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% 1-1,3-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.

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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,
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.

$$\text{CH}_3\text{C}^\circ\text{H} + \text{NAD}^+ + \text{H}^+ \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{NAD}$$

Acetaldehyde   Ethanol

**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 [23,24].

### 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.
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*, *Hypzsy-gus marmoreus*, or *Pholiota nameko* mushrooms were markedly decreased (Fig. 2).

The addition of *G. frondosa* influenced the production of carbon dioxide (CO₂), 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 CO₂ 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...
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>
<thead>
<tr>
<th>Mushroom used</th>
<th>Specific activity (U/mg)</th>
<th>Molecular mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. blazei</td>
<td>98.0</td>
<td>59</td>
</tr>
<tr>
<td>F. velutipes</td>
<td>15.6</td>
<td>90</td>
</tr>
<tr>
<td>P. ostreatus</td>
<td>4.6</td>
<td>70</td>
</tr>
<tr>
<td>T. matsutake</td>
<td>2.5</td>
<td>30</td>
</tr>
</tbody>
</table>

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).
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/cm² 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. After autoclaving for 30 min at a pressure of 1 kg/cm², 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.
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.

### Table 2

<table>
<thead>
<tr>
<th>Mushroom used</th>
<th>Alcohol concentration (mM (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wine</td>
</tr>
<tr>
<td><em>A. blazei</em></td>
<td>1736</td>
</tr>
<tr>
<td><em>F. velutipes</em></td>
<td>651</td>
</tr>
<tr>
<td><em>P. ostreatus</em></td>
<td>2648</td>
</tr>
<tr>
<td><em>T. matsutake</em></td>
<td>–</td>
</tr>
</tbody>
</table>

### 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.

### 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 acetalddehyde, 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 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, 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.

9. Physiological activity of alcoholic beverages produced by mushrooms

9.1. β-D-Glucan

The β-D-glucan (β-1,3-glucan) was evaluated by HPLC in a TSK-gel G5000PW column (0.75 cm ×
Fig. 10 shows that wine produced by *A. blazei* contained about 0.68% \( \beta \)-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% \( \beta \)-d-glucan and sake produced by *A. blazei* contained about 0.03% \( \beta \)-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 \( \times \) g for 10 min, and the supernatant was applied to determine the thrombin activity. Bovine \( \alpha \)-thrombin was purchased from Mochida Pharmaceutical Co. The thrombin clotting time in a reaction mixture (37°C) containing 50 \( \mu L \) of 12.5 NIH U/ml of thrombin and 200 \( \mu L \) 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).

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 \( \mu L \)) 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

<table>
<thead>
<tr>
<th>Mushroom used</th>
<th>Fibrinolytic activity (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wine</td>
</tr>
<tr>
<td>A. blazei</td>
<td>15</td>
</tr>
<tr>
<td>F. velutipes</td>
<td>20</td>
</tr>
<tr>
<td>P. ostreatus</td>
<td>27</td>
</tr>
<tr>
<td>T. matsutake</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

*Grape juice.

**Hopped malt extract medium.

*Rice-water.

A dissolved zone on the fibrin plate is shown: (1) F. velutipes; (2) T. matsutake; (3) control (hopped malt-extract medium).

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.

Acknowledgements

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References