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Review

Discovery of alcohol dehydrogenase from mushrooms and application to alcoholic beverages

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

Saccharomyces cerevisiae is the main microorganism used in alcoholic beverage brewing, because this microbe has alcohol dehydrogenase (ADH) activity. We have recently discovered that some genera of mushrooms produce alcohol dehydrogenase, and made wine, beer and sake using mushrooms in place of *S. cerevisiae*. The highest alcohol concentrations in the wine, beer and sake were achieved with *Pleurotus ostreatus* (2648 mM, 12.2%), *Tricholoma matsutake* (1069 mM, 4.6%) and *Agaricus blazei* (1736 mM, 8.0%). In the case of wine made using *A. blazei*, the same alcohol concentration (1736 mM, 8.0%) was produced under both aerobic and anaerobic conditions. This wine produced by *A. blazei* contained about 0.68% β-D-glucan, which is known to have preventive effects against cancer. The wine made using *Flammulina velutipes* showed thrombosis-preventing activity, giving a prolonged thrombin clotting time 2.2-fold that of the control. Thus, alcoholic beverages made using mushrooms seem to be a functional food source which can be expected to have preventive effects against cancer and thrombosis.

Keywords: Alcohol dehydrogenase; Alcohol fermentation; Mushroom; Alcoholic beverage; Anti-thrombin substance

1. Introduction

The food and pharmaceutical industries are major users of enzymes of animal, vegetable and microbial origin. The application of genetic engineering, particularly recombinant DNA technology, has had a major impact on the development of new sources of industrial enzymes for the food industry [1]. Enzymes play important roles in various aspects of food processing. In some cases, they made the development of new products possible and, in other cases, the improvement of traditional products.

Enzymes used in food processing include α-amylase [EC 3.2.1.1] (*Aspergillus oryzae*, *Bacillus subtilis*) [2], catalase [EC 1.11.1.6] (beef liver) [3], cellulases [EC 3.2.1.4] (*Aspergillus niger*, *Trichoderma viride*, *Trichoderma reesei*) [4], glucoamylases [EC 3.2.1.3] (*A. niger*, *Rhizopus* sp.) [5,6], glucose isomerase [EC 5.3.1.18] (*Aerobacter* sp., *Escherichia* sp., *Lactobacillus* sp., *Streptococcus* sp., *Bacillus* sp.) [7], glucose oxidase [EC 1.1.3.4] (*A. niger*) [8], invertase [EC 3.2.1.26] (*Kluyveromyces fragilis*, *Saccharomyces cerevisiae*) [9], lipases [EC 3.1.1.3] (Porcine pancreas) [10–12] and proteases (*B. subtilis*, *A. oryzae*,

Abbreviations: ADH, alcohol dehydrogenase

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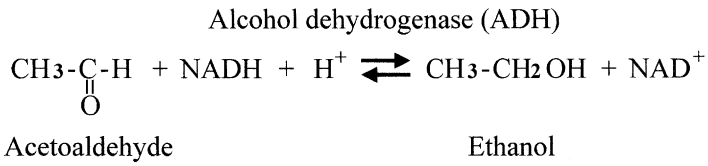

Fig. 1. The action of alcohol dehydrogenase. In the final step of alcoholic fermentation, acetaldehyde is reduced to ethanol, with NADH derived from glyceraldehydes 3-phosphate dehydrogenation providing the reducing power, through the action of alcohol dehydrogenase.

Rhizopus sp. *B. subtilis*, *B. polymyxa*, pineapple, papaya, fig, bovine, *M. pusillus*, *M. miehei*) [13].

Many familiar foods such as bread, cheese, yogurt, salamis, sour pickles, wines, beers, sake and miso achieve their characteristics as a result of microbial activity.

Wine, beer and sake are popular alcoholic beverages consumed by many people around the world. The earliest written records indicate that alcohol (ethanol) has been enjoyed in the human diet for thousands of years. Its microbial origin was only established some 150 years ago through the work of Louis Pasteur [14]. In general, *S. cerevisiae* has long been used for producing alcoholic beverages since it has potent alcohol dehydrogenase activity [EC 1.1.1.1] [15–20] (Fig. 1). We have recently discovered that some mushrooms also possess alcohol dehydrogenase [21,22]. Mushrooms such as *Pleurotus ostreatus*, *Flammulina velutipes*, *Tricholoma matsutake* and *Agaricus blazei* are commonly consumed, and are rich in fiber, protein and vitamins such as thiamin and riboflavin, in addition to having a preventive effect against cancer and thrombosis [23,24].

We therefore produced wine, beer and sake with mushrooms and expected that consumption of these alcoholic beverages could have a preventive effect against cancer and thrombosis, as well as other health benefits. In this review, we describe the discovery of alcohol dehydrogenase from mushrooms, the alcoholic beverages produced with the mushrooms and their characteristics.

2. Reason for using mushrooms as experimental materials

Thrombosis is one of the major causes of death in Japan and the western world. Presently, the three kinds of thrombolytic agents clinically used are streptokinase, urokinase, and tissue-type plasminogen activator (t-PA). However, there are still major problems with their use from the viewpoint of antigenicity, non-specific breakdown of other blood factors and the localization of the fibrin clot because of high enzymatic activity, respectively [25–27].

On the other hand, anti-coagulants of natural origin, such as heparin and anti-thrombin III, have been found in constituents of the human body [28]. Anti-thrombin III, a macromolecule substance, is an effective anti-thrombotic agent, but its activity depends on the coexistence of heparin. Okamoto and co-workers [29,30] reported a synthetic thrombin inhibitor (argatroban), 4-methyl-1-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinolinylsulfonyl-L-arginyl)-2-piperidine carboxylic acid, which is an effective drug for acute stage cerebral thrombosis [31]. However, this compound is unlikely to be satisfactory as a thrombolytic agent in the chronic stage. Therefore, the screening of inhibitory agents for thrombin which correspond to the formation of fibrin, but not that of plasminogen activators, is now desirable.

We tried to screen for microorganisms having anti-thrombin activity to obtain effective anti-thrombin substances and having fibrinolytic activity to obtain effective fibrinolytic enzymes. Some anti-thrombin and fibrinolytic activities were found from many culture broths of mushrooms [32–35]. Therefore, we used mushrooms as experimental materials to produce functional foods which could have a preventive effect against thrombosis, as well as other health benefits.

3. Bread processing experiments revealed the alcohol fermentation ability of mushrooms

We studied the processing of bread, in which mushrooms were added to some parts of the wheat flour. The wheat flour and mushrooms were blended and

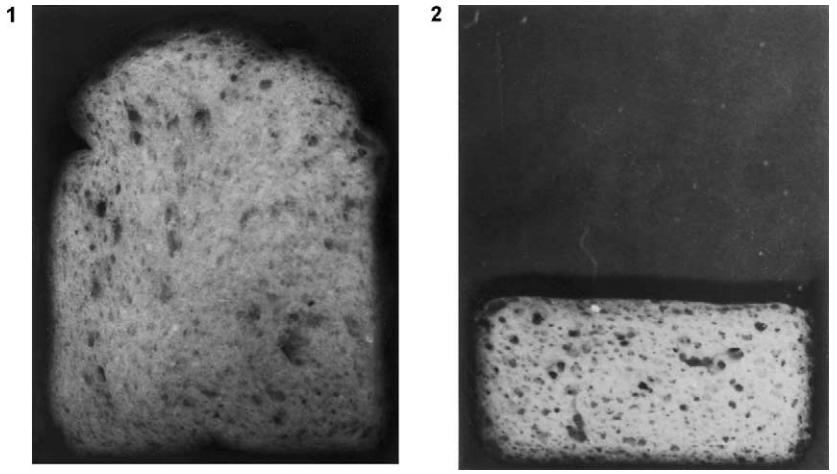

Fig. 2. Photographs of breads. Maitake was added at 10% (w/w) to wheat flour. Photograph shows the middle cutaway view of bread: (1) white bread (control); (2) maitake-added bread.

baked, and the effect of this mushroom blending on the functional properties in breadmaking was investigated [36–38]. The characteristics, such as loaf volume, of mushroom bread differed substantially from standard white bread. The loaf volume and specific loaf volume of bread containing 10% *Grifola frondosa*, *Hypsizygus marmoreus*, or *Pholiota nameko* mushrooms were markedly decreased (Fig. 2).

The addition of *G. frondosa* influenced the production of carbon dioxide (CO_2), however, there was no response to medium containing *G. frondosa* without baker's yeast. The addition of *G. frondosa* to white bread supplied carbohydrates to the baker's yeast and promoted alcohol fermentation under anaerobic conditions. This led to increased CO_2 production resulting in a sharp fall in dough containing 10% or more of *G. frondosa*.

However, before obtaining the above results, we thought that mushrooms may be able to carry out alcohol fermentation because the addition of mushrooms led to increased gas production. Therefore, we studied the alcohol dehydrogenase activity of mushrooms, and found it to be present in some.

4. Selection of mushrooms for alcohol fermentation

A. blazei MWU-C20, *F. velutipes* MWU-C3, *P. ostreatus* MWU-C1 and *T. matsutake* MWU-C21 were used in this experiment (Fig. 3). These mushrooms were purchased at a local market at Nishinomiya in Japan. Their cultures were obtained by aseptically inoculating the tissue from the fruiting bodies into a medium containing 2% malt extract (pH 5.6). Mushroom cultures that had been grown on an incline were inoculated into 200 ml of the medium in a 500 ml Erlenmeyer flask. Cultivation was carried out at 25 °C for 2 weeks under aerobic conditions with a rotary shaker (100 rpm). Mycelia were collected by centrifugation at 10,000 × *g* for 10 min and washed twice with an ice-cold saline solution. The mycelial pellet was suspended in a 10 mM Tris–HCl buffer (pH 7.5) and subjected to sonication with an ultrasonic oscillator (Branson, Sonifier 250, 20 kHz) for 16 min at below 8 °C. The undestroyed mycelia and debris were discarded after centrifuging at 10,000 × *g* for 10 min. The supernatant solution obtained was used as the cell-free extract.

5. Mushroom alcohol dehydrogenase

5.1. Enzyme assay

The standard reaction mixture contained 200 μmol of ethyl alcohol, 1 μmol of NAD^+, 200 μmol of a Tris–HCl buffer (pH 7.5), and the cell-free extract in a final volume of 1.0 ml. The substrate was replaced with water in a blank mixture. Incubation was conducted

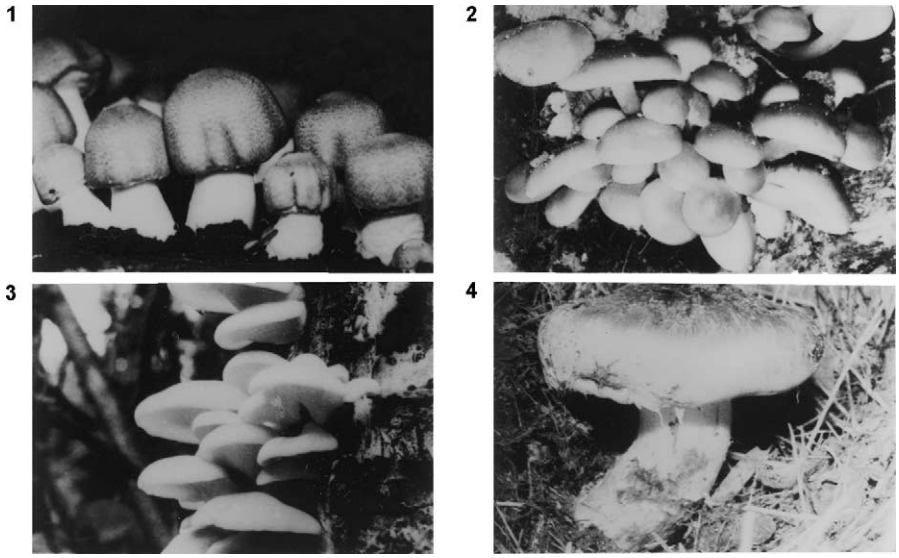

Fig. 3. Mushrooms used in the production of alcoholic beverages: (1) *A. blazei*; (2) *F. velutipes*; (3) *P. ostreatus*; (4) *T. matsutake*.

at 30 °C in a cuvette with a 1 cm light path. The reaction was started by adding NAD^+ and monitored by measuring the initial change in absorbance at 340 nm with a Hitachi 150-20 double-beam spectrophotometer equipped with a thermostatically controlled cuvette holder and continuous chart recorder. One unit of enzyme activity is defined as the amount that catalyzed the formation of 1 μmol of NADH per minute during the reaction. Specific activity is expressed in units per milligram of protein. Protein was measured by the method of Lowry et al. [39], with crystalline bovine serum albumin being used as the standard.

The ADH activity in cell-free extracts of the three types of mushroom was examined. As shown in Table 1, potent activity was found in the extract of *A. blazei* (98.0 U/mg), whereas low activity was found in the extracts of *F. velutipes* (15.6 U/mg), *P. ostreatus* (4.6 U/mg) and *T. matsutake* (2.5 U/mg).

Table 1
Specific activity and molecular mass of alcohol dehydrogenase

Mushroom used	Specific activity (U/mg)	Molecular mass (kDa)
A. blazei	98.0	59
F. velutipes	15.6	90
P. ostreatus	4.6	70
T. matsutake	2.5	30

5.2. Electrophoresis

Gel electrophoresis of the native enzymes was conducted on 7.5% polyacrylamide gel by using the method of Davis [40]. Alcohol dehydrogenase (ADH) activity staining was performed in a solution containing a 50 mM Tris–HCl buffer (pH 7.5), 1.25 mM NAD^+, 10 mM ethanol, 0.4 mM phenazine methosulfate, and 0.5 mM nitroblue tetrazolium.

Polyacrylamide gel electrophoresis (PAGE) of native ADH from *A. blazei* and subsequent active staining is shown in Fig. 4. The electrophoresis of purified ADH from *A. blazei*, *F. velutipes*, *P. ostreatus* and *T. matsutake*, which had been obtained by gel filtration in a TSK gel G3000SW column with an HPLC system and by extraction from the active staining gel after PAGE, showed a single band upon activity staining.

5.3. Measurement of the molecular mass

The molecular mass was measured by gel filtration in a TSK gel G3000SW column (0.75 cm × 30 cm) at a flow rate of 700 μl/min with 0.01% β-mercaptoethanol and 10% glycerol. A calibration curve was constructed with the following proteins: glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa).

Fig. 4. Active staining after polyacrylamide gel electrophoresis of (ADH) of *A. blazei*. The purified enzyme (2 μg of protein) was electrophoresed on a 7.5% polyacrylamide slab gel at a current of 2.5 mA according to the method of Davis [40]. The arrow indicates the position of ADH: (1) ADH of *A. blazei*; (2) ADH of *F. velutipes*; (3) ADH of *P. ostreatus*; (4) ADH of *T. matsutake*.

As shown in Table 1, the molecular masses of the enzymes were estimated to be about 59 kDa (*A. blazei*), 90 kDa (*F. velutipes*), 70 kDa (*P. ostreatus*) and 30 kDa (*T. matsutake*) by gel filtration.

6. Production method of alcoholic beverages

6.1. Wine brewing

Wine was brewed following conventional method, except that mushrooms were used in place of *S. cerevisiae*. Mushroom mycelia, cultured by the method described in Section 4 were used. Grapes (Kyohou) were washed with water, mashed in a mixer, and then adjusted to an 11% sugar concentration with water and to pH 5.8. The resulting grape juice was autoclaved for 30 min at 1 kg/cm^2 of pressure. Two grams of the mycelia of mushrooms was added to 30 ml of autoclaved grape juice in an Erlenmeyer flask, which was incubated at 20 °C ± 1 °C for 40 days. In order to maintain the sugar concentration at 11%, autoclaved conc. grape juice was added to the flask every time a sample was taken (the total sugar concentration was 30–45%). The same grape juice without inoculating the mycelia was prepared as a control.

6.2. Beer brewing

Beer brewing was performed following conventional methods except that mushrooms were used in place of *S. cerevisiae*. Two grams of mushroom mycelia was added to autoclaved hopped malt extract medium (pH 5.8) containing 10% malt extract and 0.1% hop extract in an Erlenmeyer flask, and incubated at 20 °C ± 1 °C for 14 days. The same hopped malt extract medium without inoculation was prepared as a control (hopped malt extract).

6.3. Sake brewing

Sake brewing was performed by conventional methods except that mushrooms were used in place of *S. cerevisiae* and *A. oryzae*. Thirty grams of rice (Koshihikari, Toyama-prefecture, Japan) was thoroughly washed with water, placed in an Erlenmeyer flask and soaked in water overnight at room temperature. The water was then drained off and the flask sealed with aluminum foil. After autoclaving for 30 min at a pressure of 1 kg/cm^2, the rice was cooled, inoculated with mycelia (1 g) obtained by the method described in Section 4, and incubated at 25 °C ± 1 °C for 15 days. When the spawned rice had thoroughly colonized, 120 ml of autoclaved water was added to the flask. The same rice–water without inoculation was prepared as a control (rice–water). Finally, the brewing was continued at 20 °C for 29 days. It was previously reported that mushrooms produced amylase [41]. Furthermore, we reported that the mushrooms used in this experiment can convert starch (rice) into sugar and amylase was produced both outside and inside the cells [42].

7. Alcohol concentration of alcoholic beverages produced by mushrooms

7.1. Wine

The alcohol (ethanol) concentration was measured by HPLC in a TSK-gel Oapak-A column (0.78 cm × 30 cm) at a flow rate of 1 ml/min, using water with RI.

Table 2
Alcohol concentration of alcoholic beverages produced using mushrooms

Mushroom used	Alcohol concentration (mM (%))		
	Wine	Beer	Sake
A. blazei	1736 (8.0)	–	1736 (8.0)
F. velutipes	651 (3.0)	651 (3.0)	629 (2.9)
P. ostreatus	2648 (12.2)	–	–
T. matsutake	–	1069 (4.6)	651 (3.0)

Four genera of mushrooms possessing ADH activity were used for alcohol brewing. As shown in Table 2, the highest alcohol concentration in the resulting wine was achieved using *P. ostreatus* (2648 mM, 12.2%), while the *F. velutipes* wine (651 mM, 3.0%) had a low alcohol content. The alcohol concentration in the wine produced by *A. blazei* was 1736 mM (8.0%). In the case of *A. blazei*, the same alcohol concentration was also produced under aerobic conditions with a rotary shaker at 100 rpm as shown in Fig. 5.

7.2. Beer

The highest alcohol concentration in the beer was achieved using *T. matsutake*, and was 1069 mM (4.6%), while the *F. velutipes* beer (651 mM, 3.0%) had a low alcohol content. The flavor of beer produced by *T. matsutake* was the same as that of the fruit-body.

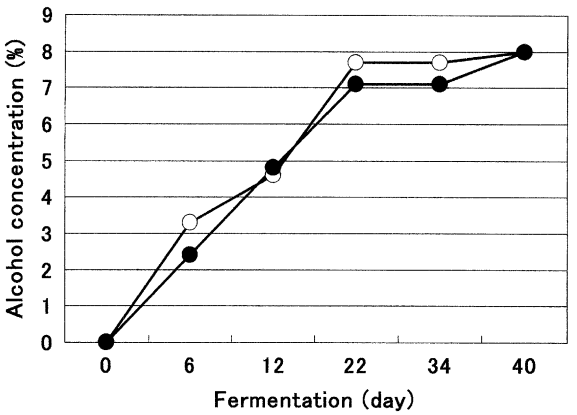

Fig. 5. Time-course plot for the production of alcohol (ethanol) by *A. blazei*. *A. blazei* was incubated at 25 °C in grape juice under aerobic conditions with a rotary shaker (100 rpm) and under anaerobic conditions (standing culture). The alcohol concentration is shown under aerobic (○) and anaerobic (●) conditions.

7.3. Sake

The highest alcohol concentration in the sake was achieved using *A. blazei*, and was 1736 mM (8.0%), while the *F. velutipes* sake (629 mM, 2.9%) and *T. matsutake* sake (651 mM, 3.0%) had low alcohol contents. The flavor of sake produced by *T. matsutake* was the same as that of the fruit-body.

8. Characteristics of alcohol fermentation of mushrooms

8.1. Mechanism of alcohol fermentation

As shown in Fig. 5, *A. blazei* produced alcohol in both the aerobic and anaerobic conditions, although *S. cerevisiae* cannot achieve this in the aerobic condition.

In general, the conversion of carbohydrate into ethanol requires the action of alcohol dehydrogenase produced by the yeast *S. cerevisiae* during ethanol production, and therefore *S. cerevisiae* has traditionally been used to make alcoholic beverages such as wine, beer and sake. Louis Pasteur first showed a balance between the amount of glucose fermented by yeast (*S. cerevisiae*) and the alcohol and carbon dioxide formed.

It is known that glucose is converted to pyruvate via four different pathways such as the Embden–Meyerhof–Parnas (EMP) pathway, hexose monophosphate (HMP) pathway, Entner–Doudoroff (ED) pathway and the phosphoketolase pathway.

The reaction sequence from glucose to pyruvate in yeast involves the EMP pathway (Fig. 6), in which pyruvate is converted into acetaldehyde, and then ethanol in the aerobic condition [43].

However, in the present study, alcoholic beverages were produced using only mushrooms because they also have alcohol dehydrogenase activity.

The ED pathway is one of the most recently described pathways discovered by Entner and Doudoroff during metabolic studies of *Pseudomonas saccharophila* (Fig. 7). It has since been found in a number of other microorganisms [44–50]. It was thought that oxygen may play an important role in the selec-

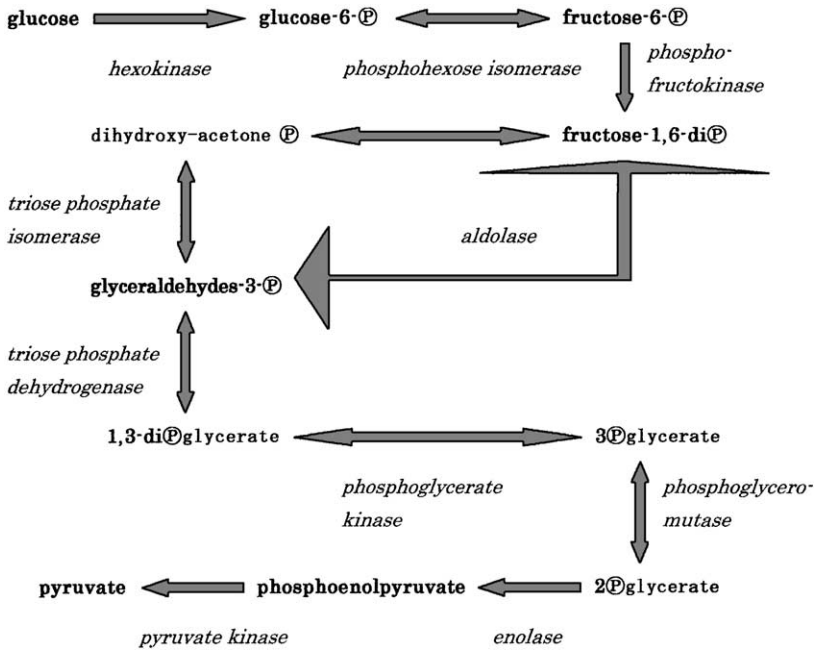

Fig. 6. The reaction of the Embden–Meyerhof–Parnas pathway.

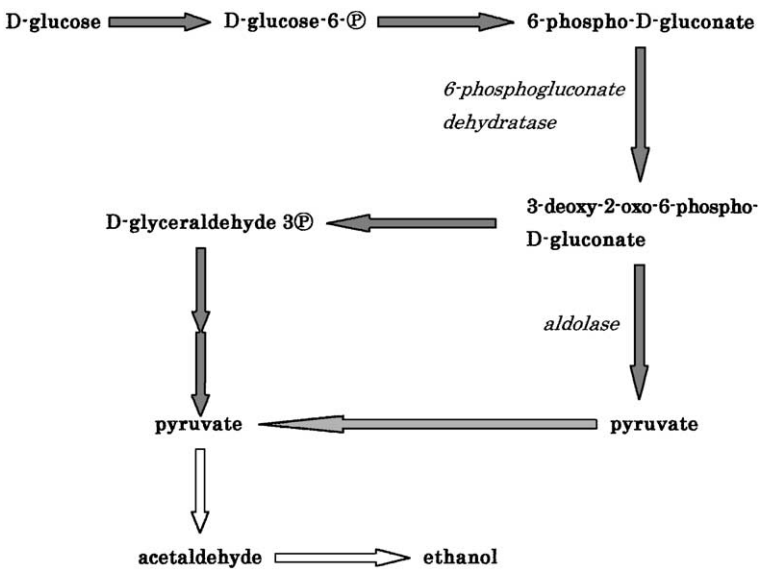

Fig. 7. The reaction of the Entner–Doudoroff pathway.

tion of pathway usage. The majority of anaerobic microorganisms were found to contain the EMP pathway; facultative aerobes were found to contain a combination of the EMP and HMP pathways, and strict aerobes were found to contain almost exclusively the ED pathway, although the findings that the strict anaerobe *Zymomonas mobilis* can only use the ED pathway and that homofermentative lactobacilli can use the EMP pathway even under aerobic conditions cast some doubt upon the early assumptions.

A. blazei produced alcohol in both the aerobic and anaerobic conditions, although *S. cerevisiae* cannot achieve this in the aerobic condition. It therefore seems that alcohol fermentation by *A. blazei* depended on both the EMP pathway and ED pathway, while only the EMP pathway is used by *S. cerevisiae* (Fig. 8).

8.2. Saccharification and alcohol fermentation in the production of sake

Sake is the traditional alcoholic beverage in Japan and still one of the most popular Japanese drinks. The raw material used in sake brewing is “koji”, a culture of *A. oryzae* on steamed rice, which is subjected to parallel fermentation. Koji, which is comparable to the malts used for beer brewing, is used for saccharification of starch and decomposition of protein contained

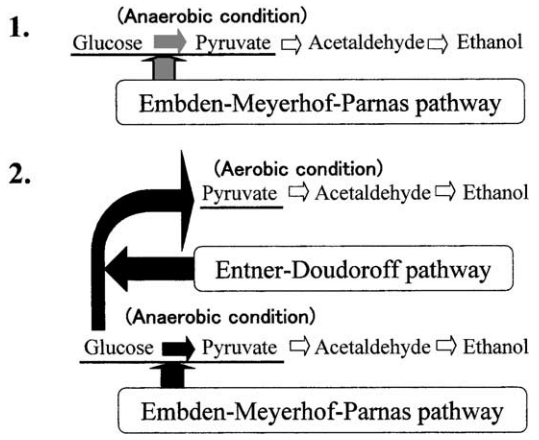

Fig. 8. Scheme of the mechanism of alcohol fermentation. Alcohol fermentation by *S. cerevisiae* depended on only the Embden–Meyerhof–Parnas pathway. On the other hand, alcohol fermentation by *A. blazei* depended on both the Embden–Meyerhof–Parnas pathway and Entner-Doudoroff pathway: (1) yeast (*S. cerevisiae*); (2) mushroom (*A. blazei*).

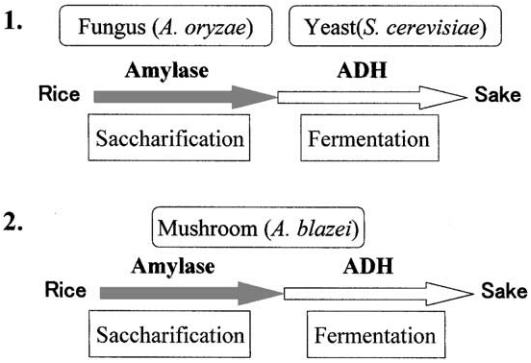

Fig. 9. Scheme of method of sake production. In general, the conversion of carbohydrate (rice) into fermentable sugars is performed by the action of amylase produced by a fungus, *A. oryzae*, during sake production, and *S. cerevisiae* (alcohol dehydrogenase) and *A. oryzae* (amylase) have been used to make sake. On the other hand, sake was produced using only mushrooms because they have both alcohol dehydrogenase and amylase activity. (1) General method; (2) method in this experiment using mushroom.

in the raw material, rice grains. Whereas fermentation takes place after filtration of the mash in beer brewing, in the sake mash, which is called “moromi”, sugars liberated from rice grains are fermented successively by yeast. Parallel fermentation represents the combination of progressive decomposition of starch and of other substances, and slow fermentation at a low temperature.

Therefore, the conversion of carbohydrate (rice) into fermentable sugars is performed by the action of amylases produced by a fungus, *A. oryzae*, during sake production, and *S. cerevisiae* (alcohol dehydrogenase) and *A. oryzae* (amylase) have been used to make sake. On the other hand, in this research, sake was produced using only mushrooms because they have both alcohol dehydrogenase and amylase activity (Fig. 9). This may be a simple method to produce sake because the process only requires inoculation of mushrooms in rice and water.

9. Physiological activity of alcoholic beverages produced by mushrooms

9.1. β-D-Glucan

The β-D-glucan (β-D-1,3-glucan) was evaluated by HPLC in a TSK-gel G5000PW column (0.75 cm ×

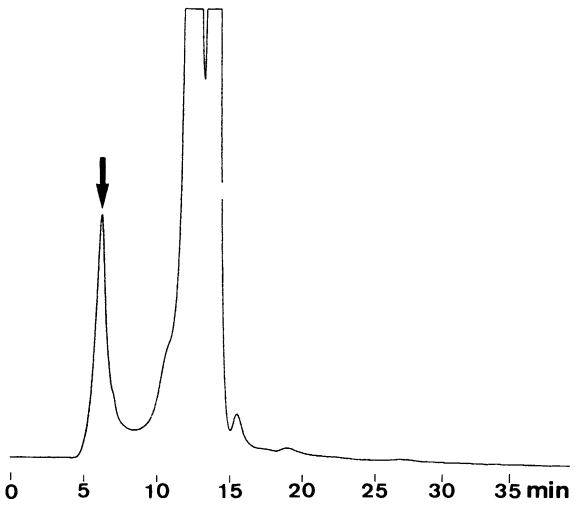

Fig. 10. β-D-Glucan of alcoholic beverages produced by *A. blazei*. The arrow indicates the position of β-D-glucan.

30 cm) at a flow rate of 1 ml/min, using water with RI.

Fig. 10 shows that wine produced by *A. blazei* contained about 0.68% β-D-glucan, which has been reported to have preventative activity against cancer (e.g. it has been shown to have a preventative effect against cancer of the Sarcoma 180/ICR mouse) [23]. Drinking wine produced using *A. blazei* may thus provide anti-cancer benefits.

Beer produced by *T. matsutake* also contained about 0.17% β-D-glucan and sake produced by *A. blazei* contained about 0.03% β-D-glucan.

9.2. Thrombin time

The coagulability was tested by using the thrombin time (TT), the elapsed time until the fibrin formation of thrombin, by the method described by Kinoshita and Horie [51]. After the fermentation, the wine, beer and sake were centrifuged at 10,000 × *g* for 10 min, and the supernatant was applied to determine the thrombin activity. Bovine α-thrombin was purchased from Mochida Pharmaceutical Co. The thrombin clotting time in a reaction mixture (37 °C) containing 50 μl of 12.5 NIH U/ml of thrombin and 200 μl of 0.33% bovine fibrinogen was measured by a KC1A coagulometer (Heinrich Amelung).

The effects of the alcoholic beverage samples produced by mushrooms on thrombin time are summarized in Table 3. The three wines produced with *F. velutipes*, *A. blazei*, and *P. ostreatus* showed anti-coagulative activity on TT. The TT value for wine produced by *F. velutipes* was longer than that by *A. blazei* or *P. ostreatus*. The thrombin clotting time of the wine produced by *F. velutipes* was determined to be more than 2.2 times longer than that of the control (grape juice).

Table 3
Effect on thrombin time of alcoholic beverages produced using mushrooms

Mushroom used	Thrombin time (s)		
	Wine	Beer	Sake
A. blazei	310	–	212
F. velutipes	358	303	282
P. ostreatus	320	–	–
T. matsutake	–	600[a]	313
Control	161[b]	268[c]	144[d]

[a] More than 600 s.
[b] Grape juice.
[c] Hopped malt extract medium.
[d] Rice–water.

On the other hand, the TT of beer produced by *T. matsutake* was longer than that of *F. velutipes*. The thrombin clotting time of the beer produced by *T. matsutake* was determined to be more than 2.3 times longer than that of the control (hopped malt extract medium).

That of sake produced by *T. matsutake* was longer than those of *F. velutipes* and *A. blazei*. The thrombin clotting time of the sake produced by *T. matsutake* was determined to be longer than 2.2 times that of the control (rice–water).

9.3. Fibrinolytic activity

In order to determine the fibrinolytic activity, the method of Astrup and Mullertz [52] using fibrin plates was employed. An artificial thrombus was prepared on a disk by coagulating 0.4% bovine fibrinogen with thrombin, the wine (30 μl) was poured onto the fibrin plate, and the potency required to dissolve the thrombus was determined after 1 h.

Table 4 shows the fibrinolytic activity of the alcoholic beverage samples produced by mushrooms. The wines produced by *A. blazei*, *F. velutipes* and *P. ostreatus* all showed fibrinolytic activity on the fibrin

Table 4
Fibrinolytic activity of the alcoholic beverages produced using mushrooms

Mushroom used	Fibrinolytic activity (mm^2)		
	Wine	Beer	Sake
A. blazei	15	–	0
F. velutipes	20	0	0
P. ostreatus	27	–	–
T. matsutake	–	36	0
Control	0[a]	0[b]	0[c]

[a] Grape juice.
[b] Hopped malt extract medium.
[c] Rice–water.

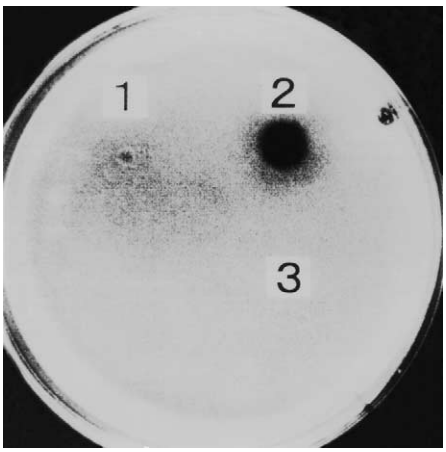

Fig. 11. Fibrinolytic activity of beer produced using mushrooms. A dissolved zone on the fibrin plate is shown: (1) *F. velutipes*; (2) *T. matsutake*; (3) control (hopped malt-extract medium).

plate, although the control did not. The beer produced by *T. matsutake* showed fibrinolytic activities on the fibrin plate, although that of *F. velutipes* and control did not (Fig. 11).

Thus, alcoholic beverages produced with these mushrooms may have a preventive effect on thrombosis [53].

10. Conclusions

In general, the conversion of carbohydrate into ethanol requires the action of alcohol dehydrogenase produced by the yeast *S. cerevisiae* during ethanol production, and therefore *S. cerevisiae* has traditionally been used to make alcoholic beverages such as wine, beer and sake. However, in the present study, alcoholic beverages were produced using only mushrooms because they also have alcohol dehydrogenase activity. *A. blazei* produced alcohol in both the aerobic and anaerobic conditions, although *S. cerevisiae* cannot achieve this in the aerobic condition. It therefore seems that alcohol fermentation by *A. blazei* depended on both the EMP pathway and ED pathway, while only the EMP pathway is used by *S. cerevisiae*.

Furthermore, the conversion of carbohydrate (rice) into fermentable sugars is performed by the action of amylases produced by a fungus, *A. oryzae*, during sake production, and *S. cerevisiae* (alcohol dehydrogenase) and *A. oryzae* (amylase) have been used to make sake. In this research, sake was produced using only mushrooms because they have both alcohol dehydrogenase and amylase activity.

Furthermore, lactic acid bacteria, particularly *Lactobacillus* and *Streptococcus* genera, have long been used in making cheese since they are potent lactate dehydrogenase [EC 1.1.1.27] producers [54,55]. A milk-clotting enzyme is also necessary to make cheese [56]. Rennet preparations from the stomachs of young ruminants are the traditional coagulants used [57]. Recently, we discovered that some mushrooms, including *Schizophyllum commune* [58], possess lactate dehydrogenase and a milk-clotting enzyme. We therefore produced a cheese-like food with *S. commune* in anticipation that its consumption could help to inhibit cancer and thrombosis, as well as having other health benefits [59].

Miso is a traditional fermented food in Japan, and *A. oryzae*, *Pediococcus halophilus* and *Saccharomyces rouxii* are used to make miso because they have a potent ability to produce amylase and protease, lactate dehydrogenase, and alcohol dehydrogenase, respectively. Recently, we have discovered that some mushrooms also possess amylase, protease, lactate dehydrogenase and alcohol dehydrogenase [42,60,61]. We produced a miso-like food with mushrooms because we hypothesized that this miso may have a preventative effect against thrombosis and cancer, as well as other health benefits [62].

Fermented foods, whether from plant or animal origin, are an intricate part of the diet in all parts of the world. The diversity of raw materials used as substrates, methods of preparation and qualities of the

finished products are fascinating as one begins to learn more about the eating habits of various cultures. The preparation of many indigenous or "traditional" fermented foods and alcoholic beverages remains today as a household art.

Therefore, utilization of different fermentative microorganisms such as mushrooms could fuel the development of new fermented foods that have attractive functional properties.

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