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EFFECT OF CHELATING AGENTS ON THE BIOSYNTHESIS OF RIBOFLAVIN BY CANDIDA GUILLIERMONDII

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The effect of chelating agents on riboflavin production, cell dry weight and glucose consumption by *C. guilliermondii* in shake-flask cultures was studied. The complexing agents tested were: ethylenediamine-tetracetic acid (EDTA), diaminocylohexane-*N,N*-tetra acetic acid (CDTA), diethylene-triaminepenta acetic acid (DTPA), and nitrilotriacetic acid (NTA). The amount of chelating agents added to the medium was 2.02 to 18.36 mM.

Of all the chelating agents, EDTA gave highest yield of riboflavin and their stimulatory effect was in the decreasing order of EDTA, CDTA and DTPA. NTA at all levels, however, did not stimulate riboflavin production. The stability constants of the metal chelates and their structural formulae were important factors in stimulating riboflavin formation.

The selected species of *Candida*, such as *C. guilliermondii* and *C. flareri* produce riboflavin. Tanner *et al.*[1,2] have reported that riboflavin formation by *Candida* species is very sensitive to iron concentration, the tolerance limit of iron being 0.005–0.01 μg/ml. Media containing organic nutrients, therefore, need to be freed of excess iron. The conventional methods use ion-exchange resins[3] and treatment with a chloroform solution of 8-hydroxyquinoline.[4] These methods are exacting, laborious and subject to failure by contamination of the medium from dirt and walls of the vessels. Choudhary and Pirt[5,7] have reported a new and as yet little used method based on adding metal-chelating agents to the medium so as to decrease the concentration of free metal ions to the desired values. The significance of the method is that the metal complexes act as a 'metal buffer' which reversibly dissociates so as to replace free metal ions as they are consumed by a growing organism, or to combine with metal ions added to the system. For wider aspects of the subject, the symposium in Federation Proceedings[8] should be consulted. The metal-complexing agents are available in large number and their physiological effects have been little investigated and need to be better known if metal buffers are to be used more widely. The purpose of the present study was to investigate the effect of metal chelates as metal buffer on the biosynthesis of riboflavin by *Candida guilliermondii* in shake flasks.

The theory of metal-complexing agents, which is analogous to that of hydrogen-ion buffers has been described by Chaberek *et al.*[9] The dissociation of a simple metal complex MX is represented by

$$MX^{2-n} \rightleftharpoons M^{+2} + X^{-n}$$

where M^{-2} is the metal ion and X^{-n} is amount of chelating agent of valency n. The stability constant K is given by

$$K = \frac{(MX^{2-n})}{(M^{+2})\ (X^{-n})}$$

where the brackets indicate molar concentrations. The stability constant is used as a measure of the degree of chelation. Values of the stability constants for metals and chelating agents used in the work are given in Table 1.

Table 1.—Log 'K' Values (Stability Constants) of Metal Chelates.*

Metal	NTA	EDTA	CDTA†	DTPA
Fe^{3+}	15.87	25.1	31.0	28.6
Cu^{2+}	12.68	18.8	21.3	21.1
Zn^{2+}	10.45	16.5	18.67	18.3
Fe^{2+}	8.84	13.33		16.5
Mn^{2+}	7.44	14.04	16.78	‡
Mg^{+2}	7.00	8.69	10.32	‡

*All values from Bjerrum *et al.*[10] except † from Simons *et al.*[11] ‡Not known.

Methods

Organism.—The yeast strain of *Candida guilliermondii* ATCC-9058 was used throughout present investigation. It was maintained on agar medium containing (g/l): glucose 40; agar 20; KH_2PO_4 0.5; $MgSO_4.7H_2O$ 0.5; $(NH_4)_2SO_4$ 2.0; asparagine 2.0 and biotin 1.0 mg/l.

Inoculum Preparation.—The yeast, after growing on agar medium at 30° for 3 days was inoculated

into 50 ml fermentation medium, described below, in a 300-ml conical flask. The inoculum was grown for 48 hr at 30°C on a rotary shaker. The yeast cells were separated from the culture medium by centrifuging at 3000 rev/min for 10 min. The cells were washed three times with sterile distilled water and a suspension of these cells was made in sterile distilled water. The optical density of the suspension was maintained at 0.205 throughout the investigation, using a Hilger colorimeter with a blue filter.

Media.—The composition of the fermentation medium was (g/l): glucose 40; KH_2PO_4 0.5; $MgSO_4.7H_2O$ 0.5, (Mg^{+2} 2.028 mM), $(NH_4)_2SO_4$ 2.0; asparagine 2.0 and biotin 1.0 mg/l. All reagents were of analytical grade and glass-distilled water was used for the preparation of solutions. All media, unless otherwise stated, were sterilized at 121°C for 15 min. Fermentation medium was divided into two parts in glass bottles for sterilization: (1) glucose (2) organic and inorganic salt solutions both were four times concentration. Each part of the fermentation medium was washed in a separatory funnel with chloroform containing 8-hydroxyquinoline by the method of Waring and Werkman.[4] After washing, pH of the salt solution was adjusted to 5.0. Air was also passed from the solution in order to remove last traces of chloroform before sterilization. Sterile solutions of glucose and salts were then mixed. Variable concentration of iron as ferrous sulphate was added afterwards to the sterile medium.

For shake flask culture 50 ml medium including 1 ml yeast cell suspension was held in a 300-ml conical flask plugged with cotton wool. The flasks in duplicate were shaken on a rotary shaker (through 1½ in, 125 cycle/min) and incubated at 30°C for 7 days.

Chelating Agents.—The chelating agents such as ethylenediamine-tetracetic acid (EDTA), diaminocyclohexane-*N*,*N*-tetraacetic acid (CDTA), diethylenetriaminepentaacetic acid (DTPA) or nitrilotriacetic acid (NTA) were dissolved and brought to pH 5.0 by adding NaOH. The solutions were autoclaved at 121°C for 15 min.

Analytical Methods.—The yeast cells from each flask were separated by centrifugation at 3000 for 20 min. The cells were twice washed thoroughly with distilled water followed each time by centrifuging. The cells were dried at 105°C overnight for dry weight determination.

Glucose was estimated by the reduction method of Fujita and Iwatake [12] and riboflavin spectrophotometrically at 445 mμ. All glass containers brought in contact with solutions and fermentation medium were cleaned with chromic acid and rinsed with glass-distilled water.

Results

Effect of Iron Concentration.—The biosynthesis of riboflavin by *C. guilliermondii* was examined in the presence of different amounts of iron (0–2.0 μM) and the results are given in Table 2. The yield of riboflavin and glucose consumption were decreased with the increase in the concentration of iron. The cell dry weight, however, was slightly increased by increasing iron concentration. In the absence of iron, riboflavin formation was negligible indicating that its presence is essential for riboflavin synthesis by the yeast (see Table 2).

TABLE 2.—EFFECT OF IRON ON THE PRODUCTION OF RIBOFLAVIN CELL DRY WEIGHT AND GLUCOSE CONSUMPTION BY *Candida guilliermondii* ATCC–9058.

Iron conc. μM	Dry weight g/l	Glucose used g/l	Riboflavin mg/l
0.00	7.50	23.41	2.50
0.18	8.82	27.75	18.15
0.90	9.62	22.34	14.00
1.80	10.71	20.57	11.60
9.00	11.60	19.22	5.00

Effect of Chelating Agents.—The riboflavin formation in shake flask cultures, by *Candida* yeast, was greatly increased by the addition of EDTA; CDTA or DTPA (Table 3). The stimulatory effect of complexing agents was in decreasing order of EDTA, CDTA, CDTA and DTPA. In cultures containing 0.18 μM Fe^{+2}, the amount of riboflavin produced in the presence of 2.03 μM EDTA (Metal: EDTA 1:1) was 103 mg/l and it was increased to 123 and 135 mg/l when cultures were grown with EDTA concentration of 6.09 and 18.27 mM respectively. The chelation of trace metals particularly of iron at higher levels i.e. 1.8 and 9.0 μM by EDTA also increased the yield of riboflavin, significantly. That is, the toxic effect of iron concentrations on riboflavin synthesis, as compared with control cultures, was greatly reduced by complexing the metal ions. The production of riboflavin at all levels of iron was maximum when metal-chelate ratios were maintained at 1:9, i.e. 18.36 mM of EDTA, CDTA or DTPA. The effect of EDTA and

TABLE 3.—EFFECT OF CDTA, DTPA, EDTA OR NTA, ADDED AT THE TIME OF INOCULATION, ON THE PRODUCTION OF RIBOFLAVIN BY *C. guilliermondii* IN BASAL MEDIUM.

Conc. of chelating agents mM	Metals: chelate	0.18 µM Fe+2 Riboflavin mg/l			Conc. of chelating agent mM	Metals: chelate	1.8 µM Fe+2 Riboflavin mg/l			Conc. of chelating agent mM	Metals: chelate	9.0 µM Fe+2 Riboflavin mg/l		
		CDTA	DTPA	EDTA	NTA			CDTA	DTPA	EDTA	NTA			CDTA	DTPA	EDTA	NTA
0.00	1:0	18.15	18.60	18.20	18.30	0.00	1:0	12.0	11.54	11.8	11.45	0.00	1:0	5.64	5.50	5.42	5.43
2.03	1:1	93.00	40.80	103.0	18.22	20.03	1:1	93.0	38.65	104.0	11.51	2.04	1:1	95.5	40.20	106.0	5.38
6.09	1:3	108.0	48.50	123.0	18.35	6.09	1:3	107.5	48.00	124.0	11.48	6.12	1:3	105.5	48.00	124.0	5.35
18.27	1:9	118.0	56.50	135.0	18.10	18.27	1:9	115.0	56.00	137.0	11.44	18.36	1:9	117.0	56.00	136.0	5.42

CDTA on riboflavin synthesis, however, was more pronounced than DTPA. In constrast, NTA (2.03–18.36 mM) had no effect on the yield of riboflavin, it remained like that in control cultures.

The effect of all chelating agents was also studied on the glucose consumption and cell dry weight and both of them were little affected by the complexing agents.

Discussion

The present work shows that chelation of iron in particular, magnesium and other unknown trace metals present due to contamination from the glass surface, etc. by complexing agents increased riboflavin formation. Moreover, the presence of iron is also essential in the medium for riboflavin formation. All chelating agents, except NTA, showed stimulatory effect on the yield of riboflavin and it was decreased in the order of EDTA, CDTA and DTPA. The constants of the metal chelates (Table 1) show EDTA to be weaker than CDTA or DTPA, yet EDTA gave highest yield of riboflavin. The possible explanation may be the difference in their structural formulae. NTA, on the other hand, is also a weaker chelating agent than EDTA, CDTA and DTPA, the availability of iron and other metals would have been greater in the culture medium containing this complexing agent. Thus no stimulation of riboflavin formation was found at all levels of NTA (0–18.36 mM). Moreover, it is impossible to have a system consisting entirely of a single oxidation state of iron. The ferrous chelate is readily oxidized to the ferric chelate. An iron chelate system, therefore, will comprise a mixture of the two oxidation states with the trivalent state predominating. Ferric form will form a neutral complex with NTA. Such a neutral complex as suggested by Chaudhary and Pirt[7] would be expected to be lipid soluble and therefore able to diffuse through the plasma membrane into the cell and thus make the iron available. This point suggests that iron concentration in the presence of NTA may be largely responsible for its nonstimulatory effect. A similar effect of NTA as compared with EDTA, CDTA and DTPA on citric acid production by *Aspergillus niger* has also been reported by Choudhary and Pirt.[7]

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