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# Comparison of Sucrose-Hydrolyzing Enzymes Produced by *Rhizopus oryzae* and *Amylomyces rouxii*

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***Rhizopus oryzae* produces lactic acid from glucose but not efficiently from sucrose, while *Amylomyces rouxii*, a species closely related to *R. oryzae*, ferments these sugars equally. The properties of two sucrose-hydrolyzing enzymes purified from culture filtrates of *R. oryzae* NBRC 4785 and *A. rouxii* CBS 438.76 were compared to assess lactic acid fermentation by the two fungi. The substrate specificity of the enzymes showed that the enzymes from strains NBRC 4785 and CBS 438.76 are to be classified as glucoamylase and invertase respectively. The entity of the enzyme from strain NBRC 4785 might be a glucoamylase, because eight residues of the N-terminal amino acid sequence coincided with those of the deduced protein from the *amyB* gene of *R. oryzae*. The enzyme from NBRC 4785 was more unstable than that from strain CBS 438.76 under conditions of lower pH and higher temperature. These observations mean that the culture conditions of *R. oryzae* for lactic acid production from sucrose should be strictly controlled to prevent inactivation of the glucoamylase hydrolyzing sucrose.**

**Key words:** glucoamylase; $\beta$-fructofuranosidase; *Rhizopus oryzae*; *Amylomyces rouxii*

Sugar beet is a principal rotation crop in Hokkaido, the northernmost island of Japan. It is used in the manufacture of crystallized sucrose, but circumstances surrounding sugar beet become more and more severe on the domestic sugar industry. Annual consumption of sugar is decreasing in Japan, having reached to 3 million tons in 1976 because of expanded use of high fructose corn syrup, increasing import of sugar-containing foods, and dietary changes of consumers to less sweet tastes. To find a way out of these difficulties, valuable products synthesized from sucrose are required. Hence, we have paid attention to lactic acid as a source of such products.

Lactic acid is widely used in the food, cosmetics, chemical, and pharmaceutical industries.[1] The functional applications of lactic acid as a food ingredient include its use as a natural acidulant, preservative, and flavor enhancer. One of the most important uses is as a raw material for polylactide, which can partly replace conventional plastics synthesized from fossil resources as a biodegradable polyester.

Microbial production of lactic acid is usually conducted by anaerobic fermentation of lactic acid bacteria in a medium containing mono- or disaccharides supplemented with complex nitrogen sources. The addition of natural components, such as yeast extract or peptone, increases the culture costs and complicates the purification procedures for lactic acid. Among fungal strains aerobically forming organic acids, *Rhizopus oryzae* is known as an alternative microorganism for lactic acid fermentation.[2,3] The production yield of lactic acid by *R. oryzae* is lower than that of lactic acid bacteria, and this fungus primarily synthesizes L-lactic acid in the presence of ammonium salts as sole nitrogen source.[4,5] The costs of recovery and purification of lactic acid produced by fungi are less than those by lactic acid bacteria. *R. oryzae* converts starchy materials to lactic acid by the action of external amylases but does not degrade sucrose as efficiently.[6]

*Amylomyces* is a monotypic genus containing the single variable species *A. rouxii*, which is closely related to *R. oryzae*,[7] as shown by the formation of rhizoids, stolons, and black-pigmented sporangia.[8] A distinct morphological characteristic of *A. rouxii* is the enormous number of chlamydospores produced in the aerial and substrate mycelium. Ellis *et al.*[8] reported that five strains of *A. rouxii* grew on glucose and sucrose equally, although *A. rouxii* was inferior to *R. oryzae* in growth[8] and the production rate of lactic acid.[9] We have surveyed the strains of *R. oryzae*, and we found that some strains ferment sucrose vigorously. In the present experiments, sucrose-hydrolyzing enzymes of selected strains of *R. oryzae* and *A. rouxii* were purified to compare their properties for the efficient and stable production of lactic acid from sucrose by *R. oryzae*.

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## Materials and Methods

*Organisms.* Twenty-six strains of *R. oryzae* accumulating lactic acid predominantly were obtained from the NITE Biological Research Center (Chiba, Japan). The NBRC numbers of these strains are as follows: 4705, 4706, 4707, 4716, 4744, 4758, 4780, 4783, 4785, 4798, 4804, 4809, 5318, 5319, 5378, 5379, 5380, 5384, 5413, 5418, 5438, 5440, 5780, 6155, 9364, and 31005. *A. rouxii* CBS 438.76 was used as the reference strain.

*Culture.* Cells were grown on potato dextrose agar prepared horizontally in a standing test tube. The mycelium was taken from the surface of the agar and inoculated in 100 ml of a medium containing 10% sugar, 0.2% $(NH_4)_2SO_4$, 0.065% $KH_2PO_4$, 0.025% $MgSO_4 \cdot 7H_2O$, and 2.5% $CaCO_3$ in a 300-ml Erlenmeyer flask. Sugar and other components of the medium were autoclaved separately and mixed just before inoculation. After cultivation of *R. oryzae* for 6 d and *A. rouxii* for 14 d at 30 °C with shaking (150 rpm), unless otherwise stated, the filtrate of the medium was dialyzed against distilled water and used as the crude enzyme.

*Enzyme assays.* The reaction mixture for the assay of the sucrose-hydrolyzing enzyme contained a 100 mM acetate buffer (pH 5.0), 150 mM sucrose, and the crude enzyme in a total volume of 0.25 ml. The sucrose was replaced with 10 mg/ml of soluble starch or 20 mg/ml of inulin when necessary. After incubation at 30 °C for 30 min, the reaction was stopped by the addition of a 3,5-dinitrosalicylic acid reagent, and the reducing sugars produced were determined.[10] The activity hydrolyzing *p*-nitrophenyl α-D-glucopyranoside was measured by the formation of *p*-nitrophenol as α-glucosidase activity. One unit was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to glucose or *p*-nitrophenol per min under the above conditions.

*Molecular weight.* SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in 7.5% gel to estimate the molecular weight of the enzyme.[11] The protein bands were visualized by silver staining. The standards for molecular weight determination were obtained from Bio-Rad Laboratories (Hercules, CA).

*Analytical procedures.* Lactic acid and ethanol were determined by high-performance liquid chromatography (HPLC).[12] Soluble sugars were analyzed by thin-layer chromatography (TLC) using a solvent system of 1-butanol/2-propanol/acetic acid/water (7:5:2:4, v/v). Spots were visualized by spraying the plate with an anisaldehyde-sulfuric acid reagent.[13] Protein was measured by the method of Bradford.[14] The N-terminal amino acid sequence of the enzyme was analyzed by automated Edman degradation in a protein sequencer (Procise 494, Applied Biosystems, Foster City, CA).

*Reagents.* Fructooligosaccharides, which contain 1-kestose, nystose, and $1^F$-β-fructofuranosyl nystose,[15] and a purified preparation of glucoamylase from *Rhizopus* species with an approximate molecular weight of 70,000 were obtained from Wako Pure Chemical Industries (Osaka, Japan).

*Analysis of DNA sequences.* The primers, designed based on the *amyB* gene sequence (DQ219822) of *R. oryzae* NRRL 395, were BF10 (5′-CACTGAAACCGGGAAATATGAC-3′) and BR14 (5′-TAGAAGACATCAAGCCACTCAC-3′). These were used in the polymerase chain reaction. Fragments of about 2.1 kb amplified from the genomic DNA of strains NBRC 4785 and NBRC 5384 (= NRRL395) were sequenced on both strands with an automated DNA sequencer.

## Results

*Production of lactic acid from sucrose and other sugars*

Twenty-six strains of *R. oryzae* were cultured in a medium containing 10% sucrose as the principal carbon source for 10 d. All strains except for two, NBRC 4798 and NBRC 5418, produced lactic acid to some extent, but no sucrose-hydrolyzing activity was detected in the culture filtrate (data not shown).

Strain NBRC 4785, which reproducibly produced lactic acid, was selected as a representative strain of *R. oryzae* and compared with *A. rouxii* CBS 438.76 at 30 °C for lactic acid fermentation of some sugars (Table 1). Appreciable amounts of ethanol accompanied the formation of lactic acid in the two strains, as usually observed.[12] Lactic acid was produced from glucose, sucrose, maltose, fructooligosaccharides, and soluble starch by *R. oryzae* NBRC 4785, and from glucose, sucrose, raffinose, fructooligosaccharides, and soluble starch by *A. rouxii* CBS 438.76. The latter strain produced a detectable amount of lactic acid from inulin. These fermentation profiles suggested differences in the hydrolysis of fructosyl-linkage by the two strains.

**Table 1.** Production of Lactic Acid and Ethanol from Sugars by the Two Fungal Strains

| Component | *R. oryzae* NBRC 4785 | | *A. rouxii* CBS 438.76 | |
|---|---|---|---|---|
| | Lactic acid (mg/ml) | Ethanol (mg/ml) | Lactic acid (mg/ml) | Ethanol (mg/ml) |
| Glucose | 77.8 | 6.5 | 71.2 | 6.0 |
| Sucrose | 77.3 | 5.7 | 73.1 | 4.9 |
| Maltose | 74.1 | 8.2 | <0.1 | <0.1 |
| Raffinose | <0.1 | <0.1 | 38.5 | 1.8 |
| Fructooligosaccharides | 24.6 | <0.1 | 70.8 | 5.1 |
| Inulin | <0.1 | <0.1 | 1.5 | 0.5 |
| Soluble starch | 70.1 | 3.8 | 66.2 | 5.5 |

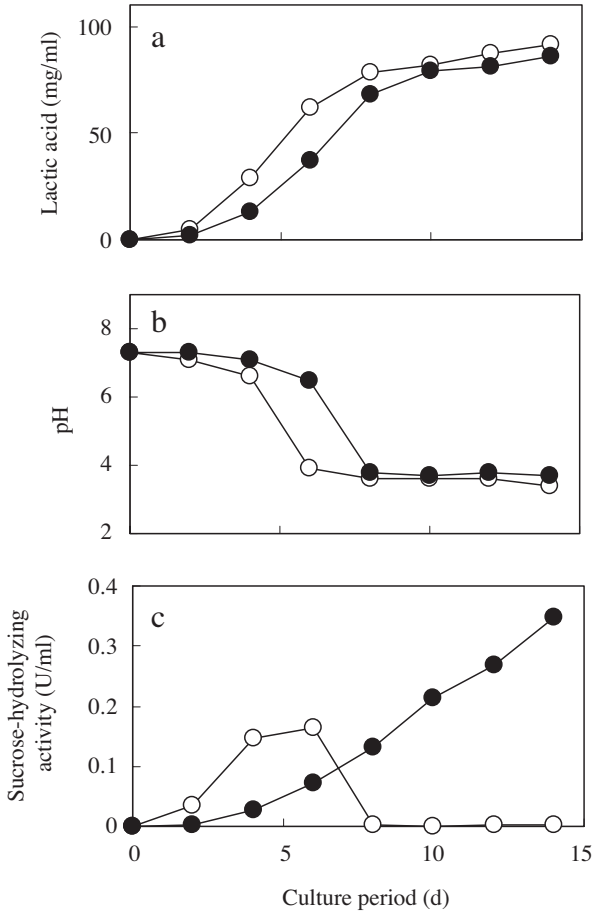


**Fig. 1.** Lactic Acid Production (a), pH (b), and Sucrose-Hydrolyzing Activity (c) in the Medium Containing Sucrose during Growth of the Two Fungal Strains.
Symbols: ○, *R. oryzae* NBRC 4785; ●, *A. rouxii* CBS 438.76.

*Synthesis of sucrose-hydrolyzing enzymes*

Lactic acid production and sucrose-hydrolyzing activity were followed during the growth of *R. oryzae* NBRC 4785 and *A. rouxii* CBS 438.76 in the medium containing 10% sucrose (Fig. 1). Strain NBRC 4785 formed lactic acid more rapidly than strain CBS 438.76, with a pH reduction. There was a remarkable difference in the change in sucrose-hydrolyzing activity between the two strains. The activity of strain NBRC 4785 increased with a slight lag period after inoculation and disappeared for 8 d. On the other hand, strain CBS 438.76 continued to synthesize the sucrose-hydrolyzing enzyme throughout the culture periods. A decrease in sucrose in the media of both strains was accompanied by the appearance of monosaccharides, which were consumed, but not completely (Fig. 2). An external pH below 4.0 might inhibit further fermentation of monosaccharides for both strains. In strain CBS 438.76, the rate of sucrose hydrolysis appeared to be higher than that of sugar consumption, resulting in an accumulation of monosaccharides during 2 to 6 d of cultivation. Strain NBRC 4785 consumed the monosaccharides formed from sucrose more rapidly than strain CBS 438.76

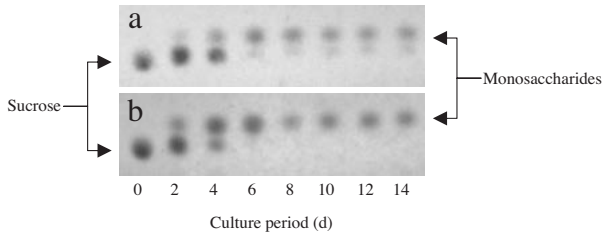


**Fig. 2.** Residual Sugars in the Media Containing Sucrose during Growth of *R. oryzae* NBRC 4785 (a) and *A. rouxii* CBS 438.76 (b). One μl of the culture filtrate after 10-fold dilution was applied to TLC.

and left a detectable amount of sucrose that was not hydrolyzed by the loss of sucrose-hydrolyzing activity. Incomplete hydrolysis of sucrose might cause defects in the industrial production of lactic acid from sucrose by *R. oryzae*. From these reasons, the sucrose-hydrolyzing enzymes of the two fungi were purified in order to compare their properties.

*Purification of sucrose-hydrolyzing enzymes*

The culture filtrate (1,560 ml) of *R. oryzae* NBRC 4785, which included maximal sucrose-hydrolyzing activity, was dialyzed against distilled water and concentrated 80-fold by ultrafiltration. The concentrated filtrate was mixed with a 1.0 M acetate buffer (pH 5.0) to adjust it to 20 mM, and was passed through CM-Toyopearl 650 M in a column (1.5 × 13 cm). The column was washed with the buffer and eluted with a linear gradient of an NaCl concentration from 0 to 500 mM in the buffer. The active fractions eluted with 130 mM NaCl were collected and saturated with solid ammonium sulfate to 40%. The enzyme solution was applied to a Butyl-Toyopearl 650 M column (1.5 × 13 cm) equilibrated with a buffer containing 40% saturated ammonium sulfate. The column was washed with the same buffer and eluted with a decreasing gradient of 40% to 0% saturated ammonium sulfate. The enzyme eluted with 18% saturated ammonium sulfate was recovered.

The culture filtrate (1,610 ml) of *A. rouxii* CBS 438.76 grown for 14 d was purified according to the methods described above. The dialyzed and concentrated filtrate was mixed with 1.0 M Tris–HCl (pH 8.5) to adjust it to 20 mM and was passed through a DEAE-Toyopearl 650 M column. The active fractions eluted with 160 mM NaCl were collected and applied to a Butyl-Toyopearl 650 M column. The active fractions eluted with 22% saturated ammonium sulfate were put on a column of Toyopearl HW-55 (1.5 × 90 cm) equilibrated with a 20 mM phosphate buffer (pH 6.8) containing 50 mM NaCl.

A single peak of the activity was found in each chromatograph of the two enzymes. By these procedures, the enzymes were purified, 3.5-fold for strain NBRC 4785 and 7.4-fold for strain CBS 438.76

**Table 2.** Purification of the Sucrose-Hydrolyzing Enzymes Produced by the Two Fungal Strains

| Strain | Step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| *R. oryzae* NBRC 4785 | Culture filtrate | 215 | 19.3 | 11.1 | 1.0 | 100 |
| | CM-Toyopearl | 135 | 7.77 | 17.4 | 1.6 | 62.8 |
| | Butyl-Toyopearl | 76 | 1.96 | 38.8 | 3.5 | 35.3 |
| *A. rouxii* CBS 438.76 | Culture filtrate | 652 | 3.91 | 167 | 1.0 | 100 |
| | DEAE-Toyopearl | 157 | 0.448 | 350 | 2.1 | 24.1 |
| | Butyl-Toyopearl | 81 | 0.117 | 698 | 4.2 | 12.5 |
| | Toyopearl HW55 | 49 | 0.0398 | 1,231 | 7.4 | 7.5 |

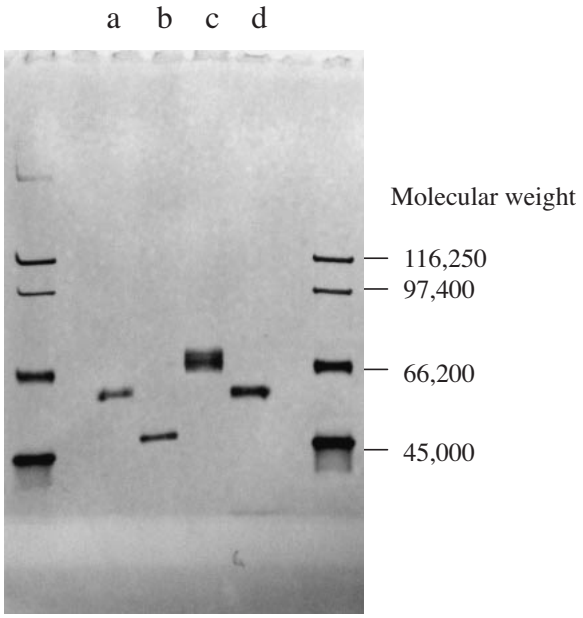


**Fig. 3.** SDS-Polyacrylamide Gel Electrophoresis of the Purified Enzymes from the Two Fungal Strains.

The purified enzymes of *R. oryzae* NBRC 4785 (0.083 μg) and *A. rouxii* CBS 438.76 (0.144 μg) before (a, c) and after (b, d) treatment with endoglycosidase H, respectively, were electrophoresed on 7.5% gel.

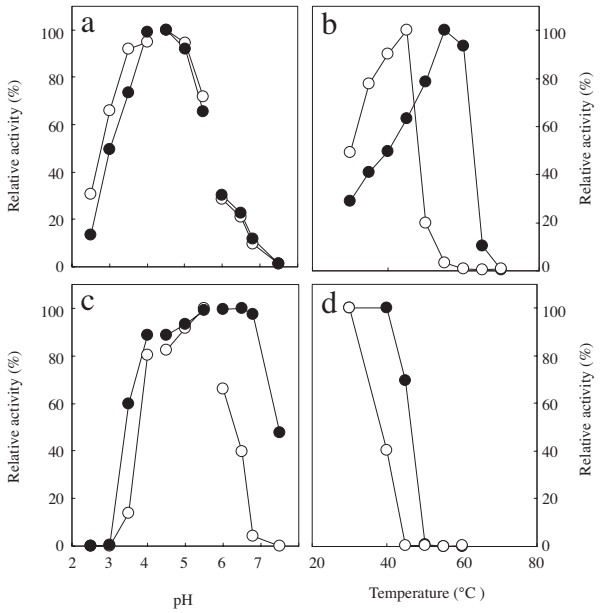


**Fig. 4.** Effects of the pH (a) and Temperature (b) on the Activity of the Enzymes and Those of pH (c) and Temperature (d) on the Stability of the Enzymes.

Buffers used: citrate, pH 2.5 to 3.8; acetate, pH 4.0 to 5.5; phosphate, 6.0 to 7.2. Symbols: ○, *R. oryzae* NBRC 4785; ●, *A. rouxii* CBS 438.76.

(Table 2). The enzymes were dialyzed against a 20 mM phosphate buffer (pH 6.8) and used in subsequent experiments.

*Properties of the purified enzymes*

Each purified enzyme gave a single band on SDS–PAGE with molecular weights showing homogeny through electrophoresis (Fig. 3). The molecular weights of the enzymes from strains NBRC 4785 and CBS 438.76 were estimated to be 59,000 and 69,000, and fell to 47,000 and 60,000 after treatment with endoglycosidase H, respectively.

Sucrose-hydrolyzing activity was assayed in a pH range of 3.1 to 7.5 at 30 °C and in the range of 30 °C to 70 °C at pH 5.0 (Fig. 4a, b). The two enzymes gave maximal activities at pH 4.5 at 30 °C, while the optimal temperature for the enzyme from strain CBS 438.76 was higher than that from strain NBRC 4785. Before the assay, the enzymes were incubated at 40 °C for 30 min at various pHs and temperatures for 30 min in a 20 mM phosphate buffer (pH 6.8) (Fig. 4c, d). The enzyme from strain NBRC 4785 was more unstable than that from CBS 438.76.

The effects of various concentrations of sucrose on the enzyme activity were examined. The values of $K_m$ (mM) and $V_{max}$ (U/mg protein) were calculated to be 200 and 103 for the enzyme from strain NBRC 4785, and 52.4 and 1,710 for that from strain CBS 438.76, respectively.

The products of sugars incubated with the purified enzymes were analyzed by TLC (Fig. 5). The enzyme from strain NBRC 4785 split both sucrose and maltose into monosaccharides almost completely and produced monosaccharides from fructooligosaccharides and soluble starch. The terminal moiety of glucose appeared to be hydrolyzed from fructooligosaccharides by this

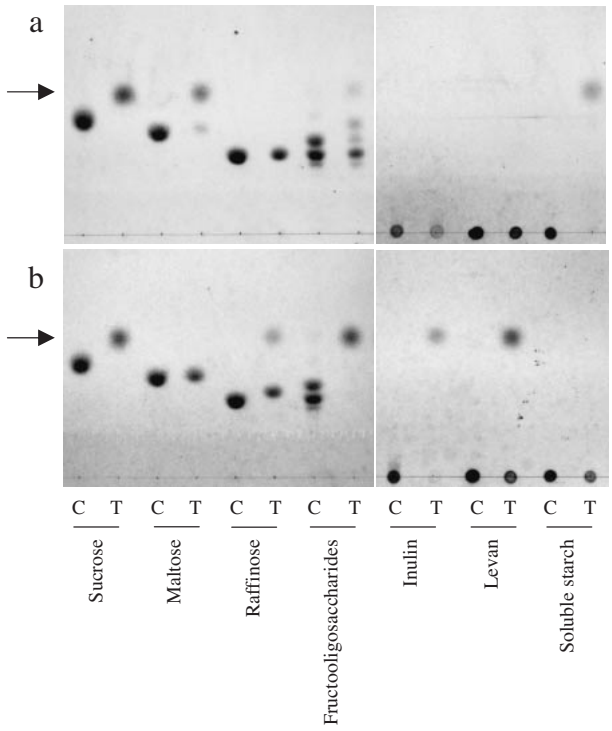


**Fig. 5.** Hydrolysis Products of Sugars Due to the Purified Enzymes from *R. oryzae* NBRC 4785 (a) and *A. rouxii* CBS 438.76 (b).

The reaction mixture contained a 100 mM acetate buffer (pH 5.0), 5 mg/ml of each sugar, and the purified enzyme (15 U) in a total volume of 0.10 ml. After incubation of the mixture at 30 °C for 20 h, the reaction was stopped by placing the mixture in boiling water for 5 min. One µl of each mixture before (C) and after (T) the reaction was applied to TLC. The arrow indicates the position of the monosaccharides.

enzyme, leaving residual oligosaccharides. When compared with the commercial enzyme preparation, the specific activities (U/mg protein) for sucrose, soluble starch, and *p*-nitrophenyl α-D-glucopyranoside were 35.9, 1.26, and 0.01 in the enzyme from strain NBRC 4785, and 0.04, 3.09, and 0.04 in the purified enzyme of glucoamylase from the *Rhizopus* species, respectively. The enzyme from strain NBRC 4785 can be classified as glucoamylase irrespective of its high activity for sucrose.

The enzyme from strain CBS 438.76 bore monosaccharides from sucrose, raffinose, fructooligosaccharides, inulin, and levan, but not maltose or soluble starch (Fig. 5). The complete hydrolysis of fructooligosaccharides indicates that this enzyme hydrolyzed the terminal moiety of fructose exogenously. The ratio of the activity on inulin *versus* sucrose was 0.013, indicating a higher preference for sucrose.

The N-terminal amino acid sequence of the enzyme from strain NBRC 4785 was SKPATFPT, which agreed with that of the protein from the *amyB* gene (DQ219822) of *R. oryzae* NRRL 395 (= NBRC 5384).[16] DNA sequence analysis of *amyB* genes from strains NBRC 4785 (AB444724) and NBRC 5384 (AB444726) revealed that the deduced amino acid sequences were identical except at position 90. As for the enzyme from strain CBS 438.76, determination of the N-terminal amino acid sequence was unsuccessful.

## Discussion

There are several types of microbial enzymes that can be involved in the degradation of sucrose.[17] The first enzyme is invertase (β-fructofuranoside fructohydrolase: EC 3.2.1.26), hydrolyzing sucrose and other β-fructofuranosides, and the second enzyme is inulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7), hydrolyzing inulin. These enzymes commonly attack the substrates from the fructose moiety, but show distinguishable properties. The ratio of the activity on inulin *versus* sucrose was found to be less than 0.02 for invertase and higher than 0.1 for inulinase.[18] Fungal invertases have been purified and characterized from *Aspergillus ficuum*,[18] *Aspergillus nidulans*,[19] *Aspergillus niger*,[20–23] *Aspergillus oryzae*,[24] and *Aspergillus ochraceus*.[25] However, invertase has not been found in the genus *Rhizopus*, and inulinase has been reported to participate in the hydrolysis of sucrose in spite of its lower affinity.[26] The third enzyme is α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20), which hydrolyzes maltose and its oligosaccharides from the glucose moiety. α-Glucosidases from different origins vary in substrate specificity, and the enzyme that preferentially hydrolyzes sucrose has been alternatively called glucoinvertase or glucosidosucrase.[17] Exogenous maltose has been incorporated into the cells of *Saccharomyces cerevisiae*[15] and *Candida albicans*[27] and hydrolyzed by α-glucosidase. The enzyme from the yeast *Torulaspora pretoriensis* showed much higher affinity to sucrose than to maltose.[28]

In the present experiments, sucrose-hydrolyzing enzymes produced by *R. oryzae* NBRC 4785 and *A. rouxii* CBS 438.76 were assumed to be distinct types based on the results of lactic acid production from some sugars. The profiles of sugar hydrolysis showed that the enzymes from strains NBRC 4785 and CBS 438.76 are to be classified as glucoamylase and invertase respectively.

The N-terminal amino acid sequence and molecular weight confirmed that the entity of the enzyme from strain NBRC 4785 might correspond with one of the glucoamylases encoded by the *amyB* gene in addition to the *amyA* gene in *R. oryzae* NRRL 395.[16] The AmyA and AmyB proteins share homologous amino acid sequences, but the AmyB protein lacks a starch-binding domain, differently from the AmyA protein. The commercial glucoamylase from the *Rhizopus* species used in the present experiments might be the AmyA protein[16] based on its molecular weight of 70,000. Loss of a starch-binding domain appears to cause drastic changes in the substrate specificity of the AmyA protein. The physiological role of the AmyB protein is not understood, because the *amyB* gene has not been highly

expressed in shake-flask cultures with various carbon sources, and no glucoamylase activity has been detected when recombinant expression of the *amyB* gene was conducted in *Pichia pastoris*.[16)]

The enzyme from *R. oryzae* NBRC 4785 was more sensitive to external pH and temperature than that from *A. rouxii* CBS 438.76. Acidification of the medium for 8 d after inoculation was severe in the enzyme secreted from strain NBRC 4785, and might have limited the activity. The other *R. oryzae* strains used in the present experiments also appeared to possess an AmyB protein carrying sucrose-hydrolyzing activity, because no activity was detected after 10 d of cultivation. The presence of invertase in strain CBS 438.76 does not delimit the species *A. rouxii*, because some strains accepted in *A. rouxii* did not grow on sucrose or raffinose.[8)] At least, it can be stated that AmyB protein plays a crucial role in the lactic acid fermentation of sucrose in *R. oryzae*. The lower expression of the *amyB* gene and instability of the AmyB protein can explain why the fermentation of sucrose by *R. oryzae* lacks efficiency, as reported previously.

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