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EFFECT OF pH ON EXOCELLULAR RIBOFLAVIN PRODUCTION BY *EREMOTHECIUM ASHBYII*

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SUMMARY

The production of riboflavin by *Eremothecium ashbyii* grown in a chemically defined medium in batch culture was affected by pH of the medium. Highest yields were obtained at constant pH of 4.5 and 5.5, while little or no riboflavin was detected at either pH 3.5 or 8.5. The medium pH also affected cell morphology. When the organism was grown in a stirred tank reactor and an airlift vessel at pH 4.5 very similar levels of riboflavin were obtained.

INTRODUCTION

Riboflavin overproduction occurs in several fungi including *Eremothecium ashbyii* and *Ashbya gossypii.* Although its biosynthetic pathway is well understood [Demain, 1972], information on the physiology of overproduction and influence of culture parameters on yields is not so complete [Kapralek, 1962; Ozbas and Kutsal, 1986 a&b]. There have been many studies on the effect of medium composition on riboflavin yields in these organisms [Ozbas and Kutsal, 1986b], but in these pH was not controlled at a constant value during fermentation, and therefore its importance or influence is not clear. Kapralek [1962] showed that during biomass production in *E. ashbyii*, the pH dropped, but then rose during the phase of riboflavin formation. This decrease appeared to be the result of initial accumulation of pyruvate in the culture medium, which was then utilised. Ozbas and Kutzal [1986 a&b] confirmed these observations with a different medium. They also demonstrated that highest riboflavin productivity in both *E. ashbyii* and *A. gossypii* occurred at an initial pH of 6.5, although they did not report subsequent changes in the medium pH over the fermentation period.

Therefore, this study undertook measurement of riboflavin production by *E. ashbyii* grown at constant pH values in a fermenter. A comparison was also made of the behaviour of *E. ashbyii* grown in two reactor configurations, a stirred tank and an air lift vessel.

MATERIALS AND METHODS

E.ashbyii ATCC (12995) was maintained on the glucose peptone yeast extract agar described by Ozbas and Kutsal [1986b]. It was subcultured monthly, and slopes stored at 4°C. For experimental purposes, inocula were obtained by scraping cells off the agar surface and inoculating them into the same medium dispensed as 50 ml aliquots in 300 ml Erlenmeyer flasks. These were incubated at 28°C for 22 hours in an orbital incubator (Paton Industries, South Australia) at 180 r.p.m. Cells were harvested by centrifugation, washed three times in sterile water and finally resuspended in water to provide an inoculum for the fermentation studies. The fermentation runs were carried out using an LH Series 500 fermenter and 2 l vessel, with an operating volume of 1.8 l. Temperature was maintained at 28°C, a stirrer speed of 500 rpm was used, air flow set at 1 vol vol^{-1} min^{-1} with a rotameter, and pH control achieved using an Ingold pH probe and an LH Series 505 pH control unit, which added either 2N HCl or 2N NaOH as required to maintain the set point. For experiments using the air lift vessel, an LH series 500, 2.75 l capacity air lift fermenter was used [Stasinopoulos and Seviour, 1992] with the culture parameters controlled as described above. The medium for all these experiments was the chemically defined medium described by Osman and Chenouda [1965], but glucose was present at 3% (w/v) and the tribasic form of ammonium citrate was used at 0.3% (w/v). Light was excluded by completely enclosing the vessels with alluminium foil to prevent photochemical degradation of riboflavin. Duplicate samples (10 ml) removed aseptically from the reactors were analysed. Methods used for determining biomass, and medium levels of residual NH_4^+ and glucose have been described previously [Seviour and Kristiansen, 1983]. Pyruvate levels in the culture media were measured using the Sigma Kit (procedure No. 726-UV). Riboflavin analysis on culture filtrates was carried out using a fluorescence attachment on a Varian DMS 80 spectrophotometer, with excitation at 460nm and the emission filter OG570 [Welcher, 1966]. All experiments were repeated, and the values given are the means of four separate analyses.

RESULTS AND DISCUSSION

Production of riboflavin by *E. ashbyii*, in the stirred tank reactor with no pH control, using the chemically defined medium described (Fig.1), showed a different pattern to that reported in other studies where complex media were employed [Kapralek, 1962; Ozbas and Kutsal, 1986 a&b]. In particular, the initial drop in pH, corresponding to the phase of biomass production, was not then followed by a substantial pH increase as noticed in these earlier studies. Whether this can be related to the much lower riboflavin levels with this medium compared to the others [Ozbas & Kutsal, 1986 a&b] will be discussed later. However these pH profiles may also be explained in terms of the different nitrogen sources used. For example, Kapralek [1962] related this pH increase to the eventual utilisation of complex organic nitrogen sources present in the medium, which were

not used in experiments reported in this paper. The contribution to medium pH changes by the accumulation and subsequent re-utilisation of pyruvate recorded by others [Kapralek, 1962] may also be important. Formation of biomass and riboflavin release into the medium initially closely followed each other, but then riboflavin levels in the medium continued to increase after biomass production slowed down. These changes parallelled the utilisation patterns of NH_4^+ and glucose in the medium, neither of which became completely exhausted. Morphological variations in *E. ashbyii* were also seen, especially the gradually increasing appearance of swollen hyphal cells and the formation of ascospores, although it was not possible to correlate these changes with either onset or cessation of riboflavin elaboration.

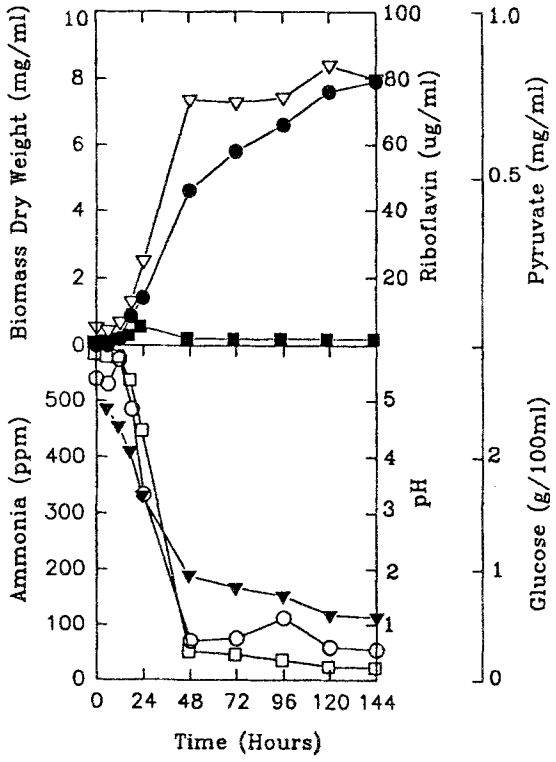

Fig 1: Riboflavin production by *E. ashbyii* grown in stirred tank fermenter. All other details are described in text. Biomass dry weight (▽),Riboflavin (●), pH (▼), Residual glucose (□), Residual ammonia (○), Pyruvate (■).

A series of experiments was undertaken where the culture medium pH was controlled, and its effect on riboflavin production determined. Results obtained (Fig. 2) reveal that culture pH had a profound effect on riboflavin levels detected in the media. For example, no riboflavin was found at

pH 3.5 and only very low levels at pH 8.5. In both cases, there was a considerable lag phase before biomass production and substrate utilisation occurred, although biomass level then reached similar or higher concentrations to those found at the other pH values tested. The highest yields of riboflavin in the medium occurred at constant pH of 4.5 and 5.5 where 270 μg ml^{-1} was eventually obtained. Pyruvate also accumulated in the medium at 36-48 hours, the onset coinciding with the onset of riboflavin production. At pH 6.5 similar levels of pyruvate to those at pH 5.5 were detected, although eventual riboflavin levels were much less. Interestingly, low accumulation of both pyruvate and riboflavin occurred at pH 3.5 and 8.5, observations which are consistent with those of Kapralek [1962].

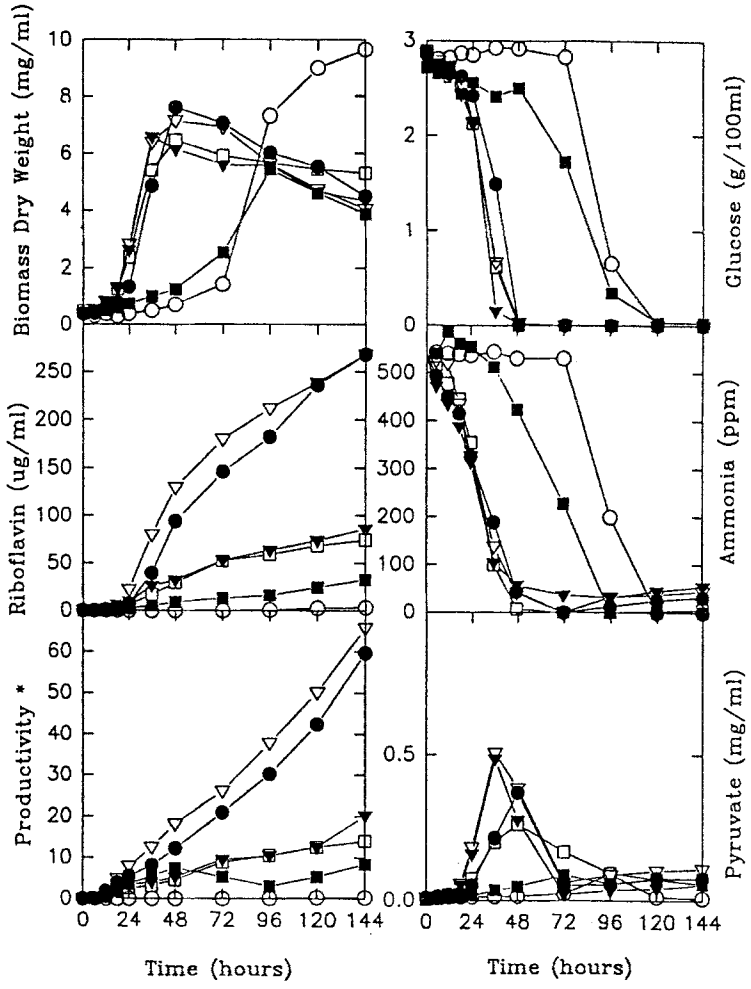

Fig 2: Riboflavin production of *E. ashbyii* grown at constant pH. pH 3.5 (○), pH 4.5 (●), pH 5.5 (▽), pH 6.5 (▼), pH 7.5 (□), pH 8.5 (■) * Productivity = mg/g dry weight biomass For other details see Fig 1.

Therefore it would appear that *E. ashbyii* prefers more acidic conditions for maximum riboflavin elaboration than suggested from earlier studies [Ozbas and Kutsal, 1986 a&b]. However it is possible that although lower pH favours riboflavin synthesis, a high pH may encourage its release from the cells. This possibility was not examined here but medium pH certainly had a striking effect on culture morphology in *E. ashbyii*. For example, as culture pH increased, hyphae became less even, and cells more bloated and pleiomorphic until at pH 8.5 they were almost all granular and highly irregular. At pH 3.5, although thin and regularly branched, the hyphae were pelleted and aggregated. Some of these morphological features are shown in Fig. 3. Although not quantified, it appeared that riboflavin yields were higher at pH values like 4.5 and 5.5 where more asci and ascospores were produced, which again agrees with other observations [Kapralek, 1962].

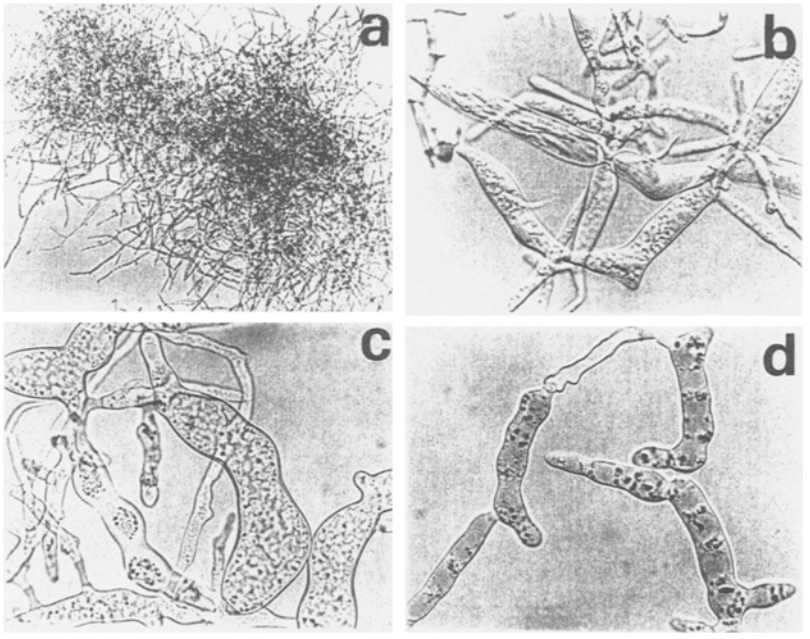

Fig 3: Typical morphology of *E. ashbyii* grown at different constant pH values. a) pelleted growth at pH 3.5 (x100), b) asci and swollen cells at pH 6.5 (x400), c) distorted cells at pH 7.5 (x400) d) vacuolated and burst cells at pH 8.5 (x400)

The lack of riboflavin elaboration by *E. ashbyii* at pH 3.5 when it grew as aggregated mycelial clumps suggested there might be some relationship between morphology and riboflavin synthesising ability. This was examined by growing it in a low shear air lift system, where such a culture morphology might be encouraged, at the optimal pH of 4.5 found in the stirred tank vessel. Results obtained are given in Fig. 4. Although *E. ashbyii* did grow as aggregated clumps of mycelium, riboflavin yields were comparable to those seen in the stirred tank reactor. However, its rate of appearance in the medium was much slower than in the stirred tank vessel. This may relate to its release from the cells, a process which could occur less readily under conditions of low shear where cell leakage might be lower. This requires further investigation.

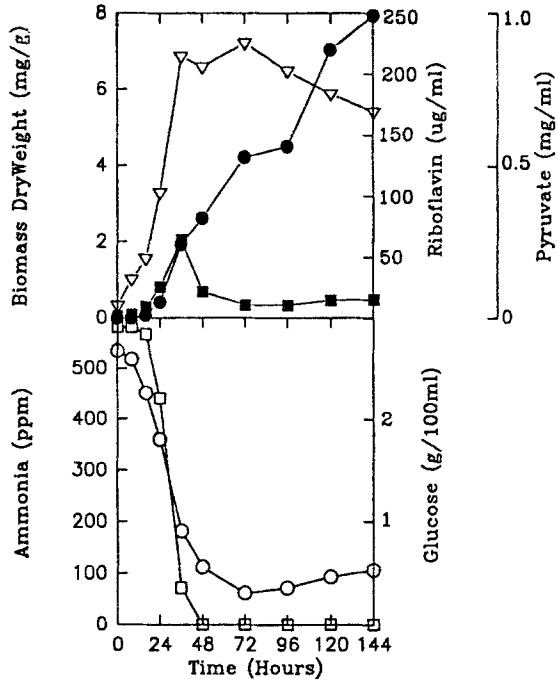

Fig 4: Riboflavin production by *E. ashbyii* grown in air lift fermenter at pH 4.5. Symbols and other details are given in Fig 1.

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