

# Improving the Yield of Glucoamylase and $\alpha$ -amylase in Solid-state Co-culture

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**Abstract.** Steamed rice inoculated with *Aspergillus oryzae*, called Koji, is an essential ingredient for making *amazake* or brewing *sake*. However, *A. oryzae* usually offers low enzyme yield, especially in case of glucoamylase. Co-culture of *Aspergillus* and *Rhizopus* strains in Koji increased glucoamylase and  $\alpha$ -amylase activities. The ratio of initial spore counts of *A. oryzae* and *R. oryzae* influenced the activity of amylolytic enzymes. When this ratio was 1:1,  $\alpha$ -amylase showed maximum activity (573 U/g-substrate, 95 h), and when this ratio was 200:1, glucoamylase showed maximum activity (180 U/g-substrate, 95 h).

## 1 Introduction

Amylases are among the most important enzymes, with great significance in biotechnological applications, constituting around 25% of the enzyme industry [1]. The amylase family has two major classes: glucoamylase and  $\alpha$ -amylase.

Glucoamylase ( $\alpha$ -1,4-glucan glucohydrolase or amyloglucosidase, EC 3.2.1.3) consecutively hydrolyzes the  $\alpha$ -1,4-glycosidic and  $\alpha$ -1,6-glycosidic bonds at the non-reducing ends of starch, glycogen, and similar carbohydrates, thereby resulting in glucose production [2].

$\alpha$ -Amylase ( $\alpha$ -1,4-glucan 4-glucohydrolase, EC 3.2.1.1) are extracellular endoenzymes that randomly cleave the 1,4- $\alpha$ -d-glucosidic linkages between adjacent glucose units in the linear amylose chain [3].

These amylolytic enzymes are widely used in the Japanese fermentation industry, e.g., for the manufacture of sake, soy sauce, miso, and amazake [4-6].

*Amazake*, a traditional non-alcoholic beverage in Japan, is produced from a mixture of rice *Koji* and boiled rice that is incubated at 55 °C to 60 °C for 8 h<sup>4</sup>. During this process, the glucoamylase and  $\alpha$ -amylase produced by *Aspergillus oryzae*, a fungus found in the rice *Koji*, hydrolyze starch to dextrin and glucose; the chief sweet component in *amazake* is glucose [4, 7, 8].

The surface of rice grains contains many proteins; the proteolytic enzymes from *A. oryzae* hydrolyze these proteins to generate large amounts of amino acids and peptides [7].

Therefore, glucoamylase and  $\alpha$ -amylase production by *A. oryzae* is important for the production of amazake.

However, the production of glucoamylase by the *Aspergillus* species is lower than that of  $\alpha$ -amylase.

Many fungal species are capable of producing glucoamylase under different conditions and techniques of fermentation. Among them, *Rhizopus oryzae*, assigned the “generally regarded as safe” (GRAS) status, is commonly used in the industrial production of glucoamylase [9]. Moreover, much work has been undertaken to enhance the activity of the glucoamylase isolated from this fungus, such as optimization of the culture conditions and identification of the genes coding for glucoamylase.

Mertens *et al.* [10] reported that the glucoamylase produced by *Rhizopus* can be divided into two different taxonomic groups, namely, Type-I and Type-II, on the basis of the differences in phenotype (e.g., acid production), rDNA internal transcribed spacer regions, and genomic structure.

Glucoamylase-I can absorb raw starch and has high raw starch-digesting activity, while glucoamylase-II cannot absorb raw starch and has extremely weak raw starch-digesting activity. Glucoamylase-I contains starch-binding sites in the N-terminal region, whereas glucoamylase-II is formed by protease digestion of the N-terminal region of the glucoamylase-I and has reduced ability to digest starch [11, 12].

Solid-state fermentation is mostly undertaken with mixed cultures. Mixed culture fermentations are those in which the inoculum consists of two or more organisms and are widely used in many processes, including the production of antibiotics, enzymes, and several types of fermented food, composting, dairy fermentation, and bioconversion of apple distillery and domestic wastewater sludge [13]. The mixed culture may offer

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internal regulation of growth and product formation, which might be difficult to achieve otherwise. Several reports describe the co-culture of two strains for enhanced enzyme production [14, 15]. For example, the synergistic interaction of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA in a submerged fermentation system allowed more efficient cellulose degradation than in monoculture with either *T. reesei* or *A. niger*, owing to the complementary interactions between the cellulases from *T. reesei* and the glucosidases from *A. niger* that allowed complete cellulose hydrolysis [16]. In addition, Yun-wei and Yan [17] reported that acid protease activity was enhanced by the co-culture of *A. oryzae* AS3042 and *A. niger* SL-09. The inoculation of *A. niger* SL-09 significantly enhanced enzyme production, primarily because of the synergetic interaction between the strains.

Building upon these previous findings, we developed a solid-state co-culture involving two fungi for glucoamylase and  $\alpha$ -amylase production. *A. oryzae* and *R. oryzae* were used for  $\alpha$ -amylase and glucoamylase production, respectively.

## 2 Materials and Methods

### 2.1 Microorganisms

Two fungal strains, namely, *R. oryzae* NBRC 4716 and *A. oryzae* NBRC 5238, were used as producers of glucoamylase and  $\alpha$ -amylase, respectively. The strains were maintained on potato dextrose agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) slants at 30 °C for 1 week.

### 2.2 Rice *Koji* preparation

Material rice (*Yumetukushi*) was obtained from Fukuoka (Japan) and polished to 90%. Steamed rice was inoculated with the spore suspension (final concentration,  $2.5 \times 10^4$  spores/g-substrate; initial water content, 38%) and cultured for 20, 45, 70, 95, or 120 h. This system was incubated at 31 °C at 90% moisture for the initial 20 h. Then, it was moved to 38 °C at 85% moisture.

### 2.3 Enzyme extraction from rice *Koji*

The crude enzyme was extracted from 10 g of rice *Koji* by using 50 mL of sterile water for 3 h at room temperature. It was filtered through the filter paper (No. 2; diameter, 125 mm; Tokyo Roshi Kaisya, Ltd., Tokyo, Japan) to remove the *Koji*; the resulting filtrate was used as the crude enzyme solution.

### 2.4 Co-culture system with pre-culture time

The co-culture system was set up for a controlled culture time. For pre-culture, the spores (final concentration,  $\sim 1.25 \times 10^4$  spores/g-substrate) of *A. oryzae* or *R. oryzae* were added to steamed rice, and cultured for 20 h (initial water content, 38%). The inoculated rice was incubated at 31 °C at 90% moisture for the initial 20 h. The pre-

culture *Koji* was inoculated with the spore suspension (final concentration,  $1.25 \times 10^4$  spores/g-substrate) of other strains and cultured for 20, 40, 65, 90, 115, and 140 h. This system was incubated at 31 °C at 90% moisture for 20 h. It was subsequently moved to 38 °C at 85% moisture.

### 2.5 Co-culture system with controlled spore count

The co-culture system was set up with controlled number of spores. *R. oryzae* and *A. oryzae* (from the slants) were separately added to 10 mL of sterile water. Spore suspensions containing different ratios of *R. oryzae* and *A. oryzae* spores (1:200, 1:50, 1:10, 1:1, 10:1, 50:1, or 200:1) were prepared, and the total spore count was adjusted to  $2.5 \times 10^4$  spores per gram of substrate.

### 2.6 Determination of glucoamylase, $\alpha$ -amylase, and acidic protease activities

Glucoamylase activity was measured using the method described by Morita et al. [18]. The reaction mixture for glucoamylase activity assay was composed of 1 mL of 2% (w/v) soluble starch (Nacalai Tesque Co., Kyoto, Japan) solution in 0.1M acetate buffer (pH 4.5) and 1 mL of the enzyme solution. The reaction progressed at 40 °C for 20 min and it was stopped by heating the test tubes in a boiling water bath for 10 min. The amount of liberated glucose was determined using the glucose oxidase method [19], by using a commercially available glucose test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). One unit of glucoamylase activity was defined as 1 microgram of glucose liberated from soluble starch per minute per milliliter of the enzyme solution.

$\alpha$ -Amylase activity was measured using an  $\alpha$ -amylase assay kit (Kikkoman, Ltd., Chiba, Japan), according to the manufacturer's instructions. One unit of  $\alpha$ -amylase activity was defined as 1 micromole of 2-chloro-4-nitrophenol liberated from 2-chloro-4-nitrophenyl 65-azide-65-deoxy- $\beta$ -maltopentaoside per milliliter of the enzyme solution.

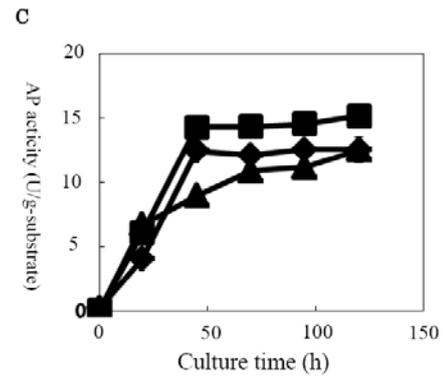
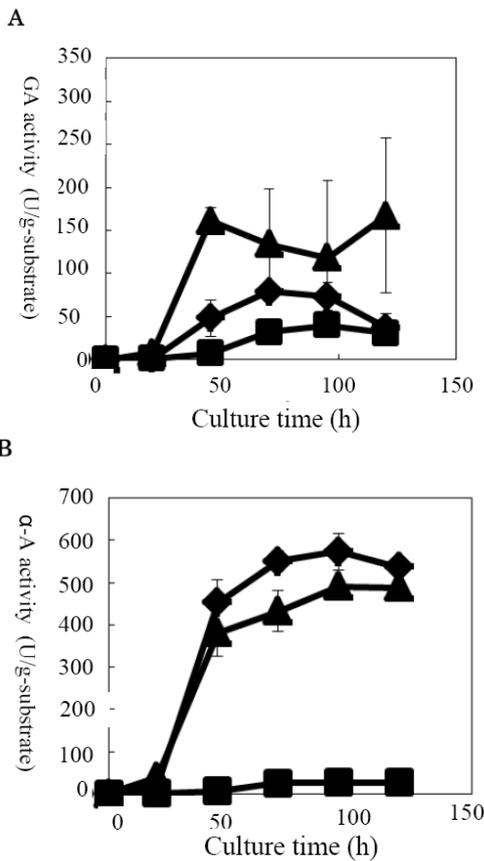
A modification of the Anson hemoglobin method [20] was used for determining acidic protease activity. Briefly, 2.5 mL of hemoglobin solution (0.6% in 0.05M acetate buffer, pH 4.5) and 1 mL of the enzyme solution were mixed and incubated at 40 °C for 30 min. The reaction was stopped by adding 5 mL of 5% trichloroacetic acid. After 60-min incubation at 40 °C, the precipitated unhydrolyzed hemoglobin was removed by filtration, and the absorbance of the solution was measured at 280 nm. One unit of the acidic protease was defined as one microgram of tyrosine per gram of solid-substrate per minute.

## 3 Results and Discussion

### 3.1. Comparison of enzyme activity in the co-culture and pure culture systems

Figure 1 shows the glucoamylase,  $\alpha$ -amylase, and acidic protease activity in *A. oryzae* NBRC 5238 pure culture, *R. oryzae* NBRC 4716 pure culture, and co-culture.

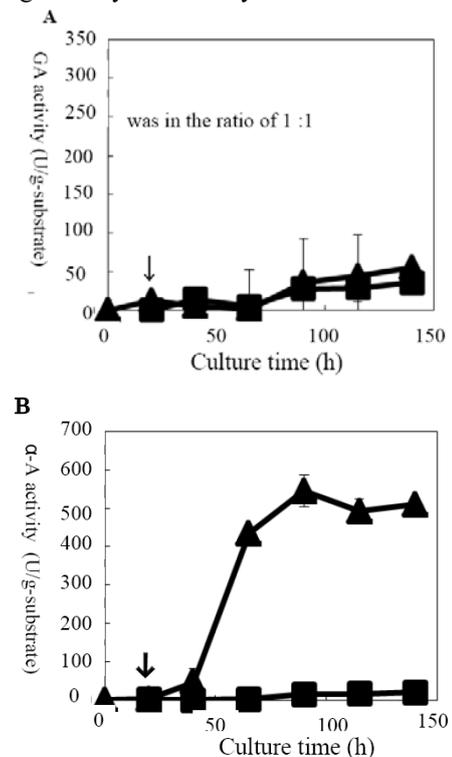
The highest glucoamylase activity was 79 U/g-substrate for 70 h and the highest  $\alpha$ -amylase activity was 573 U/g-substrate for 95 h in co-culture. The glucoamylase and  $\alpha$ -amylase activities were extremely low in *R. oryzae* pure culture. The acidic protease activity in *A. oryzae* NBRC 5238 pure culture, *R. oryzae* NBRC 4716 pure culture, and co-culture was not significantly different (Fig. 1 C). These results indicated that the highest glucoamylase activity in the co-culture system was lower than that observed in *A. oryzae* NBRC 5238 pure culture (167 U/g-substrate, 95 h). This can be attributed to the fact that it might be difficult to grow *Rhizopus* species on steamed polished rice cultures, because the enzyme produced by *Rhizopus* might not act on the heat-denatured proteins contained in rice. In particular, *Rhizopus* strains possessing low acidic carboxypeptidase activity are incapable of breaking down heat-denatured proteins. Therefore, the growth of *Rhizopus* species is delayed because of the lack of nitrogen sources [21, 22]. Moreover, Harayama *et al.*[23] reported that *R. oryzae* IFO 5418 and *Rhizopus oligosporus* NRRL 2710 showed extremely weak  $\alpha$ -amylase and glucoamylase activities in rice and soybean *Koji*. These results were in agreement with those of our study.



**Figure 1.** Enzyme activity in the co-culture system using pure cultures of *A. oryzae* NBRC 5238 and *R. oryzae* NBRC 4716  
 A. Glucoamylase (GA) activity (U/g-substrate),  
 B.  $\alpha$ -Amylase ( $\alpha$ -A) activity (U/g-substrate), and  
 C. Acidic protease (AP) activity (U/g-substrate )  
 ◆: Co-culture involving equal number of *R. oryzae* and *A. oryzae* spores (1:1), ▲: *A. oryzae* pure culture, ■: *R. oryzae* pure culture

### 3.2 Effects of a co-culture system with pre-culture time on glucoamylase and $\alpha$ -amylase production

Previous studies showed that the glucoamylase activity in the co-culture system was lower than that in the *A. oryzae* NBRC 5238 pure culture system. Therefore, we adopted the co-culture system involving pre-culture time to enhance glucoamylase activity.



**Figure 2** Influence of co-culture system with pre-culture time on glucoamylase and  $\alpha$ -amylase production  
 A. Glucoamylase (GA) activity (U/g-substrate) and  
 B.  $\alpha$ -Amylase ( $\alpha$ -A) activity (U/g-substrate )  
 ▲: pre-culture of *A. oryzae*, ■: pre-culture of *R. oryzae*, ↓: co-culture start point

Figure 2 shows the glucoamylase and  $\alpha$ -amylase activities of *A. oryzae* or *R. oryzae* during the pre-culture time. High  $\alpha$ -amylase production (545 U/g-substrate) was observed at 90 h (Fig. 2B), but glucoamylase production was reduced (Fig. 2A) in the co-culture system with pre-culture time. According to some reports, enzyme production by *A. oryzae* depended on the temperature [24, 25]. Acid protease is well produced at temperatures below 35 °C, but temperatures above 38 °C can decrease the production. The expression of *pepA* (acid protease-encoding gene) is induced at low temperatures [24]. Hisada et al. [25] reported that *glaB* (glucoamylase-encoding gene) promoter is induced by exposure to high temperature (for example, growth at 42 °C).

In this experiment, the temperature was maintained at 31 °C for 40 h during the early stages of cultivation. Thus, we assumed that glucoamylase production by *A. oryzae* was inhibited at low temperature. We found that the co-culture system with pre-culture time was not the optimal culture method.

### 3.3 Effects of a co-culture system with controlled spore count on glucoamylase and $\alpha$ -amylase productions

The above-described results showed that the glucoamylase activity in the co-culture system with pre-culture time was extremely low. Additionally,  $\alpha$ -amylase production by the seed culture of *Rhizopus* in the co-culture system was extremely low. Mikai et al. [2] have reported the co-culture of *A. oryzae* and *Rhizopus arrhizus*. Therefore, we tried another co-culture system with controlled spore count.

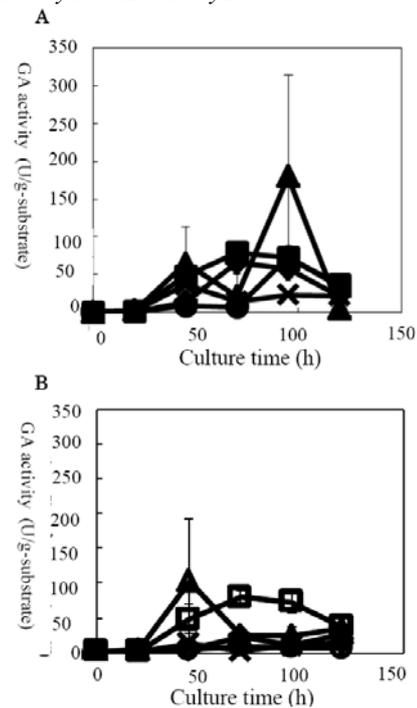
The graphs in Fig. 3 and 4 show glucoamylase and  $\alpha$ -amylase activities in the presence of controlled spore count.

The optimal initial spore count of *A. oryzae* and *R. oryzae* was in the ratio of 200:1 for glucoamylase production (180 U/g-substrate, 95 h, Fig. 3). The highest glucoamylase activity in the co-culture system (spores of *A. oryzae* and *R. oryzae*, 200:1) was higher than that observed in the *A. oryzae* NBRC 5238 pure culture (167 U/g-substrate, 120 h). The optimal initial spore count of *A. oryzae* and *R. oryzae* for  $\alpha$ -amylase production (573 U/g-substrate, 95 h) was in the ratio of 1:1 (Fig. 4).

Glucoamylase and  $\alpha$ -amylase activities improved on equalizing or increasing the initial spore ratio of *A. oryzae* and *R. oryzae* (Fig. 3A, 4A) and it reduced on increasing the initial spore ratio of *R. oryzae* and *A. oryzae* (Fig. 3B, 4B). Taken together, these data showed that the optimal conditions for the production of both glucoamylase and  $\alpha$ -amylase in a co-culture system involve equal spore count of *A. oryzae* and *R. oryzae* (1:1 ratio). Under these conditions, glucoamylase activity (167 U/g-substrate) reached ~47% of that observed in *A. oryzae* pure culture, and  $\alpha$ -amylase activity (491 U/g-substrate) reached ~117% of that observed in *A. oryzae* pure culture.

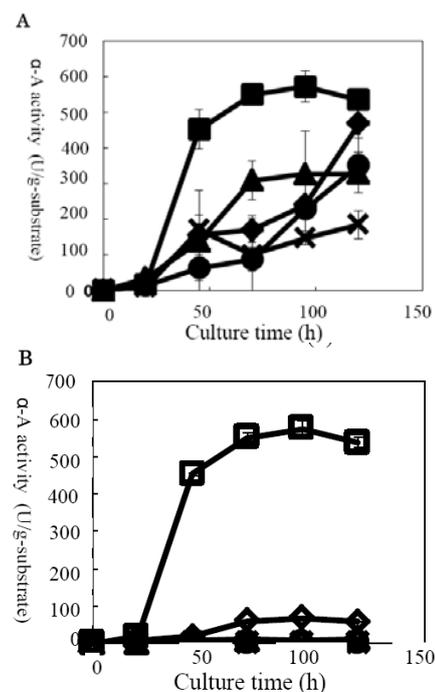
Thus, these results suggest that the production of glucoamylase and  $\alpha$ -amylase can be controlled by adjusting the numbers of *A. oryzae* and *R. oryzae* spores.

Furthermore, these data imply that increased production of glucoamylase and  $\alpha$ -amylase can be obtained by co-culturing *A. oryzae* and *R. oryzae*.



**Figure 3** Influence of co-culture system with a controlled spore count on glucoamylase production

A. *A. oryzae*:*R. oryzae*: 1:1, ■: 10:1, ◆: 50:1, ●: 100:1, ×: 200:1, ▲  
 B. *A. oryzae*:*R. oryzae*: 1:1, □: 1:10, ◇: 1:50, ○: 1:100, ×: 1:200, Δ



**Figure 4** Influence of co-culture system with a controlled spore count on  $\alpha$ -amylase production

A. *A. oryzae*:*R. oryzae*: 1:1, ■: 10:1, ◆: 50:1, ●: 100:1, ×: 200:1, ▲  
 B. *A. oryzae*:*R. oryzae*: 1:1, □: 1:10, ◇: 1:50, ○: 1:100, ×: 1:200, Δ

## 4 Conclusions

In this study, we investigated the production of glucoamylase and  $\alpha$ -amylase by *A. oryzae* and *R. oryzae* in solid-state co-culture.

Glucoamylase activity of co-culture *Koji* was optimized 180 U/g-substrate when the ratio of initial spore counts was 200:1 and  $\alpha$ -amylase of co-culture *Koji* was optimized 573 U/g-substrate when the ratio of initial spore counts was 1:1. These results indicated that amylolytic enzymes can be regulated from adjusting the numbers of *A. oryzae* and *R. oryzae* spores.

However, from a perspective in productivity of glucoamylase and  $\alpha$ -amylase, our results suggested that the co-culture system with simultaneous inoculation with equal number of spores of *A. oryzae* and *R. oryzae* represents optimal conditions for amylase production.

Further investigations are necessary to improve the production of glucoamylase and  $\alpha$ -amylase under optimal conditions (e.g., moisture, temperature) required for the co-culture system.

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