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Full Length Research Paper

Improved riboflavin production by *Eremothecium ashbyii* using glucose and yeast extract

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A detail research on the control strategies of carbon and nitrogen source was investigated for riboflavin production by *Eremothecium ashbyii*. The effects of various carbon and nitrogen sources on riboflavin biosynthesis were first employed respectively which revealed that glucose and yeast extract were the optimal medium components for riboflavin fermentation by *E. ashbyii*. To further improve the productivity of riboflavin, the shake-flask fed-batch cultivation of *E. ashbyii* was established with glucose and yeast extract, and the results demonstrate that glucose and yeast extract could significantly facilitated cell growth, but the inhibition of riboflavin biosynthesis would be caused by high concentrations of glucose and yeast extract. Based on the results obtained in shake-flask experiments, the strategies of glucose and yeast extract were developed for riboflavin fermentation in 15 L fermenter, in which glucose was fed to maintain its concentration between 0.5 to 0.8 g/100 ml during the whole fermentation process, and 60 g/L of yeast extract was continuously fed at 40 ml/h of rate from 48 to 96 h of fermentation. As a result, maximal riboflavin was 3107.59 mg/L by the fed-batch fermentation in 15 L fermenter, which was 104.77% higher than that obtained in batch fermentation (1517.59 mg/L).

Key words: *Eremothecium ashbyii*, riboflavin, biosynthesis, cell growth, glucose, yeast extract.

INTRODUCTION

Riboflavin (vitamin B_2) is a yellow, water-soluble vitamin. Due to being the precursor of coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), riboflavin plays an important role in energy metabolism and biosynthesis of a number of compounds (Lim et al., 2003). As an important biological compound, riboflavin has been widely used in the fields of feed and food additives and pharmaceuticals (Wu et al., 2007). Riboflavin has been produced commercially by chemical synthesis, fermentation and by mixed fermentation/chemical synthesis methods, and fermentation is the most recent and most cost-effective method. A number of microorganisms such as the fungi *Eremothecium ashbyii* and *Ashbya gossypii*, the yeast *Candida flaveri* and the bacteria *Bacillus subtilis*, have been successfully and widely applied to the commercial production of riboflavin (Stahmann et al., 2000; Demain and Adrio, 2008).

Many strategies have been employed to improve the production of riboflavin by *E. ashbyii*. Mehta and Modi (1981) investigated the effect of phosphate on flavinogenesis in *E. ashbyii*. Pujari and Chandra (2000) used statistical designs to determine optimal levels of medium nutrients for riboflavin production by *E. ashbyii* in shake-flask fermentation. Kalingan and Krishnan (1997) and Kalingan (1998) investigated some waste organic materials for their ability to support riboflavin production by *E. ashbyii*. Although, many efforts have been focused on improving riboflavin production, little information is available on the metabolic regulation of carbon and nitrogen source on riboflavin biosynthesis by *E. ashbyii*. In the present work, a detail research about the control strategy of carbon and nitrogen source was investigated for riboflavin fermentation by *E. ashbyii*.

MATERIALS AND METHODS

The fungus *E. ashbyii* was used for riboflavin production throughout this study. *E. ashbyii* was maintained on agar slant containing (g/L): glucose, 10; peptone, 10; KH_2PO_4, 2.0; $MgSO_4$, 1.5; agar, 20; pH 5.6 to 6.0.

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Table 1. Final results of riboflavin under four various carbon sources in shake-flask culture by *E. ashbyii*.

Carbon source	Riboflavin (mg/L)				
	n_1	n_2	n_3	Mean ± SD	Significant at 1% level[a]
Glucose	650.24	672.86	645.31	656.14 ± 14.69	A
Sucrose	375.21	398.12	352.62	375.32 ± 22.75	B
Maltose	260.09	287.43	269.47	272.33 ±13.89	C
Starch	640.69	611.53	608.33	620.18 ± 17.83	A

[a]Multiple comparisons were done by least significant difference (LSD) test; n_1, n_2 and n_3 meant the three independent determinations of riboflavin.

Media

Seed medium was composed of (g/L): glucose, 30; peptone, 15; corn steep liquor, 5; KH_2PO_4, 1.0; $MgSO_4$, 0.75; pH 5.6 to 6.0. Original fermentation medium was as follows (g/L): glucose, 50; peptone, 30; KH_2PO_4, 2.0; $MgSO_4$, 1.0; NaCl, 1.0; pH 6.0 to 7.0.

Fermentation in shake flasks

Preculture was carried out in a 250 ml Erlenmeyer flask containing 30 ml of seed medium inoculated with cells from fresh slant, and cultivated at 28°C on a rotary shaker at 180 rpm for 36 h. The seed culture was then transferred to 250 ml Erlenmeyer flask containing 30 ml fermentation medium with 10% inoculum, and incubated at 28°C on a rotary shaker at 180 rpm.

Fermentation in 15 L fermenter

Two-stage fermentation was performed for riboflavin production in a 15 L stirred tank fermenter, equipped with temperature probe, pH probe (Mettler, Toledo) and dissolved oxygen probe (Mettler Toledo). The preculture was carried out in a 1 L Erlenmeyer flask containing 100 ml sterile seed medium with inoculum from one fresh slant, and cultivated at 28°C on a rotary shaker at 180 rpm for 36 h. The pH of the fermentation medium was adjusted from 6.5 to 6.8 before inoculation. The seed culture (about 5% of the final working volume) was transferred into 15 L fermenter containing 8 L of fermentation medium. Fermentation was controlled at 28°C, and finished at 144 h. Dissolved oxygen was maintained at about 10% of air saturation during fermentation. The pH was controlled between 6.5 to 7.0 throughout fermentation by adding 2.0 mol/L NaOH or 1 mol/L HCl.

Determination of dry cell weight (DCW)

Culture broth was centrifuged at 5,000 rpm for 10 min. After washing twice with distilled water, the cell precipitate was dried to a constant weight at 105°C.

Quantification of riboflavin in the broth

Riboflavin concentration in fermentation broth was determined according to the literature (Lim et al., 2003).

Quantification of glucose in the broth

Dinitrosalicylic acid reagent was used for the determination of glucose in fermentation broth (Miller, 1959). Standard curve of glucose with different known concentrations was prepared, and. thus the amount of glucose in the culture broth was calculated.

RESULTS AND DISCUSSION

Effects of various carbon sources on riboflavin biosynthesis

For an investigation on the effect of carbon source on riboflavin biosynthesis in *E. ashbyii*, 50 g/L of four various carbon sources (glucose, sucrose, maltose and starch) were used in the fermentation medium, respectively. After 120 h of shake-flask cultivation, the final riboflavin production is listed in Table 1.

According to the experimental results of riboflavin production in the case of the four carbon sources, multiple comparisons were further performed by least significant difference (LSD) tests, as also shown in Table 1.

From the final riboflavin production and LSD tests for the four carbon sources applied to the fermentation media, it could be concluded that glucose was the optimal carbon source for riboflavin fermentation by *E. ashbyii*, in which the maximal riboflavin production (656.14 ± 14.69 mg/L) was achieved.

Effects of various nitrogen sources on riboflavin biosynthesis

The influences of 30 g/L of various nitrogen sources (beef extract, yeast extract, corn steep liquor and peptone) on riboflavin biosynthesis were further investigated in the shake-flask cultivation of *E. ashbyii*, with 50 g/L of glucose as the carbon source in the fermentation medium. The final riboflavin production (120 h) is listed in Table 2.

Based on the LSD tests of various nitrogen sources on riboflavin production (Table 2), except for no significant difference between peptone and corn steep liquor, it could be found that riboflavin productions under various nitrogen sources were statistically significant at 1% level. From the results of LSD tests, yeast extract had a

Table 2. The final results of riboflavin under four various organic nitrogen sources in shake-flask culture by *E. ashbyii*.

Nitrogen source	Riboflavin (mg/L)				
	n_1	n_2	n_3	Mean±SD	Significant at 1% level[a]
Beef extract	527.71	503.62	489.97	507.10±19.11	C
Yeast extract	1024.29	1109.07	992.73	1042.03±60.16	A
Corn steep liquor	611.36	658.76	602.29	624.14±30.33	B
Peptone	645.36	636.72	611.54	631.21±17.57	B

[a]Multiple comparisons were done by LSD test; n_1, n_2 and n_3 meant the three independent determinations of riboflavin.

significantly positive influence on riboflavin biosynthesis, in which the highest riboflavin production (1042.03 ± 60.16 mg/L) was obtained.

Establishment of fed-batch cultivation of *E. ashbyii* in shake flasks

Strategy of glucose for riboflavin fermentation

Fed-batch is generally superior to batch processing, which can change the nutrient concentrations in fermentation broth (Lee et al., 1999). Therefore, fed-batch culture has been extensively used to increase the productivity of certain primary and secondary metabolites in the fermentation process. The results (Table 1 and Table 2) show that glucose and yeast extract were the optimal components for riboflavin fermentation by *E. ashbyii*. In order to improve the productivity of riboflavin, the shake-flask fed-batch cultivation of *E. ashbyii* was established with the two components (glucose and yeast extract). The strategy of glucose was first implemented for riboflavin fermentation. To achieve the optimal strategy of glucose in shake-flask fed-batch cultivation by *E. ashbyii*, various concentrations of glucose (0, 5, 10 and 20 g/L) was fed to the fermentation broths at 48, 72 and 96 h, respectively. Figure 1 shows the kinetics of glucose consumption, biomass growth and riboflavin biosynthesis under the four runs of glucose.

As shown in Figure 1a, without glucose, the glucose concentration drastically dropped to 0.86 g/100 ml at 72 h and approximately 0 at 96 h, which indicated that additional supplement of glucose, should be operated to meet the demand for cell growth and riboflavin biosynthesis. When 5, 10 and 20 g/L of glucose were fed respectively the glucose concentrations in broths were maintained above 0 during the whole fermentation processes. It was noticeable that 20 g/L of glucose would cause glucose accumulation in the culture.

Figure1b summarizes the kinetics of biomass growth in the four fermentation processes. In consistent with glucose becoming deficient, the final DCW without glucose was significantly lower than those in the case of glucose. Furthermore, due to lack of glucose, the DCW gradually decreased after 96 h of fermentation. In contrast, with glucose added to fermentation broth, an increasing trend of DCW was obtained after 96 h. In the case of 5, 10 and 20 g/L of glucose, the maximal DCW were 9.25, 9.48 and 8.80 g/L, respectively.

Time course of riboflavin production was presented in Figure 1c. Among the four runs of glucose, riboflavin biosynthesis under 5 g/L of glucose showed a remarkable enhancement after 96 h, which indicated the highest riboflavin production (1363.52 mg/L) obtained. It was noticeable that during 72 to 120 h of fermentation, the riboflavin production without glucose was higher than those obtained in the case of 10 and 20 g/L of glucose, thus, revealing that high glucose concentration in broth had negative influence on riboflavin biosynthesis. Although, the highest DCW under 10 g/L of glucose was achieved, the final (144 h) riboflavin production was only 1093.72 mg/L which was slightly higher than that obtained without glucose (1076.18 mg/L). Furthermore, the lowest riboflavin production was achieved in the condition of 20 g/L glucose.

According to the above results, it could be concluded that glucose could accelerate the level of cell growth by avoiding glucose deficiency. However, the concentration of glucose above 5 g/L was far from the optimal range, which would cause negative influence on riboflavin biosynthesis. Hence, adjusting glucose concentration in broth was a key and sensitive parameter for riboflavin biosynthesis. Therefore, the strategy with low glucose concentration could not only avoid the insufficiency of glucose in the fermentation broth for cell growth, but also reduce riboflavin biosynthesis inhibition by high glucose concentration.

Strategy of yeast extract for riboflavin fermentation

As aforementioned (Table 2), when yeast extract was used as the organic nitrogen for fermentation medium, a significant enhancement of riboflavin production was observed. In order to improve the productivity of riboflavin, yeast extract was manipulated in the shake-flask cultivation of *E. ashbyii*, in which six various amounts of yeast extract (0, 1, 2, 3, 4 and 5 g/L) was fed to the fermentation broths at 48, 72 and 96 h, respectively. The final cell biomass and riboflavin production

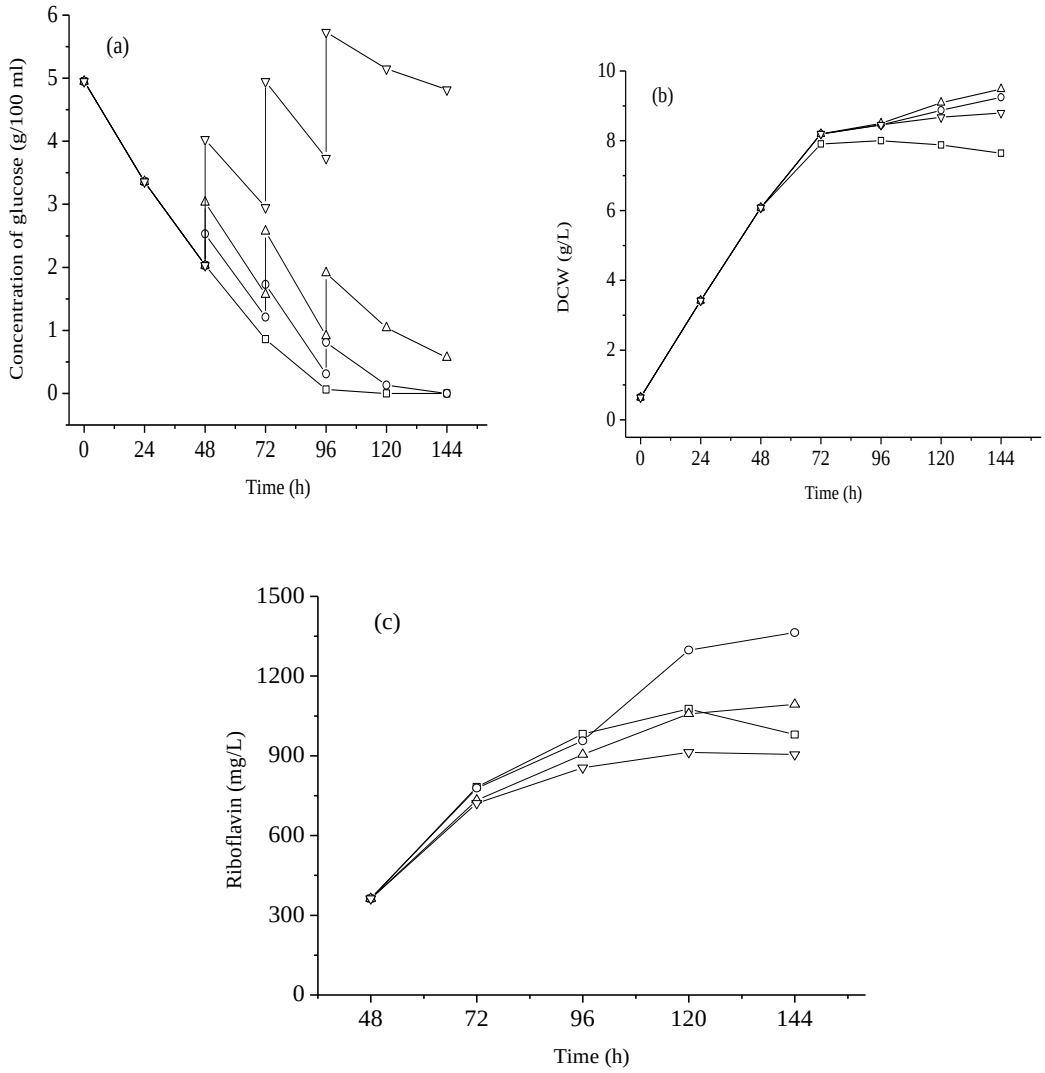

Fig.1 Time courses of glucose consumption (a), cell growth (b) and riboflavin production (c) in shake-flask cultivation of *E. ashbyii* by various concentrations of glucose feeding. Symbols for the concentrations of glucose feeding (g/L): 0 (□); 5 (○); 10 (△); 20 (▽)

Table 3. Effects of various amounts of yeast extract on cell growth and riboflavin production in shake-flask cultivation by *E. ashbyii*.

Result	Amount of yeast extract added to fermentation broths at 48, 72 and 96 h, respectively / (g/L)					
	0	**1**	**2**	**3**	**4**	**5**
DCW (g/L)	9.30 ± 0.14	10.31 ± 0.23	11.74 ± 0.16	12.14 ± 0.11	12.64 ± 0.13	13.18 ± 0.19
Riboflavin (mg/L)	1347.00 ± 41.64	1467.75 ± 61.07	1735.03 ± 47.09	1953.02 ± 60.56	1743.56 ± 39.65	1608.98 ± 19.70

(144 h) are listed in Table 3.

From Table 3, without yeast extract the final DCW and riboflavin production were both significantly lower than those under yeast extract. When the concentration of yeast extract increased from 1 to 5 g/L, the corresponding DCW presented an obviously increasing trend, which demonstrated that yeast extract had a positive influence on cell growth. However, the final riboflavin production was not in consistent with the cell biomass. As summarized in Table 3, the maximal riboflavin (1953.02 ± 60.56 mg/L) was obtained when 3 g/L of yeast extract was fed at 48, 72 and 96 h, respectively. With the enhancement of yeast extract from 3 to 5 g/L, the riboflavin production gradually decreased.

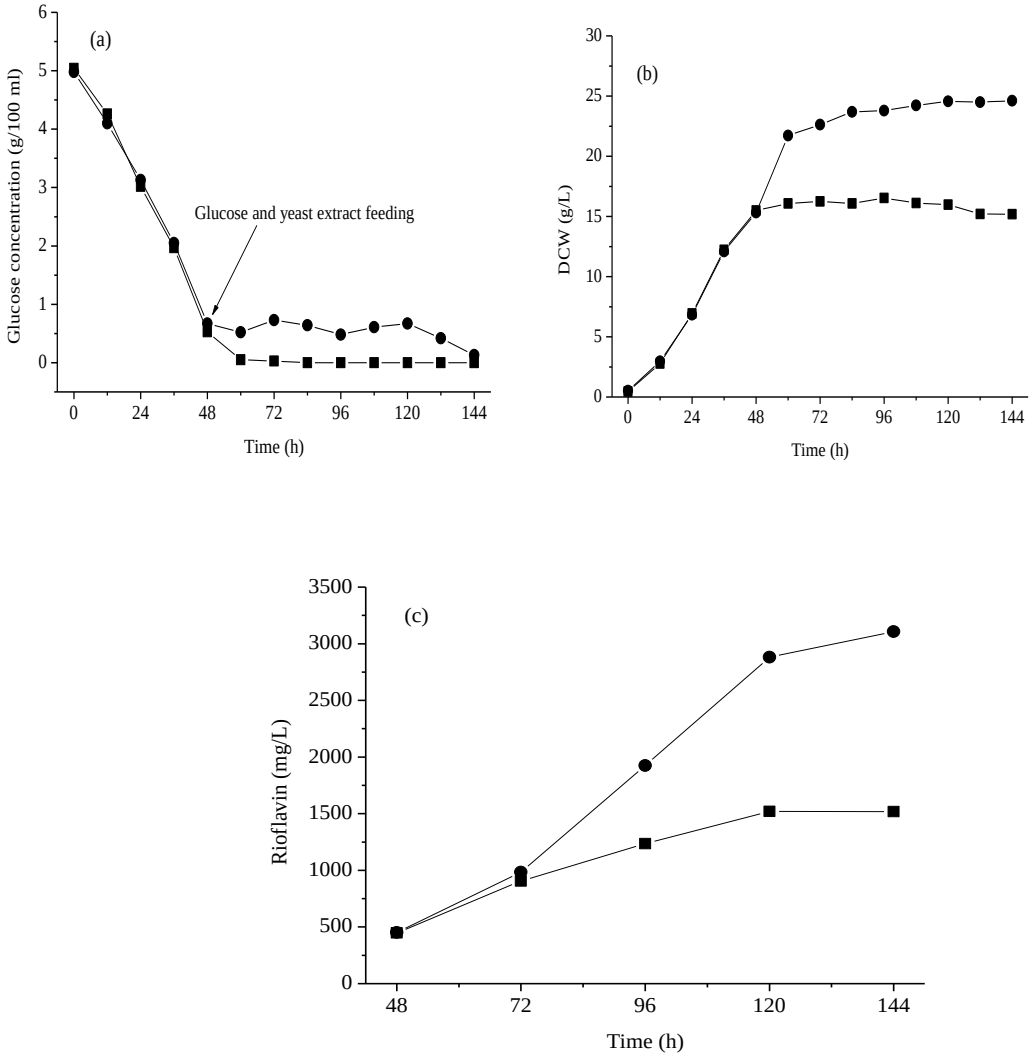

Fig. 2 Time course of glucose consumption, cell growth and riboflavin biosynthesis during *E. ashbyii* fermentation in 15-L fermenter. Symbols: -■- batch fermentation; -●- fed-batch fermentation

Based on the above results, it could be concluded that a certain amount of yeast extract could significantly facilitate cell growth, but the inhibition of riboflavin biosynthesis would be caused by high concentration of yeast extract. Therefore, the concentration of yeast extract was another crucial parameter for riboflavin biosynthesis by *E. ashbyii*.

Development of fed-batch cultivation of *E. ashbyii* in 15 L fermenter

Based on the above results obtained in shake-flask experiments, the strategies of glucose and yeast extract were implemented for riboflavin fermentation in 15 L fermenter. When glucose concentration in the broth dropped to approximately 0.5 g/100 ml, it was maintained at 0.5 to 0.8 g glucose/100 ml during the whole fermentation process. In addition, 60 g/L of yeast extract was continuously fed at 40 ml/h rate from 48 to 96 h of fermentation. Figure 2 shows the typical time course of batch fermentation and fed-batch fermentation of *E. ashbyii*.

From Figure 2a, as the fermentation progressed, glucose concentration of the batch fermentation sharply dropped to approximately 0 at 60 h. Due to lack of glucose for cell growth, a slight increase of DCW was observed from 48 to 120 h, which only maintained at 15.50 to 16.50 g/L, as shown in Figure 2b. Compared to batch fermentation, when glucose and yeast extract was manipulated at 48 h, a remarkable enhancement of DCW was observed after 48 h, which was possibly due to the positive effects of supplied glucose and yeast extract. As a result, the maximal DCW obtained in the fed-batch

fermentation was 24.63 g/L. In consistent with the cell growth, for glucose and yeast extract, a significantly higher level of riboflavin production than that obtained in batch fermentation was observed after 72 h of fermentation (as shown in Figure 2c). As a result, the maximal riboflavin under the two runs was 3107.59 and 1517.59 mg/L, respectively.

The above results illuminated that glucose and yeast extract were an efficient and suitable strategy for riboflavin fermentation, which not only avoided the insufficiency of carbon source and nitrogen source in the fermentation broth for cell growth and riboflavin biosynthesis, but also reduced the inhibition of riboflavin biosynthesis by high concentrations of glucose and yeast extract.

Conclusion

The present study investigates the metabolic regulation of carbon source and nitrogen source on riboflavin fermentation by *E. ashbyii*. It was clearly demonstrated that glucose and yeast extract had a positive promotion on cell growth, but a negative effect on riboflavin biosynthesis would be cause by their high concentrations. Consequently, glucose and yeast extract during fermentation processes was an effective control strategy to increase the final riboflavin production, which could not only avoid substrate-associated growth inhibition, but also reduce riboflavin biosynthesis inhibition by high accumulating concentrations of glucose and yeast extract. As a result, an efficient and suitable implementtation of glucose and yeast extract was established for *E. ashbyii* fermentation in 15 L fermenter.

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