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ORIGINAL PAPER

Indigestible dextrin stimulates glucoamylase production in submerged culture of *Aspergillus kawachii*

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Abstract Submerged batch cultures of *Aspergillus kawachii* grown on indigestible dextrin were investigated for potential improvements in glucoamylase (GA) production. In flask culture, specific GA productivities per dry weight biomass using dextrin and indigestible dextrin were 11.0 and 56.1 mU/mg-DW, respectively. Indigestible dextrin was a poor substrate for enzymatic hydrolysis. Rates of glucose formation from dextrin and indigestible dextrin by enzymatic hydrolysis were 0.477 and 0.100 mg-glucose/ml/h, respectively. For this reason, residual glucose concentrations in batch cultures grown on indigestible dextrin remained below 1.32 mg/ml where glucose-limiting conditions were easily maintained. Batch culture using indigestible dextrin had the same residual glucose profile as dextrin fed-batch culture, and nearly the same GA activity was obtained after 42.5 h of growth. However, between 42.5 and 66 h, the GA production rate of the indigestible dextrin batch culture (11.5 mU/ml/h) was higher than that of the dextrin fed-batch culture (6.5 mU/ml/h). During this period, a high amount of residual maltooligosaccharide was detected in the culture supernatant grown on indigestible dextrin. The high GA productivity observed in the indigestible dextrin batch culture may have resulted from the absence of glucose and the simultaneous presence of maltooligosaccharides throughout growth.

Keywords Indigestible dextrin · Glucoamylase · Submerged culture · Glucose-limiting culture condition · *Aspergillus kawachii*

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Introduction

The industrial filamentous fungus *Aspergillus kawachii* is currently commercially used for the production of the traditional Japanese spirit *shochu*. This *koji* mold is considered to be an albino mutant of *Aspergillus awamori*, which secretes a variety of enzymes into the culture medium. The starch-hydrolyzing amylolytic enzymes α-amylase and glucoamylase (GA) are noteworthy. GAs of *Aspergillus* sp. are widely recognized for having granular starch-hydrolyzing activity, and are important industrial enzymes used to produce products such as bio-ethanol from starch substrates derived from grains and cereals [8, 20, 24]. Generally, amylolytic enzyme production in *Aspergillus* sp. is strongly repressed in the presence of glucose via the protein CreA [1, 3, 4, 9, 11, 21]. To achieve efficient industrial production of these enzymes, the process must be operated at a low glucose concentration or the repression caused by CreA must be diminished or abolished [13]. On the other hand, starch or related maltooligosaccharides such as maltose and dextrin are also known to induce amylolytic enzyme production. Recently, isomaltose was found to act as a trigger for amylase production thorough the transcriptional activator AmyR [12]. However, it is difficult to achieve a high rate of enzyme production because of the rapid metabolism of such inducible maltooligosaccharides to glucose, which triggers catabolic repression. A number of approaches have been considered to overcome this problem. These include supplying the inducer at a low and continuous rate [2], using poorly metabolized derivatives such as α-methyl-D-glucose, a synthetic analogue of maltose, as a growth substrate [7], and use of relevant food additives as potential growth substrates [22]. Nondigestible oligosaccharides, which consist of maltooligosaccharides and associated substances resistant to digestion by human

alimentary enzymes, are particularly valuable for the production of low-calorie foods [15, 19]. Indigestible dextrin, which is one of the nondigestible oligosaccharides produced by the pyrolysis of corn starch, comprises α-1,4-, and α-1,6-glycosidic bonds, which are also present in native starch, and contain α-1,2-, and α-1,3-linkages and levoglucosan [17]. Due to these characteristics, indigestible dextrin contains well-developed, branched structures that are expected to be partially hydrolyzed by fungal enzymes. In the present study, we examined the effects of indigestible dextrin on GA production in submerged batch culture of *A. kawachii*.

Materials and methods

Microorganisms

Aspergillus kawachii NBRC 4308 was used throughout this study. Dry spores were suspended in sterile 30% glycerol and were maintained frozen at −80°C. Prior to initiating experiments, the suspension was transferred to potato dextrose agar (PDA) slants (Difco Laboratories, USA). The PDA slants were incubated for 7 days at 30°C after which spores were collected and suspended in sterile saline containing 0.1% (v/v) Triton X-100 (Wako Pure Chemicals, Japan). This spore suspension was used as an inoculum for the growth experiments.

Flask culture conditions

The liquid culture was carried out as described by Iwashita et al. [10], with minor modification. The basal medium contained, per liter: 1 g bactotryptone, 5 g yeast extract, 3 g sucrose, 1 g $NaNO_3$, 1 g K_2HPO4, 0.5 g $MgSO_47H_2O$, 0.01 g $FeSO_47H_2O$ and the additional carbon source indicated in the table legends. The additional carbon sources were glucose, dextrin, and indigestible dextrin (Fibersol-2; Matsutani Chemical Industry, Japan) and mixtures thereof. Conidia of *A. kawachii* were inoculated at 10^5 spores/ml into 50 ml of a basal medium in 200-m1 baffled flasks and cultivated with shaking at 120 rpm and 30°C for 72 h.

Batch culture conditions

Batch culture of *A. kawachii* was carried out in 1,000-ml jar fermenters (Type. BMJ-01NC, Able-Biott, Japan) at a working volume of 600 ml. Conidia of *A. kawachii* were inoculated at 10^5 spores/ml into 600 ml of a basal medium containing 12 g of glucose, dextrin or indigestible dextrin, and cultivated at 300 rpm, and an aeration rate of 300 ml/min at 30°C for 66 h.

Fed-batch culture conditions

Fed-batch culture was also performed in 1,000-ml jar fermenters. Conidia were inoculated at 10^5 spores/ml into 480 ml of basal medium without a carbon source. After inoculation, 120 ml of nutrient solution consisting of basal medium plus 12 g of glucose or dextrin were fed to the jar fermenter at a rate of 1.8 ml/h such that feeding was completed by 66 h following inoculation. Cultivation was conducted at 300 rpm at an aeration rate of 300 ml/min, at 30°C for 66 h.

Hydrolysis of oligosaccharides

The enzymatic hydrolysis rate of dextrin and indigestible dextrin was measured as follows. To prepare a substrate solution, 2 g of dextrin or indigestible dextrin were dissolved in water in a final volume of 90 ml. This solution was sterilized by autoclaving for 15 min at 121°C. The supernatant of the fungal culture grown on indigestible dextrin was used as the enzyme solution. This solution was filtered through a 0.22-μm-membrane filter prior to use (Nihon Millipore K.K., Japan). To determine the hydrolysis rate, 90 ml of the substrate solution, 5 ml of 200 mM acetate buffer (pH5.0), and 5 ml of the enzyme solution were mixed under aseptic conditions at 30°C during which glucose concentrations were monitored for 6 h.

Assays

The fungal culture was centrifuged and the supernatant was assayed for glucoamylase activity and sugar concentration. GA activity was measured using an enzymatic kit (Kikkoman, Japan). Briefly, while glucose-forming activity was measured, 4-nitrophenyl β-D-maltoside (G2-β-PNP) served as a substrate that could be hydrolyzed to 4-nitrophenyl β-D-glucoside (Gl-β-PNP) by both glucoamylase and α-glucosidase. Gl-β-PNP was immediately cleaved to 4-nitrophenol (PNP) in a coupled reaction catalyzed by β-glucosidase monitored by the increase in absorbance at 400 nm. This increase corresponded to the activities of GA and α-glucosidase (AGD). While AGD activity was being measured, 4-nitrophenyl α-glucoside (PNPG) was used as a substrate that was not recognized by GA. PNPG can be hydrolyzed to PNP only by AGD. The fractional quantification of GA activity was calculated by subtracting the absorbance corresponding to the increase in PNP concentration due to glucose-forming activity from the absorbance corresponding to the increase in PNP concentration due to AGD activity. A detailed calculation was carried out according to the manufacturer's instructions. One unit of GA activity was defined as that which released 1 μmol of PNP from Gl-β-PNP per minute at 37°C. Glucose

concentration was measured using a Glucose C2 Test kit (Wako Pure Chemicals, Japan) according to the manufacturer's instructions. Maltooligosaccharides were analyzed by high-performance liquid chromatography using a refractive index detector (RID-10A, Shimadzu, Japan) and an Aminex HPX-42A (300 × 7.8 mm I.D., Bio-Rad, Japan) column. Culture supernatants were diluted tenfold with distilled water and filtered (DISMIC-13cp filter with pore size of 0.45 μm, Advantec, Japan) prior to analysis. The mobile phase was water, the flow rate was 0.5 ml/min, the oven temperature was 80°C, and the injection volume was 5 μl. The biomass concentration was determined after filtering the culture products through Miracloth (EMD Biosciences, Germany). The wet mycelia collected were dried at 105°C for 6 h and their dry weight (DW) was measured.

Results and discussion

Enzymatic hydrolysis of indigestible dextrin

The enzymatic hydrolysis of dextrin and indigestible dextrin over a 6-h time course is shown in Fig. 1. The enzyme solution contained 275 mU/ml of glucoamylase activity was used in this experiment. With indigestible dextrin as substrate, the rate of glucose production was linear from about 0 to 1 h and then slowed but remained approximately linear from 2 to 6 h. The carbohydrate profile of indigestible dextrin in terms of degree of polymerization (DP) was 1.5% DP1, 2.5% DP2, 4.0% DP3, 12.0% DP4-6, and 80% DP7+ [17]. We speculate that small amounts of glucose were rapidly generated from the DP1 and DP2 fractions, and that the DP3, DP4-6 and DP7+ indigestible fractions were degraded more slowly. The glucose generation rate of dextrin between 2 and 6 h was 0.477 mg-glucose/ml/h whereas the rate for indigestible dextrin was 0.100 mg-glucose/ml/h.

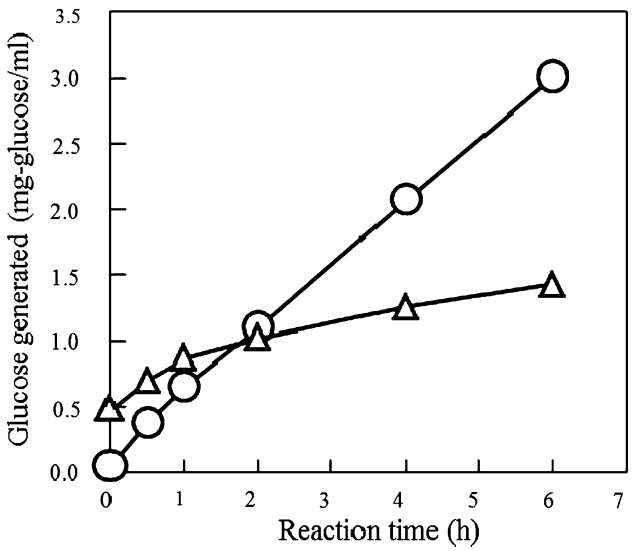

Fig. 1 Time course of glucose production during enzymatic hydrolysis of dextrin and indigestible dextrin. *Circle* dextrin, *triangle* indigestible dextrin

Effect of indigestible dextrin on enzyme productivity in flask culture

Table 1 shows GA activities in the supernatant of *A. kawachii* cultures during growth on various carbon sources. In this experiment, the cultivation by the basal medium without additional carbon source was examined as a control, and other cultivation tests were carried out by adding various carbon sources to the basal medium. Enzyme activity with 20 g/l glucose as additional carbon source was repressed while the activity was higher in 20 g/l dextrin-grown cultures relative to the activity in the basal medium without additional carbon source. GA activities with glucose and dextrin in this experiment were almost same as those in a previous report [16]. Mycelial growth was almost the same under these conditions. GA activity increased by 2.6-fold, when 20 g/l indigestible dextrin were used compared to the 20 g/l dextrin medium. The biomass decreased during growth on 20 g/l indigestible dextrin, while GA productivity per DW using dextrin and indigestible dextrin were 11.0 and 56.1 mU/mg-DW, respectively. When *A. kawachii* was cultivated with both dextrin and indigestible dextrin, a decrease in GA productivity was observed as a function of the increase in dextrin concentration. At the same time, it was found that large amounts of indigestible dextrin also repressed specific enzyme productivity.

Comparison between batch culture grown on indigestible dextrin and fed-batch culture fed glucose or dextrin in a jar fermenter

Batch cultures grown on glucose, dextrin or indigestible dextrin and fed-batch cultures fed glucose or dextrin were analyzed. Figure 2 shows time course profiles of GA activity, biomass concentration, and residual glucose concentrations. GA activities of batch cultures grown on indigestible dextrin or dextrin were 368.4 and 140.2 mU/ml at 66 h, respectively. The maximal residual glucose concentration in the indigestible dextrin batch culture was 1.25 mg/ml at 23 h. In contrast, the residual glucose concentration of dextrin batch culture increased to 5.14 mg/ml at 23 h. This large amount of GA production with indigestible dextrin was due to a small amount of glucose accumulation that likely resulted in a corresponding low level of carbon catabolite repression. On the other hand, the

Table 1 Effect of carbon sources on glucoamylase (GA) activity and cell growth of *A. kawachii*

Additional carbon source	GA activity (mU/ml)	Biomass (mg-DW/ml)	GA productivity (mU/mg-DW)
-	23.1	1.5	14.9
20 g/l Glc	1.7	9.2	0.18
20 g/l Dex	105.1	9.5	11.0
15 g/l Dex + 5 g/l IDex	153.0	9.1	16.8
10 g/l Dex + 10 g/l IDex	134.2	7.8	17.2
5 g/l Dex + 15 g/l IDex	281.2	6.5	43.2
20 g/l IDex	275.2	4.9	56.1
50 g/l IDex	206.0	8.2	25.1
100 g/l IDex	111.1	10.1	11.0

Submerged culture was carried out in 50 ml of a basal medium containing the indicated additional carbon sources and concentrations. The culture without an additional carbon source was indicated as (−). The additional carbon sources used were glucose (*Glc*), dextrin (*Dex*), indigestible dextrin (*IDex*). Cultures were grown at 120 rpm and 30°C for 72 h

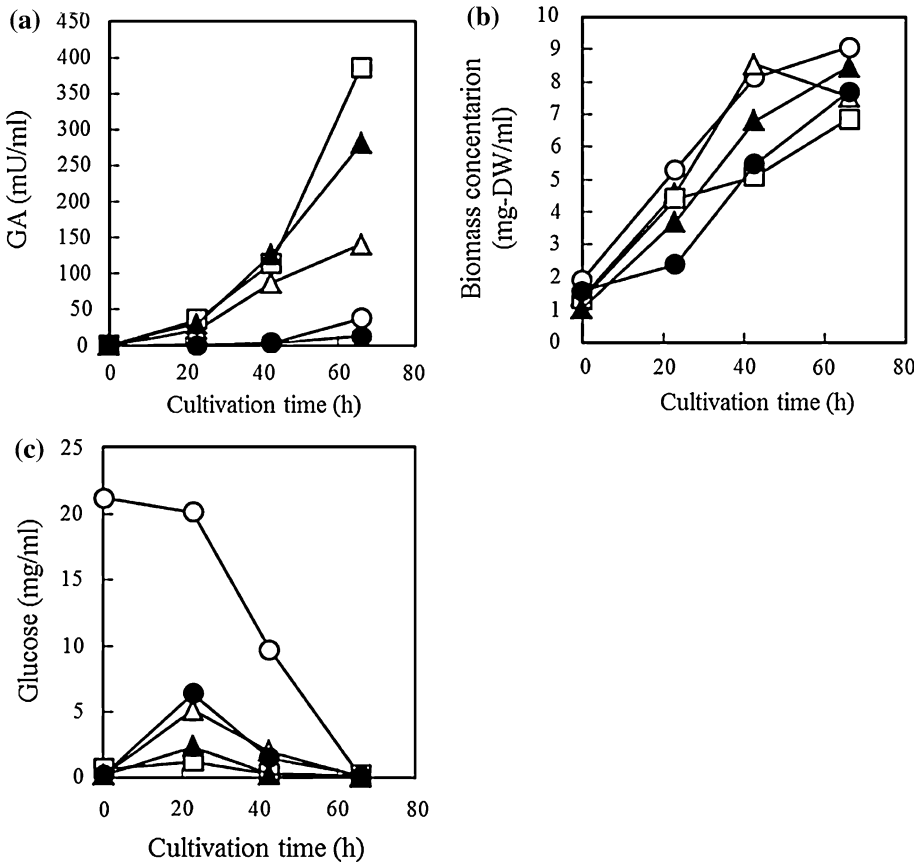

Fig. 2 GA production profiles in batch and fed-batch cultures of *A. kawachii*. **a** GA activity. **b** Biomass concentration. **c** Residual glucose concentration. *Open circle* glucose batch culture, *filled circle* glucose fed-batch culture, *open triangle* dextrin batch culture; *filled triangle* dextrin fed-batch culture, *square* indigestible dextrin batch culture

fact that GA production was not higher in the dextrin batch culture suggests that the amount of glucose present in the early stage of growth would have a profound effect. Therefore, fed-batch culture using glucose or dextrin as feeding substrates was tested as a means of diminishing the carbon catabolite repression that presumably limited GA production during growth on dextrin. In fed-batch culture using glucose, the residual glucose concentration was the same as that observed in dextrin batch culture, peaking at 6.39 mg/ml at 23 h. However, GA activity observed at 66 h was 12.8 mU/ml, less than that detected in the dextrin batch culture. We speculated that optimal GA production would occur in the presence of an inducer such as maltose and the simultaneous absence of glucose. In contrast, higher GA production was obtained in fed-batch culture using dextrin. Residual glucose concentrations were maintained at the same low level as observed in the indigestible dextrin batch cultures, with a maximal

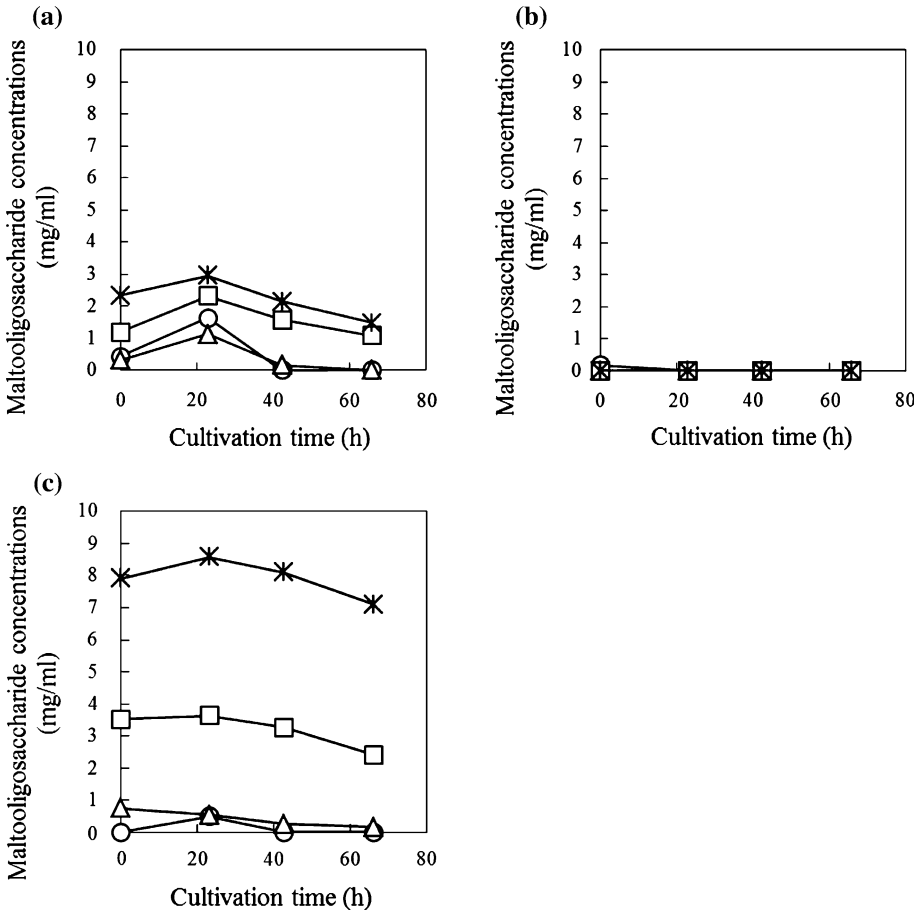

Fig. 3 Maltooligosaccharide concentrations in batch and fed-batch *A. kawachii* cultures grown on dextrin and on indigestible dextrin and in fed-batch cultures of *A. kawachii* grown on dextrin. Batch and fed-batch cultures of *A. kawachii* were grown as shown in Fig. 2. Maltooligosaccharide concentrations in the culture supernatants were analyzed by high-performance liquid chromatography as described in the Materials and methods section. **a** Dextrin batch culture. **b** Dextrin fed-batch culture. **c** Indigestible dextrin batch culture. *Open circle* DP2, *open triangle* DP3, *open square* DP4-6, *asterisk* DP7+

concentration of 2.38 mg/ml measured at 23 h. The GA production profile was almost the same as for the indigestible dextrin batch culture after 42.5 h. At 66 h, 280.4 mU/ml of GA activity was obtained, representing a 2.0-fold increase relative to the dextrin batch culture. However, between 42.5 and 66 h, the GA production rates of the indigestible dextrin batch culture and dextrin fed-batch culture were 11.5 and 6.5 mU/ml/h, respectively, indicating a 1.7-fold higher rate in the indigestible dextrin batch culture.

Figure 3 shows the maltooligosaccharide profiles of the culture supernatants. In the dextrin fed-batch culture, a small amount of maltooligosaccharides were detected because the uptake rate of dextrin by *A. kawachii* was limited by the feeding rate. On the other hand, large amounts of maltooligosaccharide remained in the indigestible dextrin batch culture because the decelerating rate of hydrolysis of the indigestible dextrin likely determined the rate of fungal uptake. The fact that a large amount of DP3–DP7+ fraction was detected in the indigestible dextrin batch culture suggests that a sufficient amount of DP2 fraction might be supplied continuously in the indigestible dextrin batch culture rather than in the dextrin fed-batch culture. Relative to the question of GA induction by the transcription factor AmyR [6, 18, 23], isomaltose has been implicated. Hydrolysis of indigestible dextrin, which contains α-1,4-glycosidic bonds, likely generates isomaltose from the DP2 fraction. Hence, it is expected that transcription of the AmyR-regulated glucoamylase gene would be induced continuously in the indigestible dextrin batch culture. Furthermore, in the culture method described here, it is likely that the glucose generated from the hydrolysis of indigestible dextrin did not greatly exceed the needs of *A. kawachii* and that a glucose-limiting condition was easily achieved. In contrast, the dextrin batch culture generated a condition of excess glucose in spite of the amount of maltooligosaccharides that remained (Figs. 2c, 3a). We speculate that the derepression caused by CreA and the induction caused by AmyR might occur simultaneously with indigestible dextrin used as a culture substrate, resulting in greatly increased GA production.

Figure 4 shows relationships between the specific rate of GA production, the specific yield of GA production and the biomass concentrations in batch and fed-batch cultures. When glucose was used as the substrate for the batch or the fed-batch cultures, the specific rate of GA production and the specific yield of GA production were very low. In the dextrin batch culture, they increased compared with the

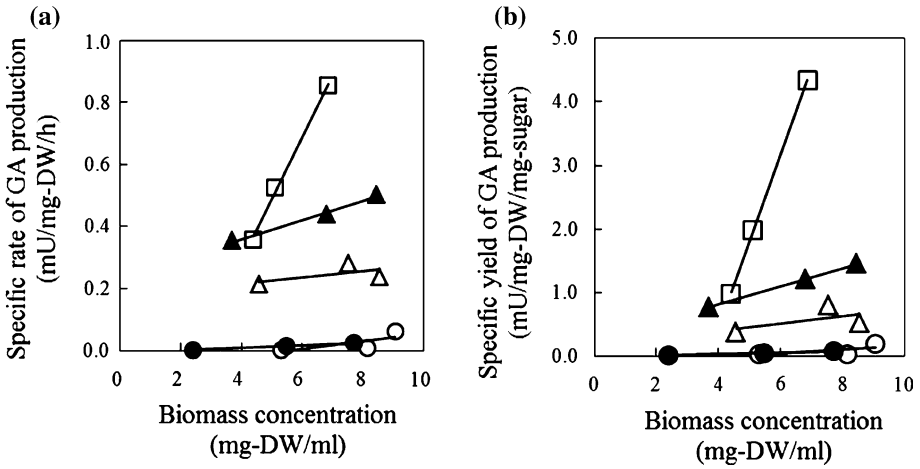

Fig. 4 Relationships between the specific rate of GA production, the specific yield of GA production and the biomass concentration in batch and fed-batch cultures of *A. kawachii*. **a** Relationship between the specific rate of GA production and biomass concentration. **b** Relationship between specific yield of GA production and biomass concentration. *Open circle* glucose batch culture, *filled circle* glucose fed-batch culture, *open triangle* dextrin batch culture, *filled triangle* dextrin fed-batch culture, *square* indigestible dextrin batch culture

glucose-grown culture. In the dextrin fed-batch culture, these values were approximately two-fold higher than in the dextrin batch culture and increased slightly when the biomass concentration increased. Surprisingly, the specific rate of GA production and the specific yield of GA production in the indigestible dextrin batch culture increased significantly when the biomass concentration increased. Although the GA activities at 42.5 h were almost same between the dextrin fed-batch culture and the indigestible dextrin batch culture (Fig. 2a), GA production by *A. kawachii* in the indigestible dextrin batch culture at 42.5 h (5.5 mg-DW/ml) was already different than that in the dextrin fed-batch culture. Masuda et al. [14] demonstrated that when whole barley was used as a carbon source in submerged *A. oryzae* culture, carbohydrate metabolism was slow owing to slow sugar release from the raw material. This results in a slow, continuous supply of sugar over a long period. As a consequence, nitrogen metabolism is maintained, and this allows the biosynthesis of amino acids from nitrate, contributing to high amylolytic enzyme production. The present study and their report share certain similarities in that the flux variation in carbon metabolism has an influence on enzyme production. With respect to the transcriptional response to glucoamylase production, an in-depth characterization of the *A. niger* glucoamylase (*glaA*) promoter has been reported, clearly demonstrating that maltodextrin is a good inducer of *glaA* transcription, and that a low-maltose feeding protocol enabled control without accumulation of extracellular glucose, resulting in increased induction [5]. In terms of the supply of carbon substrates to the mycelia, indigestible dextrin batch culture has certain advantages. Further investigation into the physiological status of the mycelia in the indigestible dextrin batch culture by using metabolome and transcriptome analysis will provide new perspectives on the technology of amylolytic enzyme production.

In conclusion, we suggest that two conditions must be met simultaneously to achieve high GA production by *A. kawachii*. The first is the absence of glucose and presence of an inducer. The second is that adequate amounts of inducer must be supplied to the culture medium continuously. When glucose concentrations in the culture medium are kept low, the presence of a much higher concentration of maltooligosaccharides is advantageous for high GA productivity. Indigestible dextrin was an excellent substrate for GA production because it was degraded slowly, releasing less glucose than dextrin. Additionally, the maltooligosaccharides that remained functioned as inducers late in the culture. This newly developed batch culture system based on use of indigestible dextrin appears to have the potential to greatly enhance production of starch-hydrolyzing enzymes by industrially relevant fungal species.

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