

Research on Production of Cellulosic Ethanol – Creation of Novel Saccharifying Enzyme –

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ABSTRACT

The cost of saccharifying enzymes is one of the factors hindering the practical use of cellulosic bioethanol. In an attempt to address this issue, research has been conducted to create a novel saccharifying enzyme, in parallel with efforts to reduce the quantity of the enzyme used and the cost of production of the enzyme.

Reduction of the quantity of the enzyme used involved the creation of an original saccharifying enzyme mixture that did not include any unnecessary enzymes. In addition, genetic engineering techniques were applied in order to substitute an amino acid sequence in the enzyme that formed the main constituent, increasing enzyme activity.

Reduction of the cost of production of the enzyme involved utilizing the biomass employed in the ethanol production process as a carbon source, and employing an onsite enzyme production process exploiting solid-state fermentation, a high-efficiency production method. An original enzyme-producing strain of *Aspergillus oryzae* was created in order to realize this process.

The two outcomes discussed above represent new findings with the potential to reduce the cost of saccharifying enzymes for bioethanol production.

1. Introduction

The use of bioethanol represents one response to concerns over the depletion of fossil fuels and the necessity for the reduction of CO₂ emissions. The first generation of bioethanols were produced using starch from the edible parts of plant materials. Research is proceeding in order to make practical the use of second-generation cellulosic bioethanol, produced using the inedible parts of plant materials. Saccharifying enzymes are protein catalysts that catalyze the reaction that breaks down the cellulosic biomass and forms the glucose that fermentative bacteria use to produce ethanol. Cellulose has a strong structure, and high quantities of enzymes are needed in order to break it down. Despite years of research and development efforts by specialist manufacturers, the cost of these enzymes is 10 times or more that of the saccharifying enzymes used in the production of conventional starch-based bioethanol^{(1), (2)}. The high cost of saccharifying enzymes is a factor that hinders the practical use of cellulosic biofuels.

The authors therefore advanced research towards the development of a novel and innovative saccharifying

enzyme able to reduce costs.

In addition to explaining the aims and characteristics of the research process, this paper will discuss the performance of the developed novel saccharifying enzyme and saccharifying enzyme-producing microorganism, how their use reduces costs, and the findings obtained in the research process.

2. Development Goals

The cost of saccharifying enzymes can be easily visualized by terming the cost for producing 1L of ethanol as C (Unit: Yen/L-ethanol). The cost of the enzymes can then be determined using the following equation (Fig. 1):

Here, A: Quantity of enzymes used (g/kg-biomass);

$$C = \frac{\text{Enzyme cost for 1 kg biomass (¥)}}{\text{Ethanol produced from 1 kg biomass (L)}} = \frac{A \times B \times G}{D \times E \times F}$$

Fig. 1 Breakdown of enzyme cost

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B: Unit cost of enzyme production (Yen/g-protein); D: Sugar content of biomass (kg-monosaccharide equivalent/kg-biomass); E: Saccharification rate (kg-recovered/kg-origin); F: Fermentation efficiency (kg-ethanol/kg-glucose); G: Specific gravity of ethanol (kg/L). The sections of the equation shaded in pink show the amount of ethanol produced from 1kg of biomass. This figure is constant, determined by the necessary conditions for the process, including the rate of conversion of biomass to glucose (the saccharification rate), and physical constants. The variable section, which can be modified in order to reduce the cost of the enzymes, is therefore the area shaded in blue, the product of the quantity of enzymes used and the unit cost of enzyme production. Two orientations, the reduction of quantity used and the unit cost of production, are available for reducing the cost of the enzyme.

The research discussed in this paper focused on both orientations in conducting biochemical research and development. Specifically, in seeking to reduce the quantity of enzymes used, the research attempted to increase the catalytic activity of the saccharifying enzyme, while in seeking to reduce the unit cost of production of the enzymes, the research attempted to increase the productivity of the enzyme-producing microorganisms. From the next chapter onwards, this paper will discuss the details of the research and its outcomes.

3. Measures to Reduce Quantity of Enzyme Used

3.1. Manufacture of Novel Enzyme Mixture

Saccharifying enzymes are secreted by microorganisms (bacteria and fungi) to enable them to consume biomass as a source of nutrition. These microorganisms are cultured on a large scale industrially, and the saccharifying enzymes that they produce are isolated and concentrated. Natural microorganisms secrete more than 30 types of enzymes simultaneously. These enzymes can be broadly divided into five groups with different functions, as shown in Fig. 2 and Table 1.

Multiple types of enzymes fall into the same groups, due to the fact that enzyme groups with slightly differing functions are necessary in order to respond to the diverse range of biomass sources occurring in the natural world. However, it is normally the case that when bioethanol is produced industrially, the biomass employed as the raw material is limited to a single type. Considerably fewer enzyme types may thus be required than the over 30 types found in natural microorganism. The present research therefore set out to reduce the quantity of enzymes used by omitting the enzymes that were unnecessary for the target biomass type. Rice straw in which the saccharification process had been promoted through immersion in ammonia water was used in the experiments as biomass.

In order to verify the working hypothesis that omitting unnecessary enzymes would make it possible

to reduce the quantity of enzymes used, unnecessary enzymes were omitted from enzyme groups secreted by three microorganisms (*Acremonium*, *Thermoascus*, and *Thermotoga*) to create an enzyme mixture tailored to rice straw. For the selection of the enzymes, a process of fractionation based on differences in the surface charge of the molecules in each enzyme and the subsequent selection of fractions shown to be effective in rice straw saccharification experiments was repeated (Fig. 3).

As a result of this process, one enzyme was selected from each of the enzyme types shown in Fig. 2 and Table 1 to create an enzyme mixture using a total of five enzymes. In addition, the ratio of constituent enzymes was changed and further rice straw saccharification experiments were conducted, in order to determine the optimum ratio. Figure 4 shows the saccharification activity of the mixture. The biomass saccharification activity of the synthetic mixture was approximately 2.5 times greater than that of a natural *Acremonium* culture solution.

These results demonstrated the validity of the initial working hypothesis that it would be possible to reduce the quantity of enzymes used by omitting unnecessary enzymes.

3.2. Enzyme Amino Acid Substitution

The research next sought to increase the activity of the enzymes making up the synthetic mixture. Amino acid substitution was employed as the method. Enzyme molecules are formed from polymers that take fixed three-dimensional structures, as shown in Fig. 5. These polymers are proteins, and their component monomers are amino acids. There are 20 types of amino acids that make up enzymes, and the three-dimensional structure and catalytic activity of the enzyme are determined by their arrangement. It is known that the substitution of part of the amino acid sequence of a natural enzyme into another sequence can result in either a decline in performance or increased activity. Tests were conducted focusing on CBH1, the main constituent (weight composition: 47%; see Table 1) of the novel saccharifying enzyme mixture discussed in the previous section. Figure 5 shows the molecular structure of CBH1. CBH1 is a polymer made up of 550 linked amino acids, with a central hollow section resembling a tunnel. Multiple polar and highly hydrophobic amino acids exist on the surface of this hollow section. The formation of non-covalent bonds with these amino acids

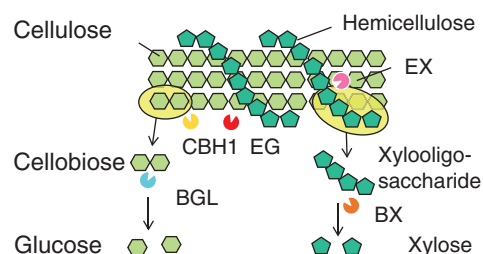


Fig. 2 Functions of components in enzyme mixture

Table 1 Composition of enzyme mixture

Enzyme group	EG: Endoglucanase	CBH1: Cellobiohydrolase 1	BGL: Beta-glucosidase	EX: Endoxylanase	BX: Beta-xylosidase
Function	Cuts the surface of crystalline cellulose to create nicks	Cleaves out cellobiose at reducing end of cellulose chain	Cuts cellobiose into glucose	Cuts hemicellulose into xylooligosaccharide	Cuts xylooligosaccharide into xylose
Original organism	<i>Acremonium</i>			<i>Thermoascus</i>	<i>Thermotoga</i>
Weight percentage (%)	24	47	24	4	1

incorporates cellulose chains into this hollow section of the enzyme, cleaving the chains. The number of possible amino acid substitutions is 19^{50} , ruling out a test of each one. Thirty sites at which amino acids on the surface of the hollow section of the enzyme were in contact with the cellulose chain were therefore identified using computer

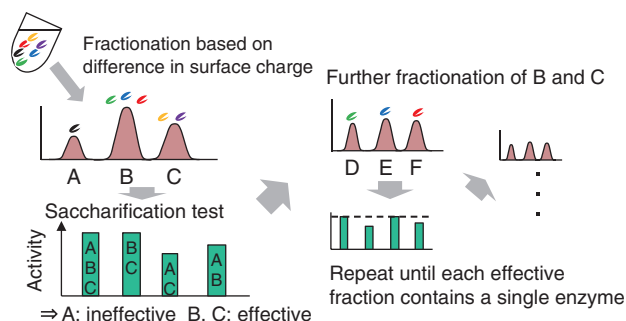


Fig. 3 Scheme of enzyme selection

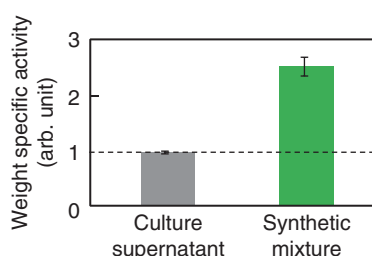


Fig. 4 Specific activity of novel enzyme mixture

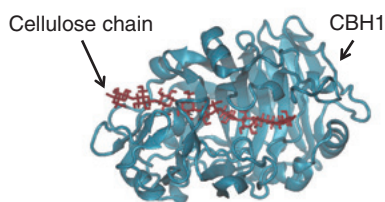


Fig. 5 Molecular structure of CBH1 and cellulose chain

modelling, and each amino acid was replaced by alanine, a separate amino acid. Changes in activity resulting from these substitutions were investigated. It was thought that because alanine did not possess the polarity or high level of hydrophobia of the amino acid prior to the substitution, the substitution of alanine would increase activity in cases in which the pre-substitution amino acid had a negative effect on activity. As Fig. 6 shows, of the 30 substitution products, three displayed higher activity than the pre-substitution wild type (WT). Figure 6 shows the six highest values for activity. With regard to nomenclature, N062A, for example, indicates that the 62nd asparagine molecule (N) from the end of the sequence has been substituted with an alanine molecule (A). This was the substitution product displaying the highest level of activity, approximately 7.8 times higher than that of the WT.

The results of a molecular dynamics simulation of the process of incorporation of the cellulose chain into the hollow section of CBH1 showed that the substitution of the amino acid that formed hydrogen bonds with the cellulose chain in the WT with alanine promoted the incorporation of the cellulose chain by the interior of the enzyme molecule, and that this was a factor in the increased activity of the substitution product.

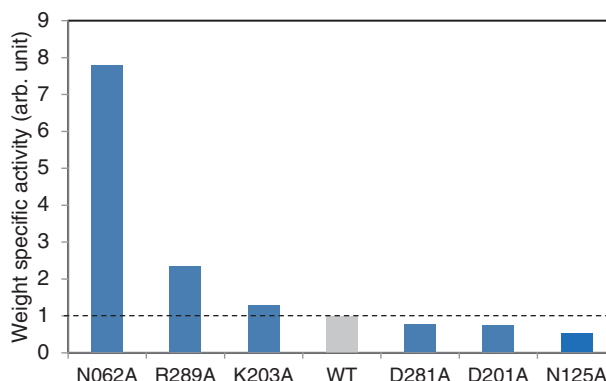


Fig. 6 Specific activity of CBH1 mutants

4. Measures to Reduce Unit Cost of Enzyme Production

4.1. Concept for Onsite Production Process

The production process shown in Fig. 7 was selected in order to make it possible to produce the novel enzyme discussed in the previous chapter at a low cost. In the normal process, a homogeneous sugar solution is used as a source of nutrition for the culturing of the enzyme-producing microorganisms, and the microorganisms are cultured in a liquid state. This technique helps to enable the mass production of enzymes of a uniform quality. Given that the purpose of the present research was to produce enzymes that would be used in the process of production of ethanol from biomass, it was thought that it would be possible to use the biomass that formed the raw material for the ethanol as a source of sugar for the microorganisms, rather than a high-cost homogeneous sugar solution, by means of annexing the enzyme production line to the main process (onsite production). In addition, because the biomass is solid, solid culturing of the microorganisms in the biomass itself would be more suited to onsite production than liquid culturing. Given that solid culturing can be conducted in an area smaller by the volume of the liquid medium than liquid culturing, solid culturing would make it possible to reduce equipment costs.

4.2. Creation of Original Enzyme-producing Microorganism

A suitable microorganism was necessary to make possible the onsite production process discussed above. *Aspergillus oryzae* was selected for the research under discussion. Solid culturing techniques using *A. oryzae* are well advanced in Japan as a result of their application in

Table 2 Comparison of candidates for enzyme production host

Organism	<i>E. coli</i>	<i>B. subtilis</i>	<i>P. pastoris</i>	<i>A. oryzae</i>
Capable of phenotypic transformation?	Yes	Yes	Yes	Yes
Capable of solid-state fermentation?	No	Yes	No	Yes
Capable of CBH1 production?	No	No	No	Yes

the production of fermented foodstuffs such as sake and miso. In addition, techniques for genomic modification and phenotypic transformation of *A. oryzae* have basically become established, making it possible to engineer the production of foreign enzymes not originally secreted by the organism⁽³⁾.

As numerous varieties of *A. oryzae* exist, research first focused on screening. Fifteen varieties of *A. oryzae* were obtained. The enzyme secretion volume of these varieties in a solid rice straw culture was compared, and the variety with the highest secretion volume was selected.

Next, CBH1, the amino acid discussed above, was introduced to the genomic DNA of this strain of *A. oryzae* using a technique known as homologous recombination, and it was determined that the organism was capable of producing it (Table 2). Three other microorganism varieties employed as a comparison did not produce CBH1, further demonstrating the validity of selecting *A. oryzae* as a novel enzyme-producing microorganism.

The enzyme secretion volume of this strain of *A. oryzae* was further increased by reading its entire genome sequence and, based on the information obtained, artificially deleting

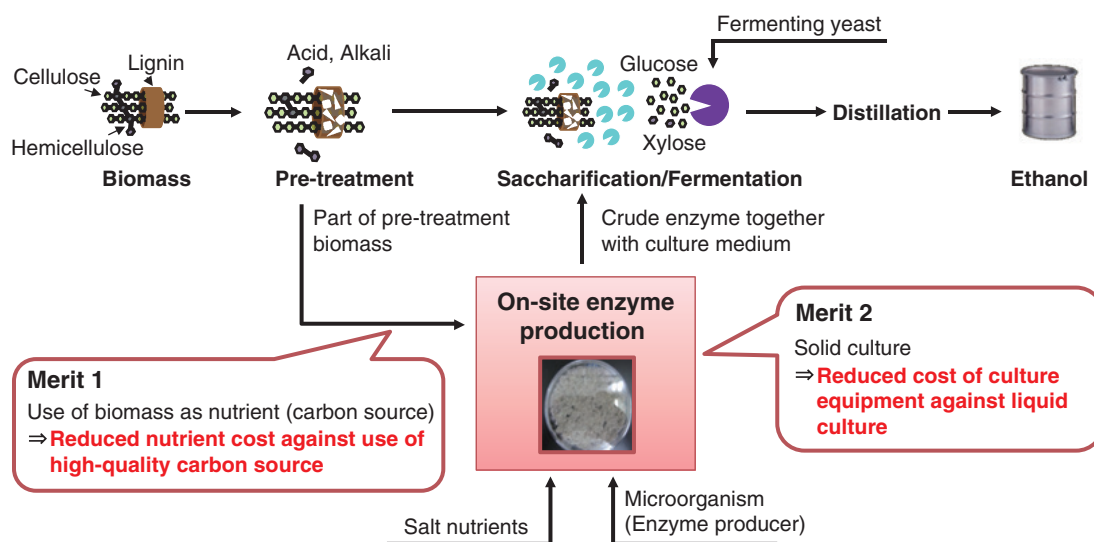


Fig. 7 Scheme of enzyme production

three genes considered to impede enzyme secretion. As a result, as shown in Fig. 8, it was possible to create a strain able to produce 1.5 times the CBH1 volume of RIB40, the standard strain of *A. oryzae* used in making sake⁽⁴⁾.

In addition, when the characteristics of the new strain were investigated, it was found to display tolerance to the inhibitory effect on the growth of microorganisms of the low-molecular-mass compound furfural, which detaches from lignin, a component of the biomass. Figure 9 shows a comparison of the furfural tolerance of the developed novel enzyme-producing microorganism with that of RIB40, using the volume of production of CBH1 as the index. RIB40's production of CBH1 declines to approximately 40% in the presence of furfural, while furfural has almost no effect on the novel organism. The characteristics displayed by the novel enzyme-producing microorganism can be considered to represent a considerable advantage for solid culturing of the organism in biomass.

An investigation of factors producing the organism's furfural tolerance based on the results of a comprehensive analysis of its genome and intracellular substances (RNA analysis, metabolome) indicated the possibility that there had been changes in its capacity to adapt to intracellular oxidative stress, as well as changes in the speed of the protein synthesis reaction. These results can be considered important findings about the molecular biology of *A. oryzae*.

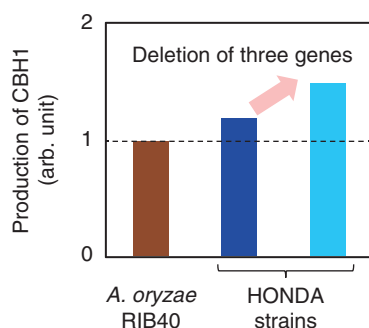


Fig. 8 Production of CBH1 by HONDA strains

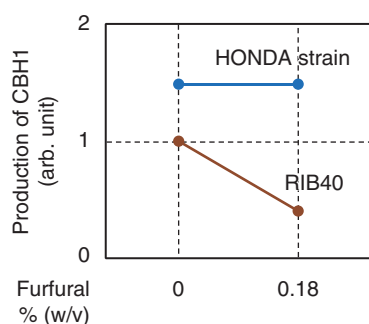


Fig. 9 Tolerance against furfural of original strain

5. Effect on Cost

Measures to reduce the volume of enzyme use and production cost have been discussed above. These measures have been evaluated from the perspectives of saccharification activity and production volume respectively. The higher the values of these parameters, the lower the volume of enzymes used and the lower the production cost, and therefore ultimately the lower the cost (Fig. 1).

While investigating the level of performance against the protein mass of standard market enzymes used in the production of cellulosic bioethanol, the present research found that the highest level of performance was approximately equivalent to that of a natural *Acremonium* culture solution. The novel enzyme mixture created in this research displayed 2.5 times the level of activity of a natural *Acremonium* culture solution (Fig. 4). A simple calculation indicates that it would be necessary to use 1/2.5th the quantity of this enzyme to realize the same level of saccharification performance as the highest-performing market enzyme. Assuming, because *Trichoderma*, the market enzyme-producing microorganism, is the same type of filamentous bacterium as the novel enzyme-producing microorganism (a fungus), that the unit price is the same (from Fig. 1), the cost of the enzymes produced by the novel microorganism will also be 1/2.5th that of enzymes produced by *Trichoderma*.

The amino acid-substituted mutant created in this research that displayed the highest level of activity in relation to CBH1 realized a level of activity approximately 7.8 times that of the WT (Fig. 6). Because the CBH1 WT represents 47% of the total mass of the enzyme mixture (Table 1), calculations show that the use of the amino acid substitution product displaying the highest level of activity would reduce the amount of enzyme used by 41% ($=47 - 47 / 7.8$) while maintaining the performance of the enzyme mixture. Based on the considerations indicated above, this should also reduce costs by 41%. In actuality, the volume of production of the amino acid substitution product displaying the highest level of activity by *A. oryzae* was low, and its effect on reducing the quantity of the enzyme mixture required has not been verified.

The cost-effect ratio of the use of the novel enzyme-producing microorganism cannot be discussed directly, because it is dependent on the production process. However, assuming that the same production process is employed, the enzyme-producing microorganism created in this research produces 1.5 times the CBH1 volume of RIB40, the standard *A. oryzae* strain used widely in the food industry. A simple calculation shows that the unit cost of enzyme production for the novel organism is 1/1.5.

Based on the above, the novel enzyme mixture and novel enzyme-producing microorganism created during this research can be expected to contribute to reducing the

cost of saccharifying enzymes used in the production of bioethanol.

6. Conclusion

Research has been conducted towards the realization of an innovative low-cost enzyme able to assist in the practical use of cellulosic bioethanol. This research produced the following outcomes.

- (1) A novel enzyme mixture created by removing unnecessary enzymes from a mixture produced by natural organisms displayed approximately 2.5 times the saccharification activity of a natural *Acremonium* culture solution. In addition, amino acid sequence substitution in CBH1, the main constituent of the enzyme mixture, increased catalytic activity by approximately 7.8 times.
- (2) A novel enzyme-producing strain of *A. oryzae* able to be solid-cultured in biomass was created in order to make it possible to realize an onsite production process. This strain of *A. oryzae* was able to produce 1.5 times the volume of the enzyme as RIB40, the standard strain.

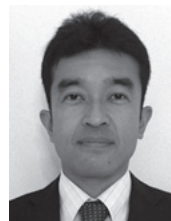
These results demonstrate that a technology has been developed that will make it possible to reduce the cost of saccharifying enzymes in bioethanol production.

The techniques employed during this research were highly reliant on genetic engineering techniques that have displayed striking progress from the 1980s onwards. In the future, the value of applying these techniques in fields other than those of the environment and energy can be expected to further increase.

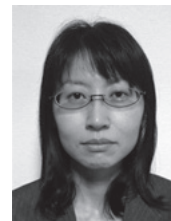
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