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Simultaneous Increase of Glucoamylase and \( \alpha \)-Amylase Production in Submerged Co-culture of *Aspergillus* and *Rhizopus* Strains

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Our aim was to assess the increase in amylase production in a novel submerged-culture system (involving co-culture of filamentous fungi) and the effect of medium composition on the amylolytic activity. Filamentous fungi, *Aspergillus oryzae* (HI-G strain) and *Rhizopus arrhizus* (P20 strain) were used as \( \alpha \)-amylase and glucoamylase-producing organisms, respectively. The initial concentration of nitrogen and carbon sources, and the ratio of spores to mycelial weight of *A. oryzae* and *R. arrhizus* were found to influence the activity of amylolytic enzymes. Maxim enzymatic activity was achieved when initial maltose and ammonium acetate concentrations were 3% and 1.29% (w/v), respectively. Under these cultivation conditions, the cell concentration was 0.62 g per 100 mL, and glucoamylase and \( \alpha \)-amylase activities were 675 and 4.68 U/mL, respectively. In addition, agitation speeds (50, 125, 200, 275, 350, and 500 rpm) in a 5-L jar fermenter affected the enzyme activity; an increase in glucoamylase production was obtained at 200 rpm after 120 h. These results indicate that high production of glucoamylase and \( \alpha \)-amylase can be attained in a system of submerged co-culture of *A. oryzae* and *R. arrhizus*.

**Keywords:** co-culture, glucoamylase, \( \alpha \)-Amylase, Aspergillus oryzae, Rhizopus arrhizus

1. Introduction

Glucoamylase (GA; \( \alpha \)-1,4-glucan glucohydrolase or amyloglucosidase, EC 3.2.1.3) consecutively hydrolyzes \( \alpha \)-1,4-glycosidic and \( \alpha \)-1,6-glycosidic bonds at the non-reducing ends of starch, glycogen, and similar carbohydrates thereby resulting in production of glucose; this enzyme is widely used in the Japanese fermentation industry, e.g., for the manufacture of sake, soy sauce, and miso [1,2]. The enzyme is produced by filamentous fungi such as *Rhizopus* and *Aspergillus*, which are considered the most important genera for the commercial production of glucose. Filamentous fungi can be used for solid-state and submerged fermentation to produce GA from wheat bran and cassava starch substrates [3-6]. There are 2 major culture methods for filamentous fungi: solid-state culture, which involves inoculation of a raw material with fungal conidia (with pretreatment), and submerged culture, which involves inoculation of a submerged medium (containing a raw material and other sources of nutrients, in water) with fungal conidia or precultured fungal hyphae [7].

For sake brewing, steamed rice, koji (*Aspergillus oryzae*), and water are basic raw materials. Alcohol fermentation requires a 3-stage process for the mash: liquefaction of steamed rice by \( \alpha \)-amylase (\( \alpha \)-A), saccharification of the liquefied starch by GA to sugars, and fermentation using *Saccharomyces cerevisiae* during sake brewing. Therefore, GA and \( \alpha \)-A production by *A. oryzae* are important production stages and affects quality of sake. GA production using *A. oryzae* is usually carried out in a solid-state culture system because solid-state culture is useful for the production of many types of enzymes that are required in large amounts for sake brewing. Biesebeke et al. [8] reported that the glucoamylase A (glaA) gene from *A. oryzae* strain ATCC16868 is expressed in both solid and submerged culture, whereas the glucoamylase B (glaB) gene is not expressed in submerged culture. In addition, the promoter region of glaB mediates the induction of transcription by starch, by high temperature, low enzyme activity in water, and by physical barriers to hyphal extension [9]. In contrast, submerged culture generally decreases enzyme yields [7], especially the GA yield. Nevertheless, submerged culture systems are superior to solid culture, because the components of the
medium can be controlled easily and because submerged culture offers high production efficiency.

Masuda et al. [7] reported that submerged culture, with modifications of raw material- processing conditions using whole barley, resulted in higher GA and acid-stable α-A production by Aspergillus kawachii NBRC 4308. In addition, Imai et al. [10] reported high GA and α-A production that was obtained when the specific glucose consumption rate of A. oryzae RIB 642 was 0.015 g of glucose per gram of cells per hour in fed-batch culture, in a rotary draft tube fermenter. Furthermore, Fujio and Morita [11] reported high GA secretion by Rhizopus sp. A-11 in submerged culture involving liquefied cassava starch, which was produced in a reaction of α-A with gelatinized cassava starch in the presence of heat and metal ions. Previously, we developed a method for increased GA production based on co-culturing of Bacillus amyloliquefaciens NBRC 14141 and Rhizopus cohnnii P5; we found that ammonium acetate promotes the production of GA [12]. In particular, we showed that ammonium acetate in the medium causes the mycelial weight of R. cohnnii to increase and prevents the acidification of culture. Moreover, ammonium acetate inhibits the initial growth of B. amyloliquefaciens and reduces the production of protease. Our results indicate that one organism modulates the growth and production of metabolites by the other organism in the co-culture of fungi and bacteria [12].

A bacterial strain, however, cannot be used for sake brewing, because it can cause contamination as well as deterioration of taste and quality. Building upon these earlier findings, here, we developed a novel system of submerged co-culture of 2 fungi and optimized the medium composition for GA and α-A production. A. oryzae (HI-G strain) and Rhizopus arrhizus (P20 strain) were used as α-A and GA-producing organisms, respectively.

2. Materials and Methods

2.1 Microorganisms

Seven Rhizopus strains and 11 Aspergillus strains were used as producers of glucoamylase and α-amylase, respectively, and the strains were maintained on potato dextrose agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) slants at 30°C for 1 week. Then, these strains were allowed to ferment a liquid medium, as described by Fujio et al. [11] with modification, at 30°C and 200 rpm for 96 h. As a result, R. arrhizus P20 and A. oryzae HI-G were chosen for further analysis because they produced higher levels of GA and α-A, respectively, than did the other strains.

2.2 Culture conditions

The composition of the SLS medium used in the culture, as described by Fujio et al. [11], was as follows: 1% (w/v) rice for sake brewing (Yamada Nishiki, 70% rice-polishing ratio), 0.43% (w/v) CH₃COONH₄ (ammonium acetate), 0.1% (w/v) K₂HPO₄, 0.05% (w/v) MgSO₄, 0.1% (w/v) KCl, 0.001% (w/v) FeSO₄·H₂O, 0.0003% (w/v) ZnSO₄·H₂O, 0.021% (w/v) CaCl₂, and 0.33% (w/v) citric acid monohydrate. The pH level was adjusted to 6.0 with 1 M KOH solution. In addition, various concentrations of the following substances were added to the basal medium: as nitrogen sources, ammonium acetate or sodium nitrate (0%, 0.14%, 0.43%, 1.29%, 2.58%, or 3.87% [w/v]) was used. For carbon sources, rice, for sake brewing (Yamada Nishiki, 70% rice-polishing ratio) or maltose or sucrose (0%, 1%, 2%, 3%, or 4% [w/v]) was used. A 500-ml shaking flask containing 100 ml of the medium was autoclaved at 121°C for 20 min to ensure sterility before culture. The pure culture was inoculated with 2 ml of a spore suspension (final concentration = 2.0 × 10⁸ spores per ml), and incubated at 30°C and 200 rpm for 96 h.

2.3 The co-culture method

The co-culture was carried out with controlled volume of the broth. For preculture, R. arrhizus and A. oryzae from slants were added separately to 10 ml of sterile water, and the SLS medium was inoculated with 2 ml of a spore suspension (final concentration = 2.0 × 10⁸ spores per ml). The preculture medium was incubated at 30°C and 200 rpm for 24 h. For the co-culture, precultures containing mycelia were measured with a sterile 250-ml graduated cylinder and combined in the following combinations: 10 ml, 50 ml, or 90 ml of R. arrhizus preculture with 90 ml, 50 ml, or 10 ml of A. oryzae preculture, respectively. The co-cultures were placed in sterile 500-ml shaking flasks for mixing, and then the liquid mixtures were incubated at 30°C and 200 rpm shaking for 96 h.

Another co-culture system was implemented with a controlled number of spores. For preculture, R. arrhizus and A. oryzae from slants were added separately to 10 ml of sterile water. Spore suspensions containing various ratios of R. arrhizus spores and A. oryzae spores (1 : 200, 1 : 50, 1 : 10, 1 : 1, 10 : 1, 50 : 1, or 200 : 1) were created,
and the total number of the spores was adjusted to 2.0×10^7 spores per mL. Each spore suspension was simultaneously added to the SLS medium and incubated at 30°C and 200 rpm for 96 h. The experiments were performed in triplicate (the error bars in figures represent standard deviation).

2.4 Laboratory-scale fermentation

Batch cultivation was conducted using a 5-L jar fermenter (model MD-N 500 B.E. Marubishi Co., Ltd., Tokyo, Japan). The medium composition was the same as in section 2.2. The carbon source, however, consisted of 3% (w/v) maltose, and the ammonium acetate concentration was changed to 1.29% (w/v). The pH level was adjusted to 6.0 with KOH. The above-mentioned spore-controlled co-culture system was implemented. For preculture, *R. arrhizus* and *A. oryzae* from slants were added to 10 mL of sterile water. The suspensions of *R. arrhizus* spores (1.7×10^6) and *A. oryzae* spores (3.3×10^6) were simultaneously added to the SLS medium. The basal culture conditions were as follows: 3-L working volume, agitation speed 200 rpm, an aeration rate of 1 L/min, temperature 30°C, and incubation for 120 h. The experiments were performed once.

2.5 Preparation of an enzyme solution and measurement of dry mycelial weight

The culture broth was filtered through filter paper (No. 2: Tokyo Roshi Kaisya, Ltd., Tokyo, Japan) to remove the mixed mycelia of *R. arrhizus* and *A. oryzae*; the resulting filtrate was used as a crude enzyme solution. The pH level of the enzyme solution was measured using a pH meter. The total dry mycelial weight (DMW) was measured and served as a growth index, according to the method described by Morita et al. [13]. The mycelia were dried at 105°C for 24 h. Total DMW was defined as mycelial weight per 100 mL of a liquid culture medium.

2.6 Determination of GA and α-A activity

The GA activity was measured using the method described by Morita et al. [13]. The reaction mixture for the GA activity assay was composed of 1 mL of 2% (w/v) soluble starch (Nacalai Tesque Co., Kyoto, Japan) solution in 0.1 M acetate buffer (pH 4.5) and 1 mL of an enzyme solution. The reaction was carried out at 40°C for 20 min and was stopped by heating the test tubes in a boiling water bath for 10 min. The amount of liberated glucose was determined by means of the glucose oxidase method [14], using a commercially available glucose test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). One unit of GA activity was defined as 1 μg of glucose liberated from soluble starch per minute per milliliter of an enzyme solution.

The α-A activity was measured using an α-A assay kit (Kikkoman, Ltd., Chiba, Japan), according to the manufacturer’s instructions. One unit of α-A activity was defined as 1 μmol of 2-chloro-4-nitrophenol liberated from 2-chloro-4-nitrophenyl 6°-azide-6°-deoxy-β-maltopentaoside per milliliter of an enzyme solution.

3. Results and Discussion

3.1 Production of amylases in the co-culture with controlled volume of broth

Enzymatic activity and DMW were measured in mixtures of the culture broth containing *R. arrhizus* and/or *A. oryzae* (Fig. 1). When the quantities of *R. arrhizus* and *A. oryzae* broths were 10 mL and 90 mL, respectively, the activity of α-A increased from the time point 24 h to 120 h, but there was a low rise in GA activity. In contrast, when the quantities of *R. arrhizus* and *A. oryzae* broth were 50 mL each, the activity of α-A slightly increased from the time point 24 h to 120 h, but the activity of GA increased very little.

Nevertheless, when the mixture was adjusted to contain 90 mL and 10 mL of *R. arrhizus* and *A. oryzae* broth, respectively, the GA activity increased significantly from the time point 24 h to 120 h. The α-A activity also increased slightly from the time point 24 h to 120 h. High GA activity in koji is very important for quality of sake. Glucose concentration in moromi can be maintained at high level, and yeast activity was enhanced by koji with high GA activity. There was an effect on the increase of fermentation rate in moromi and a characteristic flavor component of sake. Therefore, GA is the most important enzyme for sake brewing and serves as a quality indicator of koji. Consequently, based on the highest GA activity, we used the co-culture with specific volumes of *R. arrhizus* broth (90 mL) and *A. oryzae* broth (10 mL) for further experiments. Optimum time duration of the co-culture was 96 h and resulted in highest α-A activity.

Next, we compared time course of enzymatic activity in co-culture containing specific volumes of *R. arrhizus* broth (90 mL) and *A. oryzae* broth (10 mL) for 120 h with pure culture of *R. arrhizus* or *A. oryzae* for 96 h because the co-culture was performed the preculture for 24 h. The GA activity in the pure culture of *R. arrhizus* increased quickly during the time window of 24–48 h and...
reached the maximum at 96 h (580 U/mL), whereas GA activity was virtually undetectable in the pure culture of *A. oryzae* throughout the culture period. In contrast, the lowest \( \alpha \)-A activity was observed throughout the period of pure culture of *R. arrhizus*, but the highest \( \alpha \)-A activity was detected in the pure culture of *A. oryzae*. In the pure culture of *A. oryzae*, \( \alpha \)-A activity reached the maximum of 4.2 U/mL at 96 h.

With respect to co-culture of *R. arrhizus* and *A. oryzae*, the level of \( \alpha \)-A activity was different in the pure culture of *A. oryzae*, but GA activity was similar in the pure culture of *R. arrhizus*. We can conclude that the secretion of \( \alpha \)-A into the liquid medium gradually increases, because the maximum activity is 1.5 U/mL at 96 h. Nonetheless, \( \alpha \)-A activity in the co-culture at 72 h was approximately one-third of that in the pure culture of *A. oryzae*, and reached a maximum. On the other hand, we assumed that the secretion of GA into the liquid medium significantly increased during the period 0–24 h, similar to that in the pure culture of *R. arrhizus*, and at 48 h, the GA activity was 412 U/mL. Furthermore, we observed that DMW values were similar (Fig. 1C) under all culture conditions, *R. arrhizus* pure culture, *A. oryzae* pure culture, and co-culture of *R. arrhizus* and *A. oryzae*.

### 3.2 Effects of nitrogen sources on amylase production

Kundu *et al.* [15] evaluated the effect of nitrogen sources on amylase synthesis by *A. oryzae* E. I. 212 and found that ammonium nitrate, sodium nitrate, l-asparagine, peptone, and tryptone increase mycelial growth: 0.05% sodium nitrate (NaNO\(_3\)) is the best nitrogen source. Accordingly, we carried out co-culture (quantities of the broth of *R. arrhizus* and *A. oryzae* were 90 mL...
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and 10 mL, respectively, culture duration 96 h), each experiment involving ammonium acetate in the SLS medium and sodium nitrate as nitrogen sources and optimized the concentration. Based on activity levels, we concluded that the production of both GA and α-A was low at each concentration of sodium nitrate, but a slight increase in both GA and α-A activity was observed in the co-culture compared to pure culture of R. arrhizus or A. oryzae (Fig. 2).

Furthermore, both GA and α-A activity in the co-culture depended on DMW. Enzymatic activity and DMW increased to 0.43% but decreased when the concentration was > 1.29%. At the optimal concentration of sodium nitrate (0.43%, w/v), GA and α-A activities were 201 U/mL and 1.3 U/mL, respectively.

Figure 3A shows that ammonium acetate is a better nitrogen source than sodium nitrate for induction of maximal production of GA. When the concentration of ammonium acetate was 0.43% and 1.29% (w/v), the GA activity for both R. arrhizus pure culture and co-culture was noticeably higher than under other culture conditions; however, when the concentration was greater than 2.58% (w/v), GA activity was inhibited. The increase in α-A activity in the co-culture was dependent on the concentration of ammonium acetate, and the maximum activity was 2.7 U/mL when the concentration of ammonium acetate was 2.58% (w/v). In contrast, at the maximum α-A activity in co-culture, the GA activity was extremely low; therefore, the optimal concentration of ammonium acetate seems to be 1.29% (w/v). The decrease in GA activity at 2.58% (w/v) ammonium acetate can be attributed to the degradation of R. arrhizus mycelia. The difference in the effects of nitrogen sources may be explained by autotrophic assimilation. Ammonia is assimilated into amino acids in a 1-step reaction within the cell [16]. In contrast, the nitrate ion is assimilated into an amino acid after reduction to the ammonium ion [17]. Therefore, it is likely that the difference in assimilation of ammonia and nitrates involves an effect on enzyme production.

Judging by these results, 1.29% (w/v) ammonium acetate is the optimal nitrogen source; thus, we used this concentration and this source for further experiments.

![Figure 2](image_url)
3.3 The co-culture system with a controlled number of spores

Previously, we developed the following novel co-culture method: first, \textit{B. amyloliquefaciens} cell suspension is added to a liquid medium and cultured for 24 h; then, co-culture is initiated by inoculating the culture with \textit{R. coh-nii} spores. As a result, the yields of GA increases, and a maximal GA activity (740 U/mL) is obtained [12]. Consequently, in the present study, we also evaluated similar culture methods. First, spores ($10^7$) of \textit{A. oryzae} were added to the optimal SLS medium and cultured for 12 or 24 h. Then, the seed culture of \textit{A. oryzae} was inoculated with an \textit{R. arrhizus} spore suspension ($10^5$); thus, this step started the co-culture. The GA production in the seed culture of \textit{A. oryzae} was extremely low (data not shown). The reason is likely to be the decrease of enzyme yields (especially GA) in submerged cultures of \textit{A. oryzae} [7].

Moreover, the co-culture system with a controlled volume of the broth is not practical for the fermentation industry because this system is time-consuming and necessitates the use of 3 sterile fermenters. Therefore, we tried another co-culture system with a defined number of spores. This method can be used by the Japanese fermentation industry because this approach involves it would use an existing fermenter and is much easier than the co-culture methods described above in this paper. Therefore, we identified the optimal initial spore number of \textit{R. arrhizus} and \textit{A. oryzae}. The GA activity, \textit{a-A} activity, and DMW at different spore numbers are shown in Fig. 4. The GA activity increased directly with the increasing number of \textit{R. arrhizus} spores. By contrast, the \textit{a-A} activity decreased with the increasing number of \textit{R. arrhizus} spores. These results indicate that the production of GA and \textit{a-A} can be controlled by adjusting the numbers of \textit{R. arrhizus} and \textit{A. oryzae} spores. Taken together, these data show that the optimal conditions for both GA and \textit{a-A} production in co-culture involve equal numbers of \textit{R. arrhizus} and \textit{A. oryzae} spores ($10^7$, 1:1 ratio). Under these culture conditions, the GA activity
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Fig. 4 The influence of the number of spores on (A) glucoamylase (GA) activity (U/mL), (B) α-Amylase (α-A) activity (U/mL), and (C) dry mycelial weight (DMW; grames per 100 mL) in the co-culture of *Rhizopus arrhizus* and *Aspergillus oryzae* during 96 h. The number of the total spores was adjusted to 2.0×10^7.

![Graphs showing enzyme activity and dry mycelial weight](image)

(420 U/mL) reached ~ 70% of that of pure culture of *R. arrhizus*, and the α-A activity (3.6 U/mL) reached ~ 77% of that of *R. arrhizus* pure culture. These data imply that high production of GA and α-A can be obtained simultaneously by co-culturing *A. oryzae* and *R. arrhizus*. In addition, no significant differences in DMW were observed under the different spore conditions, but the optimal conditions contributed slightly to mycelial growth compared to the other conditions (Fig. 4C).

Thus, our results suggest that the co-culture system with simultaneous inoculation with equal spore numbers of *R. arrhizus* and *A. oryzae* represents the best conditions for amylase production.

### 3.4 Effects of carbon sources on amylase production

Extensive research has been conducted on the effect of carbon sources on amylase production by filamentous fungi. Kundu *et al.* [15] showed that glucose, maltose, and starch contribute to good growth and amylase production by *A. oryzae* E. I. 212 in submerged culture; they found that 2% (w/v) starch is the best inducer. Moreover, Pavezzi *et al.* [1] reported that in submerged culture, GA production by *S. cerevisiae* strain C468 (containing wild-type GA cDNA from *Aspergillus awamori*) is induced by gelatinized starch but not raw starch. Thus, we carried out the co-culture (quantities of the broth of *R. arrhizus* and *A. oryzae* were 90 mL and 10 mL, respectively, culture time 96 h) using rice for *sake* brewing and using maltose and sucrose as carbon sources; we examined the effect of different substrates on amylase production (Fig. 5). Compared to other substrates, when maltose was added to the modified SLS medium, GA activity was higher. Under these conditions, the increase in GA activity was dependent on the maltose concentration, and the maximal activity at 3% (w/v) was 675 U/mL. The activity of GA when using sucrose also depended on the increasing concentration, but sucrose did not significantly contribute to GA production because GA activity was lower with sucrose than with other carbon sources. On the other hand, GA activity was higher with 1% (w/v) rice for *sake* brewing than with 1% (w/v) sucrose but...
gradually decreased, being inversely dependent on the rice concentration. In addition, under the culture conditions with maltose or rice, α-A activity was higher than with the other substrates and was not dependent on their concentration. These results show that high production of GA and α-A can be achieved when maltose serves as a carbon source.

Gomi et al. [18] reported that the production of GA and α-A by filamentous fungi, such as A. oryzae, A. niger, A. nidulans, and A. kawachii is responsive to maltose, and synthesis of these amylases is regulated at the transcriptional level by the AmyR gene. Therefore, we assumed that amylase production in co-culture of R. arrhizus and A. oryzae is also affected by AmyR, because maltose functions as an inducer of AmyR. Nonetheless, the influence of maltose on mycelial growth was similar to that of sucrose; DMW was highest with rice for sake brewing. This result suggests that rice degradation products (which are composed of ingredients such as starch, proteins, lipids, and cellulose) that are generated by R. arrhizus and A. oryzae enzymes are likely to promote mycelial growth.

Some enzymes produced in solid-state culture have been shown to be trapped in the cell wall in submerged culture [19]. In addition, Oda et al. [19] demonstrated that the majority of proteins trapped in the cell wall of A. oryzae strain RIB40 in submerged culture are α-A and β-glucosidase, and the secretion of these enzymes is controlled posttranslationally via trapping in the cell wall under submerged conditions. As stated by Kato, Shimoi, and Ito [20], in submerged culture, more than 10–20% of α-A, 50% of GA, and 80% of α-glucosidase are trapped in the cell wall of A. oryzae RIB 40 or A. kawachii IFO 4308; however, in solid culture, more than 80% of GA and α-glucosidase is released into the solid medium, and most of the α-A that is produced is secreted. On the basis of these previous findings, we determined the amount of trapped GA and α-A in the cell wall of R. arrhizus and A. oryzae in submerged co-culture. Activity of neither GA nor α-A was detected among the trapped enzymes; this result contradicts the above reports.

Popolo et al. [21] reported that the cell wall is not a static shield in yeast, but its highly dynamic structure
can change according to the physiological needs of the cell. Therefore, *A. oryzae* and *R. arrhizus* may also alter the structure of the cell wall under these culture conditions, and almost all GA and α-A may be secreted into the liquid medium.

### 3.5 Production of amylase in the 5-L jar fermenter

Figure 6 shows time course of GA activity, α-A activity, glucose concentration, and pH for such cultures. The medium composition was the same as that in section 2.2, except the carbon source consisted of 3% (w/v) maltose, and the ammonium acetate concentration was changed to 1.29% (w/v). The suspensions of *R. arrhizus* spores (1.7 × 10^7) and *A. oryzae* spores (3.3 × 10^6) were simultaneously added to the medium. The optimal initial spore number of *R. arrhizus* and *A. oryzae* was 1:1 ratio in flask scale and GA activity decreased unless spore number of *R. arrhizus* was the same or more than spore number of *A. oryzae*. GA activity was no significant difference when the initial spore number of *R. arrhizus* and *A. oryzae* was 1:1 ratio and 5:1 ratio at 200 rpm in the jar fermenter. When agitation speed was 350 rpm, GA activity of the 5:1 ratio was about two times higher than that of 1:1 ratio (data not shown). Thereafter, we used the co-culture with the initial spore number of *R. arrhizus* and *A. oryzae* at the rate of 5:1 in the jar fermenter. The GA and α-A activities at high agitation speeds (350 or 500 rpm) stopped increasing after 72 h of culture but continued to increase for a longer period at lower agitation speeds (50, 125, 200, and 275 rpm). Moreover, the GA and α-A activities in the co-culture were generally higher than in pure culture of *R. arrhizus* or *A. oryzae* and were almost equal to the activities of amylase achieved in the 500-mL shaking flask. The maximal GA activity at each agitation speed was obtained at 120 h. The GA activity decreased depending on the agitation speed when the latter was > 200 rpm, and the highest activity was obtained at 200 rpm. Nonetheless, the GA activity at 50 or 125 rpm was low, in spite of the lower agi-
tation speed. On the other hand, at 350 or 500 rpm, the low GA activities could be caused by the destruction of hyphae by shear stress. Kasai et al. [22] reported that low filter paper activity of Trichoderma reesei in a fermenter at 500 rpm is caused by the destruction of the hyphae by shear stress.

Furthermore, Farid et al. [23] showed that GA production in submerged culture of A. awamori is maximal when the agitation speed is 200 rpm; at 300 rpm, the production is markedly decreased. In contrast, the α-A activity increased in their study proportionately with the agitation speed, and the highest activity was obtained at 350 rpm; however, at 500 rpm, the activity decreased similarly to that of GA. Nevertheless, between 200 and 500 rpm, a marked difference in α-A activity was not observed [23]; furthermore, the production was highest under these culture conditions. These results suggest that GA activity is significantly inhibited in comparison with α-A in co-culture of R. arrhizus and A. oryzae likely because R. arrhizus is more easily affected by shear stress than A. oryzae is. In addition, Figs. 6A–B show that the higher enzymatic activities were obtained at 120 h when pH increased significantly, judging by pH measurements over the time course as shown in Fig. 6D.

According to some reports [7, 19], high concentrations of glucose in culture broth inhibit amylase synthesis by filamentous fungi. In co-culture, however, the glucose concentration in the broth is not significantly linked to either GA or α-A activity (Fig. 6C).

**4. Conclusions**

We previously reported [12] the development of a novel system for GA production in submerged co-culture of B. amyloliquefaciens and R. cohni and found that ammonium acetate promoted the production of GA, helping to achieve a high GA activity (740 U/mL). According to the present study, ammonium acetate and maltose facilitate amylase production as nitrogen and carbon sources, respectively, in co-culture of R. arrhizus and A. oryzae. The maximal enzymatic activity is obtained when initial maltose and ammonium acetate concentrations are 3% (w/v) and 1.29% (w/v), respectively. Under these cultivation conditions, GA and α-A activities are 675 U/mL and 4.68 U/mL, respectively. In this study, we obtained high GA and α-A activity in submerged co-culture of the fungi R. arrhizus and A. oryzae. Consequently, our findings are applicable to GA production in liquid culture and for brewing of sake in submerged culture, because this culture method is simple and seems to be more efficient than the conventional solid culture.

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Aspergillus 属菌と Rhizopus 属菌の混合液体培養における
グルコアミラーゼおよび α-アミラーゼ生産の増強

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従来の清酒醸造では、種々の加水分解酵素を多量に
分泌する Aspergillus oryzae（黄麹菌）を蒸米上に培養
させて造る。固体麹を原料として使用している。一方で,
黄麹菌の培養制御や品質管理の容易さなどの観点から,
原料および栄養源を含有した液体培地で黄麹菌を培養
した、液体麹による清酒醸造が望まれる。しかし、液
体麹において黄麹菌の糖化酵素（グルコアミラーゼ）
分泌が固体麹に比べて著しく低下するため、液体麹を
清酒生産に用いるためにはグルコアミラーゼ生産の増
強が課題として挙げられる。

そこで本研究では、液体麹において高いグルコアミ
ラーゼ生産性を有する Rhizopus 属菌と Aspergillus oryzae
の混合培養系によりグルコアミラーゼ生産の増強を目的
とし、清酒醸造への実用化に向けて検討を行った。

Rhizopus arrhizus P20 と Aspergillus oryzae (HI-G) の
至適酵素生産条件確立のために、初発添加細胞数比およ
び培地成分の変化におけるグルコアミラーゼ活性
（GA）および α-アミラーゼ活性（α-A）を測定した。
その結果、R. arrhizus と A. oryzae の初発添加細胞数比
を1:1に制御し、培地中の窒素源および炭素源として
酵酸アンモニウム 1.29%, マルトース 3%をそれぞれ
同時に添加することで、プラスコスケールにおいて
GA: 675 U/ml, α-A: 4.7 U/ml の高活性を得ることを
見出した。そこで、至適酵素生産条件において5 L 容
ジャーファーメンター（実容量 3 L）内で共培養を実施
したところ、プラスコスケールと同等の高い GA, α-A
が認められた。さらに、ジャーファーメンターのかく
はん速度がグルコアミラーゼおよび α-アミラーゼ生産
に大きく関係し、かくはん速度 200 rpm においてとく
にグルコアミラーゼ生産に適することを見出した。