THE GROWTH AND PIGMENTATION OF ACTINOMYCES COELICOLOR AS AFFECTED BY CULTURAL CONDITIONS

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The use of characteristic pigments as criteria in species differentiation among the actinomycetes has been hampered by the comparative lack of knowledge of the conditions under which these pigments are produced. The striking red-blue indicator pigment of *Actinomyces coelicolor* (Müller) Lieske has been described (Conn, 1943) as a possible taxonomic character. The present investigation was designed to determine the cultural conditions which affect the formation of this pigment, with particular regard to carbon nutrition, nitrogen nutrition, and pH relations.

MATERIALS AND METHODS

The culture of *A. coelicolor* used has been described by one of us (Conn, 1943) as isolate B-3. The basal medium used throughout contained cp glucose, 10 g per L; Difco asparagine, 0.5 g per L; cp K_2HPO_4, 0.5 g per L; cp $MgSO_4 \cdot 7H_2O$, 0.25 g per L; Difco yeast extract, 0.5 g per L; and a minor element mixture composed of salts of Zn, Fe, Cu, B, Mn, and Mo in the amounts recommended by Robbins and Ma (1942). In this medium the sugar was found to be 85 to 90 per cent utilized in 18 days at 25 C; amounts of asparagine in excess of 0.5 g per L did not increase dry weight with glucose at 10 g per L. The organism was grown on the surface of 50 ml of liquid medium in 125-ml Erlenmeyer flasks. In still culture this and other actinomycetes make normal growth only on the surface; the flocculent subsurface mycelium so often noted in liquid cultures is characterized by a very slow and irregular growth rate and by poor sugar utilization.

In experiments on carbon and nitrogen nutrition, replicate cultures were harvested at 6, 12, and 18 days after seeding; data from the last period only are reported. The dry weight was determined by drying the mycelium overnight at 70 C on tared filter paper. All pH measurements were made with the glass electrode.

To compare pigment production under different conditions, the filtered culture fluid was adjusted to pH 7.0 and diluted with 4 volumes of phosphate buffer (pH 6.98). The intensity of pigmentation was then read in a Fisher electrophotometer against a similarly adjusted and diluted sterile medium, using a filter with peak transmittance at 650mμ.

All cultures were run in triplicate. Dry weight determinations were made for each flask; other measurements were made on a pooled sample from the three replicates.

CARBON AND NITROGEN NUTRITION

A condensed summary of data on the utilization of carbon and nitrogen sources is presented in table 1. Dry weight and pigment intensity were recalculated on the basis of glucose controls in the carbon source experiment, of asparagine controls in the nitrogen series.

Except with organic acids, unavailability of a particular carbon source was evidenced not only by low relative growth but by a high final pH, resulting from the utilization of asparagine as a source of energy. Examples of this were the media having as carbon source sorbose, inulin, dulcitol, and sorbitol. With neutralized organic acids as energy sources, a rise in pH was in many cases evidence of utilization.

Study of the growth data indicates that xylose, mannose, and glycerol were better carbon sources than glucose; arabinose proved to be much poorer, probably because of the production of acid. Sucrose was only slightly utilized; trehalose and lactose were slowly available. Among the organic acids, acetic, lactic, fumaric, succinic, malic, and gluconic acids were able to support growth in excess of that in the no-carbon control. Tartaric and citric acids did not support measurable mycelial growth, although the former was evidently attacked sufficiently to cause an increase in alkalinity.

Pigment formation was proportional to mycelial growth with one general exception: when the final pH was either high or low, pigment intensity was not so great as would be expected from the dry weight. This is particularly notable among the organic acids; in the poorly buffered basal medium the utilization of neutralized lactic, fumaric, succinic, and malic acids resulted in final pH values above 8.7, and no pigment was formed. Utilization of acetate and gluconate was not accompanied by such extreme alkalinity, and some pigment developed. A further exception to the general proportionality of growth and pigmentation was noted in glycerol media. The greater relative pigment intensity was not the result of reaction changes; it appears that glycerol specifically favors the production of pigment.

Turning to the nitrogen nutrition data of table 1, it is evident that all of the four amino acids tested were utilizable; urea also proved an adequate source of nitrogen. On the other hand, neither nitrate nor ammonium salts supported growth comparable to that with asparagine; in the case of nitrate, the dry weight was no higher than in the glucose yeast extract control. With ammonium salts, the low final pH is the cause of poor growth; this acidity results undoubtedly from preferential absorption of the ammonium ion. Supplementary experiments with ammonium phosphate at several concentrations showed that satisfactory growth occurs if the pH can be held above 6.0. In a poorly buffered medium it is impossible to supply enough ammonium nitrogen for utilization of 1 per cent glucose without the development of too acid a reaction. Failure of growth in the nitrate medium may be associated with the observed heavy accumulation of nitrite.

The several peptones tested supported excellent growth and pigmentation. The latter fact shows that so-called "synthetic" media are not necessary for the

formation of the red-blue indicator pigment. As in the case of carbon sources, the intensity of pigmentation was roughly proportional to growth except in media with an unfavorable reaction. This is not always clear from the 18-day

TABLE 1

The utilization of carbon and nitrogen sources by A. coelicolor

CARBON SOURCE*	CONCENTRATION	RELATIVE GROWTH†	RELATIVE PIGMENT INTENSITY†	FINAL pH	NITROGEN SOURCE‡	CONCENTRATION	RELATIVE GROWTH§	RELATIVE PIGMENT INTENSITY§	FINAL pH
	g/L					g/L			
None		17	0	8.4	None		30	32	6.7
d-Glucose	10.0	100	100	7.2	*l*-Asparagine	0.50‖	100	100	6.7
d-Mannose	10.0	202	200	7.0	Glycine	0.29‖	83	36	7.0
d-Galactose	10.0	84	97	7.1	*l*-Leucine	1.00‖	76	36	7.0
d-Fructose	10.0	79	96	7.0	*l*-Tryptophane	0.78‖	98	82	6.9
d-Xylose	10.0	143	121	7.2	Urea	0.24‖	86	51	7.0
l-Sorbose	10.0	26	0	8.5	$NaNO_3$	0.64‖	18	0	6.0
l-Arabinose	10.0	65	46	5.2	$(NH_4)_2HPO_4$	0.50‖	57	0	5.7
Starch	10.0	107	87	7.0	Ammonium acetate	0.58‖	18	0	5.6
Inulin	10.0	32	0	8.5	Peptone	1.00	146	118	6.8
Trehalose	10.0	90	38	8.1	Tryptone	1.00	91	106	7.0
Cellobiose	10.0	81	95	6.7	Casitone	1.00	116	100	7.0
Maltose	10.0	62	43	7.3	Pepticase	1.00	175	118	6.4
Lactose	10.0	105	64	6.8	Casamino acids	1.00	116	129	6.6
Sucrose	10.0	34	0	8.4	Sodium caseinate	1.00	72	53	6.9
Glycerol	10.0	135	170	6.6	Gelatin	1.00	106	29	6.4
Mannitol	10.0	82	88	7.1	Egg albumin	1.00	44	35	6.0
Dulcitol	10.0	20	0	8.6					
Sorbitol	10.0	27	0	8.2					
Acetic acid	5.0	33	33	8.4					
Lactic acid	5.0	60	0	8.8					
Fumaric acid	5.0	69	0	9.1					
Succinic acid	5.0	47	0	8.9					
dl-Malic acid	10.0	60	0	8.9					
Tartaric acid	5.0	20	0	8.7					
Citric acid	5.0	24	0	8.2					
Gluconic acid	5.0	82	25	8.2					

* Basal medium (g/L): asparagine—0.5, yeast extract—0.5, K_2HPO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.25, and minor elements.

† Dry weight and pigment intensity of glucose control taken as 100.

‡ Basal medium (g/L): glucose—10.0, yeast extract—0.5, K_2HPO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.25, and minor elements.

§ Dry weight and pigment intensity of asparagine control taken as 100.

‖ Total nitrogen, 0.106 g/L.

data alone; thus, the relatively low pigment intensity of glycine and leucine media was associated with an acid reaction (about pH 6.0) earlier in the growth cycle. The same was true of the gelatin medium.

The data suggest that the nitrogen sources most favorable for growth and pigmentation—asparagine, tryptophane, and the peptones—are so not because

of the greater availability of nitrogen per se, but because the utilization of these materials is not accompanied by drastic pH changes. This, rather than specific nutrilite effects, may explain also the growth-promoting properties of yeast extract.

If this is indeed the case, an excess of any nitrogen source, if unbalanced by an increase in the glucose level, should cause a reaction unfavorable for pigment formation. The experiment summarized in table 2 affords confirmation of this hypothesis. Failure of pigment to develop in high peptone media was associated with a high pH, but there was no measurable reduction in growth. Experiments not reported in detail showed that the deleterious effect of high peptone levels on chromogenesis can be eliminated by increasing the concentration of glucose.

TABLE 2

*The effect of peptone concentration on growth and pigmentation of A. coelicolor**

NITROGEN SOURCE	CONCENTRATION	pH		DRY WEIGHT	RESIDUAL SUGAR	COLOR†	PIGMENT INTENSITY
		Initial	Final				
	g/L			*mg*	*mg/100 ml*		
None		6.62	7.01	36.2	306.4	P	12.5
Asparagine	0.5	6.94	6.90	68.3	138.1	P	26.0
Peptone	0.5	6.86	6.99	54.0	303.2	P	23.0
Peptone	1.0	6.85	6.98	87.2	33.4	P	26.8
Peptone	2.0	6.88	7.69	83.1	8.4	B	39.3
Peptone	5.0	6.84	8.46	81.7	13.9	0	

* Basal medium (g per L): glucose—10.0, yeast extract—0.5, K_2HPO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.25, and minor elements. Duration of experiment 18 days.

† 0—none, P—purple, B—blue.

From these considerations it follows that the apparent utilization of a given source of nitrogen in part depends on the nature of the carbon source used and its concentration, since these factors affect the pH of the medium.

THE INFLUENCE OF PH ON GROWTH AND PIGMENTATION

Changes in reaction have been postulated as the explanation of several phenomena of nutrition, especially with regard to the utilization of organic acids, peptone, and ammonium salts. An experiment, summarized in table 3, tested the effect of variations in the initial pH of the culture medium on growth and pigment formation.

Growth occurred in media adjusted initially to pH 5.0 to 10.9, with the maximum dry weights at pH 6.9 to 7.7. The range in which pigment formed was somewhat narrower, pH 6.0 to 9.9, the optimum for pigment intensity being pH 7.3 to 7.7. It is evident that the organism is able substantially to lower the pH of alkaline media when glucose is the source of carbon; in order to obtain a final pH comparable to that attained in high peptone media, the medium had to be initially at pH 11.0. The difference between the pH limits of growth and

those of pigment formation explains the failure of pigmentation to accompany growth in media which become acid or alkaline during metabolism.

TABLE 3

*The influence of the pH of the medium on growth and pigment prouction of A coelicolor**

MEDIUM	pH		DRY WEIGHT	COLOR‡	PIGMENT INTENSITY
	Initial†	Final			
			mg		
A	3.70	3.63	−0.2	0	
B	4.21	4.24	0.3	0	
C	4.98	4.92	27.5	0	
D	6.00	6.30	65.0	R	12.5
E	6.90	6.59	89.7	P	23.6
F	7.31	6.97	87.5	P	32.9
G	7.70	6.97	82.0	P	34.9
H	8.08	7.40	67.0	B	19.7
I	8.30	7.46	61.5	B	22.9
J	9.00	7.50	52.0	B	21.2
K	9.42	7.81	62.5	B	18.8
L	9.92	7.94	54.7	B	8.9
M	10.92	8.61	30.0	0	
N	11.20	11.06	0.3	0	

* Basal medium (g/L): glucose—10.0, asparagine—0.5, yeast extract—0.5, K_2HPO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.25, and minor elements. Glucose added aseptically after sterilization. Duration of experiment 18 days.

† Adjusted with HCl or NaOH, pH measured after sterilization.

‡ 0—none, R—red, P—purple, B—blue.

DISCUSSION

The controlling factor in the production of pigment by a vigorously growing culture of *A. coelicolor* is the reaction of the medium. Within the range which permits chromogenesis, the actual color of the pigment is again determined by pH. Of the variety of carbon and nitrogen sources tested, there was none which supported growth but not chromogenesis except those compounds the presence of which or the utilization of which caused the pH to drop below about 6.0 or to rise above about 8.5.

Within the pH range favorable for chromogenesis there is a discernible effect of reaction on the amount of pigment formed. The pH range for optimum production of pigment is somewhat narrower than the range of maximum mycelial growth. For this reason it is possible to effect changes in the amount of color without changing the final dry weight of the culture.

The possible taxonomic value of the pigments of actinomycetes has been discussed elsewhere (Conn and Conn, 1941; Conn, 1943). The present work emphasizes the need for careful control of certain environmental factors, and demonstrates that such control makes it possible to obtain reproducible results. Parenthetically, it may be mentioned that the strain of *A. coelicolor* used has been

carried in culture for 8 years with no detectable change in the pigment or in other characters.

SUMMARY

The growth of *Actinomyces* (*Streptomyces*) *coelicolor* Müller in surface culture has been studied with particular reference to the formation of the pigment characteristic of this species.

In a survey of carbon sources, mannose, xylose, and glycerol were found to support the heaviest mycelial growth. The organism is able to utilize a wide range of sugars, polyatomic alcohols, and organic acids. Compounds not utilized included sorbose, inulin, sorbitol, dulcitol, tartaric acid, and citric acid; sucrose is only slightly utilized.

Satisfactory nitrogen sources for growth include several amino acids and peptones, urea, casein, and gelatin. Ammonium salts of weak acids support normal growth only in a buffered medium; in a poorly buffered medium the acidity arising from preferential absorption of the ammonium ion interferes with growth. Nitrate is absorbed but undetermined secondary effects make it unsuitable under the conditions tested.

The optimum pH for growth is pH 6.9 to 7.7; the lower limit of growth is pH 4.2 to 5.0, the upper limit pH 11. Pigment is formed in media having an initial pH of 6.0 to 9.9, the optimum being pH 7.3 to 7.7.

Regardless of the specific compounds used to supply carbon and nitrogen, pigment develops in any medium able to support mycelial growth, provided that the course of metabolism does not shift the final reaction to either side of the range pH 6.0 to 8.5. Any medium, "synthetic" or not, which supports growth without drastic pH changes also supports chromogenesis.

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