gram of biomass, respectively. This result suggested that COSLIF can increase substrate accessibility by 33-fold and yield a cellulosic product with high substrate digestibility mediated by cellulase and a fast enzymatic hydrolysis rate even at a low enzyme loading.

Bamboos, giant woody tree-like perennial evergreen grasses, are one of the fastest growing woody plants in the world. It is estimated that approximately 26,650 culms are produced per hectare in a bamboo plantation during a 10-year growing cycle (Shanmughavel and Francis, 2001). The average yearly biomass productivity in a 10-year growth cycle is approximately 95 tons of dry biomass per year per hectare (i.e., ~39 tons/acre/year), in which culms account for ~80% of total biomass (Shanmughavel and Francis, 2001). The above results indicate that bamboo are among the highest biomass producers as compared to other bioenergy plants in terms of tons of dry weight per acre per year, such as switchgrass (~4–8), dedicated forests including poplar (~8), Miscanthus (~10–25), *Phragmites australis* (common reed, ~18–28) (Sathitsuksanoh et al., 2009), and sugarcane (~25–30) (Zhang, 2008). In addition, existing systems for bamboo plantation, harvesting, and transportation would provide advantageous opportunities to build bamboo-based refineries as compared to other potential bioenergy plants, such as switchgrass and Miscanthus.

Current fungal cellulase cost per gallon of cellulosic ethanol may range from ~30 cents to more than 100 cents, based on a typical cellulase loading (15 filter paper units per gram of diluted acid-pretreated glucan) (Himmel et al., 2007; Zhang et al., 2006b). Obviously, ethanol production costs would reduce greatly as specific activity of cellulase increases (Fig. 3). When a typical cellulase loading of 15 FPUs/g glucan was decreased to 1 FPU/g glucan, the ethanol yield decreased from 88.1 to 83.6 gallons per ton of bamboo. Trade-off points between enzyme saving and ethanol revenue loss ($2 and $3 per gallon of ethanol) are shown in Fig. 3. If the selling price of ethanol was $3 per gallon and cellulase selling prices were greater than ~$0.16 of cellulase per gallon, a 15-fold reduction in cellulase loading would generate a more positive revenue increase than a loss of ethanol revenue. The enzyme-saving advantage (e.g., 84 cents per gallon) was far greater than the ethanol loss (e.g., 0.15 cents per gallon) when the cellulase cost was 90 cents per gallon. The above economy analysis remained relatively simple because a lot of factors, such as, large-size reactors needed for longer hydrolysis time, more utilities for mixing and cooling/heating, and waste treatment are not accounted. In general, cellulase is

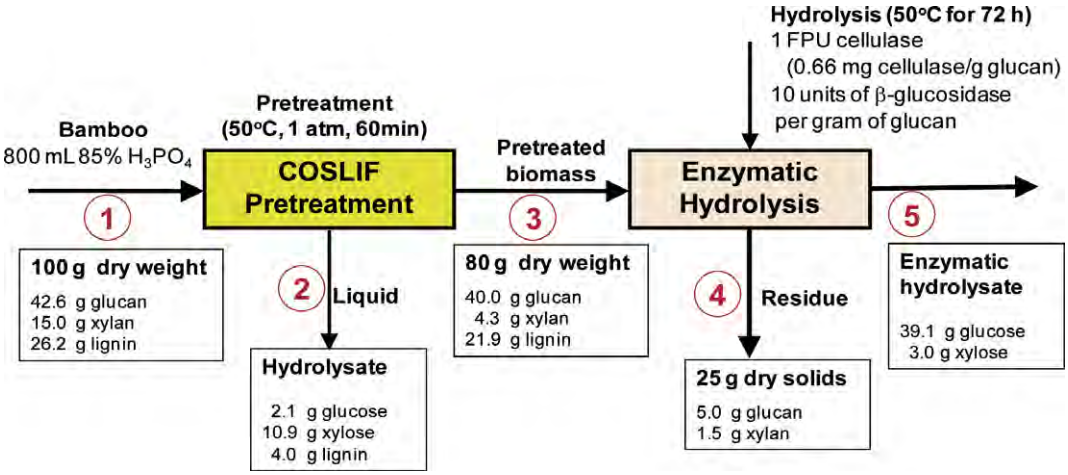

Fig. 2. Mass balance for bamboo pretreated by COSLIF followed by enzymatic hydrolysis by 1 FPU of cellulase and 10 units of beta-glucosidase per gram of glucan.

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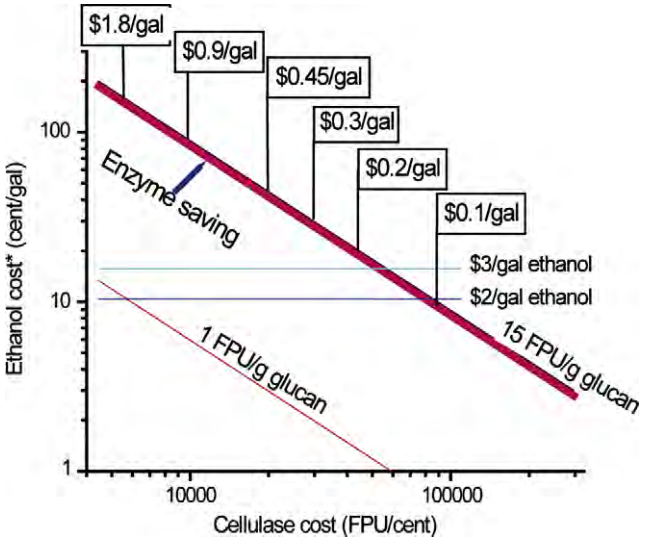

Fig. 3. Cost analysis of ethanol production* (involving only cellulase costs and ethanol yield loss due to a decreased cellulase loading) in terms of cellulase production costs. It was estimated that 29,665 FPUs were needed for one gallon of cellulosic ethanol produced based on an assumption that 15 FPUs per gram of glucan could cost ranging from 3 to 200 cents. The bamboo-to-ethanol yields were 88.1 and 83.6 gallons per ton of bamboo at 15 and 1 FPU per gram of glucan, respectively, based on 90% ethanol fermentation yields.

still one of the dominant cost fractions of the whole process (Himmel et al., 2007; Zhang, 2008; Zhang et al., 2006b).

4. Conclusions

The modified COSLIF pretreatment effectively disrupt recalcitrance of bamboo, generating highly reactive cellulosic materials as shown in a high glucan digestibility of 88.2% with 1 filter paper unit per gram of glucan at hour 72. A 15-fold reduction in cellulase usage will be of great importance in profitable production of cellulosic ethanol.

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References

Gratani, L., Crescente, M.F., Varone, L., Fabrini, G., Digiulio, E., 2008. Growth pattern and photosynthetic activity of different bamboo species growing in the Botanical Garden of Rome. Flora 203, 77–84.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315, 804–807.

Hong, J., Ye, X., Wang, Y., Zhang, Y.H.P., 2008. Bioseparation of recombinant cellulose binding module-protein by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. Anal. Chem. Acta 621, 193–199.

Hong, J., Ye, X., Zhang, Y.H.P., 2007. Quantitative determination of cellulose accessibility to cellulase based on adsorption of a nonhydrolytic fusion protein containing CBM and GFP with its applications. Langmuir 23, 12535–12540.

Kuo, C.-H., Lee, C.-K., 2009. Enhancement of enzymatic saccharification of cellulose by cellulose dissolution pretreatments. Carbohydr. Polym. 77, 41–46.

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat. Biotechnol. 26, 169–172.

Moxley, G., Zhang, Y.-H.P., 2007. More accurate determination of acid-labile carbohydrate composition in lignocellulose by modified quantitative saccharification. Energy Fuels 21, 3684–3688.

Moxley, G.M., Zhu, Z., Zhang, Y.-H.P., 2008. Efficient sugar release by the cellulose solvent based lignocellulose fractionation technology and enzymatic cellulose hydrolysis. J. Agric. Food Chem. 56, 7885–7890.

Sathitsuksanoh, N., Zhu, Z., Templeton, N., Rollin, J., Harvey, S., Zhang, Y.-H.P., 2009. Saccharification of a potential bioenergy crop, *Phragmites australis* (common reed), by lignocellulose fractionation followed by enzymatic hydrolysis at decreased cellulase loadings. Ind. Eng. Chem. Res. 48, 6441–6447.

Shanmughavel, P., Francis, K., 2001. Physiology of Bamboo. Scientific Publishers, Jodhpur, India.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2006. Determination of Structural Carbohydrates and Lignin in Biomass. Laboratory Analytic Procedure LAP-002 <http://devafdc.nrel.gov/pdfs/9572.pdf>.

Wyman, C.E., 2007. What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol. 25, 153–157.

Zhang, Y.-H.P., 2008. Reviving the carbohydrate economy via multi-product biorefineries. J. Ind. Microbiol. Biotechnol. 35, 367–375.

Zhang, Y.-H.P., 2009. A sweet out-of-the-box solution to the hydrogen economy: is the sugar-powered car science fiction? Energy Environ. Sci. 2, 272–282.

Zhang, Y.-H.P., Ding, S.-Y., Mielenz, J.R., Elander, R., Laser, M., Himmel, M., McMillan, J.D., Lynd, L.R., 2007. Fractionating recalcitrant lignocellulose at modest reaction conditions. Biotechnol. Bioeng. 97, 214–223.

Zhang, Y.-H.P., Cui, J.-B., Lynd, L.R., Kuang, L.R., 2006a. A transition from cellulose swelling to cellulose dissolution by *o*-phosphoric acid: evidences from enzymatic hydrolysis and supramolecular structure. Biomacromolecules 7, 644–648.

Zhang, Y.-H.P., Himmel, M., Mielenz, J.R., 2006b. Outlook for cellulase improvement: screening and selection strategies. Biotechnol. Adv. 24, 452–481.

Zhang, Y.-H.P., Lynd, L.R., 2006. A functionally-based model for hydrolysis of cellulose by fungal cellulase. Biotechnol. Bioeng. 94, 888–898.

Zhang, Y.H.P., Berson, E., Sarkanen, S., Dale, B.E., 2009. Sessions 3 and 8: pretreatment and biomass recalcitrance: fundamentals and progress. Appl. Biochem. Biotechnol. 153, 80–83.

Zhu, Z., Sathitsuksanoh, N., Vinzant, T., Schell, D.J., McMillan, J.D., Zhang, Y.-H.P., 2009. Comparative study of corn stover pretreated by dilute acid and cellulose solvent-based lignocellulose fractionation: enzymatic hydrolysis, supramolecular structure, and substrate accessibility. Biotechnol. Bioeng. 103, 715–724.

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