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Physio-Morphological Changes in a Riboflavin Producer *Eremothecium ashbyii* DT1 and UV Mutants in Submerged Fermentation

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Abstract By UV-irradiation of *Eremothecium ashbyii* DT1, a highly flavinogenic mutant (UV-18-57) and a nonflavinogenic mutant (UV-85) were obtained. The physio-morphological characteristics of these three strains were studied on glucose medium in submerged fermentation. Glucose utilization and mycelial growth occurred in 0–2 days of fermentation. By the third day, the biomass had declined. Extracellular riboflavin excretion was distinct from the second day, reaching a maximum rate by the fourth day. The hyphae of the highly flavinogenic mutant UV-18-57 were broader than DT1, while the nonflavinogenic UV-85 hyphae were very thin. Riboflavin accumulation was high in UV-18-57 (extracellular riboflavin, 825 μg/ml, and intracellular, 490 μg/ml) and caused the mycelia to swell into bulbous forms. Riboflavin accumulation was less in DT1 (108 μg/ml extracellular and 24 μg/ml intracellular) and correspondingly its hyphae were thinner than those of UV-18-57 and swollen bulbous mycelia were not prominent. UV-85 was nonflavinogenic and, accordingly, its morphological characteristics included long thin filaments with no intracellular riboflavin accumulation. A large number of greenish fluorescence spores were seen in UV-18-57, whereas DT1 had less spores and UV-85 was nonsporulating. Sporulation is correlated with riboflavin production. UV-18-57 had better mycelial integrity and lysis started only by the seventh day, whereas DT1 and UV-85 started to lyze earlier by 4–5 days. By the late stage of fermentation (eighth day), DT1 had a few long, thin filaments indicating some secondary growth, whereas UV-85 showed a compact pellet form of mycelia. Most mycelia of UV-18-57 still appeared intact.

Key words: *Eremothecium ashbyii*, glucose, morphology, physiology, riboflavin, UV mutants

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The filamentous hemiascomycete *Eremothecium ashbyii* is a biotechnologically important producer of riboflavin. Much work has been carried out on *E. ashbyii* in examining its nutritional requirements and in studying its mechanism of biogenesis of riboflavin [1, 16, 23]. Very little attention has been paid to the physiology of this organism in connection with riboflavin production [7, 8, 15, 18, 19]. There are no reports on time-course studies to correlate morphological changes with different phases of riboflavin fermentation. Therefore, it is of scientific interest to systematically further characterize this unique organism with respect to its morphology, physiology, and regulation of riboflavin production.

The present work describes the morphological changes of *E. ashbyii* DT1, and its highly flavinogenic (UV-18-57) and nonflavinogenic (UV-85) mutants during glucose fermentation in submerged culture.

MATERIALS AND METHODS

Organisms

Eremothecium ashbyii NRRL 1363 wild-type was obtained from the National Center for Agricultural Utilization Research (NCAUR), Illinois, U.S.A. From this, a flavinogenic *E. ashbyii* DT1 was isolated by plating on potato dextrose agar medium containing 0.1% of sodium dithionite. Sodium dithionite allows selection of yellow flavinogenic colonies easily. By repetitive UV mutagenesis of *E. ashbyii* DT1 mycelia in sterile distilled water (Phillips 15W UV tube, at 15 cm distance, and 60–120 sec. exposure), a highly flavinogenic mutant (UV-18-57) and a nonflavinogenic mutant (UV-85) were obtained [17]. The three cultures (DT1, UV-18-57, and UV-85) were used in the present study. Cultures were maintained on potato dextrose agar (PDA) slants containing (g/l): potato mash, 200.0; dextrose, 20.0, and agar, 20.0, cultured for

5 days at 30°C, stored at 4°C, and subcultured once a fortnight.

Riboflavin Fermentation in Glucose Medium

Mycelia collected from agar slants were inoculated into 50 ml glucose-peptone-yeast extract (GPY) medium (in a 250 ml amber colored flask) containing (g/l): glucose, 20.0, peptone, 10.0, and yeast extract, 4.0. The initial pH of the medium was adjusted to 6.0 with 1 N NaOH before sterilization. The cultures were incubated for 48 h at 30°C on a rotary shaker at 200 rpm.

The mycelia from GPY broth were transferred (1%, v/v) to 50 ml of glucose-peptone-yeast extract mineral base (GPYMB) medium (in a 250 ml amber colored flask) containing (g/l): glucose, 30.0; peptone, 8.0; yeast extract, 2.0; KH_2PO_4, 2.0; NaCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; and Tween-80, 1.8% (v/v) [10]. The initial pH of the medium was adjusted to 6.0 with 1 N NaOH before sterilization. The cultures were incubated for 7–8 days as above. At various time intervals, mycelia were collected by filtration through a G2 filter, washed, and wet mounts (without staining) were observed under a bright field microscope (Nikkon Labophot-2 fitted with a Nikkon-FDX-35 camera).

Analytical Procedures

Extracellular riboflavin: Assayed exactly as per IS [6]. The culture broth, after filtration through Whatman filter paper 1, was taken in each of two test tubes labeled A and B. One ml of working standard solution of riboflavin was added to tube A and 1 ml of distilled water was added to tube B. The solutions were then acidified by the addition of glacial acetic acid (1 ml). Then, 0.5 ml of 4% (w/v) $KMnO_4$ was added to oxidize the impurities. After exactly 2 min, 0.5 ml of 3% H_2O_2 solution was added to both the tubes to decolorize the solutions. The fluorescence of the solutions was measured using a fluorometer with 366 nm primary and 475 nm secondary filters (Elico CL-53). To tube B, about 10 mg of sodium dithionite was added and the fluorescence was measured within 10 sec. The riboflavin is measured in terms of the difference between the fluorescence before and after chemical reduction by sodium dithionite.

Intracellular riboflavin: Measured as per Kapralek [7]. The dried mycelium, along with filter paper, was shredded into small pieces in 0.02 N HCl, autoclaved at 121°C for 20 min, and centrifuged at 3,000 rpm. Riboflavin extracted into the supernatant was assayed fluorimetrically as above.

Glucose utilization: Followed using a YSI Biochemistry analyzer (YSI 2700 Select, Yellow Springs Instruments, U.S.A.).

Biomass: Estimated by filtering the mycelia from the broth using Whatman filter paper 1, and then washing and drying the mycelium at 80°C for 24 h.

pH: of the fermentation broth was measured with a Precision pH meter (Philips, PR 9405 M).

RESULTS AND DISCUSSION

Glucose fermentation to riboflavin appeared to be in three stages. In the early stage of 0–2 days, the *E. ashbyii* parental strain DT1 utilized around 40% glucose with peak biomass production (4 mg/ml) (Fig. 1a), while the two UV mutant derivatives (UV-18-57 and UV-85) consumed 99% glucose corresponding to near doubling in biomass (8.6 mg/ml)(Figs. 1b and 1c). The biomass steeply declined by the third day in DT1 but only gradually in the UV mutants (Figs. 1a-1c). A second biomass peak was indicated in 6–7 days in DT1, possibly due to the residual glucose, but it was not prominent in the UV mutants where the substrate was exhausted. Total glucose consumption was reported within 36 h in *E. ashbyii* and *A. gossypii* (a closely related riboflavin producer) [7, 8, 20, 21]. However, strain DT1 appeared to differ as it utilized only about 60% glucose in eight days, while its UV mutants consumed 99% in 24 h.

Extracellular riboflavin production was evident from the second day in the mutant UV-18-57 and was nearly 8-fold higher (825 µg/ml) than the parental strain DT1 (108 µg/ml). UV-85 was nonflavinogenic (10–15 µg/ml). The intracellular riboflavin in UV-18-57 was also considerably higher (490 µg/ml) than in DT1 (24 µg/ml) (Figs. 1a and 1b). Riboflavin is a natural cell metabolite and its excretion is known to occur after peak biomass production, which is typical of a "pseudosecondary metabolite" [1].

By day one, the pH decreased by 1.3 units in both the UV mutants and by 0.6 units in DT1 which corresponded with its less glucose consumption (Figs. 1a-1c) The initial pH drop was possibly due to the accumulation of pyruvate or acetic acid, and increased to 6.1–6.6 later by reutilization of pyruvate and complex organic nitrogen sources.

The microscopic morphology of the parental *E. ashbyii* and the mutants differed from each other under the shake flask fermentation conditions (Fig. 2 and Table 1), and the changes could be related to their physiological status. The highly flavinogenic mutant UV-18-57 showed very broad hyphae initially, which transformed into very swollen bulbous hyphae fragments filled with yellowish fluid and dark yellow crystals, presumably riboflavin. Mycelial integrity appeared stable without lysis even upto the seventh day. In contrast, the UV-85 mutant started to lyze very early by the fifth day. DT1 showed characteristics mostly intermediate between flavinogenic UV-18-57 and nonflavinogenic UV-85 with less thick hyphae, moderate filament stability and lysis.

The swelling of UV-18-57 hyphae into bulbous forms (0–2 days) and their decrease in size later (Fig. 2h) concomitant with increase in extracellular riboflavin and decrease in intracellular riboflavin (Fig. 1b) suggested initial riboflavin accumulation which was released extracellularly by lysis. In this context, a recent work by Forster *et al.* [2] reports that riboflavin accumulation in the vacuoles of *A. gossypii* and that inactivation of the *VMA1* gene causes the

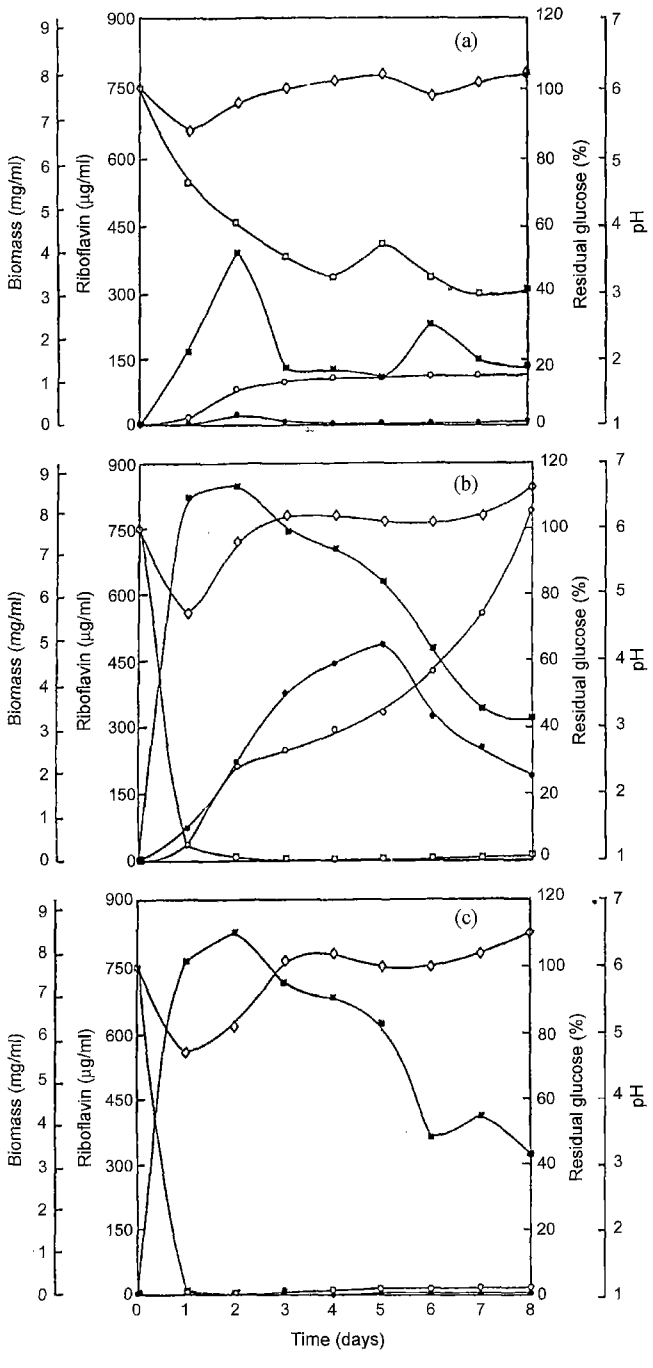

Fig. 1. Time course of riboflavin production during growth on GPYMB medium by *E. ashbyii*.
(a) DT1, (b) UV-18-57, and (c) UV-85 (○, extracellular riboflavin; ●, intracellular riboflavin; □, residual glucose (%); ■, biomass; ◇, pH).

vacuole to lyze and release riboflavin. Further investigations on intracellular organelles of *E. ashbyii* are necessary to draw any conclusions on similarity here. It was interesting to note that UV-18-57 hyphae also contained numerous hyaline droplets in the early stage (Fig. 2 and Table 1). Schmidt *et al.* [20] and Stahmann *et al.* [21] have indicated formation and degradation of lipid bodies in *A. gossypii* prior to riboflavinogenesis. Lipid accumulation during growth on glucose was also suggested in *E. ashbyii* [22]. Vijayalakshmi and Chandra [24] reported riboflavin production utilizing various lipids (sunflower oil, olive oil, and triolein) as carbon sources and also observed lipase activity. The production of lipids and lipases by fungi is a frequently described phenomenon with some promise for industrial applications [3, 13, 14, 25]. The hyaline droplets could be the lipid bodies accumulating prior to riboflavin synthesis. This interpretation is supported by the lack of these bodies in the nonflavinogenic mutant UV-85.

Recently, *E. ashbyii* was reported to have thin cell walls compared to the thick, rigid double-layered cell walls of *Trichoderma reesei* or *Penicillium chrysogenum* [9]. This could explain rapid protoplast formation in *E. ashbyii* [9, 10]. The formation of swollen bulbous mycelial fragments in UV-18-57 in the early stage of riboflavin fermentation possibly represents the response of this organism to osmotic tension changes due to riboflavin synthesis. Owing to the thin cell walls, the mycelia swell up. Very few bulb forms were seen in DT1 and nonflavinogenic mutant UV-85, because of nonaccumulation of riboflavin reinforcing the above inference.

Similarly, Yu *et al.* [26] observed that *Trichoderma reesei* forms long, thin hyphae, and branching at 24 h; long thick hyphae with optimum cellulase production between 48-100 h; thin and shorter hyphae at 120 h; and hyphal fragmentation and autolysis at 148 h. The onset of antibiotic in actinomycetes was also closely related to the mycelium morphological changes [11]. Huh and Choi [4] have observed the effect of environmental stress on morphological transition from unicellular yeast to a pseudohyphae-like morphology in *Hansenula anomala* B-7 strain. Lee *et al.* [12] studied the effect of ammonium phosphate on the mycelial growth and exopolysaccharide production of *Ganoderma lucidum*. At low levels of ammonium phosphate, there was improved mycelial growth by maintaining a more filamentous morphology than at high concentrations with a pellet form of growth.

UV-18-57 was highly sporulating while DT1 was poor in sporulation, and UV-85 was nearly nonsporulating (Fig. 2 and Table 1). Earlier workers have correlated sporulation with riboflavin overproduction [7, 22]. Nonsporulating *E. ashbyii* produced less riboflavin [1]. Mutant UV-18-57 is highly sporulating and produces more riboflavin, in agreement with these reports.

Our study is the first attempt to link the physiological status of the *E. ashbyii* parental and mutant strains to their morphological variations in submerged cultivation. Understanding the physio-morphological changes would enhance a better control over the riboflavin fermentation process, apart from being of fundamental scientific interest on *E. ashbyii*s phylogenetic relationships.

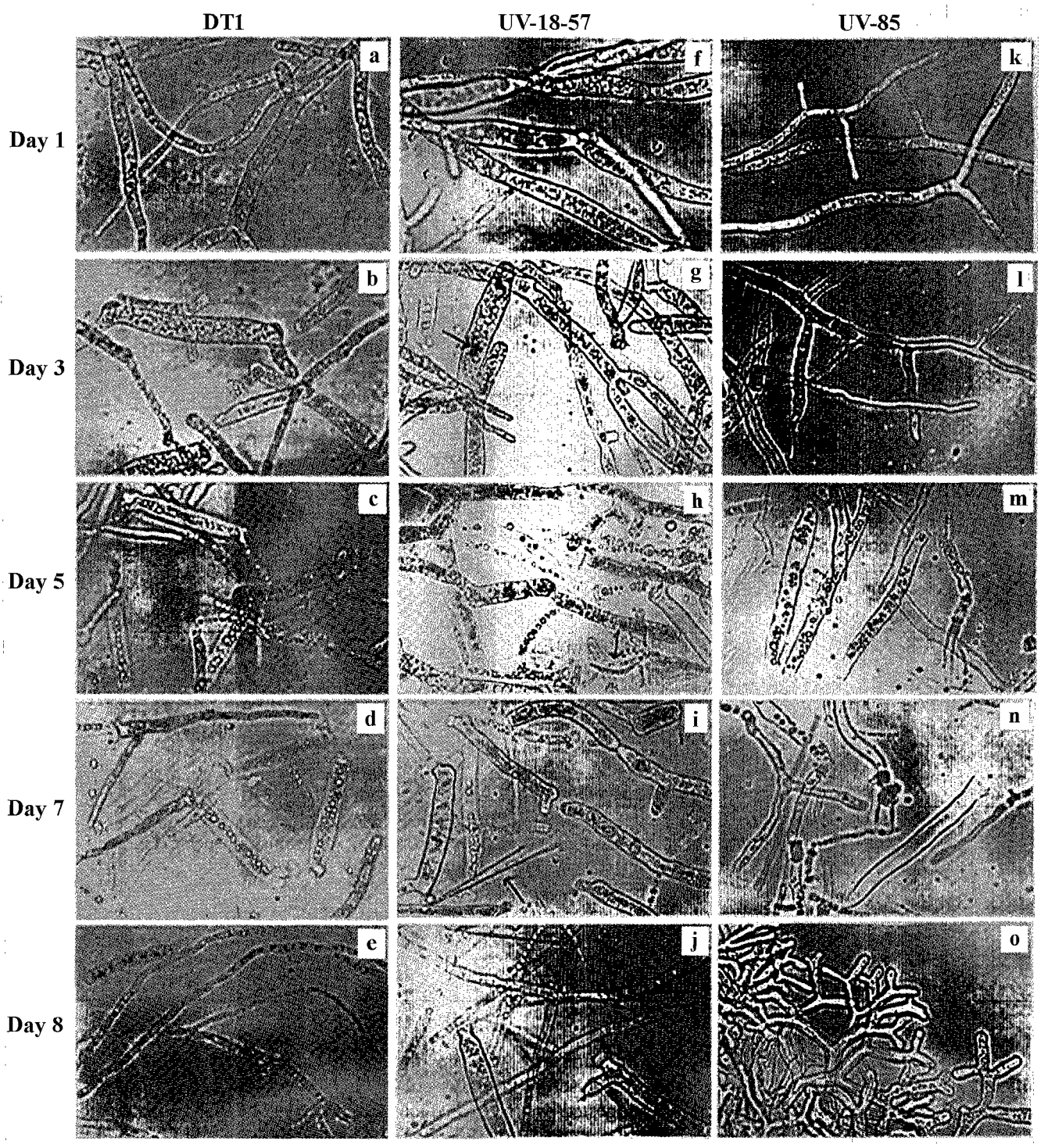

Fig. 2. Morphological variations in the *E. ashbyii* DT1 and UV mutants mycelia at different stages of fermentation in shake flasks (Bright-Field Microphotographs (x400)). (Please refer to Table 1 for description).

It is evident that swollen bulbous-hyphae forms as in UV-18-57 are good indicators of riboflavin oversynthesis. Any attempts to trigger the formation of yellow bulbous hyphae and delay the onset of lysis is likely to significantly improve riboflavin production. Fermentative synthesis of riboflavin has various applications such as animal feed supplement, probiotic growth promoters, etc. Another scope of the study was to enable rapid screening for high yielding riboflavin strains by mere microscopic observation.

Table 1. Morphological variations in *E. ashbyii* which correlate with its physiological status during glucose fermentation.

Time (days)	*E. ashbyii* parental DT1	*E. ashbyii* mutant UV-18-57	*E. ashbyii* mutant UV-85
0–2	Medium size filaments	Long broad filaments	Very thin long filaments
	Hyaline droplets	Numerous hyaline droplets	Very few hyaline droplets
	Lateral growth formation (Fig. 2a)	Lateral growth (Fig. 2f)	Lateral growth (Fig. 2k)
	Peak biomass	Peak biomass	Peak biomass
	Only 40% of glucose utilized	99% glucose utilized	99% glucose utilized
	Extra- and intracellular riboflavins synthesized	Extra- and intracellular riboflavins synthesized	Negligible extra- and intracellular riboflavins synthesized
	pH decreased (Fig. 1a)	pH decreased (Fig. 1b)	pH decreased (Fig. 1c)
3–5	Moderate swelling of filaments but distinct bulbous forms not seen (Fig.2b)	Highly swollen bulbous mycelial fragments (Fig. 2g)	No swelling or bulbous forms; filaments very thin and appear empty (Fig. 2l)
	Yellowish fluid accumulation not distinct	Hyaline droplets convert to yellowish fluid	No yellowish fluid accumulation
	No riboflavin crystals	Riboflavin crystals seen inside bulbous forms (Fig. 2g) (indicated by arrow)	
	Few cells start to lyze (Fig. 2c); few hyaline spores	Lysis not seen; numerous greenish fluorescence spores (indicated by arrow) (Fig. 2h)	Start of lysis distinct (Fig. 2m); no spores
	Rapid decline of biomass	Slow decline in total biomass	Slow decline in total biomass
	Slow increase in extracellular riboflavin; negligible intracellular riboflavin	Peak intracellular riboflavin accumulation; increase in extracellular riboflavin	No extracellular or intracellular riboflavin
	pH stabilized at 6.0 to 6.1 (Fig. 1a)	pH stabilized at 6.3 (Fig. 1b)	pH stabilized at 6.3 (Fig. 1c)
7	Lysis distinct; fragmented empty hyphae and few intact hyphae (Fig. 2d)	Start of lysis (Fig. 2i)	Lysis advanced with almost empty and fragmented hyphae (Fig. 2n)
	Second biomass peak evident (Fig. 1a)	Biomass declines (Fig. 1b)	Biomass declines (Fig. 1c)
		Steep increase in extracellular riboflavin and crystals seen in the broth (Fig. 2i) (indicated by arrow); decline in intracellular riboflavin (Fig. 1b)	
8	Appearance of long thin mycelia (result of secondary growth?) (Fig. 2e)	Lysis advanced; few dispersed filaments and some swollen forms persist; yellowish fluid is found in few cells (Fig. 2j)	Compact, short, thick, highly branched, whitish filaments (Fig. 2o)
	No further increase in extracellular riboflavin.	Further increase in extracellular and decline in intracellular riboflavins (Fig. 1b)	
	40% residual glucose available (Fig. 1a)		

If quick and precise methods for the determination of differentiation were available, they would be very useful for the successful control of fermentation, as suggested by Lee [11]. He showed that physiological differentiation was evident through mycelium morphological changes, which was closely associated with the onset of secondary metabolite (antibiotic) production [11]. The nonflavinogenic mutant UV-85 would be useful for metabolic comparisons with flavinogenic UV-18-57. Similar kind of block mutants were developed in *Streptomyces peucetius* ATCC27952 and used in determining the biosynthetic intermediates by feeding the intermediates or its analogues followed by analysis of the final product [5].

In conclusion, the physiological characteristics of the *E. ashbyii* UV mutants, UV-18-57 and UV-85, differed from the parental DT1 in terms of increased glucose utilization and higher biomass production. While UV-18-57 produced 8-9-fold higher riboflavin than DT1, UV-85 was nonflavinogenic. The mutants also differed in morphological characteristics. While UV-18-57 showed long broad hyphae and later swollen bulbous forms during riboflavin production, the parental DT1 and nonflavinogenic UV-85 maintained thinner

filamentous mycelia. Sporulation and mycelial integrity were better in UV-18-57.

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