

Research on Production of Cellulosic Ethanol – Development of High-Efficiency Yeast Strain using Mutagenesis and Selection and Self-Cloning, and Study of Entire Process –

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ABSTRACT

A fermentation organism capable of producing a high fermentation yield in the presence of inhibitors, necessary for the realization of increased efficiency in the process of production of second-generation bioethanol from inedible plant biomass, has been developed. The construction of the organism involved a combination of mutagenesis and selection and self-cloning, and results indicated the effectiveness of this combination in the creation of fermentation organisms. Self-cloning is a form of genetic modification implemented within a scope that makes it possible to treat the organism as non-genetically-modified.

Mutagenesis and selection using biomass hydrolysates increased both the organism's tolerance to inhibitors in hydrolysates and its ethanol fermentation yield. Using the selected strain as a base, genes for the expression of transaldolase and alcohol dehydrogenase were enhanced by self-cloning. The developed strain was able to produce a higher concentration of ethanol.

An examination of the entire process led to the discovery of a biomass pre-treatment condition that would control the production of phenols, which act as inhibitors. A process using this pre-treatment condition and the developed fermentation organism would be able to produce 213 L of ethanol from 1 dry-ton of corn stover.

1. Introduction

The replacement of oil refineries by bio-refineries is advocated as a response to concerns over the depletion of fossil fuels and the necessity for the reduction of CO₂ emissions. Bio-refineries are processes that produce useful substances from biomass, a renewable resource, as the starting material. One of the most highly developed of these processes is the process of production of bioethanol. First-generation bioethanol is produced using the edible parts of sugar cane and corn plants; research is now proceeding to commercialize second-generation bioethanol, cellulosic bioethanol produced using the inedible parts of plant materials. A number of facilities able to produce several tens of millions of gallons of bioethanol per year by means of saccharification and fermentation (microbial fermentation of sugars obtained from the breakdown of biomass) were established throughout the world between 2014 and 2015. The authors conducted research on the production of cellulosic bioethanol by means of saccharification and fermentation, and constructed a fermentation organism

displaying increased tolerance to inhibitors in order to increase the efficiency of the process as a whole.

2. Overview of Ethanol Production Process

2.1. Composition of Biomass

Plant biomass is mainly composed of cellulose, lignin, and hemicellulose (Fig. 1). Cellulose is a polymer formed

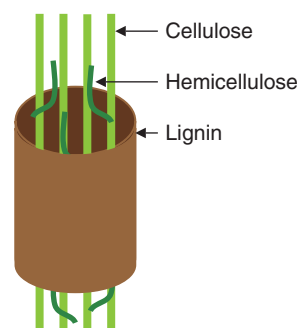


Fig. 1 Illustration of biomass structure

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by strong bonding between hexose (glucose) units via β -1,4 linkages. It supports the structure of plants as a fibrous material. Few organisms produce cellulase, the enzyme that degrades cellulose, and breaking the substance down therefore represents a challenge.

Lignin is a polymer containing numerous six-membered rings. It is strongly integrated and wraps around cellulose, forming plant structures. Hemicellulose functions to bond lignin and cellulose. Part of the hemicellulose structure contains pentose.

2.2. Composition of Process: Pre-treatment, Saccharification, Fermentation, and Condensation

Figure 2 shows a schematic overview of the bioethanol production process.

Sucrose and glucose in the juice extracted from pressed sugar cane is able to be directly used by numerous fermentation organisms. Starches in the edible parts of corn plants contain glucose units weakly bonded by α -1,4 linkages, and fermentation organisms are able to use the substances following treatment of the biomass with the low-cost enzyme amylase. Because of this, the first-generation bioethanol production process is comparatively simple.

In the second-generation bioethanol production process (termed “second-generation process” below), the integrated structure formed by cellulose, lignin, and hemicellulose is destroyed, and the cellulose is broken down and used as glucose. The challenges presented by second-generation processes originate in the strong structure of the biomass.

Second-generation processes can be broadly divided into gasification, and saccharification and fermentation methods. The saccharification and fermentation method is further divided into a number of processes.

Lignin is broken down in the pre-treatment process. Thermochemical methods are effective in the pre-treatment process, and hot water treatments, alkali treatments, and acid treatments are generally used.

In the saccharification process, cellulase is added as an aqueous solution, and cellulose is broken down into glucose. The high cost of cellulase is one of the issues of the saccharification process.

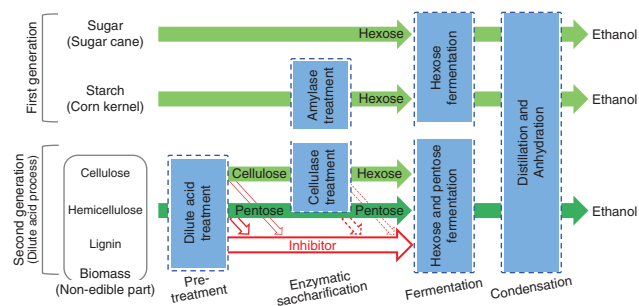


Fig. 2 Schematic diagram of bio-ethanol production process

Table 1 Characteristics of fermentation inhibitors

Category	Description	Inhibition mechanism	Countermeasure		
			Pre-treatment	Fermentation condition	Genetic modification
Phenols	Hydrophobic	Damage to membrane	Suppress production	–	–
Aldehydes	Oxidative	Oxidation of enzymes, etc.	–	–	Overexpression of ADH to reduce aldehydes
Organic Acids	Dissociation depends on pH	Damage due to change in intracellular pH	–	Fermentation pH control	Overexpression of TAL subject to damage by acetic acid

Pentose is also formed in the pre-treatment and saccharification process, chiefly originating in hemicellulose. In order to make maximum use of the biomass and increase the efficiency of the processes, it is considered necessary to use pentose for fermentation in the second-generation process. Because most organisms are unable to efficiently use pentose, this necessitates genetic modification by means of techniques such as genetic modification and mutagenesis and selection. In the fermentation process, hexose and pentose are converted into ethanol.

The condensation process involves distillation and anhydration. Because distillation uses a considerable amount of heat energy, it is desirable that the concentration of ethanol in the fermentation liquor should be high.

2.3. Fermentation Inhibitors

During the pre-treatment process, lignin and hemicellulose become denatured into a variety of substances. Some of the pentose and hexose produced becomes excessively degraded. These chemical substances include inhibitors that strongly impede the functioning of microorganisms during the fermentation process. Figure 3 shows a schematic diagram of the paths to formation of inhibitors. Because inhibitors cause a considerable decline in the yield from the fermentation process, they have a considerable effect on the ethanol production process as a whole. Table 1 provides an overview of types of fermentation inhibitors and their characteristics.

Among fermentation inhibitors, acetic acid is known

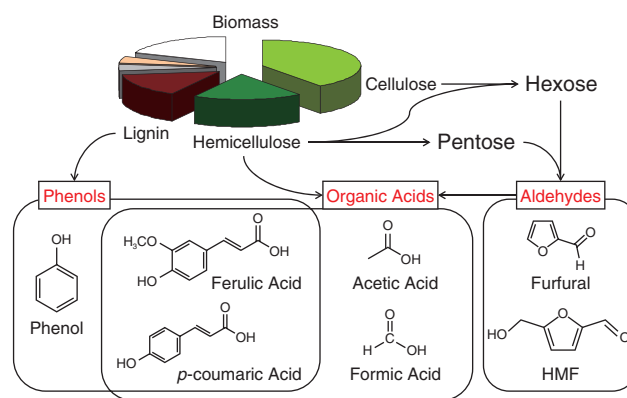


Fig. 3 Schematic diagram of production of fermentation inhibitors

to reduce the activity of transaldolase (TAL), an important enzyme in the assimilation of pentose. At the same time, the enzyme alcohol dehydrogenase (ADH) is known to reduce and attenuate aldehydes⁽¹⁾.

2.4. Types of Biomass

Research on biomass to form the raw material for the second-generation process has focused mainly on agricultural residue, including rice straw and wheat straw, corn stover (corn leaves and stalks), and bagasse (strained sugar cane lees).

In the US, where research on the second-generation process has been most vigorously advanced, expectations are high for the use of the agricultural residue corn stover (corn stalks and leaves). Estimates from data published by the United Nations Food and Agriculture Organization⁽²⁾ indicate that corn stover is the third most abundant agricultural residue in the world after rice straw and wheat straw, and it can be considered to possess considerable potential to become the main raw material for the second-generation process.

2.5. Pre-treatment Methods

A variety of pre-treatment methods are available, including hot water treatment, alkali treatment, and dilute acid treatment. Research is being advanced on dilute acid treatment methods in the US. While dilute acid treatment promotes the breakdown of hemicellulose, making the use of degradative enzymes unnecessary, it is also known to produce high quantities of the fermentation inhibitor acetic acid. High quantities of aldehydes are also observed as a result of dilute acid treatment, and pyrolysis simulations have shown this to be a result of excessive degradation of pentose and hexose.

When the volume of sugars obtained from the biomass in the pre-treatment process and the subsequent saccharification process is increased, the concentration of inhibitors also tends to increase. The fact that the pre-treatment and saccharification processes, and the inhibitor resistance of the fermentation organisms, contribute to this trade-off relationship with the fermentation yield while being mutually related necessitates the realization of fully integrated modifications.

3. Subject and Methodology of Research

3.1. Research Aim

The high cost of saccharification enzymes represents a major issue for the second-generation process. With the current level of performance of enzymes on the market as a precondition, the present research sought to reduce the volume of enzymes employed. This would necessitate powerful pre-treatment, but would also increase the volume of inhibitors produced. The aim of the research was therefore to enhance the inhibitor resistance of the fermenting organisms, that is, to increase the fermentation

yield when inhibitors were present, in order to increase the efficiency of the second-generation process.

3.2. Selection of Biomass Type and Pre-treatment Method

The selection of the type of biomass and the pre-treatment method employed determines the inhibitors, sugars, and other components of the hydrolysate supplied to the fermentation process, in addition to the characteristics of the fermentation organism demanded for the fermentation process. In order to enhance the inhibitor resistance of the fermentation organism, it is necessary to determine the target biomass type and pre-treatment method.

Corn stover was the target biomass for the present research, and dilute acid treatment was selected as the pre-treatment method.

3.3. Selection of Base Organism

The ability to naturally assimilate pentose, necessary for the realization of increased process efficiency, was prioritized in the selection of the base organism for the construction of the fermentation organism. While normal microorganisms do not possess the ability to assimilate pentose, necessitating the introduction of foreign genes via genetic modification, *Meyerozyma guilliermondii* naturally possesses this ability, making it possible to construct a non-genetically-modified organism. Even if biotechnological genetic modification was conducted, if only the genes naturally present in the organism were the subject of the process, it would be defined as self-cloning, and fall outside the purview of the Cartagena Protocol on Biosafety, which regulates genetically modified organisms, and the organism could be treated as non-genetically-modified. Given the fact that this would offer global advantages, including more ready social acceptance and simpler administrative procedures, *M. guilliermondii* was selected as the base organism for the creation of a fermentation organism.

3.4. Method of Enhancing Metabolic Pathways

It is possible to modify the reactions of a fermentation organism to sugars and inhibitors by modifying the metabolic pathways within the organism. Figure 4 shows a simplified image of the ethanol fermentation pathway in *M. guilliermondii*.

The following method of enhancing the fermentation organism's resistance to inhibitors was employed in the present research: First, a mutagenesis and selection method was applied using biomass hydrolysate, and promoters were genetically modified in those areas in which further enhancement was necessary. The mutagenesis and selection method was again applied in order to adjust the disruption of the metabolic balance caused by the genetic modification. Because promoters control the degree of expression of specific enzymes in organisms, while genetic modification would make it possible to enhance the degree of expression of the desired enzymes, it could also be expected to

disrupt the balance of the organism's network of metabolic pathways. It was predicted that adjusting the disrupted metabolic balance would make it possible to obtain a high-performance fermentation organism.

3.5. Mutagenesis and Selection

In the selective breeding of plants and animals, mutations and hybridizations are used in order to select for desired characteristics. A similar modification, involving mutagenesis and selection, is also possible in the case of microorganisms. If numerous microorganisms with mutated DNA are cultured in a medium which, for example, contains only pentose and no hexose, of all the mutant strains, only mutant strains able to assimilate pentose will survive. In addition, the fact that the organisms with the most rapid assimilation speed will overwhelm the others means that it is possible to obtain the strain with the most desirable characteristics even within the same mutation.

In order to increase the speed of mutagenesis and selection, it is necessary to artificially induce mutations with a high level of frequency. The disparity mutagenesis method makes this possible. Replication errors invariably occur when DNA is replicated in living organisms. Artificial control of this correction function makes it possible to induce mutations with a high level of frequency and at random positions in the DNA using the disparity mutagenesis method. The method uses a modified DNA repair enzyme that induces replication errors at a higher frequency, or a mutagen cocktail with the same effect.

The present research created a biomass-derived hydrolysate, and employed it in a process of mutagenesis and selection. Because this made it possible to select microorganisms displaying resistance to a real biomass hydrolysate, it was possible to create microorganisms with a high level of suitability for the process. Disparity

mutagenesis was also employed in the second process of mutagenesis and selection conducted in order to adjust the organism's metabolic networks. These processes of mutagenesis and selection using the disparity mutagenesis method were conducted by the developer of the process, Neo-Morgan Laboratory, a joint research partner with Honda.

3.6. Self-cloning

In order to further increase the inhibitor resistance of the selected fermenting organism using an actual biomass hydrolysate, genetic modification falling within the category of self-cloning was employed.

TAL occurs in the metabolic pathway for fermentation of pentose into ethanol (Fig. 4), and the reduction of this enzyme's activity in the presence of acetic acid has been reported for baker's yeast, *Saccharomyces cerevisiae*⁽³⁾. Based on metabolome analysis, it was assumed that the behaviour of TAL in the presence of acetic acid would be the same for *M. guilliermondii* as for baker's yeast. The genetic sequence in *M. guilliermondii* that displayed the closest homology with the TAL sequence in baker's yeast in a database⁽⁴⁾ was selected to enhance its expression as TAL in the organism. The goal of this enhancement of TAL expression was to increase acetic acid resistance, promote growth (promote pentose assimilation), and increase the fermentation yield. To obtain the samples used in the metabolome analysis, a culture solution was drawn off at 24 and 72 hours following the commencement of fermentation in a CS hydrolysate, and methanol was added in order to stop the biological reaction. The internal standard solution method was employed to quantify the samples.

Broadly divided, eight types of ADH are known to be produced by baker's yeast⁽⁴⁾. The considerations in selecting the ADH for enhanced expression in *M. guilliermondii* were that the reaction proceeded in a forward direction, that the enzyme possessed the ability to reduce aldehydes, and that dependence on the coenzyme NADH was assumed. The genetic sequence in *M. guilliermondii* that displayed the highest level of homology with ADH1 in baker's yeast was selected. The goal in enhancing the expression of ADH in the organism was to increase aldehyde resistance and increase the fermentation yield.

The enhancement of TAL and ADH expression in the organism proceeded as follows: A strain of *M. guilliermondii* displaying auxotrophy for uridine as the result of a natural mutation was obtained from a fluoroorotic acid containing medium. It was considered that this uridine auxotrophic strain possessed a mutation of the gene *ura3*, which codes orotidine 5'-phosphate decarboxylase⁽⁵⁾. The DNA segment shown in Fig. 5 was inserted into the site in order to simultaneously help eliminate the uridine auxotrophy by repairing *ura3* and enhance expression of TAL and ADH by means of homologous recombination. A glyceraldehyde 3-phosphate dehydrogenase promoter was employed to realize steady-state and strong expression of

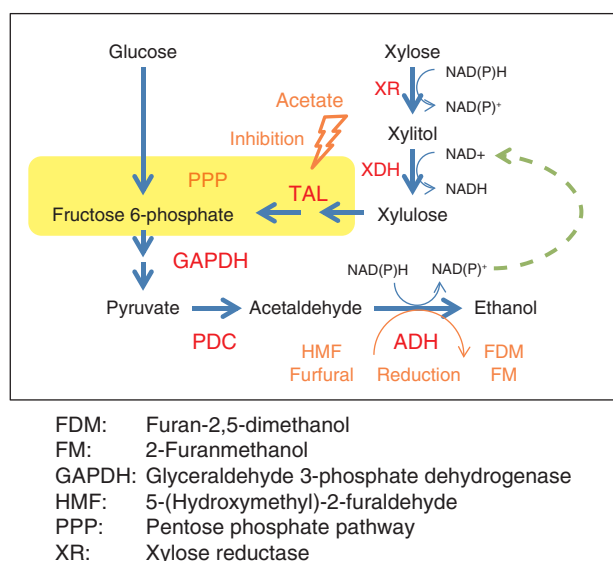


Fig. 4 Metabolic pathway of *Meyerozyma guilliermondii*

TAL. In the case of ADH, a xylose reductase promoter was employed to enhance expression of ADH in the presence of xylose. 1000 bp upstream of the initiator codon were taken as promoters, and 400 bp downstream of the termination codon were taken as terminators. Frozen-EZ Yeast Transformation II, manufactured by Zymo Research Corp, was employed for phylogenetic transformation.

3.7. Results of Mutagenesis and Selection and Self-cloning

The present research initially conducted mutagenesis and selection using a hydrolysate produced from rice straw biomass heat-treated with an aqueous ammonia solution (RS hydrolysate). This procedure produced a variety of effects, including boosting the organism's ability to assimilate pentose such as xylose and arabinose, reducing the incidence of fermentation by-products, restricting growth of yeasts and increasing ethanol production by limiting the inflow of carbon sources to the TCA cycle, and increasing expression of anti-oxidants, as determined by a metabolome analysis of the strains before and after mutagenesis and selection.

The strains produced by mutagenesis and selection were subjected to enhancement of expression of TAL and ADH realized by self-cloning. A real-time PCR analysis of the self-cloning strains showed an increase of two times in TAL expression and around 1000 times in ADH expression. In the preparation of samples for real-time PCR, an RNAqueous-4PCR Kit was used for RNA isolation and for DNase treatment, a High Capacity RNA-to-cDNA Kit was used to create cDNA, and a Fast SYBR Green Master Mix Kit was used for RNA quantification.

The metabolome analysis of the self-cloning strains indicated that the pentose phosphate pathway (PPP) had been promoted, verifying the effect in enhancing expression of TAL. The same analysis also suggested that XDH was acting as a bottleneck for PPP. This suggested that while the enhancement of ADH expression had sought to increase the supply of NAD^+ and boost XDH activity (Fig. 4), this goal had not necessarily been achieved. The fact that despite the increase of approximately 1000 times in ADH expression, the XDH bottleneck had not been eliminated, led to the conjecture that the ADH was not actually dependent on NADH.

Based on the enhanced degree of expression of ADH,

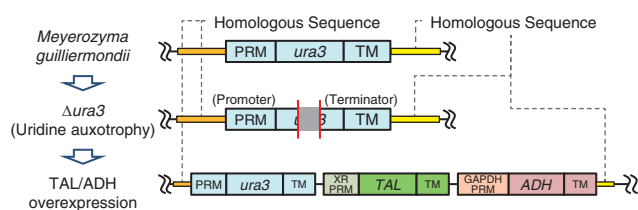


Fig. 5 Schematic diagram of DNA segment used for self-cloning

it was predicted that some imbalance would occur in the metabolism of the fermenting organism, and in fact the ethanol tolerance of all of the several self-cloning strains had declined. The mechanism of this decline in ethanol tolerance is not clear at present, but the fact that the same tendency was observed in multiple other strains displaying enhanced TAL and ADH expression strongly suggested that the self-cloning process had damaged the metabolic balance of the strains. Additional mutagenesis and selection was conducted in order to address this metabolic imbalance.

4. Performance of Fermenting Organism

4.1. Performance of Developed Fermenting Organism

In response to the decline in ethanol tolerance of the self-cloning strains, additional mutagenesis and selection was conducted with the chief aim of providing the fermentation organism with ethanol tolerance. Figure 6 shows the fermentation yield for each strain before mutagenesis and selection, after mutagenesis and selection, after self-cloning, and after the second process of mutagenesis and selection. A hydrolysate produced by applying dilute acid treatment to 20% w/w corn stover was used in fermentation tests. Because the hydrolysate contained 63.2 g/L of glucose and 34.5 g/L of xylose, including the reagent, the theoretical volume of production of ethanol was 49.96 g/L. Fermentation was conducted for 96 hr in a clear-liquid hydrolysate produced by separation of solids and fluids, and the pH was adjusted to 6.0.

The combination of positive and negative effects (promotion of the PPP, reduction of ethanol tolerance) produced by self-cloning of TAL and ADH genes alone resulted in a limited increase in the fermentation yield, but further progress was realized through the additional process of mutagenesis and selection. This demonstrated the validity of the research concept in attempting to increase the performance of the fermentation organism through a

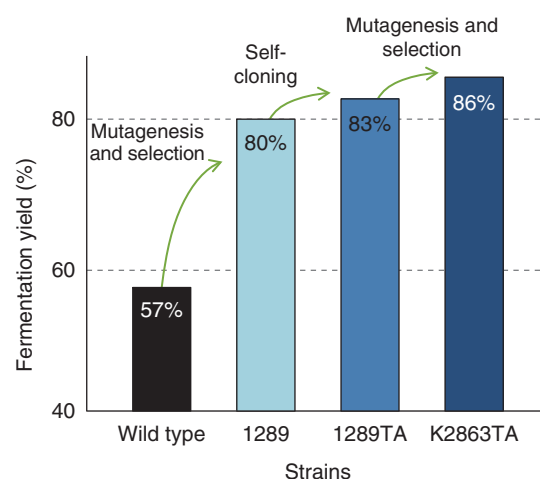


Fig. 6 Increase in fermentation yield by development of strain

combination of genetic modification and mutagenesis and selection.

4.2. Performance at Increased Scale

An ethanol production process incorporating a 5 kL fermentation tank was constructed for the research. With the increased scale of the fermentation process, factors including non-uniformity of the temperature in the tank and damage to the fermentation organisms due to agitation or high pressures at the bottom of the tank might become issues. The 5 kL process was conducted with a strain that had been selected following mutagenesis using an RS hydrolysate, like the strain 1289, and fermentation was conducted in an RS clear-liquid hydrolysate. The results obtained were almost identical to the results of a test conducted at a scale of 1 L as a contrast (Fig. 7). This result verified that the ethanol production process using the created fermentation organism could be scaled up to at least 5 kL.

4.3. Orientation of Mutagenesis and Selection and Genetic Enhancement and Realization of Increased Performance in Fermentation Organism

Fermentation tests using a single strain of the fermentation organism created via mutagenesis and selection were conducted in a slurry of corn stover subjected to dilute acid treatment. The hydrolysate with sugar concentration of more than 10% was adjusted through the addition of a reagent sugar in order to conduct tests using hydrolysates of various sugar concentrations. As Fig. 8 shows, the fermentation yield declined as the sugar concentration increased. This was due to the twin effects of an increase in the quantity of inhibitors in the hydrolysate and product inhibition, in this case inhibition by ethanol

produced by the organism. The ethanol concentration lines in the graph show the ethanol concentration in the hydrolysate following fermentation.

Figure 8 shows the line of constant production cost for the process as a whole for different fermentation yields and sugar concentrations. The closer to this line, the lower the cost at which ethanol can be manufactured. Additional mutagenesis and selection of the self-cloning strains was therefore conducted using a hydrolysate with an 8% sugar concentration, with which the fermentation yield was closest to this line. This process was successful in increasing fermentation yield at this sugar concentration.

As a response to product inhibition, mutagenesis and selection of the self-cloning strains was conducted using a hydrolysate with a sugar concentration of 11%, with tolerance to high ethanol concentrations as a selection pressure. As a result, an increase in the concentration of the produced ethanol was observed. In addition to demonstrating the necessity of setting parameters such as the composition and concentration of the hydrolysate and the concentration of the produced ethanol in advance, these cases indicate that the performance of the fermentation organism can be increased under the specified conditions.

4.4. Stress Response and Increased Fermentation Organism Performance

Integrating the results of transcriptome and DNA mutations and metabolome analyses, an analysis of the metabolic flow in the organism was conducted in order to elucidate the bottleneck in the metabolic flow. The results suggested that the developed fermentation organism went into a state of stress response when assimilating pentose. Stress response reactions seek to ensure the continued existence of the organism in the presence of stressors such as inhibitors. Product inhibition is also a cause of this stress.

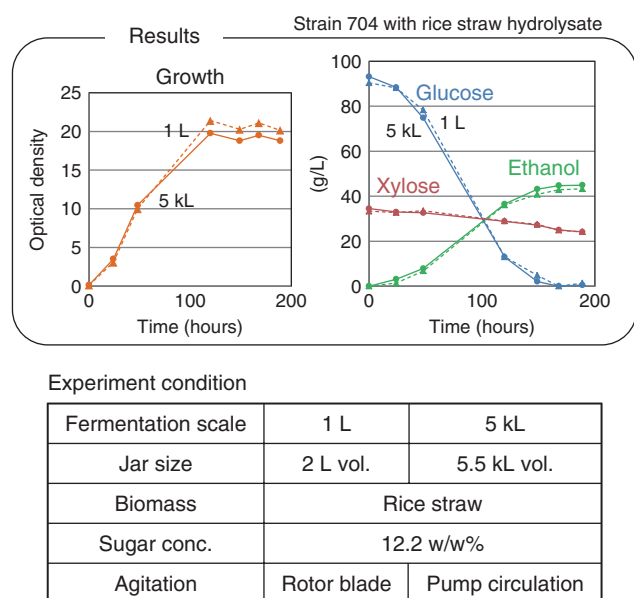


Fig. 7 Comparison of 1 L- and 5 kL-scale tests

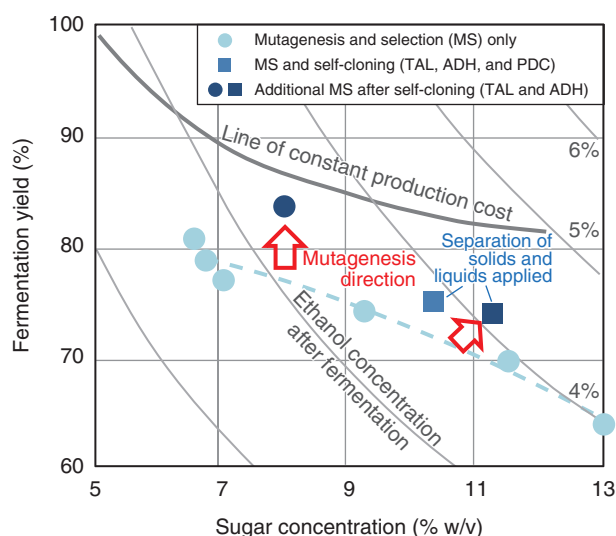


Fig. 8 Dependence of fermentation yield on sugar concentration

Death of the fermenting organisms due to the ethanol they have produced and the consequent termination of fermentation, with no reaction to product inhibition stress occurring, is a phenomenon that has been observed in baker's yeast employed in sake-making, during which fermentation produces high-concentration ethanol. It was considered that by removing this self-limitation on ethanol production due to product inhibition, that is, by forcing the organism to continue ethanol production, it would be possible to create an organism capable of the production of high-concentration ethanol. In an attempt to do so, the expression of pyruvate decarboxylase (PDC) (Fig. 4) was enhanced in addition to TAL and ADH, in order to increase the volume of ethanol production. This direction was taken because the results of a metabolome analysis had suggested that PDC represented a bottleneck following the enhancement of TAL and ADH expression. The results showed that the volume of ethanol production had been increased against the volume prior to the enhancement of PDC expression (Fig. 8), indicating the possibility that partial removal of the self-limitation on ethanol production had been realized.

It was considered that the removal of the self-limitation on ethanol production discussed above, in addition to the removal of stress responses to other inhibitors, would make it possible to create organisms that continued to ferment ethanol even in the presence of inhibitors and ethanol, and therefore to further increase the ethanol yield.

5. Fermentation Process and Overall Input and Output

5.1. Estimation of Allowable Concentration of Inhibitors and Determination of Pre-treatment Conditions

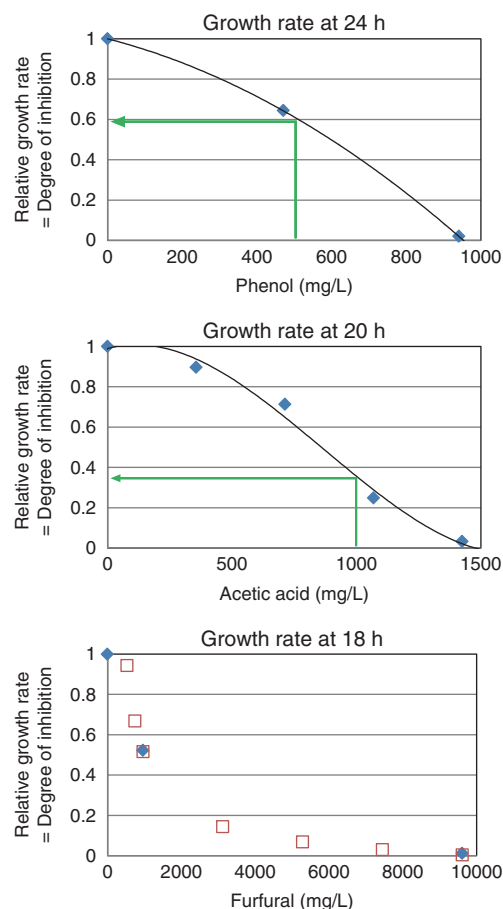
The effect of the inhibitors shown in Table 1 on retarding the growth of the fermenting organism was quantified.

First, the growth inhibition effect of each inhibitor in isolation was quantified by adding each of the inhibitors at several different levels of concentration to a synthetic culture medium considered to have no growth inhibition effect and studying the resulting degree of inhibition (Fig. 9). The fact that phenols, aldehydes, and organic acids display different inhibition mechanisms (as shown in Table 1) is reflected in the differences in the curves expressing the relationship between the concentration of the added inhibitor and the number of fermenting organisms after a fixed period of time. The effect of each inhibitor on the growth of the organisms can be read from these curves. For example, 500 mg/L of phenol added to the medium has the effect of slowing the organism's growth rate to around 0.6 times the rate in the synthetic culture medium. 1000 L of acetic acid slows the growth rate to 0.35 times the rate in the synthetic medium. The combined effect of the presence of both substances in the medium at these concentrations can be approximated as the product of these two figures,

or $0.6 \times 0.35 = 0.21$ times the growth rate in the synthetic medium. In this way, the effect of inhibitors in retarding the growth of the organisms was quantified through measurement of the concentration of the inhibitor in the hydrolysate.

This method of estimation uses the product of the concentration of each inhibitor for the sake of simplicity, and is not able to encompass the presence of all of the inhibitors. There are therefore limits to its accuracy. Because of this, an attempt was made to increase accuracy by means of parameter fitting, with parameters set as follows: First, the quantified phenols (substances that occur in a large number of types, and are therefore difficult to quantify) were multiplied by a fixed amount. The effect of Maillard reaction products, which are normally indicated as having an inhibition effect, was also set at a fixed value.

Because this estimation method made it possible to study the balance of inhibitors in a biomass hydrolysate, upper limit amounts for allowable concentrations of *p*-coumaric



Vertical axes indicate standardized growth rate.
(Culture OD with X mg/L inhibitor ÷ Culture OD without inhibitor)
Typical examples of phenols, aldehydes, organic acids are shown.
Optical density (610 nm) at ca. 20 hours was measured.
Organic acid concentration was multiplied by the dissociation constant for estimation of the concentration upper limit, in order to reflect the actual inhibition effect.

Fig. 9 Estimation of upper limit for inhibitor concentration

Table 2 Process conditions

Pre-treatment	Corn stover	Dry biomass ground to 3 mm pieces Mix at ratio of biomass : 2.5 % w/v Dilute acid = 1:2 Maintained at 170°C for 10 minutes
	Rice straw	Dry biomass ground to 3 mm pieces Mix at ratio of biomass : 25 % w/v aq. ammonia = 1:0.7 Maintained at 80°C for 3 hours
Saccharification	Substrate: 16.4 % w/w Enzyme: 0.4 mg/g-BM Maintained at 50°C for 72 hours at pH 5	
Fermentation	Substrate: 14.0 % w/w Sugar concentration: 6.2 % w/w Maintained at 30°C, at 28 mg/L/h aeration for 67 hours at pH 6	
Quasi-SSF	Saccharification and fermentation changed as follows Saccharification: 72 → 20 hours + 4 hours cooling Fermentation: 67 → 76 hours Process hours: 139 + cooling time → 100 hours	

acid and other phenols were determined. It is challenging to respond to the effects of phenols by means of fermentation conditions or genetic modifications, making it necessary to control the amount of phenols that are generated (Table 1).

The relationship between inhibitors and the amount of sugar produced for differing dilute acid treatment conditions (concentration, temperature, time) was examined via pyrolysis simulation, and pre-treatment conditions that would help to ensure that the amount of inhibitors remained below the upper limit and a high concentration of sugar was obtained were determined (Table 2).

The study of conditions in the entire process, including pre-treatment and saccharification, and measures for the enhancement of the inhibitor tolerance of the fermenting organism in parallel in this way made it possible to create a fermenting organism capable of use in the second-generation process.

5.2. Sugar Concentration of Hydrolysate and Fermentation Yield

Cost reductions in each constituent process will be necessary in order to realize practical application of the second-generation process. This necessitates increased efficiency, but in many cases efficiency in one process comes in a trade-off relationship with efficiency in another process. In addition to the relationship between inhibitor concentration and sugar concentration discussed above, other relationships within the second-generation process include the fact that it is not possible to control the total cost of the process if the quantity of saccharification enzymes is increased in order to enhance the sugar yield, and that if the sugar concentration is low, the ethanol concentration will also be low, increasing the distillation energy used.

Given this, it was decided to conduct a parameter study to determine the change in the entire cost when conditions in each process were changed, in order to identify the conditions that minimized the cost. A consideration of the line showing a constant production cost in relation to changing fermentation yields and sugar concentrations in Fig. 8 indicated that when the fermentation organisms

shown on the blue line were used, fermentation at sugar concentrations around 7 to 8% was closer to the line of constant production cost than fermentation at around 11%, and this controlled the total cost of the process. The form of the line of constant production cost shows that there is a limit to the cost reduction achievable by lowering the sugar concentration. The realization of further reductions in total cost would necessitate increasing the fermentation yield at high sugar concentrations in order to reduce distillation energy and equipment cost.

5.3. Realization of Quasi-SSF Process

Simultaneous saccharification and fermentation (SSF) is a process available for the production of bioethanol. In this process, conducting enzymatic saccharification and ethanol fermentation simultaneously in the same vessel makes it possible to reduce the time needed for the process. In addition, because fermentation is conducted using a slurry without the need for separation of solids and liquids following the saccharification process, SSF makes it possible to integrate the saccharification and fermentation tanks, and to omit the solid/liquid separation process, thus reducing initial costs.

In SSF, the conditions for saccharification and fermentation are identical. However, because there was a considerable difference between the optimum conditions for the saccharification enzyme (50°C, pH 4) and the fermentation organism (30°C, pH 6) employed in this research, saccharification and fermentation under entirely identical conditions were not practicable. The potential of a quasi-SSF process, in which saccharification and fermentation were conducted successively in the same vessel, was therefore examined. A special apparatus of 3 L was constructed for the process. Following enzymatic saccharification for 20 hr, the temperature was reduced and the pH adjusted, and the fermentation organism was added to conduct fermentation. This procedure indicated that quasi-SSF was able to reduce the time needed for the process by 39 hr and increase the efficiency of the equipment (Table 2).

5.4. Material Balance

The material balance and energy balance of the bioethanol production process is also strongly dependent on the processes other than enzymatic saccharification and ethanol fermentation. An NREL report⁽⁶⁾ was referred to in calculating the material balance of the bioethanol production process in this research. The material balance was calculated based on the assumption that all energy for the production process was supplied by methane fermentation using fermentation residues or the combustion of the residues for generation. When the total cellulose and hemicellulose content of the corn stover employed was 57% w/w, the sugar yield from enzymatic saccharification under the conditions shown in Table 2 was 63% and the fermentation yield from the quasi-SSF process was 86%.

It was calculated that following the subsequent distillation and condensation processes, 1 dry-ton of biomass would ultimately yield 213 L of anhydrous ethanol.

6. Conclusion

The research discussed in this paper produced the following results:

- (1) Using a pentose-assimilating yeast as the base organism, an ethanol fermentation organism able to be used in the second-generation process with the status of a non-genetically-modified organism was created.
- (2) Under the conditions employed in this research, including the use of the developed fermentation organism, 1 dry-ton of corn stover containing a total of 57% w/w of cellulose and hemicellulose produced 213 L of ethanol.

The research generated the following findings:

- (1) The combination of genetic modification and mutagenesis and selection is effective in the construction of fermentation organisms. The research indicated the necessity not only of modifying known enzymes, but also of modifying the entire network of metabolic pathways, including unknown enzymes.
- (2) It is not practicable to create all-purpose fermentation organisms able to respond to any hydrolysate; fermentation organisms specialized for specific biomass types and processes are necessary, and these organisms should be created following the restriction of process conditions.

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