

UDC 577.164.12:665.335.2:582.28
ISSN 1330-9862

preliminary communication

(FTB-1179)

Improvement of Riboflavin Production Using Mineral Support in the Culture of *Ashbya gossypii*

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Received: September 25, 2002

Accepted: April 24, 2003

Summary

Riboflavin production in a culture of *Ashbya gossypii* was enhanced by adding mineral support with adsorbed soybean oil. When the support in an amount of 1 % to the amount of medium was added into the culture at agitation intensity corresponding to impeller rotation rate of 600 rpm, the attained riboflavin concentration was 2.5 g/L at the culture time of 4 days, *i.e.* 1.6 times higher than that in the culture without adding mineral support. Riboflavin yield coefficient based on consumed substrate was also 1.6-fold higher than that in the culture without mineral support. In order to investigate the effect of mineral support on the riboflavin production and mycelial morphology variation, intracellular oil droplets were investigated by staining mycelia with Nile red. The *A. gossypii* cells, growing in submerged culture using soybean oil as a carbon source, were found to form intracellular micro-lipid bodies. The oil droplets became bigger as the culture time increased, and then the riboflavin leakage began, whereas lipid bodies gradually disappeared. When the soybean oil adsorbed on mineral support was added to the culture, the diameter of *A. gossypii* mycelia was much thicker and more riboflavin crystals were accumulated than in the pool of culture without mineral support; and what is more, mycelial autolysis was delayed for 2 days due to the presence of mineral support.

Key words: riboflavin, vegetable oil, mineral support, *Ashbya gossypii*

Introduction

Riboflavin, a yellow, water soluble solid, is widely distributed in plants and animals, and plays an important role in live organisms because it is the precursor of flavin mononucleotide (riboflavin 5'-monophosphate, FMN) and flavin adenine dinucleotide (FAD), which are functioning as coenzymes for a wide variety of enzymes in intermediate metabolism. A daily dose of 0.3–1.8 mg of riboflavin is essential for humans in order to avoid deficiency symptoms like dermatitis (1,2). Furthermore, the vitamin is used as animal feed supplement in the

less pure form. The amounts used in animal feed additives make more than 80 % of industrial products of riboflavin (3).

In the early 1980s, the world consumption of riboflavin was reported to be 1250 t for human and animal uses (4). At present, the amounts of riboflavin production are speculated to be more than 3000 t/year. Riboflavin is produced commercially by chemical synthesis or by biological synthesis, the latter being the preferred

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route because it is cheaper than the former (5). As a result, the current riboflavin production by fermentation using microorganisms is estimated to be about 500 t/year. This process has several merits *e.g.* economically less expensive, reducing waste and energy requirements, and using renewable resources like sugar or plant oil (3). Carbon source consumption in the oil-utilising microorganisms like *Ashbya gossypii* is different from that of glucose. Sometimes the consumption rate is low due to low solubility against water, which may be the reason why it is difficult for a mass production of riboflavin to use vegetable oil as a carbon source. If the oil consumption rate was higher, the fatty acids liberated from oil might be inhibitory to the mycelia growth and subsequently riboflavin production.

Therefore, the optimisation of oil consumption rate in the culture is a key factor to improve the riboflavin production rate. To increase oil consumption rate the oil-water interfacial surface area should be increased, which may be achieved by an increase in agitation rate or addition of surfactant into the culture. However, the increase in agitation rate is not recommended because it causes either an increase in shear stress that damages mycelial growth or operating cost. In the case of the addition of surfactant, the oil suspension may be improved, but mycelia growth and biosynthesis may be inhibited.

In this paper, the effect of mineral support on the riboflavin production and oil consumption in the culture of *A. gossypii* using vegetable oil as carbon source was investigated. As a support, a mineral one was selected, so-called »mountain leather«, which has a stratified structure in which talc is regularly loaded on alternate layers. That structure made a square tunnel of 10.0 × 3.6 Å, which was observed by electron microscope (6). Silica dioxide constituted approximately 53 % of the total components. The specific surface area was 230–380 m²/g (7), which is about 2.5 times higher than that of an active carbon. This support has both hydrophilic and hydrophobic properties (8). Moreover, since the support is highly porous, mycelia are easy to be entangled around the support. Thus, this support may be suitable to co-immobilise oil and mycelia, which may optimise either oil consumption or riboflavin production. Subsequently, we visualised the existence of intracellular oil and intracellular production of riboflavin in the presence or in the absence of mineral support in the culture.

Materials and Methods

Chemicals

Glucose, soybean oil, corn steep liquor, gelatin, potassium dihydrogenphosphate, threonine, zinc sulfate heptahydrate, cobalt (II) chloride, magnesium sulfate heptahydrate and manganese (II) chloride tetrahydrate were supplied by Wako Pure Chemical Industries Co. (Osaka, Japan). Glycine was purchased from Kanto Chemical Co. (Tokyo, Japan). Tween 80 and myo-inositol were purchased from Sigma Chemical Co. Yeast extract was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Glucose, soybean oil, corn steep liquor, gelatin, and yeast extract are the first grade, while other chemi-

cals are a special grade. Mineral support (AID PLUS ML-50D) was supplied by Mizusawa Chemical Industries Co. (Niigata, Japan) (Table 1).

Table 1. Physical properties of mineral support (ML-50D)

Chemical composition	Mg ₈ Si ₁₂ O ₃₀ (OH) ₂ ·46-8H ₂ O
Colour	Light yellow
Specific gravity	2.4
Apparent specific gravity	0.2
Specific surface area (m ² /g)	300
Cumulative pore volume (μL/g)	0.7–0.8
Oil absorption capacity (mL/g)	2.8
pH (3 % suspended solution)	8.5
Average pore size (μm)	0.2
Length of fiber (μm)	5–10
Dimension of tunnel structure (10 ⁻⁴ μm)	5.6–11.0

Strain, media and culture conditions

Strain *Ashbya gossypii* ATCC 10895 was used throughout this study. The cells were grown at pH=6 and 30 °C on solid medium containing (per liter): yeast extract 10 g; glucose 10 g; glycine 3 g and agar 20 g. After 2 days of cultivation on the solid medium the agar plates were stored at 4 °C. Subculture was carried out every 4 weeks (9). Inocula for flask cultures were grown in modified seed medium (10) containing (per liter); corn steep liquor 30 g; yeast extract 9 g and soybean oil 15 g. The pH of the medium was adjusted to 6.8. The medium was dispensed into 500 mL shaking flasks in 100 mL portions. Seed cultivation was carried out at 28 °C on the rotary shaker (Bio Shaker, TAKASAKI Sci. Inst. Co., Japan) at 200 rpm for 40 h.

Riboflavin production

In flask cultures, the amount of 2.5 mL of seed cultures was inoculated into 500 mL shaking flasks containing 50 mL of production medium (10) containing (per liter): gelatin 30 g, corn steep liquor 60 g, glycine 1.5 g, KH₂PO₄ 1.5 g, CoCl₂·6H₂O 2 mg, MnCl₂·4H₂O 5 mg, ZnSO₄·7H₂O 10 mg, MgS₂O₃·6H₂O 1 mg. With the exception of soybean oil, these ingredients were dissolved in 1000 mL distilled water. pH was adjusted to 6.8. The amount of 50 mL of medium was dispensed into 500 mL shaking flasks. To each flask, 50 g/L of soybean oil was added as a carbon source. The pH of media used throughout these investigations was adjusted with 5 N KOH and then the media were autoclaved at 121 °C for 15 min. The cultivation was performed at 28 °C on the rotary shaker at 200 rpm for 7 days.

For the production of riboflavin 5-L jar fermentor (MJC-5CB, Mitsuwa Rikaki, Osaka, Japan) was used. Mixing was achieved using an impeller mounted in the vessel of the fermentor. The diameter of the vessel and impeller is 150 and 72 mm, respectively; height of the vessel is 320 mm. The impeller is 6-flat-blade disc turbine type 17 mm in width and 13 mm in height. To increase agitation intensity two impellers are positioned at

40 and 95 mm from the bottom of the vessel on a centrally located stirrer shaft. Four baffles 10 mm in width are attached to the vessel.

One loop of mycelia grown in solid medium was used as the first seed culture, which was carried out in 500 mL shaking flasks including 50 mL of medium containing per liter: yeast extract 2 g, peptone 20 g, myo-inositol 0.6 g, and glucose 10 g (11) on the rotary shaker at 200 rpm and 28 °C for 24 h. The first seed culture of 1.0 mL was inoculated into the second seed medium, which was the same as the above-mentioned modified seed medium (10).

One hundred mL of inoculum from the second seed culture was inoculated in 2 L of production medium containing the same components as that used for the flask cultures. The production cultures were carried out using 5 L jar fermentor at the culture conditions of 28 °C, 1 vvm aeration with various agitation intensities in the range of impeller rotation speed from 400 to 700 rpm. pH was adjusted to 6.8 before autoclaving and was not controlled during the culture.

To investigate additive effect of mineral support (ML-50D, Mizusawa Co. Ltd. Niigata, Japan) on oil consumption and riboflavin production, various amounts of mineral support were added to the flask separately. Before its addition to culture broth, mineral support was washed with phosphate buffer (pH=7), rinsed twice with distilled water and dried at 105 °C overnight. Then, the defined amount of soybean oil was adsorbed and the oil-absorbed mineral supports were autoclaved. After cooling, the sterilised mineral support was added to the production medium that was sterilised separately. The mineral supports in the culture broth behaved uniformly, because they were small enough in size to be suspended in the culture.

Analytical methods

For the determination of maximum mycelial mass in the fermentor culture, culture broth was taken and filtered through the paper filters (No. 1, Advantec Co. Ltd.). The filtrate was kept at -20 °C for riboflavin assay, and the remaining collected mycelia were washed with distilled water, dried overnight at 105 °C and then weighed (12). When mineral supports were present in the culture, the dry cell mass was determined by subtracting the concentration of these supports previously added to the culture.

The residual soybean oil concentration was measured by the solvent extraction method (13). Five mL of culture broth and 5 mL of *n*-hexane were mixed and vigorously shaken for 2 min in capped Erlenmeyer flasks and then centrifuged at 3000 rpm for 15 min. The upper hexane layer was removed and dried at 80 °C for 3 h to determine the mass of the extracted oil.

For riboflavin measurements, 0.8 mL of filtrate was mixed with 0.2 mL of 1 M NaOH. A volume of 0.4 mL of the resulting solution was neutralised with 1 mL of 0.1 M potassium phosphate buffer (pH=6.0), and the absorbance at the wavelength of 444 nm was measured. Riboflavin concentration was converted using a conversion factor of 127.297 mg/1 A unit/L (14).

This conversion factor was verified by HPLC (PU-980, JASCO, Co. Ltd., Tokyo). Filtered sample was injected onto a Shimpack column (VP-ODS, Shimadzu, Kyoto), and riboflavin was identified at the wavelength of 270 nm with UV detector (UV-970, JASCO, Co. Ltd., Tokyo) (15). Authentic riboflavin was purchased from SIGMA Chem. Co. (St. Louis, Mo).

For visualisation of intracellular lipids, 1 mL of culture broth was rinsed with *n*-hexane and then with phosphate buffered saline (PBS) solution (0.1 M, pH=7.4). After the oil drops attached on mycelia were removed, the mycelia were used for visualisation of intracellular lipids. The mycelia were mixed with 0.5 mL PBS solution and 0.05 mL Nile red solution (Nile red 10 mg/acetone 1000 mL) (16). The Nile red was used for staining intracellular lipid droplets, which were detected by fluorescence (excitation, 530 nm; emission, 605 nm). After the storage of the mixture of Nile red and mycelia for 30 min in a dark place, stained lipid bodies were photographed using fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus, Tokyo, Japan). In the presence of mineral support in the culture, the mycelial morphology and riboflavin were observed as described above, but without staining with Nile red. Extra and intra oil droplets were distinguished from mineral supports or mycelia. Since riboflavin is yellow, its existence was identified in the standard microscope without any staining.

Results

Riboflavin production in the culture of *A. gossypii* in flask

Fig. 1 shows typical riboflavin production using soybean oil as a sole carbon source. Riboflavin reached its highest value of 680 mg/L at culture time of 120 h, when soybean oil was consumed almost completely. When the soybean oil was almost consumed, mycelial mass reached its maximum, but gradually decreased due to autolysis (data not shown).

Mycelia were grown at an early growth phase, and simultaneously accumulated a lot of fatty acids inside themselves due to the uptake of soybean oil (Fig. 2.). A thin mycelium at the culture time of 1 day started thick-

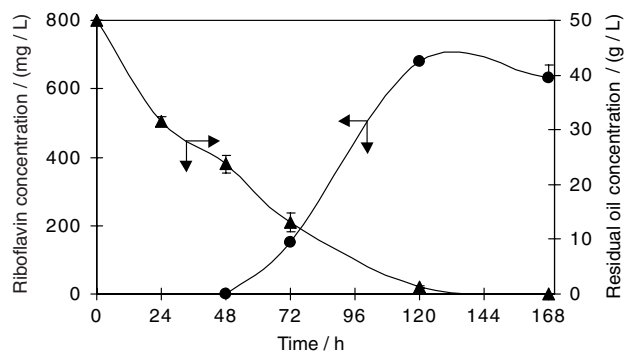


Fig. 1. Riboflavin concentration (closed circle) and residual oil concentration (closed triangle) in the flask culture of *A. gossypii*. Experiments were carried out three times and average values are shown with standard error bars

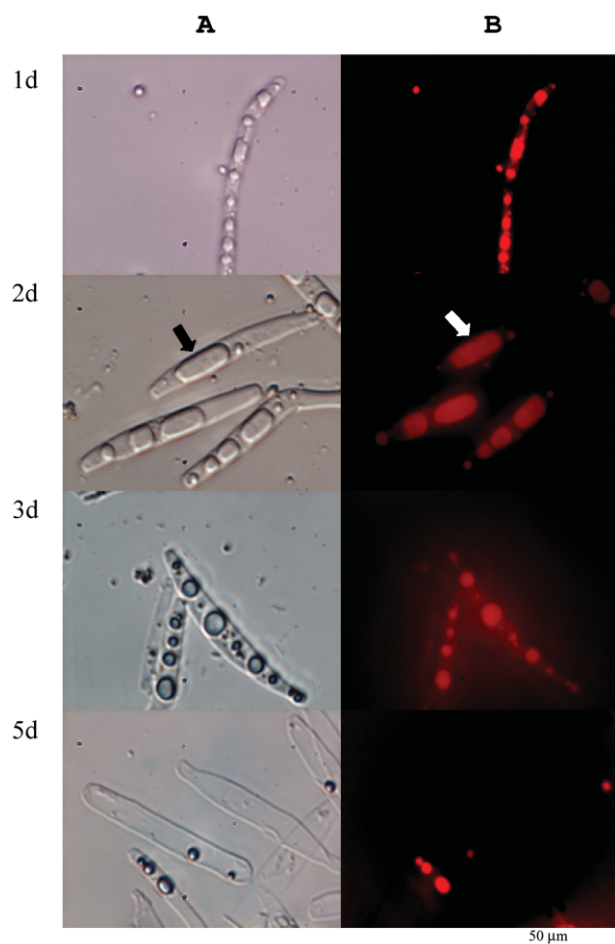


Fig. 2. Change on mycelial morphology in the flask culture of *A. gossypii*. Images of A and B were taken from the microscope and fluorescent microscope, respectively. The magnification of images was 600. Numbers denote culture time. Circles or ellipses in A and red fluorescents in B denote lipid droplets (arrows), respectively

ening at the culture time of 2 days due to the accumulation of fatty acids (2 d in Figs. 2A and 2B). This might cause an increase in the dry cell mass. With the progress of riboflavin biosynthesis intracellular fatty acids were converted to riboflavin, and the accumulated riboflavin leaked from mycelia with the progress of cultivation (see 3 d and 5 d in Figs. 2A and 2B).

Mycelia at the culture time of 5 days show a small amount of oil inside mycelia, and cytosol was empty due to the leakage of riboflavin (5 d in Figs. 2A and 2B). This might cause a decrease in dry cell mass.

Effect of the addition of mineral support

To investigate the effect of the addition of mineral support on riboflavin production in the culture of *A. gossypii*, the support was added to the culture in the amounts of 1, 2, 3 and 5 %, respectively (Fig. 3). The addition of mineral support increased the riboflavin production, although it had an unknown positive effect. With the addition of 1 % of the support, the increase was even 56 % higher than in control. Riboflavin production decreased with the increase of the concentration of mineral support over 1 %.

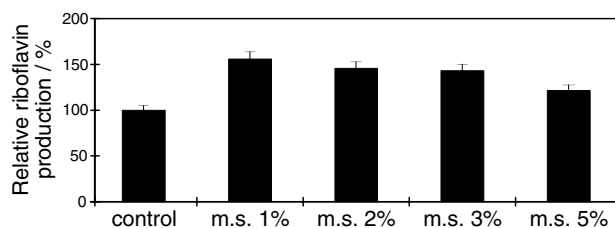


Fig. 3. Effect of mineral support on riboflavin production in flask culture. ms indicate cultures with different percentage of mineral support

Effect of agitation on riboflavin production

In the case of the riboflavin production by *A. gossypii*, plant oil showed to be advantageous with respect to glucose as a carbon source. Therefore, the enhanced oil consumption rate in culture may be a key factor in increasing riboflavin production. There are some methods to increase the oil-water interfacial surface area. Appart from the supplementation of surface-active agents, high agitation speed is another one. In contrast with bacteria, however, since fungal growth is characterised as a hyphal growth which results in formation of pellet or agglomerated filaments, high agitation may damage a mycelial growth. To cultivate fungi sensitive to shear stress caused by a mechanical agitation, it is important to optimise the agitation rate.

Table 2 shows the cell growth, rate of oil consumption and riboflavin production in the culture at different agitation intensity of impeller rotation speeds of 400, 600, and 700 rpm, respectively. Dry cell mass increased with the increase in the agitation intensity. Riboflavin concentration at the impeller rotation speed of 700 rpm was 1.60 g/L, which was slightly higher than that at 600 rpm. Maximum oil consumption rate also increased up to 600 rpm, whereas the effect of further intensification of agitation was negligible. The maximum riboflavin production rate increased proportionally with the oil consumption rate. The highest product yield coefficient based on the consumed amount of oil was found to be 3.2×10^{-2} g/g at the impeller rotation speed of 700 rpm. Hence, in order to increase the oil consumption rate in the culture, the agitation of 700 rpm was preferred. At higher agitation rate an improvement of riboflavin production cannot be expected.

Additive effect of mineral support in fermentor cultures

When 1 % of mineral support was added into the culture agitated with impeller rotation rate of 400 rpm, oil consumption rate decreased to that of control culture (A in Fig. 4 I), while the attained riboflavin concentration was similar to that of the control batch (B in Fig. 4 I). However, in the case of the cultures agitated with impeller rotation speed of 600 rpm, although the oil consumption rate was similar to or lower than that of the control batch (A in Fig. 4 II), there was a difference in the riboflavin production between the cultures in the presence and in the absence of mineral support (B in Fig. 4 II). In the culture with mineral support agitated with impeller rotation speed of 600 rpm, riboflavin con-

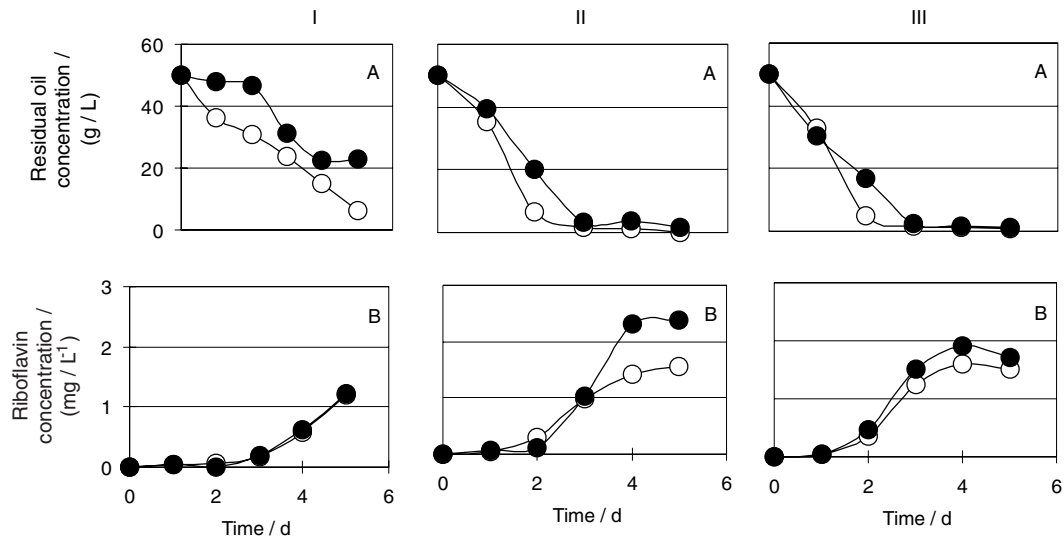


Fig. 4. Effects of agitation rates on concentrations of residual oil (A) and riboflavin (B) in jar fermentor culture with (closed circle) or without (open circle) supplementation of mineral support. Agitation rates in cultures were controlled at 400 (I), 600 (II), and 700 rpm (III), respectively. Experiments were carried out twice and average values are shown

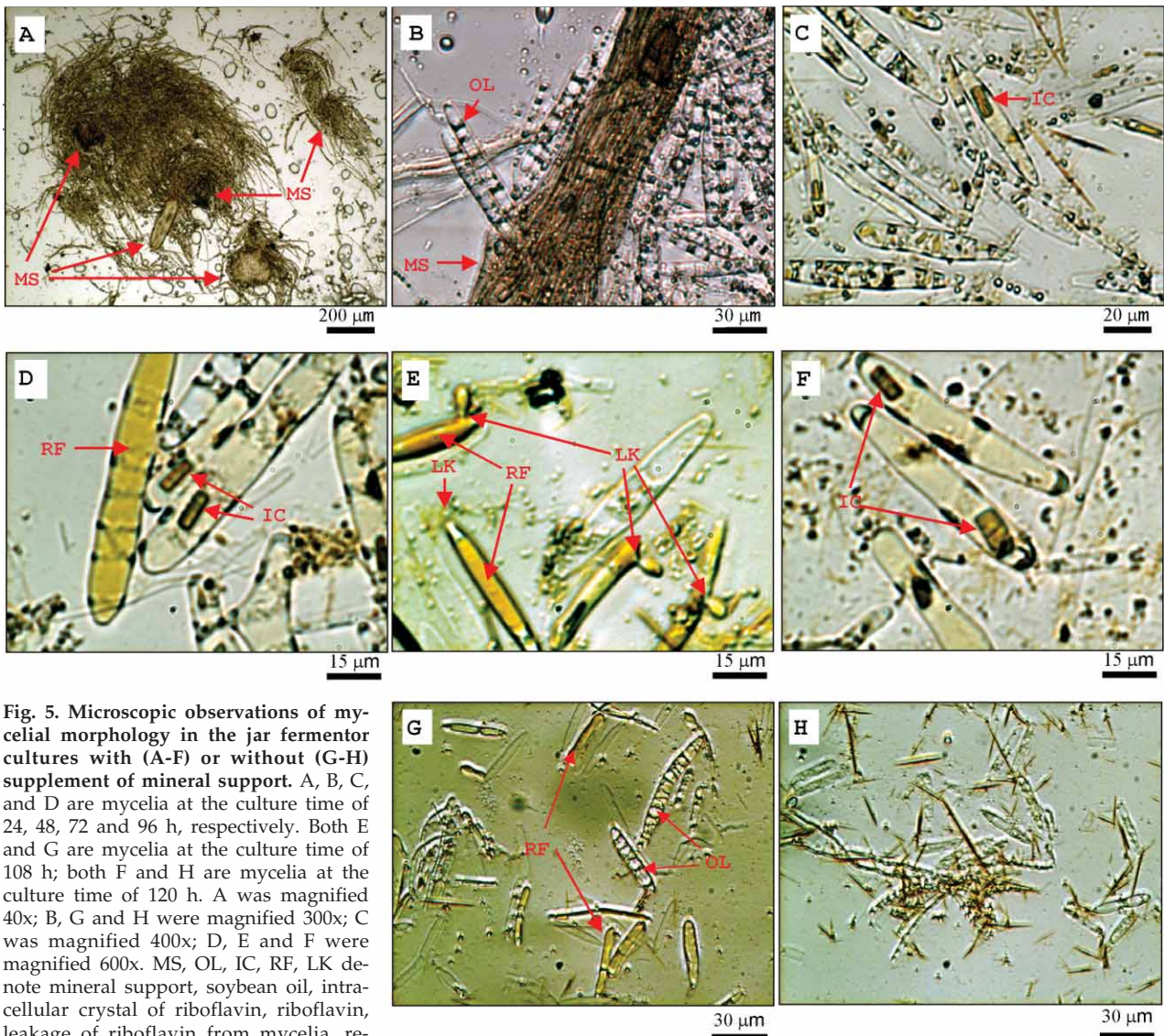


Fig. 5. Microscopic observations of mycelial morphology in the jar fermentor cultures with (A-F) or without (G-H) supplement of mineral support. A, B, C, and D are mycelia at the culture time of 24, 48, 72 and 96 h, respectively. Both E and G are mycelia at the culture time of 108 h; both F and H are mycelia at the culture time of 120 h. A was magnified 40x; B, G and H were magnified 300x; C was magnified 400x; D, E and F were magnified 600x. MS, OL, IC, RF, LK denote mineral support, soybean oil, intracellular crystal of riboflavin, riboflavin, leakage of riboflavin from mycelia, respectively

Table 2. Effect of agitation on soybean oil consumption rate and riboflavin production rate in batch culture of *A. gossypii*

Agitation: Impeller rotation speed (rpm)	400	600	700
Maximum concentration (g/L) of			
Dry cell mass	13.54	17.60	19.00
Riboflavin	1.20	1.55	1.60
Maximum rate (g/L/h) of			
Oil consumption	0.50	0.91	0.94
Riboflavin production (10^{-2})	2.60	2.90	3.70
Yield coefficient (10^{-2} g riboflavin/g consumed oil)			
	2.4	3.1	3.2

Cultures were carried out in jar fermentor using 50 g/L of soybean oil as carbon source with the same culture conditions apart from agitation rate

centration reached to 2.5 g/L, *i.e.* 60 % above that of the control. At the impeller rotation speed of 700 rpm, the culture with mineral support supplement showed 1.3 times higher riboflavin concentration than in the control batch (B in Fig. 4 III). This finding suggests that mineral support supplementation can improve riboflavin production in the culture of *A. gossypii*. The effect of mineral support addition on cell growth was not observed (data not shown).

In the control culture, oil consumption rate depended on the mechanical agitation. However, in the culture with added mineral support, oil consumption rate was slightly lower than in that without mineral support.

Observation of morphological changes in the cultures

At the culture time of 24 h grown slim mycelia clung to the mineral support, and formed big collective mycelia around the mineral supports (Fig. 5 A). However, the grown mycelia began to consume oil and accumulate it inside themselves, which made the mycelia thicken (Fig. 5 B). The accumulated droplets were verified to be oil by Nile red staining (Fig. 2 B). After 3 days of culture, clung mycelia were peeled off from the mineral support, and began to produce riboflavin, which was accumulated as intracellular riboflavin (C-D in Fig. 5). With the progress of the cultivation, most of the produced riboflavin (yellow pigment) was inside the mycelia, which were swollen (Fig. 5 D). The produced riboflavin leaked from the mycelia due to the increase of riboflavin concentration inside them (see LK in Fig. 5 E). Finally, the mycelia became empty as a mere shell of mycelia, or formed intracellular crystals of riboflavin (Fig. 5 F).

In the case of culture without mineral support (as shown in Figs. 5 G and 5 H), though there were the same culture conditions apart from mineral support addition, mycelia got thinner and the accumulated intracellular riboflavin amount was lower than that of the culture supplemented with mineral support; intracellular riboflavin crystals were not observed. At the culture time of 120 h, cell debris is shown as extracellular riboflavin (Fig. 5 H), which is not shown in Figs. 5 E and 5 F.

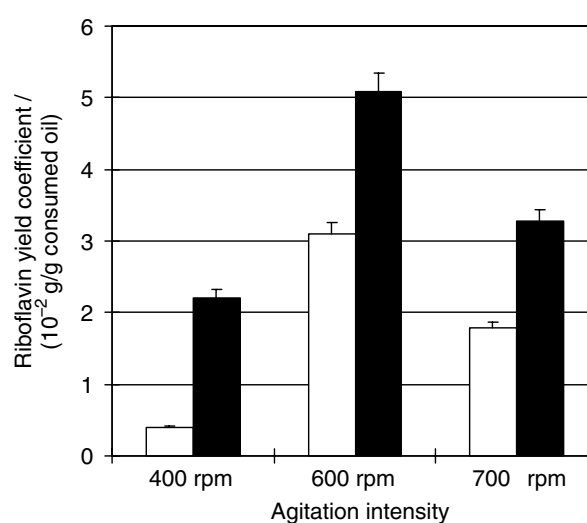


Fig. 6. Riboflavin yield coefficient between cultures with (closed bars) and without (open bars) supplementation of mineral support. Riboflavin yield coefficient (10^{-2} g riboflavin/g consumed oil) was calculated from produced riboflavin concentration on the basis of consumed soybean oil concentration

Comparison of riboflavin yield coefficient in the jar fermentor cultures with and without mineral support

Riboflavin yield coefficient in fermentor cultures with and without mineral support is shown in Fig. 6. Riboflavin production ($3.2 \cdot 10^{-2}$ and $5.2 \cdot 10^{-2}$ g/g consumed oil) was the highest at the impeller rotation speed of 600 rpm. The highest difference in riboflavin yield between cultures with and without mineral support (1.6 or 1.8 fold) was observed at the impeller rotation speed of 400 rpm. Although the amount of consumed oil was similar, riboflavin yield of culture supplemented with mineral support was at least 60 % higher than of that without mineral support. Usually riboflavin production was proportional to the oil consumption rate. Thus, in the culture supplemented with mineral support, if the oil consumption rate increased, riboflavin production would also increase. This means that mineral support has greater effect on riboflavin production than oil consumption.

Discussion

Ashbya gossypii fungus has an ability to consume plant oil as carbon source and to produce riboflavin (10, 17). Accumulation of triglycerides in the mycelia was confirmed by using Nile red staining method, as shown in Fig. 2. After 2 days of culture, oil droplets were verified, but after 3 days of culture the accumulated oil droplets decreased gradually and the riboflavin production began.

Oil consumption rate by oil utilising microorganisms may be considered to be dependent on the interfacial surface area between oil and mycelia dependent on microorganism's morphology. To increase interfacial surface area, an addition of a surfactant was considered. Tween 80 was added to the culture but mechanical agitation caused shear stress, as a result of which the stability of reactor culture decreased (data not shown). That is why the use of Tween 80 for improving oil emulsification was given up. On the other hand, agitation rate was increased to 700 rpm to improve the oil consumption rate.

Mineral support was one of the possible additions in culture of microorganisms using vegetable oil as carbon source (13). When the support was added into the culture of *Streptomyces* sp., the soybean oil was suspended as fine oil droplets, and mycelia were also attached on the support. That caused the formation of an oil-mycelia complex inside or around the support, which provided a larger specific surface area of oil. When 15 g/L of the support was used in a batch culture of *Streptomyces* sp. with 50 g/L of soybean oil, the maximum attained cephamycin C concentration was 2.8 g/L, which was 2.2 times higher than that without the support (13). This result was in agreement with the kinetic model of oil-mycelia complex around the support (18).

Similarly, in the case of the culture of *A. gossypii* the mineral support was assumed to be effective in oil consumption rate, but it was slightly slower than that without mineral support (see Fig. 4), which was the opposite result from that with the *Streptomyces* culture. Although the oil consumption rate was not improved, the riboflavin production rate and its yield were 60 % higher than those of control cultures (see Figs. 4 and 6). This suggests that the oil consumption rate of *A. gossypii* may not be limited. The reason why the mineral support improved the riboflavin production is not clear. Judging from Fig. 5 the mycelial morphology of the culture with mineral support was clearly different from that of the cultures without mineral support, enabling accumulation of intracellular riboflavin. During the cultivation, one can consider that the life cycle of mycelium would be defined as follows: mycelial growth, accumulation of soybean oil, conversion of soybean oil to the intracellular riboflavin, leakage of riboflavin, and autolysis of mycelia or sometimes spore formation.

It is significant that the conversion period of substrate to riboflavin in the mycelia can be prolonged to improve riboflavin production. This can be achieved by adding mineral support. In the culture without mineral support only few mycelia at the culture time of 108 h were observed (see Fig. 5 G).

Those mycelia were unhealthy, autolysed or empty inside. At the 120th h of cultivation without mineral support, neither mycelia nor intracellular riboflavin were observed (see Fig. 5 H). The electronic charge of the mineral support could be one possible cause for this difference. In the presence of mineral support, mycelia may be stable and riboflavin production period may be extended. Therefore, the method described in this experiment proves to be a very convenient one to improve riboflavin production in the culture of *A. gossypii*.

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Poboljšanje proizvodnje riboflavina koristeći mineralnu podlogu u kulturi *Ashbya gossypii*

Sažetak

Proizvodnja riboflavina u kulturi *Ashbya gossypii* poboljšana je dodatkom mineralne podloge na kojoj je adsorbirano sojino ulje. Ako se u kulturu doda mineralna podloga u količini od 1 % medija pri 600 o/min miješalice, dobivena koncentracija riboflavina iznosi 2,5 g/L podloge tijekom četiri dana uzgoja, što je 1,6 puta više nego kada se kultura uzgaja bez mineralne podloge. Koeficijent iskorištenja riboflavina, računajući na utrošeni supstrat, bio je isto tako 1,6 puta viši nego u kulturi bez dodatka mineralnoga supstrata. Da bi se ispitao učinak mineralne podloge na proizvodnju riboflavina i morfološke promjene micelija, ispitane su intracelularne kapljice ulja, bojeći ih s Nil crvenim. Stanice *A. gossypii*, uzgojene submerzno uz sojino ulje kao izvor ugljika, stvaraju intracelularne mikrolipidne kapljice. Uljne se kapljice povećavaju tijekom uzgoja, a kada započne otpuštanje riboflavina, one postupno nestaju. Kada se kulturi doda sojino ulje, adsorbirano na mineralnoj podlozi, promjer je micelija *A. gossypii* puno veći i više se kristala riboflavina nakuplja nego pri uzgoju bez mineralne podloge, a osim toga autoliza je micelija produžena dva dana zbog mineralne podloge.