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Repeated-batch production of kojic acid in a cell-retention fermenter using *Aspergillus oryzae* M3B9

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Abstract A cell-retention fermenter was used for the pilot-scale production of kojic acid using an improved strain of *Aspergillus oryzae* in repeated-batch fermentations. Among the various carbon and nitrogen sources used, sucrose and yeast extract promoted pellet morphology of fungi and higher kojic acid production. Repeated-batch culture using a medium replacement ratio of 75% gave a productivity of 5.3 g L^{-1} day^{-1} after 11.5 days of cultivation. While batch culture in shake-flasks resulted in a productivity of 5.1 g L^{-1} day^{-1}, a productivity of 5 g L^{-1} day^{-1} was obtained in a pilot-scale fermenter. By converting the batch culture into repeated batches, the non-productive downtime of cleaning, filling and sterilizing the fermenter between each batch were eliminated, thereby increasing the kojic acid productivity.

Keywords *Aspergillus oryzae* · Cell retention · Kojic acid · Repeated-batch culture

Introduction

Kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone) is a secondary metabolite produced by many species of filamentous fungi and is widely used in cosmetics as a whitening agent [9], in medicine as an analgesic [12] and in agriculture as a pesticide [18]. Kojic acid is also used as a raw material for the synthesis of biodegradable plastics [17]. It is produced on an industrial scale using *Aspergillus* spp and *Penicillium* spp. Carbon sources like glucose, sucrose and starch and nitrogen sources such as yeast extract, peptone and corn steep liquor are widely used for kojic acid production [2, 5, 15]. Optimal medium composition and proper culture conditions are crucial for higher kojic acid production; and the selection of optimal medium is usually case-by-case. Strategies such as fed-batch [6] and pH control [16] have been employed to enhance the yield and productivity in kojic acid fermentations.

Extending the production phase by long-term fermentation is another approach employed for enhancing kojic acid productivity. About 83 g L^{-1} kojic acid was produced from two rounds of repeated cultivation for 100 days using *A. oryzae* immobilized in Ca-alginate beads [8]. Novel bioreactor designs have also been developed for long-term kojic acid fermentation. For example, a cylindrical apparatus for membrane-surface liquid culture was developed and a steady kojic acid concentration of about 45 g L^{-1} was obtained by stably maintaining continuous cultivation for over 70 days [19]. Although cell immobilization and membrane-surface liquid culture successfully prolonged the kojic acid production period, there are still problems, such as scale-up or low production rate, which need to be overcome.

In our previous study [20], using a combination of induced mutation and protoplasting, we developed an improved strain of *A. oryzae* that is capable of accumulating high concentrations of kojic acid. In shake-flask cultures and in stirred-tank fermentations, the improved strain showed good performance not only in terms of high kojic acid concentration but also in productivity. However, the use of fed-batch culture for improving kojic acid production was found to be not as efficient as batch culture. Therefore, it was essential to develop another strategy for more efficient kojic acid production. In this study, we adopted a repeated-batch culture in a cell-retention bioreactor to extend the production phase and enhance kojic acid production.

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Materials and methods

Microorganism

An improved strain of *A. oryzae* M3B9, obtained in our previous study [20] via the combined induced mutation and protoplasting of the wild-type strain (*A. oryzae* ATCC 22788), was used as the kojic acid producer. The stock spores were harvested from potato dextrose agar (Difco Laboratories, Detroit, Mich., USA) plates with sterile water and the spore suspension was preserved at 4°C.

Shake-flask cultures

In order to investigate the effect of various nitrogen sources on cell morphology and kojic acid production, varying concentrations (1–20 g L^{-1}) of $(NH_4)_2SO_4$, yeast extract, soybean protein, tryptone, corn steep liquor (CSL), or rice bran was used along with 100 g L^{-1} glucose. Carbohydrates like glucose, fructose, sorbitol, maltose, sucrose, lactose, or starch at 100 g L^{-1} were used along with 2.5 g L^{-1} yeast extract to examine the effect of carbon source on kojic acid production by the strain. The culture medium also contained 0.5 g L^{-1} $MgSO_4$ and 1 g L^{-1} K_2HPO_4. Batch cultures were performed by inoculating appropriately 1.5×10^7 spores into 50 mL of medium in 250-mL Erlenmeyer flasks. The flasks were incubated at 30°C and 250 rpm for 8 days.

Batch and fed-batch culture

Batch cultures were carried out using a working volume of 3 L in a 5-L stirred-tank fermenter (B. Braun, Germany) equipped with two six-bladed impellers (diam. 6 cm). The seed and the main culture medium contained (per liter): 100 g sucrose, 2.5 g yeast extract, 0.5 g $MgSO_4$ and 1.0 g K_2HPO_4. Seed cultures were prepared in 1-L flasks under the same conditions as those of shake-flask culture. After inoculating with 10% seed culture, fermentation was carried out at 30°C, 300 rpm agitation and 1 vvm aeration.

Fed-batch cultures were initiated as batch cultures in a 5-L stirred-tank fermenter (B. Braun) with a working volume of 3 L. An initial sucrose concentration of 100 g L^{-1} was employed. The concentrations of other nutrients were (per liter): 2.5 g yeast extract, 0.5 g $MgSO_4$ and 1.0 g K_2HPO_4. The agitation rate was 300 rpm and the aeration rate was 1 vvm. For fed-batch cultivation, a total of 300 g of sucrose was added to the fermenter at the rate of 100 g on days 7, 10 and 13. The cultivation was continued until day 16.

Repeated-batch culture

Repeated-batch cultures were initiated as batch cultures using an initial sucrose concentration of 100 g L^{-1} in a 10-L stirred-tank fermenter (Biotop Process and Equipment, Taiwan, ROC) containing 5 L of medium. The fermenter was provided with a 60 mesh (0.25 mm) plate at the bottom for retaining the cells when the culture broth was removed through an outlet at the bottom. The original and replacement culture media and the cultivation conditions were identical to that of batch culture. Experiments were conducted by replacing 25% and 75% of the culture broth with fresh medium.

Analytical methods

Kojic acid was analyzed by the colorimetric method [3]. Glucose was measured in a glucose analyzer (YSI 2700 Select; YSI, Ohio, USA). Biomass was determined by the dry cell weight method. Culture samples were filtered on a pre-weighed filter paper (Whatman 2), washed twice with distilled water and dried at 70°C to constant weight. Cell morphology was observed by a light microscope (BX40; Olympus Optical Corp., Tokyo, Japan).

Results and discussion

Effect of nitrogen source on kojic acid production and cell morphology

The effect of nitrogen source on the kojic acid production and morphology of the improved stain M3B9 was investigated in shake-flask cultures using $(NH_4)_2SO_4$ and five other organic nitrogen sources (yeast extract, soybean protein, tryptone, CSL, rice bran) in varying concentrations (1–20 g L^{-1}). The results are shown in Fig. 1. Irrespective of the concentration of $(NH_4)_2SO_4$ used, poor kojic acid production was noticed after 8 days of cultivation as compared to the organic nitrogen sources. A biomass concentration of 5.7 g L^{-1} was obtained when 2.5 g L^{-1} $(NH_4)_2SO_4$ was used, whereas when high concentrations (5, 10, 15 g L^{-1}) of $(NH_4)_2SO_4$ were used, the biomass formation was markedly reduced (4.3, 4.5, 1.3 g L^{-1}, respectively). However, while using different organic nitrogen sources, two different trends in kojic acid production (Fig. 1) were observed. Higher kojic acid (20–41 g L^{-1}) was accumulated after 8 days of cultivation using greater amounts (10–20 g L^{-1}) of rice brain and CSL. In contrast, M3B9 produced higher concentrations of kojic acid with lower levels (2.5 g L^{-1}) of other nitrogen sources (yeast extract, tryptone or soybean protein at 2.5 g L^{-1}) in the medium.

The use of different nitrogen sources greatly influenced not only the kojic acid production pattern but also the fungal morphology. Two types of morphologies were observed when M3B9 was grown in medium with different nitrogen sources. While yeast extract favored the formation of pellets (Fig. 2a), highly entwined hyphae with irregular shapes and loose structures (Fig. 2b) were observed when rice bran was used as the nitrogen source

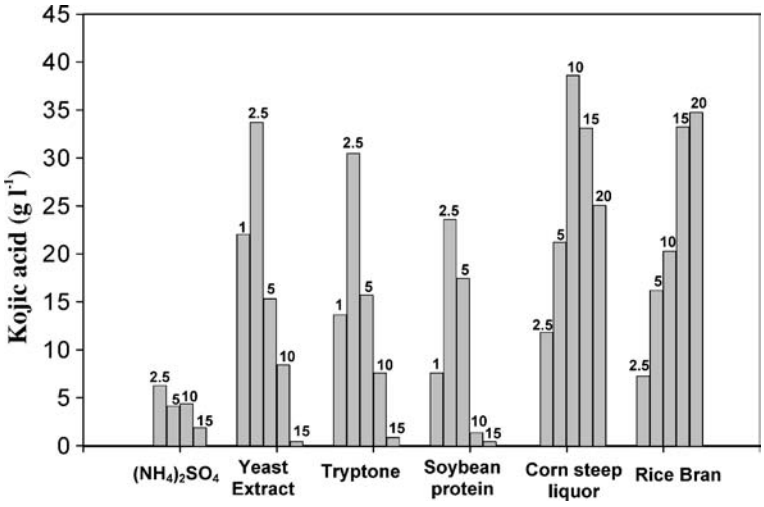

Fig. 1 Effect of nitrogen sources on kojic acid production by *A. oryzae* M3B9 in shake-flasks. Cultures were carried out in a medium containing 100 g L^{-1} glucose and varying concentrations of nitrogen sources at 30°C and 250 rpm. The *number* above each column indicates the concentration (g L^{-1}) of nitrogen source used

and this pattern of cell morphology was between filamentous and pellet-like forms. A similar phenomenon was observed in the submerged culture of *Rhizopus chinesis* when different nitrogen sources were used during antibiotic fermentation [4]. Although the actual mechanism leading to the variation in fungal morphology is still very unclear, insoluble solids or some special macromolecular components are believed to be the key factors [13].

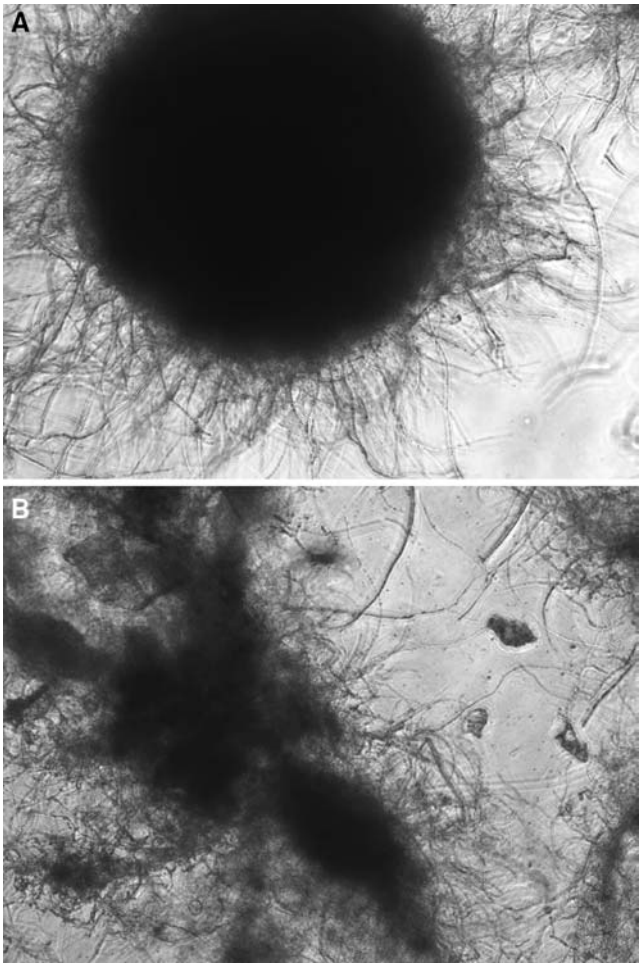

Fig. 2 Change in the cell morphology of *A. oryzae* M3B9 in shake-flask fermentations using the nitrogen sources: **a** yeast extract, **b** rice bran. Cultures were carried out in a medium containing 100 g L^{-1} glucose and 2.5 g L^{-1} yeast extract or rice bran at 30°C and 250 rpm

Fungal fermentations generally require different cell morphologies for optimizing the product yield [4]. Growth in the form of pellets lowers the broth viscosity and enhances the mixing and mass transfer properties of the suspension. It also prevents the adhesion of cells within the bioreactor [14]. Since the maximum kojic acid production levels with different organic nitrogen sources were only slightly different from each other (31–38 g L^{-1}; Fig. 1), the criterion for the selection of a better nitrogen source should be based on an appropriate morphology that would help to reduce fermentation problems. In this regard, yeast extract at 2.5 g L^{-1} was chosen as the nitrogen source for further studies in kojic acid production.

Effect of carbon source on kojic acid production

In order to find a suitable carbon source for use along with yeast extract (2.5 g L^{-1}) in the medium to sustain a pellet-like morphology of M3B9, several carbohydrates (glucose, fructose, sorbitol, sucrose, maltose, lactose, starch) were used in shake-flask cultures. High concentrations of kojic acid accumulated in the medium with all these carbohydrates, except lactose (Fig. 3). Fructose was reported to be not suitable for high kojic acid production due to the possibility that fructose in furanose form was not appropriate for direct conversion to kojic acid [15]. However, our results indicated that M3B9 effectively utilized fructose and produced a high concentration of kojic acid. The highest kojic acid accumulation of 41 g L^{-1}, corresponding to a volumetric productivity of 5.1 g L^{-1} day^{-1}, was obtained when sucrose was used. Due to the easily hydrolysable nature of this disaccharide, both glucose and fructose were simultaneously present in

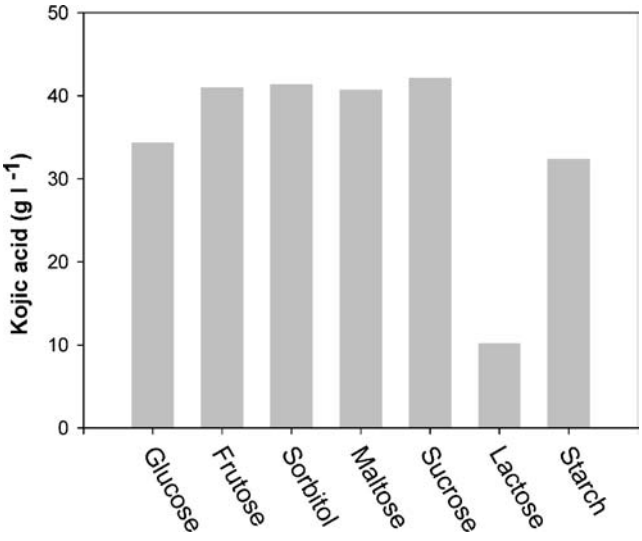

Fig. 3 Effect of carbon sources on kojic acid production by *A. oryzae* M3B9 in shake-flasks. Cultures were carried out in a medium containing 2.5 g L^{-1} yeast extract and various carbohydrates (100 g L^{-1}) at 30°C and 250 rpm

the medium and the fungus could directly consume both these monosaccharides. Kojic acid production by M3B9 was heavily suppressed by lactose, which might be due to the alteration in the metabolic regulation that prevented the formation of certain important enzymes responsible for kojic acid production. Rosfarizan and Ariff [15] also reported a similar result during kojic acid production using *A. flavus*.

Kojic acid production by batch and fed-batch culture

Pilot-scale production of kojic acid by batch and fed-batch cultures of M3B9 was investigated using a medium containing sucrose (100 g L^{-1}) and yeast extract (2.5 g L^{-1}) in a 5-L stirred tank. A typical time-course of biomass and kojic acid production during the batch cultivation is shown in Fig. 4. Growth of M3B9 was very rapid during the initial stage. A maximum cell concentration of 3.2 g L^{-1} was obtained after 1 day of cultivation. This was followed by a transition period during which the growth declined continually until day 3 of cultivation. After the transition period, the cells entered the stationary phase. A similar phenomenon was also observed during studies on the growth characteristics of *A. fumigatus* in different nutrient media [10]. The rate of kojic production was enhanced when cell growth had ceased after 1 day of cultivation (Fig. 4). This result suggested that kojic acid production by M3B9 was predominantly not growth-associated. Kojic acid continued to accumulate in the culture broth and a final concentration of 40 g L^{-1} kojic acid, equivalent to a productivity of 5 g kojic acid L^{-1} day^{-1}, was obtained after 8 days of cultivation. The concentration and productivity of kojic acid obtained in 5-L stirred tanks

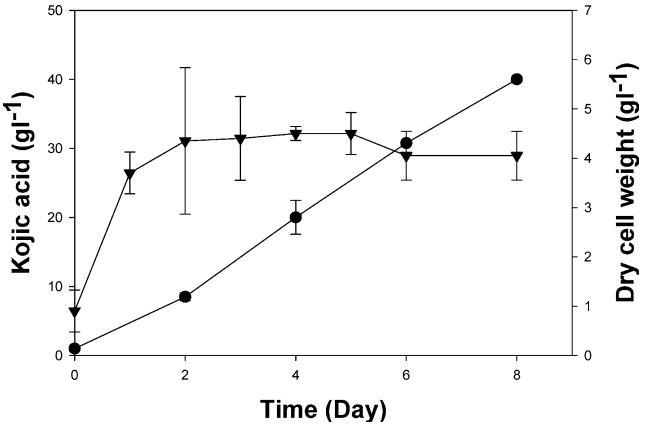

Fig. 4 Time-course of (*circles*) kojic acid and (*inverted triangles*) biomass during batch cultivation of *A. oryzae* M3B9 in a 5-L stirred-tank fermenter at 300 rpm agitation and 1 vvm aeration. Initial sucrose and yeast extract concentrations were 100 g L^{-1} and 2.5 g L^{-1}, respectively

during batch fermentations were comparable to those obtained from shake-flask cultures. The similar performances of M3B9 in shake-flasks and in the 5-L fermenter demonstrated the practicability of this strain for use in pilot-plant reactors for the large-scale production of kojic acid.

Fed-batch cultures were carried out with the addition of a carbon source (sucrose) for enhancing kojic acid production by M3B9. A fed-batch culture was initiated as a batch in a 5-L fermenter containing 3 L medium and 100 g sucrose was fed at days 7, 10 and 13 during cultivation. The time-courses of kojic acid, pH and biomass during the fed-batch culture are shown in Fig. 5. Kojic acid was produced at the rate of approximately 5 g L^{-1} day^{-1} up to 8 days of fermentation, following which it decreased to 1.5 g L^{-1} day^{-1}. Although a high

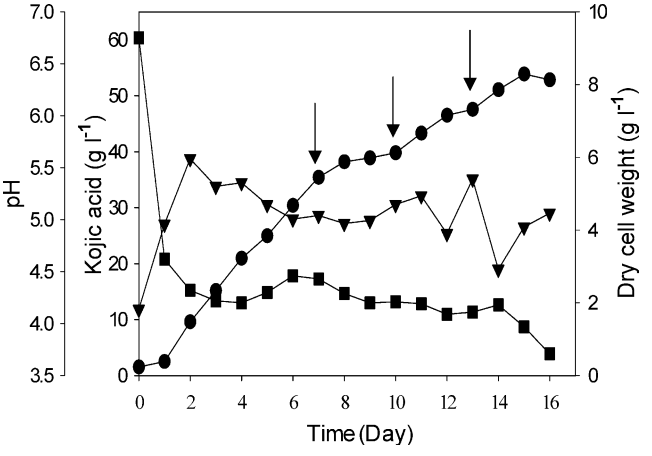

Fig. 5 Time-course of (*circles*) kojic acid, (*squares*) pH and (*inverted triangles*) biomass during fed-batch cultivation of *A. oryzae* M3B9 in a 5-L stirred-tank fermenter at 300 rpm agitation and 1 vvm aeration. Initial sucrose and yeast extract concentrations were 100 g L^{-1} and 2.5 g L^{-1}, respectively. Sucrose (300 g) was fed into the fermenter at the rate of 100 g on days 7, 10 and 13. *Arrows* indicate the time of feeding sucrose

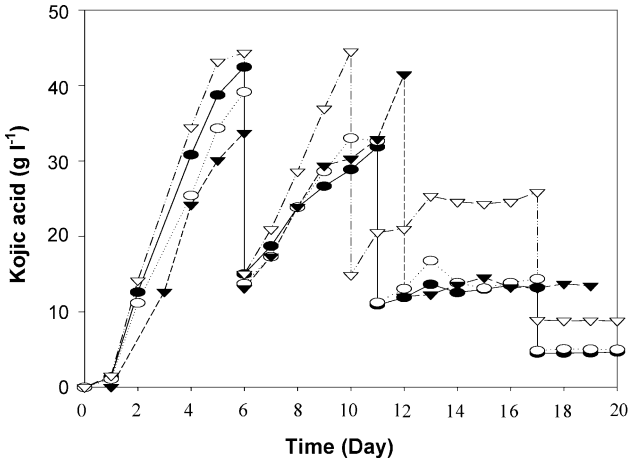

Fig. 6 Kojic acid production in repeated-batch cultures by *A. oryzae* M3B9 using different replacement media containing 100 g L^{-1} sucrose and (*filled circles*) 0-fold, (*open circles*) 0.25-fold, (*filled inverted triangles*) 0.5-fold and (*open inverted triangles*) 1.0-fold concentrations of other nutrients in shake-flasks at 30°C and 250 rpm. After 6 days, 75% of the fermented medium was replaced with fresh medium

kojic acid concentration (52 g L^{-1}) was accumulated after 15 days of cultivation, the yield (0.26 g kojic acid g^{-1} sucrose) and productivity (3.5 g L^{-1} day^{-1}) were relatively lower than those from batch culture (yield 0.44 g kojic acid g^{-1} sucrose, productivity 5 g $L^{-1}day^{-1}$). One or more factors like inhibition by the product or a change in cell morphology might have led to a lower kojic acid production in the late fermentation period. An alternate fermentation strategy was therefore developed for further enhancing kojic acid production by M3B9.

Repeated-batch culture in shake-flasks and modified stirred-tank fermenter

Repeated-batch processing is a well known method for enhancing the productivity of microbial cultures through extending the production phase of the culture by replacing a portion of the original culture with fresh substrate. In our study, repeated-batch cultures were carried out in a cell-retention fermenter, in which all the cells were retained during medium replacement. Due to the pellet-like morphology of M3B9, which mimicked immobilized cells, M3B9 could be very easily retained in the reactor.

In order to identify a suitable replacement medium for maintaining a pellet-like morphology of M3B9 during repeated-batch culture, four different media containing 100 g L^{-1} sucrose were examined in 1-L shake-flasks at 30°C and 250 rpm. Other nutrients (yeast extract, $MgSO_4$, K_2HPO_4) were maintained at 0-, 0.25-, 0.5- and 1.0-fold of that used for the batch culture. At about 6 days, 75% of the fermented medium was replaced with fresh medium and the cultivation was

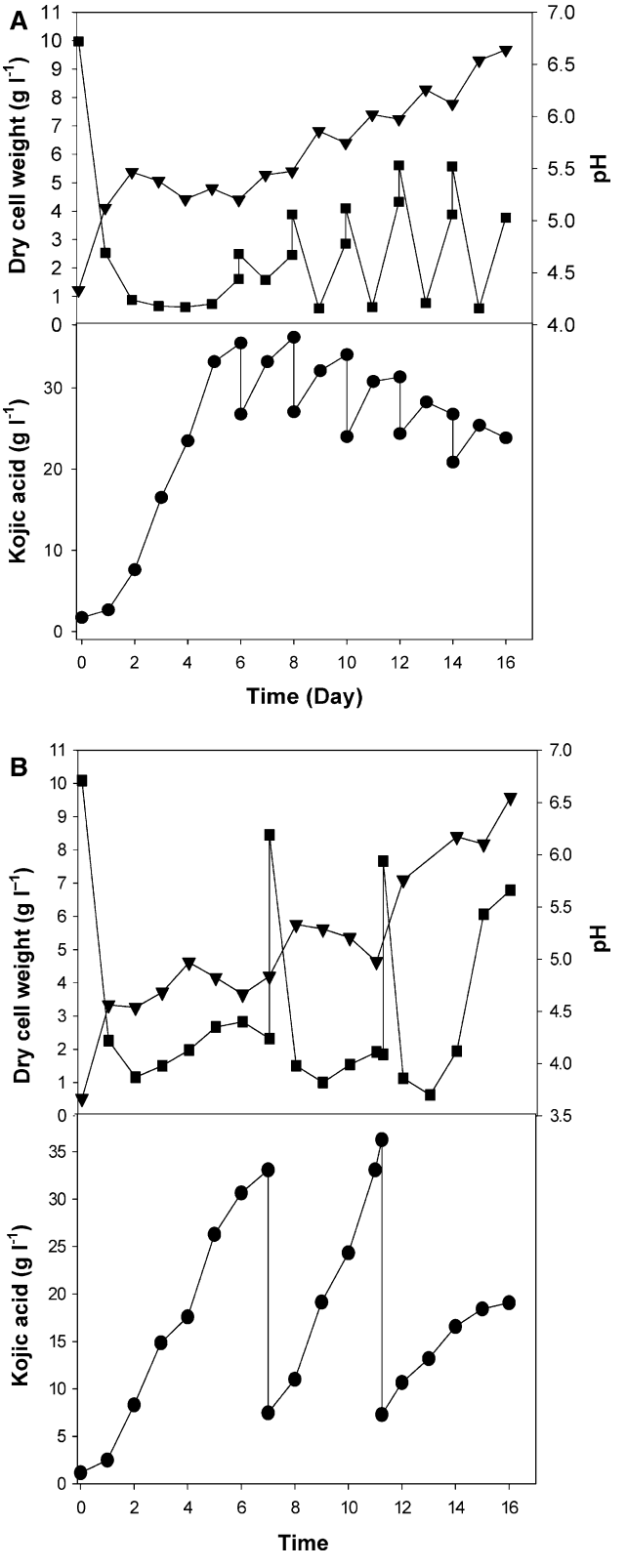

Fig. 7 Time-course of (*inverted triangles*) biomass, (*squares*) pH and (*circles*) kojic acid during repeated-batch culture of *A. oryzae* M3B9 in a 10-L cell-retention fermenter, 300 rpm agitation and 1 vvm aeration, at: **a** 25%, **b** 75% medium replacement. Initial sucrose and yeast extract concentrations were 100 g L^{-1} and 2.5 g L^{-1}, respectively

Table 1 Comparison of the overall kojic acid productivities obtained in the repeated-batch culture of *A. oryzae* M3B9 in the cell-retention fermenter with those of other methods of cultivation

Method of cultivation	Productivity ($g\ L^{-1}\ day^{-1}$)	Reference
Air-lift bioreactor	5.7	[5]
Continuous membrane-surface liquid culture	1.8	[19]
Controlled mycelial growth using immobilized cells in repeated batch	3.7	[7]
Fed-batch membrane-surface liquid culture	5.0	[11]
Resuspended mycelial system	3.6	[1]
Repeated-batch culture in cell-retention fermenter with 75% medium replacement	4.4	Present study
Repeated-batch culture in cell-retention fermenter with 25% medium replacement	4.1	Present study

continued. The results are shown in Fig. 6. The medium, which was the same as the batch (with 1.0-fold nutrients) gave the highest kojic acid yield (43 $g\ L^{-1}$) after 10 days of cultivation. Other media could not yield the same concentration of kojic acid, even after prolonged cultivation. In all cases, the yield of kojic acid drastically declined after the subsequent medium replacement.

After identifying the replacement medium for large-scale repeated-batch cultivation, it was decided to carry out repeated-batch fermentations by replacing different volume fractions of the spent medium with fresh medium to determine a suitable medium replacement ratio. A higher (75%) and a lower (25%) medium replacement were implemented in repeated-batch cultivations using M3B9 and original medium (containing 1.0-fold nutrients) in the cell-retention fermenter. During medium replacement, all the cells were retained in the reactor. The first medium replacement was carried out after 6–7 days of cultivation, when 30 $g\ L^{-1}$ kojic acid accumulated in all cases. The frequencies of replacement of the medium were as follows: every 2 days for 25% and at day 11.5 for 75%. All the repeated-batch cultivations were continued for 16 days.

The time-courses of kojic acid, biomass and pH during repeated-batch cultivation by replacing 25% and 75% of the spent medium with fresh medium are shown in Fig. 7a, b, respectively. In all cases, batch cultivation and the first cycle of repeated-batch cultivations yielded higher concentrations of kojic acid. After the first cycle, a kojic acid concentration of 37 $g\ L^{-1}$ was obtained for 25% and 75% medium replacement. However, the kojic acid concentration decreased when further cycles of repeated batches were carried out. For 25% medium replacement every 2 days (Fig. 7a), the productivity decreased after each cycle. However, for 75% medium replacement (Fig. 7b), the productivity increased after the first medium replacement on day 7. A subsequent medium replacement on day 11.5 of cultivation led to a decrease in productivity. After 11.5 days of cultivation with 75% medium replacement, 305 g of kojic acid (corresponding to an average productivity of 5.3 $g\ L^{-1}\ day^{-1}$) was obtained. In all cases, biomass recorded an increase after each cycle. The kojic acid concentration did not increase in proportion to the biomass. Furthermore, pH continuously increased during the course of repeated-batch cultivation. Based on the observed variations in pH and biomass, it was inferred that the cell physiologies were obviously different in the two fermentation phases and this led to a lower kojic acid production.

The overall kojic acid productivities obtained in the repeated-batch culture using the cell-retention fermenter are compared with those of other methods of cultivation in Table 1. A higher kojic acid production (355.47 g) and higher overall productivity (4.4 $g\ L^{-1}\ day^{-1}$) were obtained with a higher ratio (75%) of medium replacement. Repeated-batch culture with 25% medium replacement yielded a lower amount of kojic acid (324.42 g) and a lower productivity (4.1 $g\ L^{-1}\ day^{-1}$). To maintain cell physiology and reproduce the batch culture in each cycle of the repeated batch for stable productivity, the effect of medium replacement ratio and time of replacement on cell physiology and kojic acid productivity have to be further investigated. However, the similar kojic acid production profiles obtained in the repeated-batch cultures in the shake-flask and in the cell-retention fermenter (Figs. 6, 7a) indicated that the shake-flask culture simulated directly the behavior of M3B9 in the cell-retention fermenter.

Conclusions

The fungal morphology and kojic acid production of the improved strain M3B9 was greatly influenced by the type of nitrogen source used for cultivation. M3B9 demonstrated a clearly better performance in batch culture in terms of higher kojic acid yield and productivity. Although the productivity in repeated-batch culture progressively decreased after each cycle, the production efficiency of the repeated-batch culture in the cell-retention fermenter was better than that of the fed-batch. If further research is undertaken to maintain M3B9 in the production phase always, repeated-batch culture of M3B9 would be of immense potential for high kojic acid production.

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