論文の内容の要旨

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With the increasing demand for energy and the depletion of petroleum resources, bioethanol has been considered in alternative promising fuel. The second generation of bioethanol from lignocellulosic biomass becomes attractive and sustainable alternative accounts for majority biofuels worldwide because the renewable and abundant nature of biomass and its non-competitiveness with food crops, and the higher reduction in greenhouse gas emission. The key substance in lignocellulose for the production of ethanol is cellulose, which is the major constituent of plant cell wall. Cellulose is a linear polymer of D-glucose linked by β-1,4-glycosidic bond. Conversion of cellulosic materials to glucose is the key step of lignocellulosic ethanol production, which mainly depends on the degradation capacity of cellulases secreted by cellulolytic microorganisms and accessibility of these enzymes to cellulose. Filamentous fungus, *Trichoderma reesei* is a well-known source of cellulolytic enzymes and is widely used in industrial applications. Enzymatic hydrolysis of cellulose requires the synergistic reaction of three types of cellulases, the endo-β-1,4-glucanases (EG, EC 3.2.1.4), the cellobiohydrolases (CBH, EC 3.2.1.91) and the β-glucosidases (BGL, EC 3.2.1.21). Among them, the β-glucosidase is considered an important enzyme because it could relieve the cellobiose inhibition of EG and CBH. However, end-product inhibition by glucose limits the activity and cellulose hydrolysis by endogenous *T. reesei* the β-glucosidase, and this, combined with their extremely low endogenous activity, resulting in retardation of cellulose hydrolysis and subsequently insufficient conversion into ethanol. Supplementation of the β-glucosidase activity to overcome this problem is a good way to using *T. reesei* and taking advantage of its cellulolytic enzyme production capability. However, the addition of extracellular β-glucosidase, such as Novozyme 188 (the β-glucosidase from *Aspergillus niger*) approaches result in increased cost of ethanol production. Utilization of recombinant technology for increased BGL activity is a promising strategy for reducing the cost of ethanol production.

In this study, we established the ethanol production system from converge-milled Japanese cedar by using the recombinants *Aspergillus aculeatus* β-glucosidase I expressed by *Saccharomyces cerevisiae* and enzyme produced by a recombinant *T. reesei* expressing *A. aculeatus* β-glucosidase I compared to the supplementation of commercial β-glucosidase of Novozyme 188 for more economical ethanol production using *T. reesei* PC-3-7 culture supernatant.

The phylogenetic analysis showed a new *T. reesei* β-glucosidase, TrCel3B, belonging to the same cluster as *Aspergillus* β-glucosidases that exhibits high cellobiase

activity. Therefore, *cel3b* of *T. reesei* seemed like a good β-glucosidase candidate for testing expression in fermentative organism, *S. cerevisiae*. The examination of cellobiase activity of the recombinant strains using either TrCel3B and *A. aculeatus* β-glucosidase I (AaBGL1) indicated that the recombinant AaBGL1 showed higher specific activity against *p*NPGlc or cellobiose than that of TrCel3B. Moreover, the stability of AaBGL1 was also superior to TrCel3B under the various pH and thermal conditions tested. Therefore, recombinant AaBGL1 expressed in *S. cerevisiae* was selected for producing ethanol in the simultaneous saccharification and fermentation (SSF) processes. SSF has been considered an effective process for lignocellulosic ethanol production because it increases the yield of ethanol by minimizing product inhibition, eliminating the need to separate hydrolysis and fermentation processes. SSF has been shown to circumvent glucose inhibition of the hydrolytic enzymes due to rapid assimilation of sugars by the yeast used for fermentation.

Another approach to overcoming insufficient BGL activity and improving glucose yield for high efficient ethanol conversion is utilization of JN11, enzyme produced by recombinant strain X3AB1 constructed from a *T. reesei* strain expressing *A. aculeatus* β-glucosidase I (AaBGL1) under the control of *xyn3* promoter. JN11 showed over 60 times more BGL activity than *T. reesei* PC-3-7, the parental strain when grown on Avicel cellulose. Moreover, JN11 exhibited the best balance of cellulase and hemicellulase activities, therefore, is very effective for saccharification of lignocellulosic materials. These characteristics make JN11 worthy of further investigation of its potential in the conversion of lignocellulose into ethanol. Two processes of SSF (simultaneous saccharification and fermentation) and SHF (separate hydrolysis and fermentation) were used for evaluating the ethanol production of JN11 with *S. cerevisiae* wild type. In SHF, conditions for saccharification and fermentation can be optimized independently of each other, but the process is limited by product inhibition of enzymes during the hydrolysis of sugar. However, the SSF process requires scouting for the best thermal conditions that are suitable for both enzymatic reaction and fermentation of yeast, which could be a drawback for this process.

The ethanol production results of both recombinant strains indicated the use of heterologous *S. cerevisiae* or enzyme produced by recombinant strain, *T. reesei* expressing β-glucosidase I of *A. aculeatus* was as effective for ethanol production as the addition of equivalent amounts of exogenous cellobiase, Novozyme 188 on SSF or SHF processes. Examination of various amounts of *T. reesei* culture supernatant (PC-3-7) for observing in recombinant *S. cerevisiae* expressing AaBGL1 or using JN11 indicated that increases in the amount of saccharifying protein, which significantly increased the dosage of cellulases in the process, enhanced the yield and rate of cellulose hydrolysis and consequently in increased ethanol conversion. Moreover, with the same cellobiase activity of traditional enzyme mixture, *T. reesei* PC-3-7 culture supernatant supplemented by Novozyme 188, and JN11, the protein amount needed by JN11 for ethanol conversion was about 2 fold lower than for enzyme mixture, indicating that JN11 is an intrinsically economical enzyme for

bioethanol production from lignocellulosic biomass. Furthermore, the results of our experiments show that the SSF process with ethanol fermentation and concomitant enzyme activity is more efficient than SHF process for ethanol production from Japanese cedar.

The cost of cellulases used for biomass degradation is an important consideration in commercial ethanol production from lignocellulosic biomass. The addition of β-glucosidase from microbial sources increases the cost significantly. Our results demonstrated that using the heterologous AaBGL1 expressed by *S. cerevisiae* or saccharifying enzyme of recombinant AaBGL1 expressed in *T. reesei* for ethanol production can be as effective as supplementing the process with commercial cellobiase activity. Therefore, *S. cerevisiae* expressing AaBGL1 and JN11 could eliminate the cost of supplementing BGL, thereby significantly reducing the cost of industrial conversion of lignocellulosic biomass into ethanol.