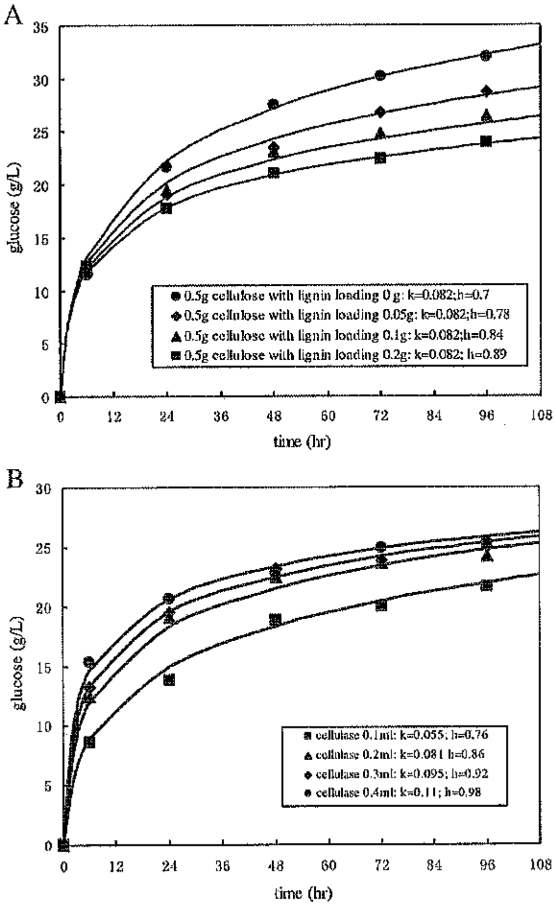

Fig. 3. Inhibition effect of lignin with different ratio of cellulose to lignin A: Effect of lignin inhibition with Avicel loading 0.5 g and Accellerase 1500 loading 0.2 ml in 10 ml reaction volume; B: Effect of cellulase loading with Avicel loading 0.5 g and lignin loading 0.1 g in 10 ml reaction volume.

adsorption capacity on various pretreated corn stover solids, even Avicel, correlates well with the hydrolysis rate at 24 h while not at 72 h (Kumar and Wyman, 2009).

A high cellulase loading has been applied to achieve a high glucose yield (Wu and Ju, 1998; Gusakov et al., 2007). The low hydrolysis efficiency caused by the presence of lignin was managed to be counterbalanced with high cellulase loading (Fig. 3B). The hydrolysis efficiency increased with the increase of cellulase loading at relative low cellulase loadings, in which increasing rate constant and fractal exponent was found at the same time. However, a further increasing cellulase loading, rate constant increased limitedly while the fractal exponent increased continuously. Thus, the lignin inhibition was only remedied limitedly by high cellulase loading. A similar phenomenon has also been reported (Sewalt et al., 1997). Product information of Accellerase 1500 also suggests that the ideal ratio of Accellerase 1500 to acid-pretreated sugarcane bagasses is about 0.24 ml/g cellulose.

3.3. Screening robust nonionic surfactant/polymer with fractal kinetic analysis

Enhancement of enzymatic saccharification of lignincellulose by addition of nonionic surfactants/polymers has been studied extensively (Park et al., 1992; Eriksson et al., 2002). For enzymatic saccharification of Avicel, enhancement (Mizutani et al., 2002; Ouyang et al., 2010) and limited effect (Zheng et al., 2008) of addition of nonionic surfactant/polymer are observed. The positive effect of nonionic surfactants/polymers aqueous solution on an enzymatic saccharification of Avicel was submerged into the experimental errors in our present work (data not shown). However, the effect of nonionic surfactant/polymer on enzymatic saccharification of Avicel containing lignin was markedly, which was evaluated with fractal kinetic analysis as shown in Fig. 4.

A fractal kinetic analysis of control (Avicel containing lignin) got the fractal kinetic parameters *k* (0.077) and *h* (0.87). An addition of polymers increased rate constant and decreased fractal exponent at the same time (Fig. 4A). An addition of nonionic surfactants also exhibited a similar result as that of addition of polymers (Fig. 4B). It should be stressed that some polymers, such as PEG 4000, PEG 10000, L64, F68 etc., increased the hydrolysis efficiency by changing the fractal kinetic parameters to about their original values of enzymatic saccharification of Avicel without lignin as shown in Fig. 3A ($k = 0.082$, $h = 0.7$). Especially, the high fractal exponent caused by the presence of lignin (Fig. 3A), which was ascribed to the non-productive adsorption of cellulase onto lignin by the phenolic group content in lignin (Chandra et al., 2007), had been completely remedied. It indicated that the addition of polymers prevents the non-productive adsorption of cellulase onto lignin. A strong interaction between lignin and polymer is also confirmed

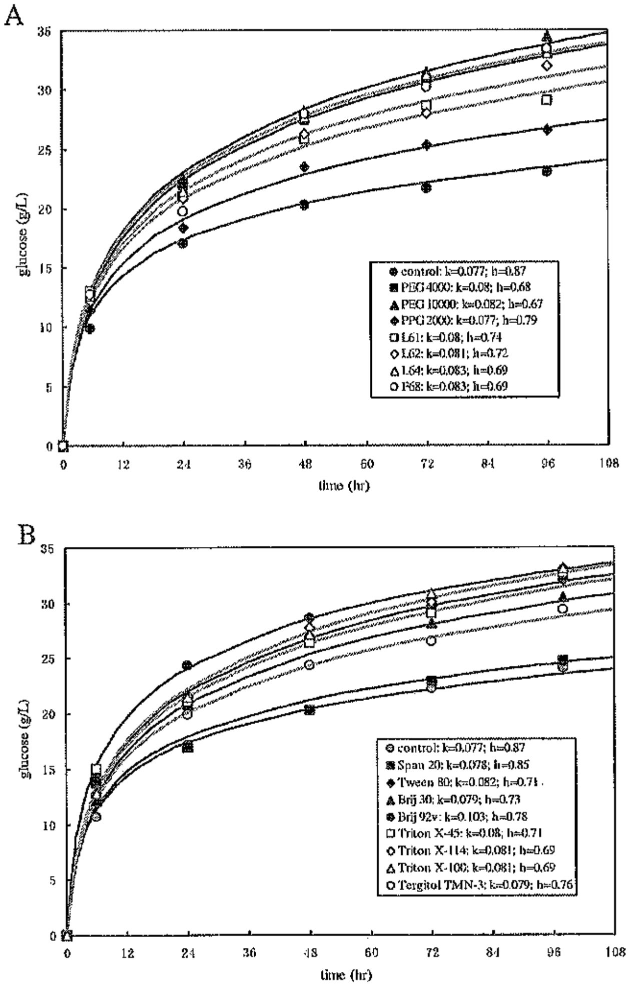

Fig. 4. Effect of nonionic surfactants/polymers on enzymatic saccharification of cellulose A: Effect of polymers with Avicel loading 0.5 g, lignin loading 0.1 g, and polymer loading 0.2 g in 10 ml reaction volume; B: Effect of nonionic surfactants with Avicel loading 0.5 g, lignin loading 0.1 g, and nonionic surfactant loading 0.2 g in 10 ml reaction volume.

by the solubilization of lignin in the L64-rich phase (Fig. 5) in the next section. A few increase of rate constant comparison to that of enzymatic saccharification of Avicel without lignin ($k = 0.082$, $h = 0.7$) had also been found. It is similar to the small positive effect of nonionic surfactants on enzymatic saccharification of crystal cellulose (Mizutani et al., 2002), which may be related to the effect of nonionic surfactant/polymer on stability or activity of cellulase (Zhang et al., 2009; Zheng, 2010).

The fractal kinetic parameters in the presence of different nonionic surfactants/polymers are summarized in Table 1. In general, the effect of insoluble nonionic surfactants/polymers, such as PPG 2000, Span 20, Tergitol TMN-3, were relatively poor. While the soluble nonionic surfactants/polymers, such as PEG 4000, PEG 10000, L64, F68, Triton X-100, Triton X-114, were relatively good. However, insoluble Brij 92v increased the rate constant markedly and a high glucose concentration was achieved. Nonionic surfactants Triton X series, no matter soluble (Trion X-100), forming cloud point system (Triton X-114) or insoluble (Triton X-45), had marked effect on elimination of the lignin inhibition. It indicates that the effect of nonionic surfactant/polymer may be related to its phase behavior in an aqueous solution and the corresponding molecular structure.

3.4. Toxicity of residual polymers/nonionic surfactants and removal of triblock copolymer L64

An enzymatic saccharification of lignocellulose is strongly depended on the pretreatment step (Wyman et al., 2005; Qing et al., 2010), while the enzymatic saccharification of lignocellulose also has strong effect on the next ethanol fermentation step. Although elimination of lignin inhibition by addition of polymers/nonionic surfactant in the enzymatic saccharification step has been confirmed, the toxicity of the additives on the ethanol fermentation strain *S. cerevisiae* should be further examined. The toxicity of nonionic surfactant/polymer, representing with the relative activity as detailed in the Section 2, was examined and summarized in table 1. Polymer PEG with nearly no toxicity has been studied extensively in extractive microbial fermentation (Sinha et al., 2000). Insoluble PPG has also been utilized for extractive microbial fermentation (Barton and Daugulis, 1992). Thus the relative activity of those polymers did not determine in the present work. Some of nonionic surfactants (such as Brij 30, Triton X-45 etc.) were toxicity to the ethanol fermentation strain *S. cerevisiae*. Some polymers with high viscosity (such as PEG 10000 etc.) also influenced the mass transfer resistances in the fermentation process (Sinha et al., 2000).

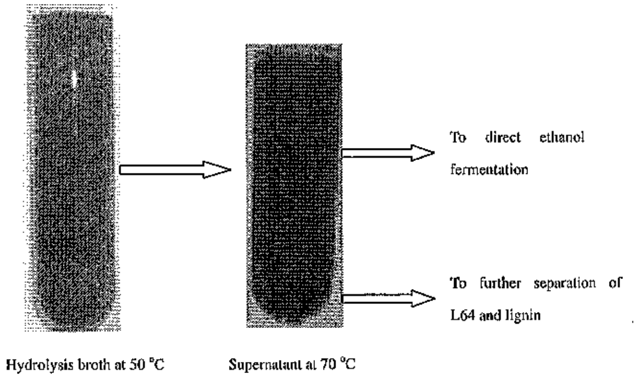

Fig. 5. Phase change of hydrolysis broth in triblock copolymer L64 aqueous solution A hydrolysate was settled in a 50 °C water bath, which was separated into a black supernatant phase containing lignin and L64 while a brown sediment phase containing residual cellulose. Then the black supernatant phase was transferred into another tube and was settled in a 70 °C water bath. The supernatant was separated into a clear upper phase containing most of glucose and the black lignin was concentrated into the lower phase containing most of L64. The clear upper phase containing most of reducing sugar can be used to ethanol fermentation while that of concentrated lignin and L64 phase may be further separated, such as acid precipitation of lignin (Gilarranz et al., 1998; Fu et al., 2010). Then the lignin can be recovered and the L64 be recycled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

An interesting phenomenon had been found in a hydrolysate of an enzymatic saccharification of cellulose containing lignin in the presence of triblock copolymer L64. When the hydrolysis broth was settled in a 50 °C water bath, the residual cellulose was deposited in the tube's bottom while the lignin was suspended in the copolymer solution (Fig. 5). The other soluble polymers/nonionic surfactants, such as PEG 4000, PEG 10000, F68, Triton X-100 etc., exhibited the same phenomenon. When the supernatant was transferred into another tube and settled in a 70 °C water bath, the triblock copolymer L64 aqueous solution was separated into a L64-dilute phase and a L64-rich phase. The lignin was concentrated into the L64-rich phase and most of glucose was remained in the L64-dilute phase (Fig. 5). The nonionic surfactants/triblock copolymers with low cloud point, such as L62, Triton X-114 etc., exhibited a similar phenomenon under the 50 °C hydrolysis conditions except that lignin, residual cellulose and polymers/nonionic surfactants were deposited in the tube's bottom. The solubilization of lignin in the L64-rich phase provides an evidence that there is strong interaction between lignin and nonionic surfactant/polymer, which is solid fact about the addition of polymers/nonionic surfactants to prevent the non-productive adsorption of cellulase onto lignin (Zheng et al., 2008). The fractal kinetic parameters (Fig. 4), relative activity (Table 1), and phase separation (Fig. 5) indicates that triblock copolymer L64 maintains the nature of nearly no toxicity, effective elimination of lignin inhibition and easy removal from the hydrolysate, respectively. Thus triblock coplymer L64 aqueous solution is potential medium in an enzymatic saccharification of lignocellulose.

4. Conclusions

Fractal kinetic model with two parameters (k and h) has been successfully applied to analyze the enzymatic saccharification of Avicel with or without lignin, in which the retarded hydrolysis rate has been represented by fractal exponent. Lignin inhibition exhibits a high fractal exponent, which can only remediate limitedly with high cellulase loading while eliminate completely by addition of nonionic surfactants/polymers. Some polymers/nonionic surfactants, such as triblock copolymer L64, also fulfills the demands of next ethanol fermentation step in the biomass based bio-ethanol industry.

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References

Bansal, P., Hall, M., Realff, M.J., Lee, J.H., Bommarius, A.S., 2009. Modeling cellulase kinetics on lignocellulosic substrates. Biotechnol. Adv. 27, 833–848.
Barton, W.E., Daugulis, A.J., 1992. Evaluation of solvents for extractive butanol fermentation with *Clostridium acetobutylicum* and the use of poly(propylene glycol) 1200. Appl. Microbiol. Biotechnol. 36, 632–639.

Bommarius, A., Katona, A., Cheben, S.E., Patel, A.S., Ragauskas, A.J., Knudson, K., Pu, Y., 2008. Cellulase kinetics as a function of cellulose pretreatment. Metab. Eng. 10, 370–381.

Börjesson, J., Engqvist, M., Sipos, B., Tjerneld, F., 2007. Effect of poly(ethylene glycol) on enzymatic hydrolysis and adsorption of cellulase enzymes to pretreated lignocellulose. Enzyme Microb. Technol. 41, 186–195.

Chandra, R.P., Bura, R., Mabee, W.E., Berlin, A., Pan, X., Saddler, J.N., 2007. Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics? Adv. Biochem. Eng./Biotechnol. 108, 67–93.

Dai, Z., Wang, Z., Xu, J.-H., Qi, H., 2010. Assessing bioavailability of the solubilization of organic compound in nonionic surfactant micelles by dose-response analysis. Appl. Microbiol. Biotechnol. 88, 327–339.

Eriksson, T., Börjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb. Technol. 31, 353–364.

Fu, D., Mazza, G., Tamaki, Y., 2010. Lignin extraction from straw by ionic liquids and enzymatic hydrolysis of the cellulosic residues. J. Agric. Food Chem. 58, 2915–2922.

Gilarranz, M.A., Rodriguez, F., Olet, M., Revenga, J.A., 1998. Acid precipitation and purification of wheat straw lignin. Separ. Sci. Technol. 33 (9), 359–1377.

Guo, G.-L., Hsu, D.-C., Chen, W.-H., Hwang, W.-S., 2009. Characterization of enzymatic saccharification for acid-pretreated lignocellulosic materials with different lignin composition. Enzyme Microb. Technol. 45, 80–87.

Gupta, R., Lee, Y.Y., 2009. Mechanism of cellulase reaction on pure cellulosic substrates. Biotechnol. Bioeng. 102 (6), 1570–1581.

Gusakov, A.V., Salanovich, T.N., Antonov, A.I., Ustinov, B.B., Okunev, O.N., Burlingame, R., Emalfarb, M., Baez, M., Sinitsyn, A., 2007. Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. Biotechnol. Bioeng. 97 (5), 1028–1038.

Kopelman, R., 1988. Fractal reaction kinetics. Science 241, 1620–1626.

Kumar, R., Wyman, C.E., 2009. Cellulase adsorption and relationship to futures of corn stover solids produced by leading pretreatments. Biotechnol. Bioeng. 103 (2), 252–267.

Ma, A., Hu, Q., Qu, Y., Bai, Z., Liu, W., Zhuang, G., 2008. The enzymatic hydrolysis rate of cellulose decreases with irreversible adsorption of cellobiohydrolase I. Enzyme Microb. Technol. 42, 43–547.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31 (3), 426–428.

Mizutani, C., Sethumadhavan, K., Howley, P., Bertoniere, N., 2002. Effect of nonionic surfactant on *Trichoderma* cellulase treatments of regenerated cellulose and cotton yarns. Cellulose 9, 83–89.

Ouyang, J., Dong, Z., Song, X., Lee, X., Chen, M., Yong, Q., 2010. Improved enzymatic hydrolysis of microcrystalline cellulose (Avicel PH101) by polyethylene glycol addition. Bioresour. Technol. 101 (17), 6685–6691.

Palonen, H., Tenkanen, M., Linder, M., 1999. Dynamic interaction of *Trichoderma reesei* cellobiohydrolases Cel6A and Cel7A and cellulose at equilibrium and during hydrolysis. Apppl. Environ. Micorbol. 65 (12), 5229–5233.

Park, J.W., Takahata, Y., Kajiuchi, T., Akehata, T., 1992. Effects of nonionic surfactant on enzymatic hydrolysis of used newspaper. Biotechnol. Bioeng. 39, 117–120.

Qing, Q., Yang, B., Wyman, C.E., 2010. Impact of surfactants on pretreatment of corn stover. Bioresour. Technol. 101, 5941–5951.

Schnell, S., Turner, T.E., 2004. Reaction kinetics in intracellular environments with macromolecular crowding: simulations and rate laws. Progr. Biophy. Mol. Biol. 85, 235–260.

Sewalt, V.J.H., Glasser, W.G., Beauchemin, K.A., 1997. Lignin impact on fiber degradation. 3. reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. J. Agric. Food Chem. 45, 1823–1828.

Sinha, J., Dey, P.K., Panda, T., 2000. Aqueous two-phase: the system of choice for extractive fermentation. Appl. Microbiol. Biotechnol. 54, 476–486.

Valjamae, P., Kipper, K., Pettersson, G., Johansson, G., 2003. Synergistic cellulose hydrolysis can be described in terms of fractal kinetics. Biotechnol. Bioeng. 84 (2), 253–257.

Wald, S., Wilke, C.R., Blanch, H.W., 1984. Kinetics of the enzymatic hydrolysis of cellulose. Biotechnol. Bioeng. 26 (3), 221–230.

Wang, Z., Feng, H., 2010. Fractal kinetic analysis of the enzymatic saccharification of cellulose under different conditions. Bioresour. Technol. 101, 7995–8000.

Wu, J., Ju, L.-K., 1998. Enhancing enzymatic saccharification of waste newsprint by surfactant addition. Biotechnol. Prog. 14 (4), 649–652.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzappe, M., Ladisch, M.R., Lee, Y.Y., 2005. Corrdinated development of leading biomass pretreatment technologies. Bioresour. Technol. 96, 1959–1966.

Xu, F., Ding, H., 2007. A new kinetic model for heterogeneous (or spatially confined) enzymatic catalysis: contributions from the fractal and jamming (overcrowding) effects. Appl. Catal. A 317, 70–81.

Yang, B., Willies, D.M., Wyman, C.E., 2006. Changes in the enzymatic hydrolysis rate of Avicel cellulose with conversion. Biotechnol. Bioeng. 94 (6), 1122–1128.

Zhang, Y.-H.P., Lynd, L.R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol. Bioeng. 84 (7), 797–824.

Zhang, Q., He, G., Wang, J., Cai, W., Xu, Y., 2009. Mechanisms of the stimulatory effects of rhamnolipid biosurfactant on rice straw hydrolysis. Appl. Energy 86, s233–s237.

Zheng, Y., Pan, Z., Zhang, R., Wang, D., Jenkins, B., 2008. Non-ionic surfactants and non-catalytic protein treatment on enzymatic hydrolysis of pretreated creeping wild ryegrass. Appl. Biochem. Biotechnol. 146, 231–248.

Zheng, Y., 2010. Application of nonionic surfactants on lignocellulosic bioethanol production. In: Wendt, P.L., Hoysted, D.S. (Eds.), Non-ionic Surfactant. Nova Science Publishers, New York, pp. 199–215.