Bacillus amyloliquefaciens と Rhizopus cohnii の混合培養系を用いたグルコアミラーゼ生産

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Glucoamylase Production in Submerged Co-Culture System of
*Bacillus amyloliquefaciens* and *Rhizopus cohnii*

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We developed novel method for producing glucoamylase (GA) from gelatinized rice flour by co-culturing *Bacillus amyloliquefaciens* NBRC 14141 and *Rhizopus cohnii* P5. In liquid culture, the acidification of the growth medium due to the growth of *R. cohnii* prevented the growth of *B. amyloliquefaciens*; however, the *B. amyloliquefaciens* protease lysed *R. cohnii* cells, which decreased the production of GA. This antagonism was repressed by high concentrations of ammonium acetate in the growth medium. However, since low pH or high ammonium acetate concentrations inhibited the initial growth of *B. amyloliquefaciens*, it needed to be precultured without ammonium acetate. However, preculturing *B. amyloliquefaciens* for 48 h led to overproduction of protease, which inhibited the growth of *R. cohnii*. As a result, the maximum GA activity (740 U/ml) was obtained when *B. amyloliquefaciens* was precultured for 24 h followed by inoculation with *R. cohnii* in the presence of 3.84% (w/v) of ammonium acetate. These results indicated that GA can be produced at high levels from gelatinized rice flour by using a submerged co-culture system.

**Key words:** glucoamylase production, co-culture, ammonium acetate

### 1. Introduction

Glucoamylase (GA, EC 3.2.1.3) is an exo-acting amylolytic enzyme that hydrolyzes \(\alpha-1,4\) and \(\alpha-1,6\) linkages of starch, glycogen, and similar carbohydrates. GA is important for the commercial production of glucose \cite{1}. Filamentous fungi, such as *Rhizopus* and *Aspergillus* strains, can be used in solid-state fermentation to produce large amounts of GA by using wheat or rice bran as substrates \cite{2-5}. This method of producing GA is superior to the production of GA from *Aspergillus niger* in submerged cultures \cite{6}. However, submerged cultures are advantageous because they allow the components of the medium to be controlled easily and the resulting enzyme product does not need to be extracted from the substrate. In most cases, the production of GA from submerged cultures requires supplemental nutrients. For instance, Fujio and Morita \cite{7} reported that high levels of GA (650 U/ml) could be produced from *Rhizopus* sp. A-11 in a liquid medium by using liquefied cassava starch, which was produced by reacting \(\alpha\)-amylase with gelatinized cassava starch in the presence of heat and metal ions. Specifically, the amount of GA (720 U/ml) that was produced by this liquid culture was about 4.4 times more than produced by solid culture with wheat bran \cite{8}. Similarly Sudo *et al.* \cite{9} reported that *A. kawachii* IFO 4308 produced large amounts of acid-stable \(\alpha\)-amylase when dextrin or maltose was used as the sole carbon source in liquid culture. In addition, Barton *et al.* \cite{10} concluded that supplementation of maltose or isomaltose induced the production of GA from *A. niger*. Although these low molecular weight carbon sources affect the production of GA, they are often too expensive for large-scale cultures because they require enzymatic pretreatment. As a result, carbon sources that do not need enzymatic pretreatment are highly desirable. In this study, we attempted to produce GA from gelatinized rice flour instead of liquefied starch by co-culturing *Bacillus* and *Rhizopus* strains. Since \(\alpha\)-amylase that is produced from *Bacillus* strains in submerged cultures has high titer and enzymatic activity \cite{11,12}, it is commonly used in many industrial applications, including brewing, production of foods, paper, and textiles, and starch liquefaction \cite{13,14}. Therefore, we hypothesized that *Bacillus* strains would effectively convert gelatinized rice starch...
to low molecular weight sugars. There have been several studies about the production of GA by co-culturing. For example, Stoilova et al. [15] reported that co-culture of Thermosascus aurantiacus and A. niger produced twice as much GA as a pure culture of A. niger from rice straw. Similarly, Wang et al. [16] showed that co-culturing increased the yield of GA by using 2 Asperillus strains in solid wheat-based medium. However, there have not been any reports about the production of GA by using a combination of fungi and bacteria in a submerged culture, despite the abundant research about the interaction between bacteria and filamentous fungi. For example, Munimbazi and Bullerman [17] showed that B. pumilus inhibits the production of aflatoxin and limits the growth of A. parasiticus. Several other reports have demonstrated that protein, peptides, and extracellular enzyme from Bacillus strains inhibit the growth or lyse fungal cells [18,19]. Moreover, enzymes from filamentous fungi inhibit the growth of Bacillus strains [20]. These studies show that, in mixed culture systems with fungi and bacteria, each organism modulates the growth and production of metabolites of the other. Therefore, in this study, we determined how ammonium acetate influences the interaction of a bacterial strain, B. amyloliquefaciens NBRC 14141, and a fungal strain, R. cohnii P5, to optimize the production of GA in a liquid culture with gelatinized rice flour as the fermentation substrate.

2. Materials and Methods

2.1 Microorganisms

Eight Rhizopus strains and 10 Aspergillus strains from our laboratory stock were used to produce GA in submerged cultures that were incubated at 30°C and 200 rpm for 3 d. In this case, we used liquefied rice flour, which was prepared by an α-amylase preparation from B. subtilis (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the method of Fujio et al. [7]. As a result, glucoamylase productivity of Rhizopus cohnii P5 was the highest than other strains.

Moreover, seven Bacillus strains obtained from National Institute of Technology and Evaluation (Chiba, Japan) were incubated in submerged cultures with gelatinized rice flour at 30°C and 200 rpm for 3 d according to the method of Fujio et al. [7] As a results, Bacillus amyloliquefaciens NBRC 14141 was the best α-amylase producer by using this liquid medium.

2.2 Medium

The liquid medium of Fujio et al. [7] with modifications was used for the submerged culture. The basal medium contained 1 g of raw rice flour (Koda shoten, Ltd., Ibaraki, Japan), 0.1 g of KHPO₄, 0.1 g of KCl, 0.05 g of MgSO₄, 0.001 g of FeSO₄ · H₂O, 0.0003 g of ZnSO₄ · H₂O, 0.021 g of CaCl₂ and 0.33 g of citric acid monohydrate that were dissolved in 100 ml of deionized water. The pH of this medium was adjusted to 6.0 with KOH, and then the medium was autoclaved at 121°C for 20 min in shaking flask. As a result, the raw rice flour was gelatinized in this liquid medium. In addition, we added various concentrations of ammonium acetate (0, 0.43%, 1.28%, 2.56%, 3.86% and 5.12% (w/v)) to this basal medium. Experiments were repeated 3 times in the maximum, and calculated as the average.

2.3 Pure culture

Mycelia and spores of R. cohnii were precultured on a potato dextrose agar slant (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 30°C for 7 d. The liquid medium was inoculated with a suspension of mycelia and spores (2 × 10⁵ spores), and then incubated at 30°C and 200 rpm for 120 h. B. amyloliquefaciens was grown on a nutrient agar slant (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and stored 4°C. The bacteria from this slant was inoculated into nutrient broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and then cultured at 37°C and 70 rpm for 1 d. Afterwards, the culture was centrifuged at 4200 ×g for 20 min, and then the cell pellet was resuspended in sterile saline. The concentration of the cell suspension was adjusted to about 10⁶ colony forming units (CFU)/ml and a 1 ml aliquot was inoculated into the liquid medium, and then incubated at 30°C and 200 rpm.

2.4 Co-culture

First, 1 ml of B. amyloliquefaciens cell suspension (1 × 10⁸ CFU) was added to the basal medium and cultured for 0, 24, or 48 h. Then, the co-culture was started by inoculating this culture with R. cohnii spores (2 × 10⁷ spores) and adding aqueous ammonium acetate. The co-culture was incubated at 30°C and 200 rpm for 120 h.

2.5 Preparation of the enzyme solution

The culture broth was filtered with filter paper (No. 2, Toyo Roshi Kaisya, Ltd., Tokyo, Japan) to remove the R. cohnii mycelia; the resulting filtrate was used as a crude enzyme solution. The pH of culture broth was measured using a pH meter (Mettler-Toledo Int. Inc., Greifensee, Switzerland) to investigate the effect for the growth of two strains.
2.6 Determination of dry mycelial weight (DMW)

The DMW was determined according to the method of Morita et al. [21]. The resulting mycelia of *R. cohnii* was dried in a 105°C oven for 24 h. The DMW was defined as the mycelial weight per 100 ml of culture broth.

2.7 Determination of bacterial cell concentration

The concentration of *B. amyloliquefaciens* cells in liquid medium was quantified by counting the number of colonies on the nutrient agar plate after incubation at 30°C for 24 h. Viable cells were defined as CFUs per ml of culture broth.

2.8 Determination of glucoamylase activity

The GA activity was measured by using the method of Morita et al. [21]. The reaction mixture for GA assay was composed of 1 ml of 2% (w/v) soluble starch (Nacalai Tesque Co., Kyoto, Japan) solution in a 0.1 M acetate buffer (pH 4.5), and 1 ml of enzyme solution. The reaction was carried out at 40°C for 20 min and was stopped by heating the test tubes in boiling water bath for 10 ml. The amount of liberated glucose was determined by the GOD method [22] by 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 that was liberated from soluble starch per min per ml of enzyme solution.

2.9 Determination of protease activity

The protease activity was determined by using the method of Kunitz [23]. The reaction mixture for GA assay was composed of 1 ml of 2% (w/v) casein (Wako Pure Chemical Industries, Ltd., Osaka, Japan) suspension in a 0.1 M phosphate buffer (pH 6.8) and 1 ml of enzyme solution. The reaction was stopped by adding 10 ml of 5% (w/v) trichloroacetic acid. After filtering the unhydrolysed casein, absorbance of the solution was measured at 280 nm. One unit of protease activity was defined as 1 μg of tyrosine that was released from casein per min per ml of enzyme solution.

3. Results and Discussion

3.1 Production of glucoamylase by a pure culture of *R. cohnii* P5

As mentioned by Fujio, Morita and Ray [7,8,24], ammonium acetate promoted GA production of filamentous fungi in submerged culture. Moreover, since Morita revealed the amount of nitrogen sources had important role for GA productivity of *Rhizopus* sp. MKU 40 [25], we optimized GA production of *R. cohnii* P5 by altering the concentration of ammonium acetate. The GA activity and DMW of a pure culture of *R. cohnii* P5 are shown in Fig. 1 and Fig. 2, respectively. Ammonium acetate markedly increased the production of GA. Specifically, the highest yield of GA was 566 U/ml when *R. cohnii* was cultured in liquid medium with 3.86% (w/v) ammonium acetate for 96 h. When the medium only contained 0.43% (w/v) ammonium acetate, the production of GA was very low while the DMW was higher than that of several reports showed [7,21]. These results showed that ammonium

![Fig. 1 Influence of ammonium acetate concentration for GA activity in a pure culture of *R. cohnii* P5. The tested ammonium acetate concentrations were ○; 0 (Basal medium), ●; 0.43, ▲; 1.28, ■; 2.56, ×; 3.84, and +; 5.12% (w/v).](image1)

![Fig. 2 Influence of ammonium acetate concentration for growths in a pure culture of *R. cohnii* P5. The tested ammonium acetate concentrations were ○; 0 (Basal medium), ●; 0.43, ▲; 1.28, ■; 2.56, ×; 3.84, and +; 5.12% (w/v).](image2)
acetate accelerates the growth of R. cohnii; however, it only enhanced the production of GA at high concentrations. However, excess ammonium acetate inhibited the growth of R. cohnii.

3.2 Production of glucoamylase by co-cultures

The GA activity of the co-cultures of R. cohnii and B. amyloliquescens are shown in Fig. 3. Unlike the pure culture of R. cohnii, the GA produced by this method did not accumulate when the basal medium was used. In addition, ammonium acetate did not affect for the production of GA in the co-cultures compared with the pure culture of R. cohnii.

![Fig. 3 Influence of ammonium acetate concentration for GA activity in co-culturing Bacillus amyloliquescens NBRC 14141 and Rhizopus cohnii P5. The tested ammonium acetate concentrations were ○; 0 (Basal medium), ◆; 0.43, ▲; 1.28, ■; 2.56, ×; 3.84, and +; 5.12% (w/v).](image)

![Fig. 4 Influence of ammonium acetate concentration for growths of Bacillus amyloliquescens NBRC 14141 and Rhizopus cohnii P5 in co-culturing them. The tested ammonium acetate concentrations were (A); 0 (Basal medium), (B); 0.43, (C); 1.28, (D); 2.56, (E); 3.86, and (F); 5.12% (w/v). Open symbol; Viable cells (CFU/ml) of Bacillus amyloliquescens NBRC 14141, Closed symbol; DMW (g/100 ml) of Rhizopus cohnii P5.](image)
As a result, we investigated the growth patterns of 2 strains in liquid medium (Fig. 4). The DMW of R. cohnii increased in both the co-cultures and the pure culture. In contrast, the growth of B. amyloliquefaciens was completely inhibited in the basal medium supplemented with 5.12% (w/v) ammonium acetate. Moreover, the growth inhibition of B. amyloliquefaciens continued for 48 h in medium that contained 0.43% (w/v) ammonium acetate and for 24 h in medium that contained 1.28%, 2.56%, and 3.86% (w/v) ammonium acetate. To elucidate the underlying mechanism of these phenomena, we measured the pH of the co-culture broth. In the pure culture of B. amyloliquefaciens in the basal medium, the pH gradually increased until it reached 7.8, whereas the pH of the co-culture broth rapidly decreased after 24 h (Fig. 5). Initially, the pH of the precultured co-culture with 0.43% (w/v) ammonium acetate decreased, but it increased after 24 h. Interestingly, the growth rate of B. amyloliquefaciens also increased as pH began to rise. Combet-blanc et al. [26] demonstrated that B. thermoamylovorans grows from pH 5.4 to 8.5 and was optimal at 7.0. Likewise, we found that B. amyloliquefaciens cannot grow in low pH. This acidification may be caused by the production of organic acids by R. cohnii [27]. However, acidification did not occur in the co-culture broth that contained more than 1.28% (w/v) ammonium acetate. Monot et al. [28] noticed a similar relationship between ammonium acetate concentration and pH in their optimization of the production of acetone and butanol from Clostridium acetobutylicum. Specifically, they showed that the production of acetic and butyric acids increased while the pH of the culture broth increased. This result suggested that ammonium acetate in the medium acts as not only a nitrogen source for R. cohnii but also a buffer.

However, the growth inhibition of B. amyloliquefaciens during the early growth phase was still observed in the presence of high concentrations of ammonium acetate (> 1.28% (w/v)), even though the pH did not decrease. This finding suggested that high concentrations of ammonium acetate may inhibit the growth of B. amyloliquefaciens. To test this hypothesis, we investigated the effect of ammonium acetate on the growth of B. amyloliquefaciens (Fig. 6). In the basal medium, the cell concentration of B. amyloliquefaciens rapidly increased during the early growth phase. However, 0.43% (w/v) ammonium acetate inhibited the initial growth of B. amyloliquefaciens. Moreover, high concentrations of ammonium acetate (1.28–5.12% (w/v)) halted the growth of B. amyloliquefaciens without decreasing the pH (data not shown). Some studies have investigated the effect of ammonium salts on bacterial growth. For example, Zhu et al. [29] similarly showed that ammonium phosphate inhibits the

![Fig. 6](image_url) Influence of ammonium acetate concentration for the growth of Bacillus amyloliquefaciens NBRC 14141 in pure culture. The tested ammonium acetate concentrations were •; 0 (Basal medium), ◆; 0.43, ▲; 1.28, ■; 2.56, ×; 3.84, and +; 5.12% (w/v).

![Fig. 7](image_url) Influence of ammonium acetate concentration added at 24 h for GA activity. Seed culture of Bacillus amyloliquefaciens NBRC 14141 was inoculated to the Rhizopus cohnii P5 spore culture at 24 h. The tested ammonium acetate concentrations were •; 0 (Basal medium), ◆; 0.43, ▲; 1.28, ■; 2.56, ×; 3.84, and +; 5.12% (w/v). †; The time of inoculation of Rhizopus cohnii P5 and of addition of ammonium acetate.
growth of *B. subtilis* and its production of tetrathydropyrrole.

These results showed that the effect of acidification or ammonium acetate on the production of GA by the co-cultures is similar to that by pure cultures of *R. cohnii*.

### 3.3 Influence of mixing times on the production of glucoamylase by co-cultures

Although high concentrations of ammonium acetate increased the GA yield and DMW of a pure culture of *R. cohnii*, it inhibited the growth of *B. amyloliquefaciens* in the co-culture. Next, we investigated the effect of preculturing *B. amyloliquefaciens* for 24 or 48 h before co-culturing them with *R. cohnii* and ammonium acetate in liquid medium to increase the cell concentration of *B. amyloliquefaciens* during the early growth phase. The GA activities from 24 h preculturing co-culture are shown in Fig. 7. In the basal medium, no GA activity was detected. Although 0.43% (w/v) ammonium acetate induced high levels of GA compared with the pure culture of *R. cohnii* (Fig. 1). However, in the presence of 1.28% (w/v) ammonium acetate, the GA activity was less than that of the pure culture of *R. cohnii*. Therefore, low concentrations of ammonium

![Fig. 8 Influence of ammonium acetate concentration added at 24 h for growths of Bacillus amyloliquefaciens NBRC 14141 and Rhizopus cohnii P5. Seed culture of Bacillus amyloliquefaciens NBRC 14141 was inoculated to the Rhizopus cohnii P5 spore culture at 24 h. The tested ammonium acetate concentrations were (A); 0 (Basal medium), (B); 0.43, (C); 1.28, (D); 2.56, (E); 3.86, and (F); 5.12% (w/v). Open symbol; Viable cells (CFU/ml) of Bacillus amyloliquefaciens NBRC 14141; Closed symbol; DMW (g/100 ml) of Rhizopus cohnii P5. ; The time of inoculation of Rhizopus cohnii P5 and of addition of ammonium acetate.](image)
acetate did not always enhance the production of GA in the 24 h precultured co-culture. In contrast, 2.56% or 3.84% (w/v) ammonium acetate promoted the production of GA. In particular, the maximum GA activity (740 U/ml) was obtained from a 120 h co-culture with 3.84% (w/v) ammonium acetate.

On the basis of these results, we investigated the growth patterns of both strains in the 24 h precultured co-culture (Fig. 8). In the basal medium, the growth of \textit{B. amyloliquefaciens} was rapid and unrestricted. However after 24 h, the growth rate of \textit{B. amyloliquefaciens} was inversely related to the concentration of ammonium acetate. When we investigated whether these effects also occurred in a pure culture of \textit{B. amyloliquefaciens} with various concentrations of ammonium acetate after 24 h, the addition of 0.43% or 1.28% (w/v) ammonium acetate inhibited the growth of \textit{B. amyloliquefaciens} during early growth phase, but it grew slowly (data not shown). In contrast, higher concentrations (2.56% or 3.86% (w/v)) of ammonium acetate inhibited the growth of these cells. Moreover the highest tested concentration (5.12% (w/v)) ammonium acetate immediately decreased the number of cells (data not shown). These results suggested that the growth of \textit{B. amyloliquefaciens} depends on the concentration of exogenous ammonium acetate.

However this effect was short-lived. In the 24 h precultured co-cultures supplemented with ammonium acetate that exhibited growth inhibition, the cell concentration recovered after 48 h in all cases except for 5.12% (w/v) ammonium acetate. These types of growth patterns, which were not observed in the pure cultures, may be due to the metabolic degradation of ammonium acetate by \textit{R. cohni}. In contrast, the growth of \textit{R. cohni} was completely inhibited in the 24 h precultured co-culture that only contained the basal medium (Fig. 8). Moreover, at low concentrations of ammonium acetate (0.43% and 1.28% (w/v)), the DMW of \textit{R. cohni} began to decrease after 48 h. However, at higher concentrations (2.56% or 3.86% (w/v)) ammonium acetate, this reduction in DMW was delayed until 96 h. In addition, \textit{R. cohni} continued to grow steadily for 120 h after its inoculation in the presence of 5.12% (w/v) ammonium acetate. Together, these results suggested that ammonium delays the decrease in DMW in a concentration–dependent manner.

More importantly, these results suggested that these growth patterns are due to \textit{B. amyloliquefaciens} since they were not observed in the pure culture of \textit{R. cohni} (Fig. 2). Therefore, it is possible that a product of \textit{B. amyloliquefaciens} decreases the DMW. Tominaga \textit{et al.} [30] showed that some enzymes from \textit{Bacillus} strains affect the growth of \textit{Rhizopus} strains. Specifically, they discovered that the cell wall of \textit{R. delemar} can be lysed by protease and chitosanase, which are produced by \textit{Bacillus} R-4. However, Liu \textit{et al.} [31] showed that ammonium salts reduce bacterial protease production in a concentration–dependent manner. Thus, it is possible that proteases from \textit{B. amyloliquefaciens} can lyse the cell wall of \textit{R. cohni}, but ammonium acetate can suppress their production. Moreover ammonium acetate may have had a combinatorial effect on the co-cultures by promoting the growth of \textit{R. cohni} and inhibiting the production of proteases by \textit{B. amyloliquefaciens}.

Fig. 9 Influence of ammonium acetate added at 24 h for protease activity. Seed culture of \textit{Bacillus amyloliquefaciens} NBRC 14141 was inoculated to the \textit{Rhizopus cohni} P5 spore culture at 24 h. The tested ammonium acetate concentrations were ●; 0 (Basal medium), ◆; 0.43, ▲; 1.28, ■; 2.56, X; 3.84, and +; 5.12% (w/v). △; The time of addition of ammonium acetate.

Fig. 10 Influence of ammonium acetate added at 24 h for protease activity in a pure culture of \textit{Bacillus amyloliquefaciens} NBRC 14141. The tested ammonium acetate concentrations were ●; 0 (Basal medium), ◆; 0.43, ▲; 1.28, ■; 2.56, X; 3.84, and +; 5.12% (w/v). △; The time of addition of ammonium acetate.
To test this hypothesis, we measured the protease activity of the culture broth from the 24 h precultured co-culture (Fig. 9). Although the synthesis of protease began to increase after 48 h in the basal medium, it tended to be inhibited as the amount of ammonium acetate increased. Interestingly, protease production was completely abolished by more than 2.56% (w/v) ammonium acetate, although B. amyloliquefaciens continued to grow. The observed relationship between the growth of B. amyloliquefaciens and the repression of protease production was consistent with Liu et al. [31] who used ammonium sulfate to inhibit protease production by B. subtilis.

Furthermore, a comparison of the protease activity (Fig. 9) with the growth pattern of R. cohnii in the basal medium (Fig. 8) showed that the growth of R. cohnii was repressed after 24 h, which coincides with the time that protease activity began to increase. In addition, the increase in protease activity and the decrease in DMW occurred simultaneously in the co-culture broth in the presence of 0.43% or 1.28% (w/v) ammonium acetate after 48 h. In contrast, R. cohnii could grow in the presence of more than 2.56% (w/v) ammonium acetate. These results suggested that high concentrations of ammonium acetate (3.84% or 5.12% (w/v)) can reduce protease production and prevent a reduction in DMW.

In the presence of 3.84% (w/v) ammonium acetate, the growth curves of B. amyloliquefaciens and R. cohnii were similar and the GA activity peaked at 740 U/ml after culturing for 120 h. However, in the 48 h precultured co-culture, high concentrations of ammonium acetate did not suppress the production of proteases by B. amyloliquefaciens (data not shown). Therefore, increasing the duration of preculturing B. amyloliquefaciens prevented the growth of R. cohnii and production of GA (data not shown), which could not be overcome by high concentrations of ammonium acetate, because protease activity was already high at the time R. cohnii was inoculated. This result also showed that ammonium acetate did not act as a B. amyloliquefaciens protease inhibitor.

4. Conclusion

Ammonium acetate promoted the production of GA by co-culturing B. amyloliquefaciens and R. cohnii. Specifically, it increased the DMW of R. cohnii and prevented the acidification of the culture broth. Moreover, it not only enhanced the growth of B. amyloliquefaciens but also reduced the production of protease that may be responsible for the lysis of the cell wall of R. cohnii. However, high concentrations of ammonium acetate inhibited the growth of B. amyloliquefaciens during early growth phase of the co-culture broth. We overcame this limitation by pre-culturing B. amyloliquefaciens in basal medium before inoculation with R. cohnii and addition of ammonium acetate. The optimum duration of the preculture was 24 h since longer periods allowed excessive accumulation of destructive proteases in the liquid medium.

Although GA productivity was optimized 566 U/ml when the pure culture of R. cohnii was conducted using gelatinized rice flour, we produced the most GA (740 U/ml) by adding 3.84% (w/v) ammonium acetate to a 24 h precultured co-culture of B. amyloliquefaciens and R. cohnii. In addition, we think that increase of gelatinized rice flour amount will lead to produce more GA by using this cultivation method. Consequently, our findings will help make the production of GA in liquid culture more cost-effective because, unlike current methods of Fujio or Morita [7,8], this method does not require the preparation or use of α-amylase.

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Glucosamylase production in submerged co-culture systems

16) X.


Bacillus amyloliquefaciens と Rhizopus cohnii の混合培養系を用いたグルコアミラーゼ生産

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本論文では，原料に糊化米粉を使用し，茨状菌と細菌を混合培養することで液化工程を省略した液体培養法によるグルコアミラーゼ生産法の構築を目的とした。混合培養を行った結果，グルコアミラーゼ生産茨状菌Rhizopus cohniiの増殖に伴って培養液pHが低下し，α-アミラーゼ生産細菌である Bacillus amyloliquefaciens の増殖が困難であった。一方で B. amyloliquefaciens 由来のプロテアーゼが R. cohnii の細胞壁溶解に関与することが明らかとなった。これらの問題に対して，酢酸アンモニウムの添加とそのタイミングが重要であることを示した。ときに B. amyloliquefaciens を 24 h 培養した時に R. cohnii 及び 3.84% (w/v) の醋酸アンモニウムを添加することで 740 U/ml と高い活性を得ることに成功した。

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