

Yellow Pigment Production by *Pseudomonas geniculata* in Maple Sap

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Received for publication 10 February 1964

To extend the study of maple sap and sirup beyond the 6- or 8-week season in which fresh material is available, maple sap is collected aseptically, transferred to sterile metal 1-gal cans, frozen, and stored at -20°C . In controlled fermentation of thawed raw sap with selected strains of *Pseudomonas geniculata*, the formation of a golden-yellow pigment in the sap was observed. This pigment appeared to be a metabolic intermediate, inasmuch as continued incubation of the culture at room temperature (25 to 27°C) resulted in a gradual disappearance of the color. The pigment was not formed during the growth of the organisms in thawed sap sterilized by autoclaving at 121°C for 15 min, but color was formed in sap sterilized by filtration through glass or membrane filters. On autoclave sterilization of the sap, which has a protein concentration of approximately 0.06%, a small amount of brown precipitate forms. The autoclaved sap also supports a heavier growth of bacteria; after 20 hr of incubation at 25°C , the final viable count in raw and autoclaved saps (initially inoculated with 2.5×10^6 organisms per ml) was 43×10^6 and 89×10^6 per ml, respectively. Yellow pigment formation was prevented by the addition of phosphate salts to the sap. The addition of sterile solutions of NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$, or Phytone (BBL) to yield a concentration of 0.05% did not affect color production, whereas the addition of $(\text{NH}_4)_2\text{HPO}_4$, $(\text{NH}_4)_2\text{H}_2\text{PO}_4$, Na_2HPO_4 , or KH_2PO_4 , in the same final concentration, resulted in microbial growth, but without color.

Pigment production can be interrupted and the disappearance of preformed pigment hastened by the addition of $(\text{NH}_4)_2\text{HPO}_4$. Amounts (100 ml) of raw sap in five sterile 300-ml Erlenmeyer flasks were inoculated with *P. geniculata* to a final concentration of 25×10^6 per ml. The flasks were incubated at 25°C for 20 hr. At selected intervals, $(\text{NH}_4)_2\text{HPO}_4$ was added to a different flask. The results (Table 1) show that addition of the salt during incubation prevented further pigment formation. After 7 hr, when the golden-yellow color was at its maximum, salt addition resulted in the disappearance of color by the twentieth hour of incubation, whereas the control fermentation was still pigmented.

To eliminate possible pH effect on pigment production, fresh raw sap with a natural pH of 6.7 was adjusted to various pH levels from 5.2 to 8.2 and inoculated with *P.*

geniculata. During 20 hr of growth at 25°C , a golden-yellow color was formed in the sap at all pH levels.

These results suggested the involvement of a cation in color formation. To check this, a series of flasks containing inoculated fresh raw sap and different amounts of the chelating agent, ethylenediaminetetraacetic acid (EDTA), were incubated at 25°C for 20 hr. No yellow color was formed with EDTA concentrations of 3.3×10^{-4} M or higher. Yellow culture filtrates were also decolorized by subsequent additions of EDTA. The addition of FeSO_4 to EDTA-treated cultures resulted in formation of yellow color. Autoclaved sap containing different concentrations

TABLE 1. Effect on pigment formation of adding $(\text{NH}_4)_2\text{HPO}_4$ at various time intervals to a culture of *Pseudomonas geniculata* in maple sap

Treatment	Time of treatment hr	Color intensity at			
		2 hr	4 hr	7 hr	20 hr
Control	—	++*	+++	++++	++++
$(\text{NH}_4)_2\text{HPO}_4$	0†	0	0	0	0
	2	++	+	0	0
	4	++	+++	++	0
	7	++	+++	++++	0

* Visual observation of color intensity, 0 = no color; ++++ = maximal color; +, ++, +++ = varying degrees of intensity.

† Time after inoculation of maple sap with *P. geniculata*.

of FeSO_4 was inoculated with *P. geniculata* and incubated at 25°C for 20 hr. Color development increased with FeSO_4 concentration, with maximal color formation occurring at 1.4×10^{-5} to 2.8×10^{-5} M Fe^{++} . Qualitative tests for Fe^{++} and Fe^{+++} were made on fresh sap (in which fermentation with *P. geniculata* does not produce the color), thawed canned sap, and autoclaved thawed canned sap. No trace of Fe^{++} was found in any of the saps. Fresh sap and autoclaved canned sap showed traces of Fe^{+++} ; canned sap indicated a high concentration of Fe^{+++} , probably dissolved from the can. Since added Fe^{++} was reactive in pigment formation, it is suggested that the bacteria reduce Fe^{+++} to Fe^{++} , which acts as the chromogen for a compound formed during metabolism.

The colored compound could not be extracted from the

culture medium with chloroform, ethyl acetate, amyl alcohol, or dioxane under acidic, neutral, or basic conditions.

The yellow color was readily removed by adsorption on charcoal, but was not eluted with acetic acid or alkali; hot pyridine eluted only a little of the color. The pigment was precipitated from the culture medium with 2 volumes of either 95% ethanol or methanol. Boiling the alcoholic solution accelerated the precipitation, although heating the medium without alcohol resulted in the disappearance of the color. Golden-yellow pigment was extracted from the alcohol precipitate with several portions of water, leaving

a brown- to orange-colored residue. The pH of the culture medium before precipitation was 6.5; the pH of the water extract of the precipitate was 8.6. On acidification to about pH 3.0 the color became red-orange. On addition of alkali to about pH 10, the yellow color intensified. At a more alkaline pH, the color disappeared. The color was reduced with dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) to the colorless state, and leuco-methylene blue was reoxidized on addition of the yellow-colored solution.

Studies are being carried out to identify the yellow pigment.