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Consolidated Bioprocessing Ethanol Production by Using a Mushroom

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1. Introduction

Plant cell walls are the most abundant biomass source in nature and are of increasing importance because worldwide attention has now focused on bioethanol production to combat global warming and to safeguard global energy. Because of competition between food and fuel production, lignocelluloses are expected to be utilized for future fuel ethanol production. One of the major problems in producing ethanol from lignocellulosic biomass is the expensive production cost. Consolidated bioprocessing (CBP) is gaining recognition as a potential breakthrough for low-cost biomass processing (Lynd, 1996; Lynd et al., 2002; Lynd et al., 2005; Van Zyl et al., 2007; Xu et al. 2009). CBP of lignocellulose to bioethanol refers to the combination of the 4 biological events required for this conversion process (production of lignocellulose-degrading enzymes, hydrolysis of polysaccharides present in pre-treated biomass and fermentation of hexose and pentose sugars) in one reactor. However, no natural microorganism exhibits all the features desired for CBP. Bacteria and yeast have been the primary candidates for CBP research and some progress has been made in this regard. Traditionally, proponents of CBP processes have identified two primary developmental pathways capable of producing industrially viable CBP microbial strains. These are category I, engineering a cellulase producer, such as *Clostridium thermocellum*, to be ethanologenic; and category II, engineering an ethanologen, such as *Saccharomyces cerevisiae* or *Zymomonas mobilis*, to be cellulolytic (Lynd, 1996; Lynd et al., 2002; Lynd et al., 2005; Van Zyl et al., 2007; Xu et al., 2009). However, the both categories have advantages and disadvantages. Cellulase producer lacks ethanol tolerance, and it is very difficult to coexpress of multiple saccharification enzyme genes in ethanol producer. Especially, heterologous expression of *Trichoderma reesei* cellobiohydrolases (cellobiohydrolase I and cellobiohydrolase II), which play the crucial role in cellulose degradation, are generally poor.

Basidiomycetes, also known as wood-rotting fungi, can achieve the complete breakdown of lignins (Cooke & Rayner, 1984; Cullen, 1997), and are considered primary agents of plant litter decomposition in terrestrial ecosystems (Thorn et al., 1996). Furthermore, some basidiomycetes produce alcohol dehydrogenases, thus allowing the production of wine using a mushroom (Okamura et al., 2000; Okamura et al., 2001). These properties of basidiomycetes appear suitable for use in CBP. In a preliminary study, we screened some edible mushrooms for their ability to produce ethanol and found that *Flammulina velutipes* is a good producer of ethanol. *F. velutipes* is a white-rot fungus that grows from spring through

late autumn on a variety of hardwood tree stubs and dead stems and is widely distributed in temperate to subarctic regions. Currently, *F. velutipes* is the most produced mushroom in bed cultivation in Japan, the annual production being 130,000 tons/year. Artificial cultivation of mushrooms in polypropylene bottles is popular in Japan. *F. velutipes* has been characterized as wide adapted strain for various kinds of substance of artificial cultivation media, thus suggesting that the strain may be useful in the conversion of a wide variety of biomass types.

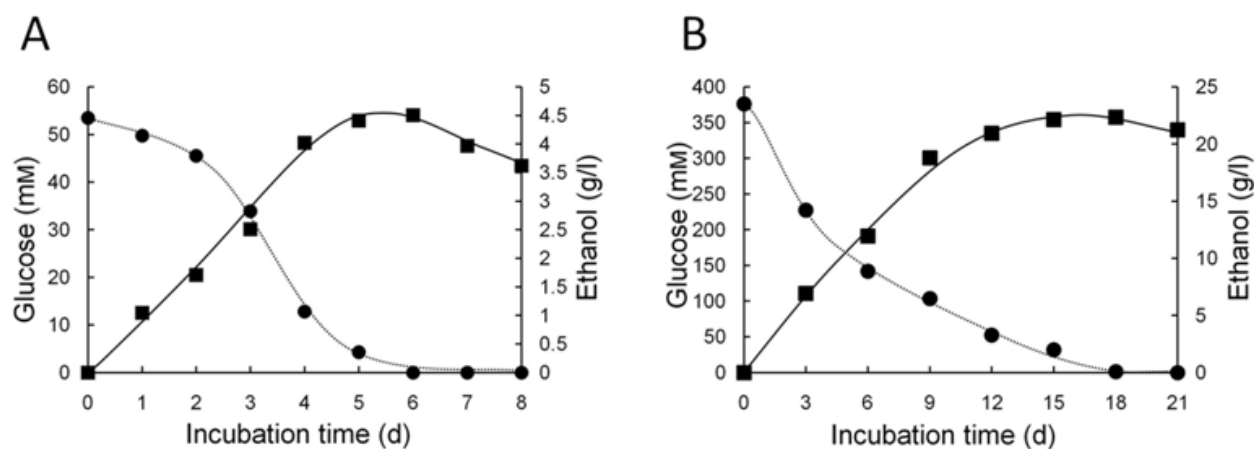
In this study, we investigated the properties of ethanol fermentation by *F. velutipes* to determine its suitability for CBP, because the use of basidiomycetes for bioethanol production is not common and the ethanol fermentation abilities of basidiomycetes are not well characterized. Furthermore, several biomass such as sorghums and rice straw were used as raw material to evaluate the detail conversion from biomass to ethanol by *F. velutipes*.

2. Properties of ethanol fermentation by *F. velutipes*

Because the use of basidiomycetes in bioethanol production is not common, and the ethanol fermentation abilities of basidiomycetes are not well characterized, we investigated the properties of ethanol fermentation by *F. velutipes* to determine its suitability for CBP (Mizuno et al., 2009b). Before the experiment, to obtain a suitable strain for CBP, 10 *F. velutipes* strains, culture stock of the Forest Institute of Toyama Prefectural Agricultural, Forestry, and Fisheries Research Center, were screened for cellulase production and ethanol fermentation. The Fv-1 strain was selected for further study because it not only produces high levels of cellulases, but also because its ability to ferment ethanol is superior to the other strains.

Firstly, fermentation of D-glucose was done by *F. velutipes* Fv-1. Figure 1A shows a conversion of 1% w/v of D-glucose to ethanol by *F. velutipes*. The consumption of D-glucose started gradually after incubation, and it was depleted after 6 d. Ethanol production correlated with sugar consumption, and it reached a maximum after 6 d. Thereafter, the amount of ethanol decreased gradually. Finally, *F. velutipes* converted 10 g/l of D-glucose to 4.5 g/l of ethanol, equivalent to a theoretical ethanol recovery rate of 88%. In the case of ethanol production from 5% w/v D-glucose, ethanol production reached a maximum, and all of the D-glucose was consumed after 18 d of incubation (Fig. 1B), and 50 g/l of D-glucose was converted to 22.4 g/l of ethanol, equivalent to a theoretical ethanol recovery rate of 87%. The conversion rate was the same as the case of 1% w/v of D-glucose. Because the incubation time to ferment 1% w/v sugar is shorter than the case of 5% w/v, we employed 1% w/v of sugar concentration in subsequent experiments.

Secondary, determination of the fermentation specificity of sugars by *F. velutipes* Fv-1 was done using various monosaccharides. As shown in Fig. 2, both D-mannose and D-fructose were converted to ethanol by *F. velutipes*. Consumption of D-mannose occurred slightly faster than that of D-glucose; it started immediately after incubation and was completely depleted after 5 d. Ethanol production from D-mannose was similar to that from D-glucose. It started during the first day of incubation and reached a maximum after 6 d. Furthermore, 4.4 g/l of ethanol was produced from 10 g/l of D-mannose, equivalent to a theoretical ethanol recovery rate of 86% (Fig. 2A). In contrast, consumption of D-fructose was slower than that of D-mannose. It started slowly after incubation and took 7 d to completely consume the D-fructose. Production of ethanol correlated with sugar consumption, and



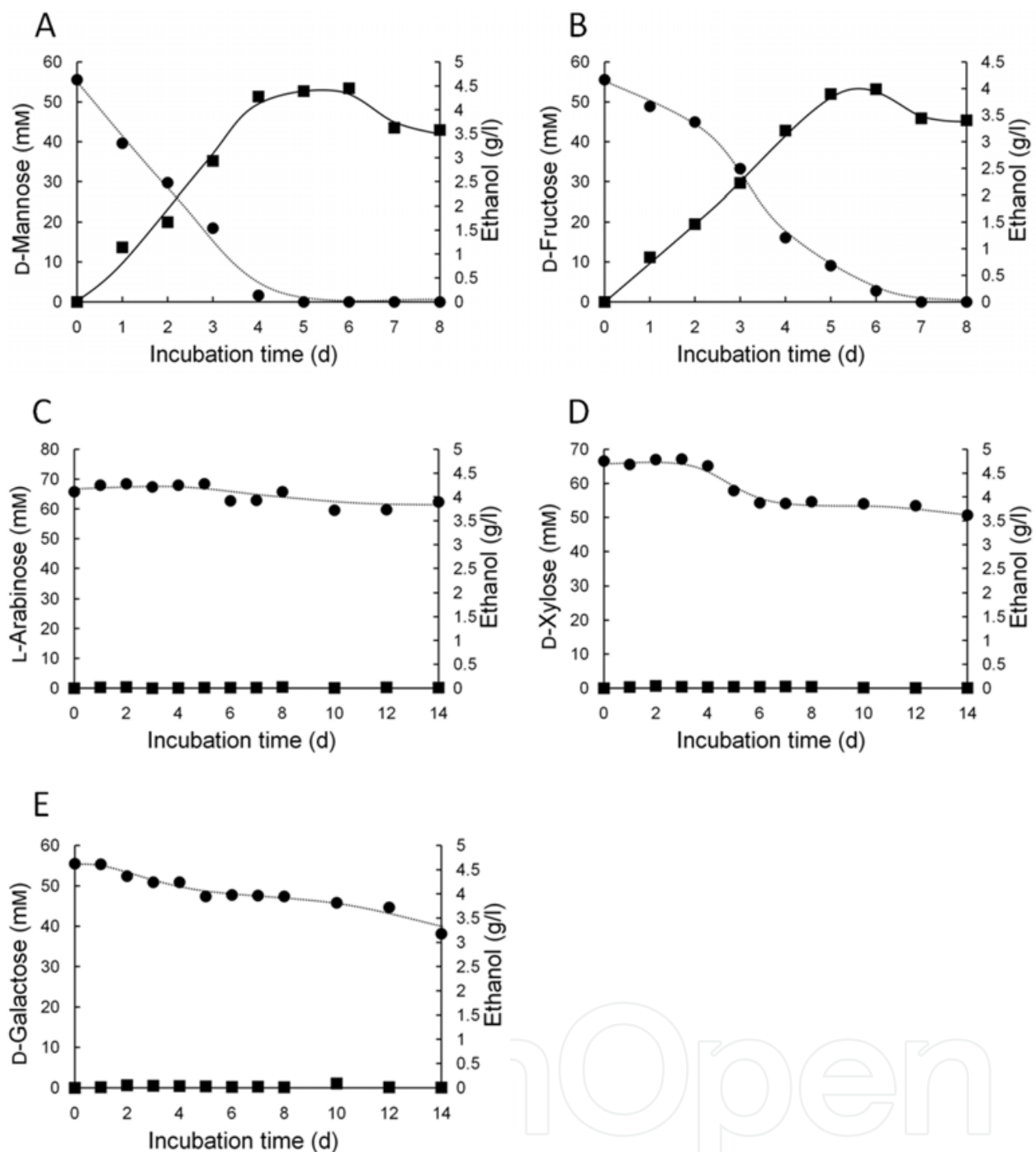
Symbols: closed circle, sugar; closed square, ethanol. The initial D-glucose concentration was (A) 1% w/v and (B) 5% w/v. (Reproduced from Mizuno et al., 2009b)

Fig. 1. Ethanol production from fermentation of D-glucose by *F. velutipes*

maximum conversion of D-fructose to ethanol was observed after 6 d. Upon completion of incubation, 4.0 g/l of ethanol was obtained from 10 g/l of D-fructose (Fig. 2B), yielding a theoretical conversion rate of 77%. In contrast to these sugars, *F. velutipes* did not convert L-arabinose, D-xylose, or D-galactose to ethanol (Figs. 2C, 2D, and 2E). Although there was slight consumption of D-xylose and D-galactose during incubation, ethanol production was not observed. In the case of L-arabinose, little sugar consumption was observed.

Next, we examined the fermentation specificity of *F. velutipes* Fv-1 toward various disaccharides. As shown in Fig. 3, *F. velutipes* possibly converted these sugars to ethanol and produced high yields. The theoretical conversion rates of these sugars were 83% and 77% from sucrose and maltose respectively. Degradation of sucrose was observed immediately after the incubation to import the sugar. The amount of reducing sugars was maximum on day 3 and was completely consumed after 7 d of incubation. Ethanol production was observed 1 d after incubation, and the amount of ethanol reached a maximum after 6 d. Finally, 4.5 g/l of ethanol was produced from 10 g/l of sucrose (Fig. 3A). In the case of maltose, degradation was observed on the first day of incubation, and the amount of reducing sugars reached a maximum after 2 d. Furthermore, the reducing sugars were completely depleted after 7 d of incubation. Ethanol production started during the first day of incubation and reached a maximum after 7 d. At the end of incubation, 10 g/l of maltose was converted to 3.8 g/l of ethanol (Fig. 3B). No conversion of xylobiose to ethanol was detected (data not shown), but a significant amount of ethanol production was observed when cellobiose was used as the carbon source (Fig. 4A). Cellobiose began degrading during the first day of incubation, and both D-glucose and cellobiose were completely depleted after 8 d. β -Glucosidase activity increased gradually during incubation. Ethanol production started after 1 d of incubation, and the amount of ethanol reached a maximum after 8 d. Upon completion of incubation, 10 g/l of cellobiose was converted to 4.5 g/l of ethanol (Fig. 4A). The theoretical conversion rate was 83%, a value similar to that of glucose and significantly higher than that of maltose. A high yield of ethanol was observed also in the higher concentration of cellobiose (Fig. 4D). Finally, 25 g/l of ethanol was produced from 50 g/l of D-glucose, and the theoretical conversion rate was 91%.

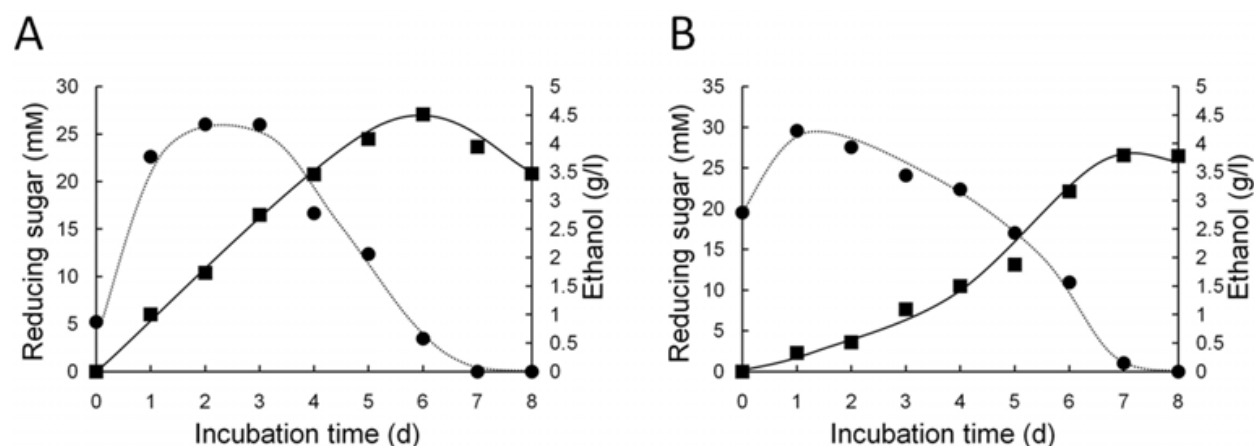
Since cellobiose was converted to ethanol at a relatively high rate, the conversions of cello-oligosaccharides to ethanol by *F. velutipes* were also investigated. Figures 4B and 4C show



Symbols: closed circle, sugar; closed square, ethanol. The initial sugar concentration was 1% w/v. (Reproduced from Mizuno et al., 2009b)

Fig. 2. Ethanol fermentation from (A) D-mannose, (B) D-fructose, (C) L-arabinose, (D) D-xylose and (E) D-galactose by *F. velutipes*

the results of the conversion of cellotriose and cellotetraose to ethanol. Both cello-oligosaccharides were effectively converted to ethanol by *F. velutipes*. During incubation, cellotriose was initially hydrolyzed to D-glucose and cellobiose, and almost 80% of the initial amount of cellotriose was hydrolyzed by 2 d. Cellotriose was not detected after 5 d of incubation, and D-glucose and cellobiose were completely depleted after 7 d. β -Glucosidase



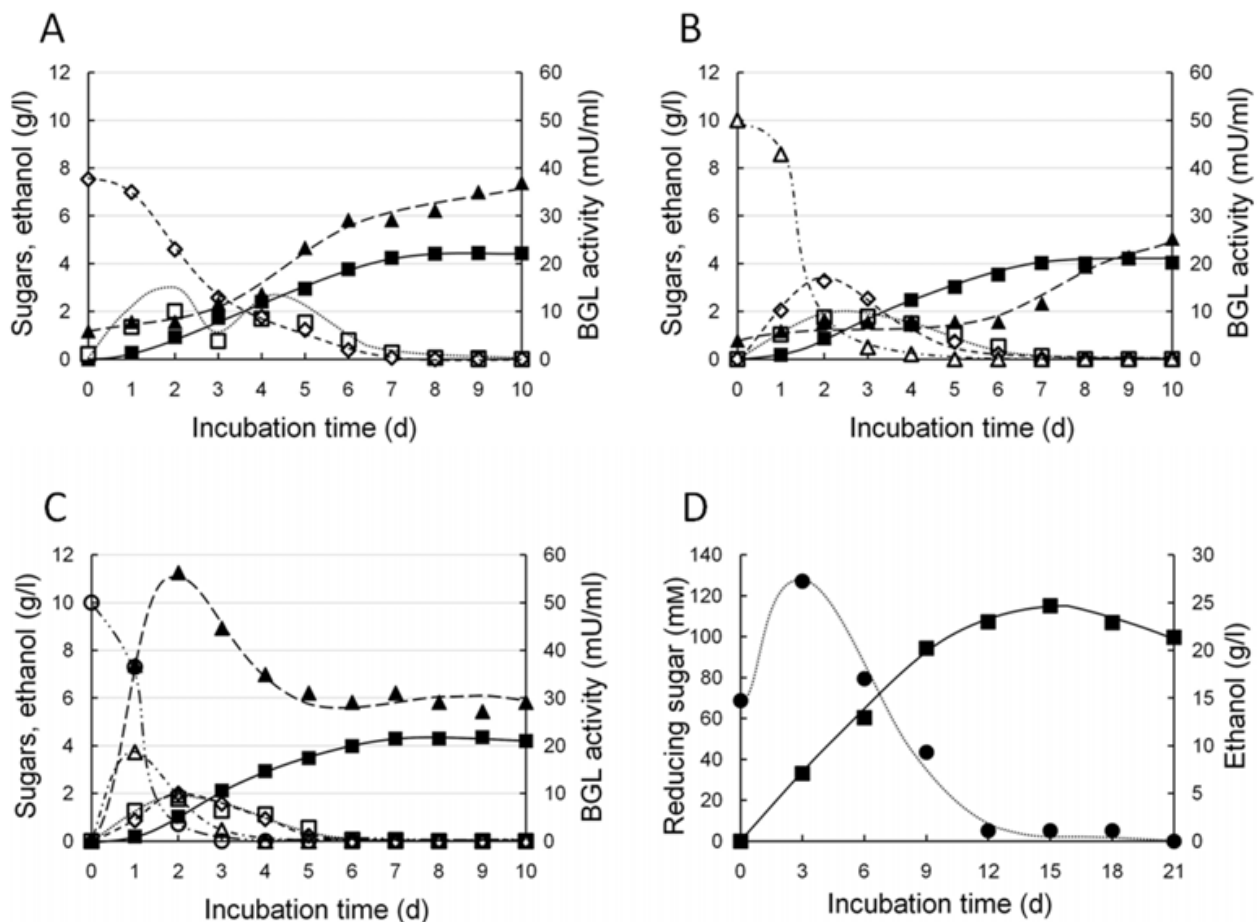
Symbols: closed circle, reducing sugar; closed square, ethanol. The initial sugar concentration was 1% w/v. (Reproduced from Mizuno et al., 2009b)

Fig. 3. Ethanol fermentation from (A) sucrose and (B) maltose by *F. velutipes*

was slightly induced by 6 d, and the activity gradually increased after 6 d. The amount of ethanol increased during incubation and reached a maximum after 7 d of incubation. *F. velutipes* produced 4.2 g/l of ethanol from 10 g/l of cellotriose, equivalent to a theoretical conversion rate of 76% (Fig. 4B). In the case of cellotetraose, it was initially hydrolyzed to cellotriose, cellobiose, and D-glucose, and more than 90% of the cellotetraose was hydrolyzed by 2 d. Cellotetraose was not detected after 3 d of incubation, and cellotriose, cellobiose, and D-glucose were completely depleted after 4, 6 and 7 d respectively. β -Glucosidase activity increased rapidly over 2 d then decreased gradually from 2 d to 5 d, and stabilized at an activity level of about 30 mU/ml. The amount of ethanol increased after incubation, and 4.4 g/l of ethanol was produced from 10 g/l of cellotetraose after 7 d of incubation (Fig. 4C). The ethanol recovery for the theoretical conversion value was 78%.

To date, many microorganisms, including *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, *Rhizopus oryzae*, and *Clostridium thermocellum*, have been reported to produce ethanol (DeMoss & Gibb, 1951; Maas et al., 2006; Ng et al., 1981; Parekh & Wayman, 1986; Weimer & Zeikus, 1977). In general, *S. cerevisiae* is the most widely used microorganism in the industry and is popular in bioethanol production, because it has high efficiency of ethanol production and high ethanol tolerance. However, we focused on basidiomycetes to develop CBP because these microorganisms have both lignocellulose degradation and ethanol fermentation abilities.

Here, we characterized properties of ethanol fermentation by *F. velutipes* Fv-1. The strain converted D-glucose to ethanol at a theoretical conversion rate of 88%, comparable to those of *S. cerevisiae* and *Zymomonas* (Swings & DeLey, 1977). On the other hand, *F. velutipes* scarcely converted pentose and D-galactose to ethanol (Fig. 2). These properties of *F. velutipes* are similar to those of *S. cerevisiae* (Barnett, 1976). Moreover, *F. velutipes* demonstrated the preferable features for CBP when oligosaccharides were used as starting materials (Figs. 3 and 4). The tested oligosaccharides were converted to ethanol at almost the same rate as that of D-glucose, and β -glucosidase activity increased during fermentation. These features are indispensable in CBP, which requires saccharification and fermentation of cellulose contained in the cell wall. It has been reported that *C. thermocellum* and *P. stipitis* can ferment cellobiose (Parekh & Wayman, 1986). Furthermore, *C. thermocellum* can also convert cellulose to ethanol directly (Ng et al., 1981; Lynd et al., 1989; Weimer & Zeikus,



Symbols: open square, D-glucose; open diamond, cellobiose; open triangle, cellotriose; open circle, cellotetraose; closed circle, reducing sugar; closed square, ethanol; closed triangle, β -glucosidase activity. The initial sugar concentration was 1% w/v (A, B, and C) or 5% w/v (D). (Reproduced from Mizuno et al., 2009b)

Fig. 4. Ethanol fermentation from (A) cellobiose, (B) cellotriose, (C) cellotetraose and (D) 5% cellobiose by *F. velutipes*

1997). However, this species cannot be used at the scene of ethanol production because fermentation of *C. thermocellum* is strongly inhibited at relatively low ethanol concentrations (5 g/l) (Herrero & Gomez, 1980). In contrast, it has been reported that basidiomycetes have tolerance of up to 120 g/l of ethanol (Okamura et al., 2001), and therefore basidiomycetes are more suitable for CBP than *Clostridium* strains. From these results, we concluded that *F. velutipes* possesses advantageous characteristics for use in CBP.

3. Properties of ethanol production from biomass by *F. velutipes*

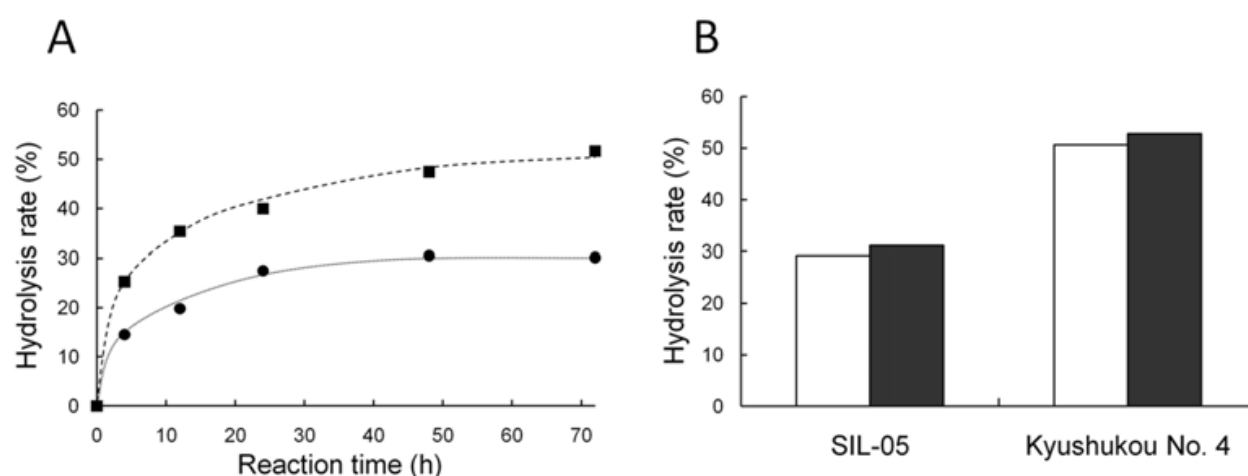
3.1 Use of whole crop sorghums as a raw material in consolidated bioprocessing bioethanol production using *Flammulina velutipes*

The ethanol fermentation abilities of basidiomycetes have not been well characterized, we evaluated the ability of *F. velutipes* in CBP. Preliminary fermentation experiments indicate that *F. velutipes* convert sugars to ethanol much more under the high concentration of biomass which close to solid state cultivation than liquid cultivation condition. Therefore, we employed solid state cultivation which usually performed in artificial cultivation of

mushrooms for the conversion of biomass to produce bioethanol. Sorghum is selected as a possible raw material to produce bioethanol by CBP using *F. velutipes*. Sorghum is a C4 crop of the grass family belonging to the genus *Sorghum bicolor* L. It is well adapted to temperate climates and can be cultured from Kyushu to Tohoku area in Japan. The plant grows to a height from about 120 to above 400 cm, depending on the variety and growing conditions, and can be an annual or a short perennial crop. Sorghum is considered to be one of the most drought resistant agricultural crops, as it is able to remain dormant during the driest periods (Xu et al., 2000). These properties of sorghum are suitable as raw material for the ethanol production. We evaluated the ability of *F. velutipes* in CBP using sorghum strains as a raw material, and solid-state CBP of ground sorghum strains (SIL-05 and Kyushukou No. 4) using *F. velutipes* was investigated. The possibility of sorghum strains as a raw material in the CBP ethanol production by *F. velutipes* is also discussed below.

We selected grinding for the pretreatment of sorghum strains. This can be used on both dry and wet materials, and the cost of grinding is one of the cheapest compared to other methods used for milling biomass. The grinding of sorghum was carried out with an ultra-fine friction grinder. Grinding was performed at room temperature, and was repeated twice. To examine the efficiency of grinding as a pretreatment, the degree of saccharification was tested using commercially available enzymes Celluclast 1.5L (Sigma, St. Louis, MO), Novozyme 188 (Sigma) and Multifect xylanase (Genencor Kyowa, Tokyo).

The saccharification yields of SIL-05 and Kyushukou No. 4 by the enzymes were 30.1% and 51.7% respectively (Fig. 5A). Kyushukou No. 4 is one of the sorghum *brown mid-rib (bmr)* mutants in which caffeic acid *O*-methyltransferase (COMT), a lignin biosynthetic enzyme, activity is reduced as compared to the wild type (Bout & Vermerris, 2003). This property of *bmr* significantly affected the hydrolysis of polysaccharides in the biomass, but there were no significant differences in the proportions of hydrolysis of the components such as cellulose and hemicellulose (Fig. 5). When the saccharification yields of cellulose and hemicelluloses were compared, degradation of hemicelluloses was slightly higher than for cellulose in both types of sorghum.



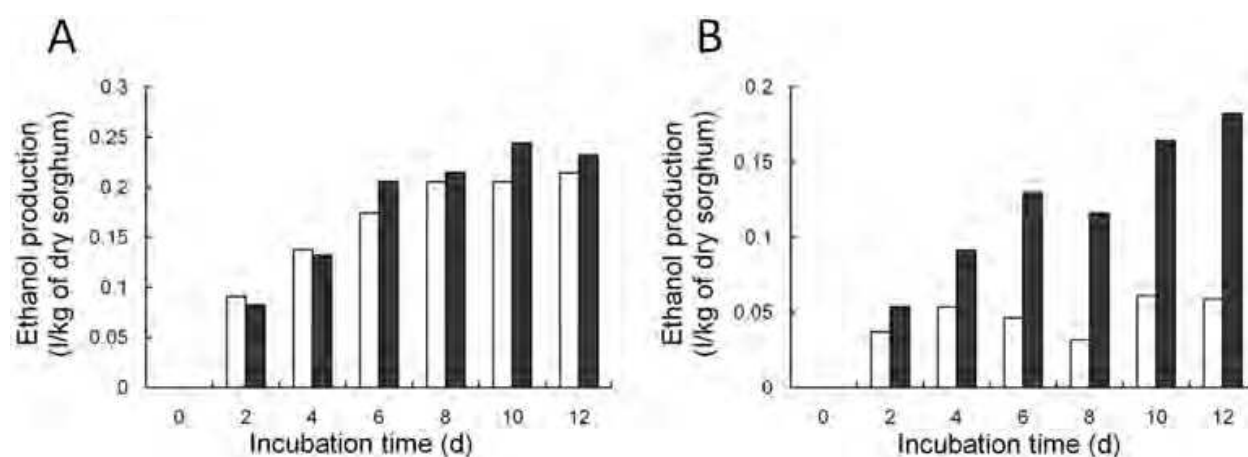
(A) Closed circle, SIL-05; closed square, Kyushukou No. 4. Broken lines were drawn by roughly following the experimental data points. (B) White, cellulose; black, hemicelluloses. (Reproduced from Mizuno et al., 2009a)

Fig. 5. (A) Time course of sorghum hydrolysis and (B) saccharification yield of cellulose and hemicellulose incubated for 72 h

Next, solid-state ethanol fermentation by *F. velutipes* was performed for both sorghum strains. Solid-state fermentation is advantageous because it carries a low ethanol production cost. Generally, sorghums contain 70–80% v/v water, corresponding to 43–25% w/v. These concentrations are necessary to obtain relatively high final ethanol concentrations.

Furthermore, it is possible to reduce the costs of many procedures, such as amount of water, concentration of biomass, treatment of waste water, and so forth, if the water concentration of the raw materials in the all ethanol production procedures is retained. The Fv-1 strain was selected for further study because it not only produces high levels of cellulases, but also because its ability to ferment ethanol is superior to the other strains. Mycelia of Fv-1 were harvested in the late exponential growth phase by centrifugation at $3,000 \times g$ and washed with sterile water. The prepared wet mycelia (20 mg of dry weight) were mixed with 100 mg of ground sorghum for solid-state fermentation.

A larger amount of ethanol was produced from SIL-05 than from Kyushukou No. 4 (Fig. 6). Because SIL-05 contained a larger amount of soluble sugars than Kyushukou No. 4 (Table 1), it should be advantageous for total ethanol fermentation. The ethanol conversion rates for the soluble sugars contained in SIL-05 and Kyushukou No. 4 were 57.2% and 38.9% respectively. The addition of saccharification enzymes was not effective for SIL-05 (Fig. 6A). This corresponded with the results of enzymatic hydrolysis (SIL-05 just hydrolyzed almost 30%) (Fig. 6). However, the ethanol conversion rate for the degraded cellulose was 85.6%, significantly higher than that for soluble sugars. In contrast, although total ethanol production was not high, ethanol production from Kyushukou No. 4 significantly increased when saccharification enzymes were added to the culture (Fig. 6B). Because the cellulose and hemicellulose in Kyushukou No. 4 were more easily hydrolyzed than SIL-05 by cellulases, significantly more ethanol was produced by the addition of the saccharification enzymes. The ethanol conversion rate for the degraded cellulose of Kyushukou No. 4 (98.3%) was much higher than that of SIL-05 (85.6%). Thus, the *bmr* mutation appears to be useful for CBP because it gives a high yield of glucose from biomass without acid or alkali pretreatment. However, the results indicate that the production of cellulases by *F. velutipes* is not sufficient for CBP, or that the saccharification enzymes are suppressed by carbon



White, no added enzymes; black, 15 µl Celluclast 1.5 L and 10 µl Multifect xylanase added.6
Reproduced from Mizuno et al., 2009a)

Fig. 6. Solid-state ethanol fermentation of (A) SIL-05 and (B) Kyushukou No. 4 by *Flammulina velutipes*

	SIL-05	Kyushukou No. 4
Water content (%)	78 (\pm 0.7)	80 (\pm 1.4)
Soluble sugar (%)	12 (\pm 0.1)	4.5 (\pm 0.0)
Cellulose content (%) ^a	5.1 (\pm 0.3)	7.4 (\pm 0.8)
Hemicellulose content (%) ^b	3.6 (\pm 0.1)	5.2 (\pm 0.5)
Other content (%) ^c	1.2 (\pm 0.2)	2.4 (\pm 0.5)

^a The amount of hexose was determined by the anthrone-sulfuric acid method.

^b The amount of pentose was determined by the orcin-Fe³⁺-hydrochloric acid method.

^c All components except for sugars. (Reproduced from Mizuno et al., 2009a)

Table 1. Compositions of SIL-05 and Kyushukou No. 4

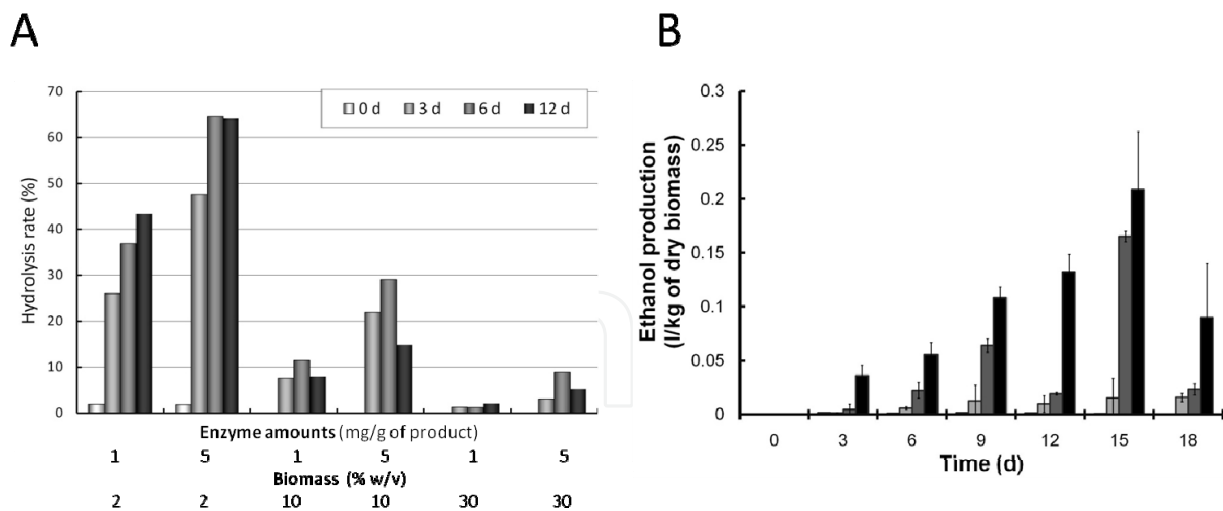
catabolites due to the existence of soluble sugars. Therefore, an effective saccharification enzyme inducing method for *F. velutipes* in the CBP is required.

In this work, we demonstrated CBP ethanol fermentation of sorghum strains by *F. velutipes* Fv-1. The procedure is quite simple and cost effective, and can reduce energy consumption, because the raw material is simply ground and then mixed with mycelia. We demonstrated the merit of high concentrations of soluble sugars and the *bmr* mutation in sorghums. Both sorghum strains can be used in CBP. The *bmr* mutation is only found in sorghums, corn, and pearl millet, giving sorghum an advantage over many other crops for ethanol production. Future studies should focus on the improvement of CBP using *F. velutipes* and the selective breeding of novel types of sorghums with high concentrations of soluble sugars and the *bmr* mutation.

3.2 Solid state fermentation of rice straw by *F. velutipes*

The solid state ethanol fermentation by *F. velutipes* was performed for ammonia treated rice straw. Solid state cultivation has a large merit to decrease the ethanol production cost. But it has demerit on the saccharification of biomass. As shown in Fig. 7A, saccharification of biomass at high concentration is quite difficult. Significant amount of cellulase is necessary to obtain enough level of saccharification, and saccharification yield do not increase in proportion to the amount of cellulase if increased the amount of cellulase. Furthermore, saccharification yield will be significantly decreased under the high substrate condition. The hydrolysis rate of 30% w/v biomass was very low (less than 10%). In contrast, ethanol yield was equivalent to 80-90% of hydrolysis rate so that the merit of our process using *F. velutipes* was proven (Fig. 7B). In the case that enzymes were not added, ethanol production by *F. velutipes* was only 0.026 l/kg of dry biomass, equivalent to a theoretical ethanol recovery rate of 5.9% from total hexose. In contrast to no enzymes addition, in the case that 1 and 5 mg/g product of enzymes were added to the fermentation, ethanol production after 15 d by *F. velutipes* was 0.26 and 0.34 l/kg of dry biomass, respectively. The ethanol conversion rates of 1 and 5 mg/g product enzymes addition were 61.6% and 77.8% for total hexose, respectively. The maximum weight loss was approximately 70% in the case that no enzymes were added to the fermentation, while the maximum weight loss for enzyme addition of 1 and 5 mg/g product were approximately 90% and 96% respectively (data not shown).

These results suggest *F. velutipes* has favourable properties for CBP. It could be expected that development of novel bioethanol production process by using *F. velutipes*.



(A) Saccharification yield of ammonia treated rice straw by enzymes. (B) Solid-state ethanol fermentation of ammonia treated rice straw by *F. velutipes*. Right gray, no added enzymes; gray, 1 mg/g product enzymes added; black, 5 mg/g product enzymes added.

Fig. 7. Saccharification and ethanol production at high biomass concentration

4. Development of a gene transfer system for *F. velutipes*

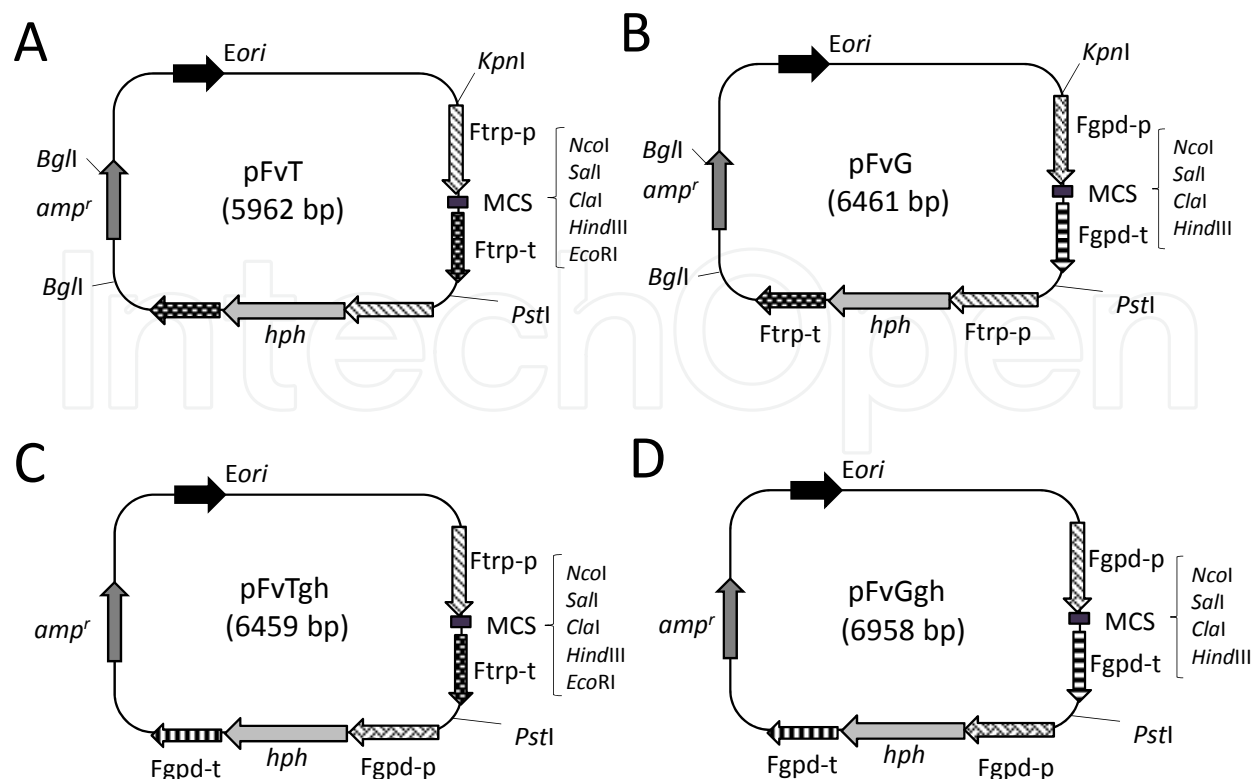
4.1 Development of a gene transfer system for the mycelia of *F. velutipes*

As shown in above, we found the edible mushroom *F. velutipes* Fv-1 strain to be an efficient ethanol producer, and, we demonstrated its preferable properties of ethanol fermentation from various sugars (Mizuno et al., 2009b), whole crop sorghums and rice straw (Mizuno et al., 2009a). However, the strain can only slightly convert pentoses, which account for approximately 20-30% of plant cell walls, into ethanol (Mizuno et al., 2009a). Therefore, genetic engineering of the pentose metabolism is necessary to make possible the ethanol fermentation from pentose. Furthermore, more efficient (low cost) conversion of biomass to ethanol could be expected if saccharification ability was strengthened by expressing cellulases. A transformation method of *F. velutipes* by the electroporation protocol for basidiospores has been reported (Kuo et al., 2004), but it requires a long period to produce basidiospores because it must go through fruiting body formation, and cannot eliminate the risk of contamination in the process of spore harvest. Since screening of many transformants is needed for improvement of the metabolic pathway by genetic engineering, the development of a simpler transformation method is desired to obtain high numbers of transformants.

Therefore, an adequate condition for protoplast preparation from mycelia of *F. velutipes* Fv-1 strain was investigated, and simpler a transformation protocol for this fungus was developed by the calcium-PEG method and the restriction enzyme-mediated-integration (REMI) method.

First, we constructed a pFvT vector for transformation of the *F. velutipes* Fv-1 strain (Fig. 8A). The vector possessed a *F. velutipes* tryptophan synthetase gene promoter and terminator (GenBank no. AB028647) to regulate expression of the constructed genes, and the hygromycin phosphotransferase gene (*hph*) from *Escherichia coli* as selection marker. The *hph* gene was obtained from pCAMBIA1201 vector (CAMBIA; <http://www.cambia.org/>).

Next, conditions to prepare protoplast from the mycelia of *F. velutipes* were optimized by modifying a method for *Phanerochaete sordida* (Yamagishi et al., 2007). The *F. velutipes* Fv-1



MCS, multiple cloning sites; Ftrp-p, *trp1* promoter from *F. velutipes*; Ftrp-t, *trp1* terminator from *F. velutipes*; Fgpd-p, *gpd* promoter from *F. velutipes*; Fgpd-t, *gpd* terminator from *F. velutipes*; *hph*, hygromycin B phosphotransferase gene; *amp^r*, ampicillin resistance gene; *Eori*, pUC19 ori. (Reproduced from Maehara et al., 2010b)

Fig. 8. Structures of the plasmids used in this study

strain was grown in PCMY (1% polypeptone, 0.2% casamino acid, 1% malt extract, and 0.4% yeast extract) medium at 25°C for 3 d. Then the mycelia were collected and incubated in enzyme solution [1.5% cellulase Onozuka-RS (Yakult Pharmaceutical, Tokyo) and 1.5% lysis enzyme (Sigma, St. Louis, MO) in 0.75 M MgOsm (0.75 M MgSO₄, 20 mM MES, pH 6.3)] at 30°C for 5 h. The protoplasts were filtered through Miracloth (Cosmo Bio, Tokyo), washed at twice with 1 M SorbOsm (1.0 M sorbitol, 10 mM MES, pH 6.3), and suspended in SorbOsm plus 40 mM CaCl₂ solution to a final concentration of approximately 10⁸ protoplasts ml⁻¹.

Genetic transformation was investigated using the pFvT vector and the protoplasts prepared as described above. The transformation procedures for *Lentinus edodes* (Sato et al., 1998) and *Schizophyllum commune* (Van Peer et al., 2009) were modified for the transformation of *F. velutipes* Fv-1. In the course of the transformation process, the effect of the structure of the plasmid DNA on transformation was evaluated using circular and linear pFvT plasmids. Approximately 6-fold transformants were obtained when the plasmid DNA was linearized (Table 2).

Because the REMI method is a popular transformation tool for fungi (Hirano et al., 2000; Maier & Schäfer, 1999; Riggle & Kumamoto, 1998; Sato et al., 1998), we evaluated the effect of REMI on the transformation for *F. velutipes* Fv-1. The *F. velutipes* Fv-1 strain was transformed by pFvT with a restriction enzyme, *BglI*, *KpnI*, or *PstI*. The addition of the restriction enzymes increased the number of transformants by about 1.6- to 5.8-fold (Table 2). This suggests that the addition of restriction enzymes enhanced the transformation efficiency of *F. velutipes*. Therefore, to find the optimum enzyme concentration for REMI, we

No. of transformants*				
Form of pFvT	Restriction enzyme (50 U)			
	none	<i>Bgl</i> II	<i>Kpn</i> I	<i>Pst</i> I
Circular	0.7 ± 1.2	7.3 ± 1.2	18.3 ± 6.1	25.7 ± 7.1
Linear	4.4 ± 0.6	12.7 ± 6.4	20.0 ± 8.7	21.7 ± 7.1

Form of DNA	Plasmid			
	pFvT	pFvG	pFvTgh	pFvGgh
Circular	11.3 ± 2.6	12.3 ± 2.5	28.3 ± 1.3	33.3 ± 2.9
Linear	11.3 ± 6.6	10.7 ± 6.9	24.7 ± 8.4	27.7 ± 11

*The values represent the average and standard deviation of triplicate. (Reproduced from Maehara et al., 2010a & 2010b)

Table 2. Numbers of transformants obtained by the REMI method

performed transformation using circular pFvT plasmid with the presence of various concentrations of *Pst*I (Fig. 9). As for the results, the number of transformants obtained was affected by the amount of restriction enzyme. The efficiency was significantly increased by the addition of *Pst*I at 25 units, by it gradually decreased when the *Pst*I amount was over 25 units, suggesting that the optimal value for transformation mediated by the *Pst*I is 25 units. In conclusion, we found a simple transformation procedure for the mycelia of *F. velutipes* Fv-1 strain by the calcium-PEG method combined with REMI. The transformation method of *F. velutipes* Fv-1 strain does not require a process of spore formation, because the mycelia could be used as starting material. Moreover, a high efficiency of transformation was obtained by the adoption of REMI.

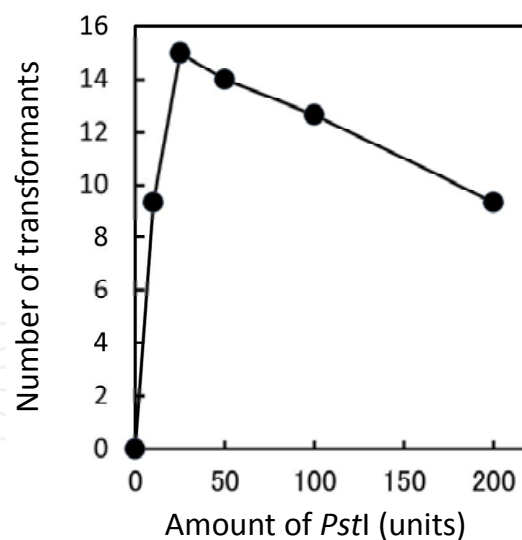


Fig. 9. Effects of the amount of *Pst*I on transformation by REMI method (Reproduced from Maehara et al., 2010a)

4.2 Improvement of the transformation efficiency of *Flammulina velutipes* Fv-1 using the glyceraldehydes-3-phosphate dehydrogenase gene promoter

To make possible genetic manipulation in *F. velutipes*, we constructed the pFvT plasmid containing the hygromycin phosphotransferase gene (*hph*) under the control of the

tryptophan synthetase gene (*trp1*) promoter, and developed an easy transformation method for *F. velutipes* by the REMI method (Maehara et al., 2010a). Here, we focused on the promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene because many tools such as promoters and selection markers are desirable for effective metabolic pathway engineering of *F. velutipes* Fv-1. The *gpd* promoters are the most frequently used constitutive promoters in basidiomycetes. GPD constitutes up to 5% of the soluble protein in *Saccharomyces cerevisiae* and other higher eukaryotic organisms (Piechaczyk et al., 1984; Punt et al., 1990), and *gpd* mRNA accounts for 2-5% of the poly (A)+ RNA in yeast (Holland & Holland, 1978).

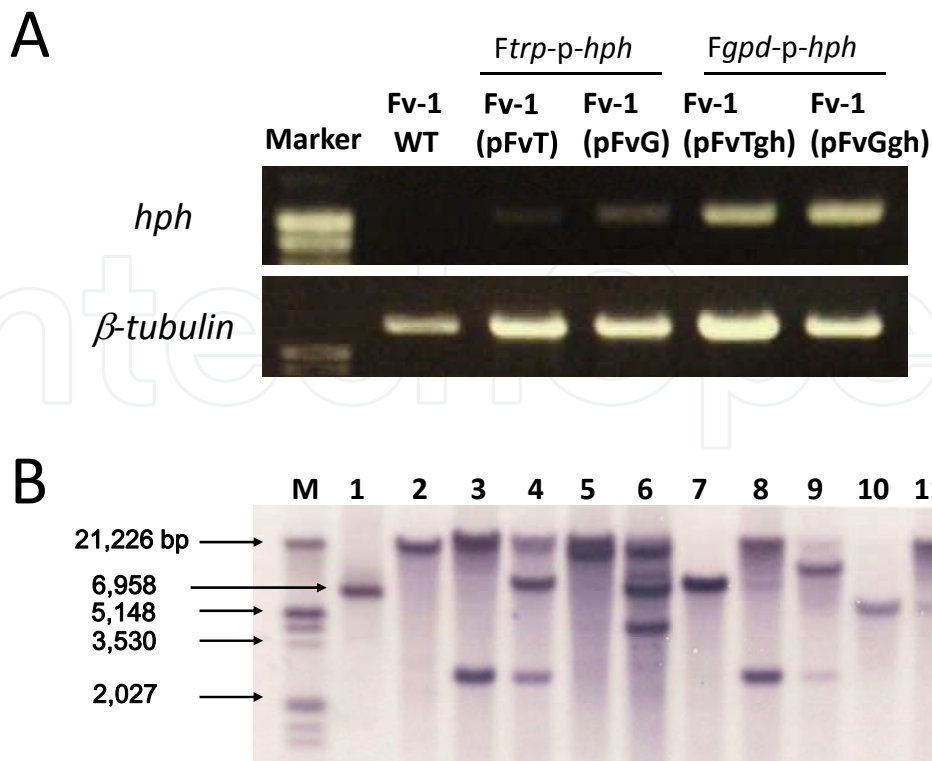
In this section, we described that construction of new plasmids having the *hph* gene from *Escherichia coli* as a selection marker, which regulated by the *gpd* promoter and the potency of the *gpd* promoter from *F. velutipes* were evaluated.

First we constructed three vectors, pFvG, pFvTgh, and pFvGgh, by modification of pFvT. The pFvT vector possessed a *trp1* promoter and terminator regulating the expression of the constructed genes, and the *hph* gene as selection marker (Fig. 8A, Maehara et al., 2010a). Vectors pFvG (Fig. 8B) and pFvGgh (Fig. 8D) contained the *gpd* promoter and the terminator of *F. velutipes* (Kuo et al., 2004) located upstream and downstream of a multiple cloning site (MCS), and both pFvTgh (Fig. 8C) and pFvGgh (Fig. 8D) contained the *gpd* promoter and the terminator located upstream and downstream of the *hph* gene (Maehara et al., 2010b).

To determine the potency of the *gpd* promoter, we compared transformation efficiency by the *gpd* promoter with that by the *trp1* promoter. Gene integrations were performed by the REMI method. Protoplasts were prepared from mycelia of the *F. velutipes* Fv-1 strain, and then plasmids were transformed into the protoplasts with *Pst*I (25 U). As shown in Table 2, about 10 transformants (10.7 to 12.3) were obtained by the transformation of pFvT and of pFvG, which contain the *hph* gene controlled by the *trp1* promoter. In contrast, as for the results of the transformation of pFvTgh and pFvGgh, the numbers of transformants were significantly increased and about 24.7 to 33.3 transformants were obtained, suggesting that the activity of the *gpd* promoter was higher than that of the *trp1* promoter in *F. velutipes* Fv-1. There is a difference of about 500-bp in the length of pFvT and pFvG, or pFvTgh and pFvGgh, but no significant difference in the number of transformants obtained by pFvT and by pFvG, and by pFvTgh and pFvGgh was not observed. It might suggest, that the difference of the sizes of these plasmids was not affected on transformation efficiency.

To compare the activity of the *gpd* and the *trp1* promoter, the expression levels of the *hph* gene in each transformant were examined by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from each set of three transformants and equal amounts of RNAs from each set of three clones were mixed and used as template for RT-PCR. As shown in Fig. 10A, the intensities of the bands of the pFvTgh and pFvGgh transformants were stronger than that of the pFvT and pFvG transformants (upper panel), suggesting that the expression level of the *hph* gene in the pFvTgh and pFvGgh transformants was higher than that in the pFvT and pFvG transformants. The results were corresponded to the transformation efficiency presented in Table 2, and strongly suggest that the *gpd* promoter is functional in the heterologous gene expression system in *F. velutipes* Fv-1 to improve the expression level of the target gene.

Finally, in order to determine whether the plasmid vector was integrated into the genomic DNA by the REMI method, the genomic DNAs of 10 randomly selected pFvGgh transformants were analyzed by Southern blot using the digoxigenin-labeled *hph* gene as a probe (Fig. 10B). Hybridization signals were detected in all the transformants, and multiple



(A) RT-PCR-based evaluation of the expression of the hygromycin phosphotransferase gene: Upper panel, *hph* gene; Lower panel, β -tubulin gene as a control. (B) Southern blotting of *Pst*I digested genomic DNA using the *hph* gene probe: Lane M, DNA molecular size markers (values on left); lane 1, pFvGgh as a positive control; lanes 2-11, genomic DNA from transformants. (Reproduced from Maehara et al., 2010b)

Fig. 10. Analysis of the transformants obtained by REMI method

hybridization signals were also detected in some transformants. There was no signal from the genomic DNA of wild-type Fv-1 as a negative control (data not shown). These results indicate that at least a single *hph* gene was introduced into all the transformants, and the *hph* gene is thought to exist as a multicopy in the genomic DNAs of many transformants (Fig. 10B, lanes 3, 4, 5, 6, 8, 9 and 11). The same size bands were detected between 2,027 and 3,530-bp in four transformants (Fig. 10B, lanes 3, 4, 8 and 9). These bands might represent about 2,700-bp of the full-length *gpd* promoter-*hph*-*gpd* terminator region. A 6.9-kb DNA fragment, corresponding to the size of the pFvGgh plasmid, was observed in the genome of only one clone (Fig. 10B, lane 6), indicating that the full length of the plasmid was successfully introduced into the transformant. Consequently, we estimate the probability of integration of full-length pFvGgh vector by the REMI method to be approximately 10%. In our previous study, the probability of integration of the full-length vector was 30% so that the frequency of REMI events of Fv-1 was 10-30% (Maehara et al., 2010a). This value seems to be the comparable level in the case of model mushroom, *Coprinus cinereus* (8-56%) (Granado et al., 1997).

In conclusion, we demonstrated that the *gpd* promoter from *F. velutipes* Fv-1 would be a useful in the transformation system of the strain. The transformation efficiency was about 3 times improved by the use of the *gpd* promoter. The vectors constructed in this study will be available to improve the genetic engineering of *F. velutipes* Fv-1 for ethanol fermentation from pentose.

5. Conclusion

In spite of CBP is gaining recognition of a low-cost biomass processing as it involves enzyme production, completely no enzyme process which does not add the saccharification enzymes have not been established. In this study, we demonstrated that *F. velutipes* can highly convert biomass to ethanol using only small amount of saccharification enzyme even in the quite high concentration of biomass such as 30% w/v. These results suggest *F. velutipes* has favorable properties for CBP. Generally, artificial cultivation of mushrooms in polypropylene bottles is performed under the condition of water content 70 to 80%. The condition must be most suitable condition to cultivate the mushrooms. Therefore, *F. velutipes* will be especially effective in situations that CBP performed under the high concentration of biomass. We believe that this point would be advantage of *F. velutipes* compared with the other microorganisms engineered for CBP and even for fungus which is possible to ferment the both pentose and hexose. In the future, we would like to develop a novel bioethanol production process by using *F. velutipes*.

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Recent studies have shown strong evidence of human activity impact on the climate of the planet. Higher temperatures and intensification of extreme weather events such as hurricanes are among the consequences. This scenario opens up several possibilities for what is now called "green" or low carbon economy. We are talking about creating new businesses and industries geared to develop products and services with low consumption of natural resources and reduced greenhouse gases emission. Within this category of business, biofuels is a highlight and the central theme of this book. The first section presents some research results for first generation ethanol production from starch and sugar raw materials. Chapters in the second section present results on some efforts around the world to develop an efficient technology for producing second-generation ethanol from different types of lignocellulosic materials. While these production technologies are being developed, different uses for ethanol could also be studied. The chapter in the third section points to the use of hydrogen in fuel cells, where this hydrogen could be produced from ethanol.

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