GROWTH AND PIGMENT STUDIES OF *PULLULARIA PULLULANS*

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ABSTRACT. — Growth and pigment formation of *Pullularia pullulans* were studied in various media. Lactose was an excellent source of carbon; the logarithmetic growth phase required 15-16 hours. The utilization of glucose by the organisms was slightly higher than that of fructose. Vitamins, especially thiamine, enhanced growth but were not essential to it. The organism did not grow in a mixture of 21% oxygen-79% helium. The addition of nitrogenous compounds to media containing maltose or glucose enhanced pigment production, but the yield was lower with the glucose system. Cultures shaken at room temperature (25°C) produced much more pigment per unit time than cultures incubated without shaking at 30°C. No pigment was formed when the organism was grown in basal media containing yeast extract alone or yeast extract plus malt extract. A procedure was devised for extracting the pigment from the cells. The pigment behaved as an indicator, changing color upon addition of acid or alkali.

The genus *Pullularia* is a yeast-like fungus widely distributed in nature (Bauer, 1938; Clark, 1957; Cook, 1958). It is important industrially because it is involved in deterioration of paint and discoloration of lumber and is injurious to plants and plant products.

The published information about the organism is scanty and conflicting. Its taxonomy is uncertain and practically all isolates are assigned to the species *P. pullulans*. The reports on its utilization of lactose are not in agreement (Negroni and Fisher, 1942; Clark and Wallace, 1958).

In the present research, utilization of carbohydrates by *P. pullulans* strain NRRL YB 4515 was investigated, and various carbonaceous and nitrogenous compounds were tested as sources of carbon and nitrogen, respectively. The effect of vitamins on growth was determined, and the extent of pigment formation was evaluated.

MATERIALS AND METHODS

The amino acids, vitamins, salts and sources of carbon were of high purity or reagent grade.

The basal culture medium contained 0.3% yeast extract plus 0.5 to 6% lactose, 5% glucose, or 5% fructose. From a 48-hr culture of *P. pullulans*, 1.5×10^6 cells were added per ml of basal medium, the initial pH being 5.4. For 15 days fifty-ml suspensions in 125-ml flasks were incubated at 30°C without shaking or were mechanically shaken at room temperature (25°C). For each of the carbohydrates a total of 64 flasks, including controls, were used. Each flask was reconstituted daily to its original volume by the addition of water. Cell counts were made hemocytometrically and by plate counting. The daily rate of carbohydrate utilization was determined by the Nelson (1944) modification of the Somogyi method (1945).

The ability of the organism to utilize various compounds containing 2,3,4,5 and 6 carbon atoms and a number of nitrogen sources was tested by employing Bacto yeast nitrogen base media (Difco 392) and

Bacto yeast carbon base media (Difco 391), respectively. The vitamin requirements were tested in Bacto yeast vitamin free base media (Difco 394). The culture in amounts of 0.10 ml containing 1.5 X 10^6 cells per ml of suspension was added to tubes containing 10 ml of the media described above. Half of the tubes were incubated at 30°C without shaking, and the remainder shaken at room temperature. Cell counts were determined by plate counting. Growth and pigment formation were measured turbidimetrically according to the Wickerham method (Wickerham and Burton, 1948; Wickerham, 1951).

Nitrogen fixation was tested in Bacto yeast carbon base medium with cells which had been transferred three times in a medium containing only maltose. Fifty-ml suspensions containing 1.5 X 10^6 cells per ml were added to 125-ml flasks with side arms provided with pressure tubing and stopcocks. The flasks were plugged at each end with sterile cotton and were exposed to 21% oxygen-79% helium. The control flasks were exposed to air. Pairs of flasks were incubated at 30°C without shaking and incubated at room temperature with shaking. Air entering the system was passed through 2 *N* sulfuric acid to trap ammonia, if any.

To study pigment formation, 1.5 X 10^6 cells from a 48-hr culture were added per ml of various media. Fifty-ml suspensions were incubated at 30°C without shaking, shaken at room temperature, or shaken at 36-40°C. The 8-day-old suspensions were centrifuged and the intensely colored supernate decanted. The cells

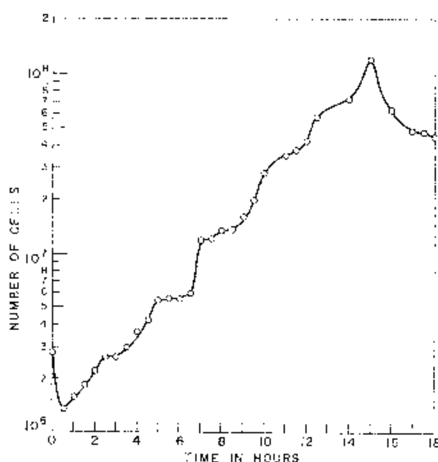

FIGURE 1.—Growth curve of *P. pullulans* on 5.0% lactose medium.

were washed three times with 0.9% sodium chloride, three times with distilled water, and were dried in thin layers. The resulting dried mass was extracted five times with 1.25 *N* sodium hydroxide, centrifuged, and the supernate decanted. The extract was neutralized with hydrochloric acid

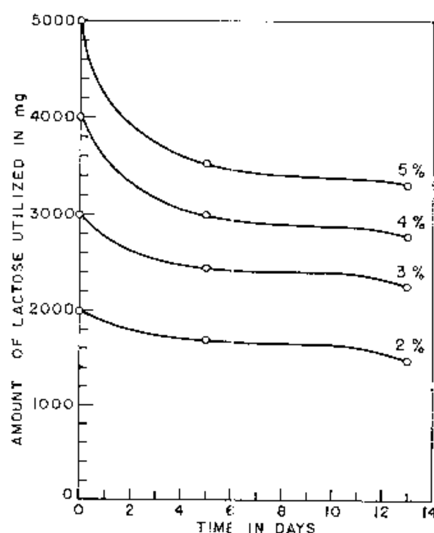

FIGURE 2.— Utilization of lactose by *P. pullulans*.

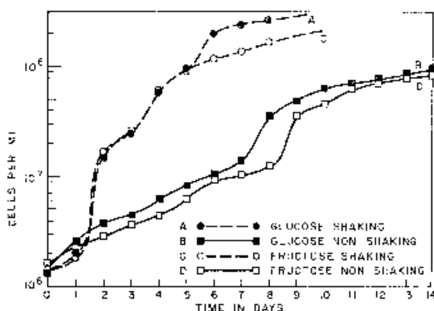

FIGURE 3. — Cell counts of *P. pullulans* in glucose and fructose media.

and dialyzed in cellophane against water until free of chloride. Excess acetone was added to the dialytic residue and the precipitated pigment removed by centrifugation.

To determine the effect of salts on pigment formation in 5% glucose or 5% maltose, NH_4Cl, $(NH_4)_2SO_4$, $MgSO_4$, K_2HPO_4, KH_2PO_4, NH_4NO_3 and KNO_3 were added singly or in mixtures, each at a concentration of 0.05% in shaken and incubated series.

RESULTS AND DISCUSSION

Growth and Carbohydrate Utilization. Maximum growth of *P. pullulans* NRRL YB 4515 and utilization of lactose occurred in media containing 0.3% yeast extract plus 5% lactose (Figures 1 and 2). The daily cell counts in media containing 0.3% yeast extract plus 5% glucose or fructose are shown in Figure 3. The lowest pH, 3.40, was found in suspensions shaken at room temperature (Figure 4). The organism utilized more of each carbohydrate when shaken at room temperature than when incubated without shaking at 30°C (Figure 5). The ability of the organism to utilize lactose was confirmed by determining the rate of lactose utilization in media containing 2 to 5% lactose and by determining its logarithmic phase (15-16 hr; Figures 1 and 2). In both the shaken and the incubated series containing glucose and fructose, slightly more of the glucose was consumed.

Carbon Compounds. When 4% sodium succinate was added to the Bacto yeast nitrogen base media (Difco 392), growth was abundant and pigment formation heavy after 120 hr. Growth was good in the presence of 2% asparagine plus 2% ammonium acetate, very poor with 2% pyruvic acid or 3% sodium glutamate plus 3% sodium formate, and absent with 2% fumaric acid plus ammonium acetate or 2% glycine plus 2% ammonium acetate. Pigment was not formed with the as-

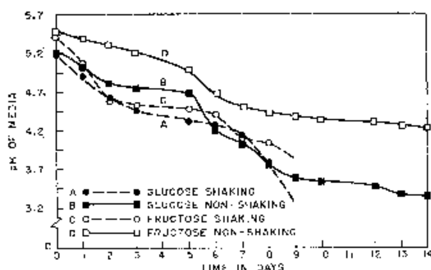

FIGURE 4. — pH changes during glucose and fructose utilization by *P. pullulans.*

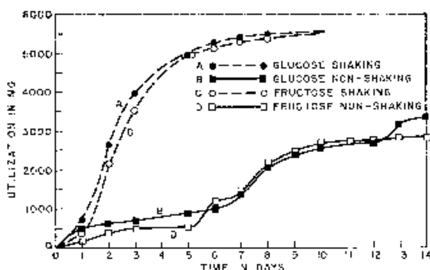

FIGURE 5. — Utilization of glucose and fructose by *P. pullulans.*

TABLE 1.—Effect of 0.05% Nitrogenous Salts on Growth and Pigment Production of *P. pullulans* in Bacto Yeast Carbon Base (Difco 391).

Test Salts	Growth								Pigment			
	Shaken at Room Temperature				Unshaken at 30°C				Shaken at Room Temp.		Unshaken at 30°C	
	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr	24 hr	96 hr	24 hr	96 hr
$(NH_4)_2SO_4$	3+	6+	8+	8+	—	3+	6+	6+	—	2+	—	1+
NH_4Cl	1+	4+	8+	8+	—	2+	6+	8+	—	2+	—	1+
NH_4NO_3	3+	6+	8+	8+	3+	5+	6+	8+	—	1+	—	—
$MgSO_4$	1+	1+	2+	2+	1+	1+	1+	1+	—	—	—	1+
KNO_3	4+	6+	8+	8+	—	1+	1+	8+	—	3+	—	—
K_2HPO_4	—	1+	1+	1+	1+	1+	1+	2+	—	—	—	—
KH_2PO_4	—	1+	1+	1+	—	1+	2+	2+	—	2+	—	1+
$NH_4Cl + MgSO_4$	3+	6+	8+	8+	—	2+	2+	8+	—	2+	—	1+
$NH_4Cl + MgSO_4 + KH_2PO_4$	3+	5+	6+	8—	2+	4+	5+	8+	—	3+	—	—
Controls	—	1+	2+	4+	—	—	1+	3+	—	—	—	—

The 8+ is maximal growth and pigment production, determined turbidimetrically;
Dash indicates no growth or pigment production.

paragine-ammonium acetate mixture. Heavy growth and pigment formation occurred with 4% glycerol or 5% sodium citrate. The 4-carbon compound fumarate, alone or with ammonium acetate, was not utilized, whereas succinate was an excellent source for growth and pigment formation.

Nitrogen Sources. When 2% asparagine, aspartic acid, cysteine, glycine, glycylglycine, leucine, lysine, glutamic acid, methionine, or creatine was added to the basal medium, growth and pigment formation were extensive after 106 hr. However with 2% tyrosine the growth rate was comparably great, but the pigment was yellow instead of black. The effect of 0.05% of various inorganic nitrogenous salts in Bacto yeast carbon base (Difco 391) is shown in Table 1. Among the nitrogenous substances tested, asparagine and glycylglycine enhanced most the growth and black pigment formation.

Vitamins. The following vitamins, added to the vitamin-free yeast base media (Difco 394), singly or in various combinations in the concentrations suggested by Wickerham (1951), produced maximum growth after 10 hr: biotin, calcium pentothenate, folic acid, inositol, niacin, riboflavin, thiamine hydrochloride, pteroylglutamic acid, and pyridoxine hydrochloride. Thiamine was the most effective single vitamin. Growth also occurred in the vitamin-free base medium but at a definitely reduced rate. The fact that the organism grew in the absence of vitamins, though more slowly, may indicate that it can synthesize them.

Nitrogen Fixation. No growth occurred after 120 hr in the flasks treated with the oxygen-helium mixture. When atmospheric air, passed through 2*N* H_2SO_4, was admitted to these flasks, growth was evident in 48 hr, and pigment in 96 hr. The control suspensions showed growth after 48 hr and pigment after 72 hr. Accordingly these findings substantiate the reported ability of *P. pullulans* to fix nitrogen from the air (Brown and Metcalf 1957).

Pigment Formation.—Of all the media tested the one containing 5% maltose plus 0.3% malt extract produced the largest amount of pigment. A lower yield of pigment resulted in the shaken series when in this medium 1% glucose, fructose, galactose or sucrose was substituted for the 0.3% malt extract. Even less pigment resulted when the maltose was decreased to 2.5% and the above named sugars were increased to 2.5%.

Minimal pigment formed in media containing 5% maltose plus the nutrient salts recommended by Wickerham (1946). Pigment was absent in media containing 5% maltose plus 0.3% malt extract, 2.5% maltose plus 0.3% yeast extract, or 2.5% maltose plus 0.3% yeast extract plus 0.3% malt extract.

Inorganic salts were found to affect pigment formation in 5% glucose or 5% maltose. In the shaken and the incubated series containing 5% glucose only slight pigment formation was noticed with NH_4NO_3 + $MgSO_4$ or NH_4Cl + KH_2PO_4 + $MgSO_4$. No pigment was formed with $MgSO_4$ alone or in an admixture with either K_2HPO_4 or KH_2PO_4. In the series with 5% maltose, pigment formation was moderate only with $MgSO_4$ alone or in an ad-

mixture with either K_2HPO_4 or KH_2PO_4, but was abundant with the other salts. Maltose containing $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3 or KNO_3 and shaken at 36-40°C produced the highest amount of pigment.

Properties of the Pigment.—The dry pigment occurred as brown to black flat scales. It was more soluble in hot than in cold water, less so in butanol and in 40% methanol, and insoluble in ether, acetone, benzene and chloroform. The addition of base to an aqueous solution produced a green coloration, and on acidification the solution changed to red. The pigment gave a positive Molish test and showed no reducing properties.

Microscopic Studies. — Pigment formation starts at the inner cell wall and spreads uniformly toward the center, filling the entire cell space in 8 to 10 days. The color *in situ* varies from light brown to jet black. The oval cells rupture at one end, and infrequently at both ends. Irregularly shaped fragments of pigment and debris are visible after several days. The older the culture, the more numerous are the pigment fragments and debris. When growth occurs to the exclusion of pigment formation, the cells are intact and only little debris is present. The locus of expulsion of the pigment can be followed distinctly.

Acknowledgments

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