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EXPERIMENTS WITH *SARCINA FLAVA*—I.
RELATIONSHIPS OF TIME AND TEMPERATURE WITH GROWTH AND PIGMENTATION

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Abstract—A survey of methods for the complete extraction of carotenoids from *Sarcina flava* has been carried out. It was shown that treatment with 2·5% H_2SO_4 in ethanol and ultrasonic treatment, followed by extraction with methanol and ultrasonic treatment, was the most satisfactory. Experiments on growth and pigmentation with time and temperature revealed that *Sarcina flava* grows optimally at 34° and produces pigment optimally at 25°.

INTRODUCTION

MOST of the investigations of the *Sarcina* group have been carried out with *Sarcina lutea*, and *Sarcina flava* has received comparatively little attention. Consequently, a series of experiments have been embarked upon to investigate the effect of temperature on the growth, pigmentation and metabolism of this micro-organism. Many methods for the extraction of pigments from micro-organisms have been published,[1-3] but in some instances, complete pigment extraction was not accomplished.[4] The severity of the extraction technique used may depend on the type of bacteria, or on the cellular localization and binding (if any) of the pigment.

It is known that most pigment-producing, non-photosynthetic bacteria tend to produce more carotenoids at lower temperatures than at those for optimum growth.[5] It was therefore decided to carry out a survey of possible carotenoid extraction procedures from *Sarcina flava* and to correlate the rate of growth and pigmentation for this bacterium, with time and temperature.

METHODS AND RESULTS

Sarcina flava (strain 7503) was obtained from the National Collection of Type Cultures, London. The stock cultures were maintained alternately on nutrient agar and in nutrient broth (Oxoid Ltd.) at 34°. The bacteria were checked microscopically, after staining, every two weeks. All experiments were carried out from agar cultures grown for varying periods of time in the dark in a standard L.T.E. incubator. These bulk cultures were grown in large aluminium foil dishes sealed in sterile nylon tubing.

Extraction Procedures

All methods were carried out on a three-day culture, the treatment given and results obtained being shown in Table 1. The results are qualitative. Method number 16 was adopted as a standard carotenoid extraction procedure for all the experiments reported below.

[1] M. M. MATHEWS, and W. R. SISTROM, *J. Bact.* 78, 778 (1959).
[2] B. H. DAVIES, *Phytochem.* 1, 25 (1961).
[3] L. R. G. VALADON, *Phytochem.* 2, 71 (1963).
[4] R. M. BAXTER, *Canad. J. Microbiol.* 6, 417 (1960).
[5] T. W. GOODWIN, *Biosynthesis of Vitamins and Related Compounds*, Academic Press, London and New York (1963).

TABLE 1. SUMMARY OF PIGMENT EXTRACTION PROCEDURES FROM *Sarcina flava*

Method No.	Technique*	Result	Comment
1	S in acetone, followed by grinding with acid-washed sand and centrifugation	−ve	—
2	S in distilled water, A at 34° (48 hr), E—acetone	−ve	—
3	S in acetone, U (5 min), centrifugation	+	Very little pigment extracted
4	S in 0·5 N HCl, boil (5 min), cool, E—acetone	+	Very little pigment extracted
5	S in 0·5 N HCl, U (5 min), E—acetone	+ +	Little pigment extracted
6	S in 0·5 N HCl, U (5 min), boil (10 min), cool, E—acetone	+ +	Little pigment extracted
7	S in N methanolic KOH, saponify (20°) under N_2 in dark (12 hr)	+ +	Little pigment extracted
8	S in N methanolic KOH, U (5 min), then as in 7	+ +	Little pigment extracted
9	S in methanol, U (5 min)	+ +	Little pigment extracted
10	As 8, then 0·5 N HCl treatment of residues, E—acetone	+ + +	Much pigment not extracted
11	S in distilled water containing egg white (lysozyme), incubate at 34° (48 hr), E—acetone	+ + +	Much pigment not extracted
12	S in 2·5% H_2SO_4 in ethanol, U (5 min)	+ + +	Much pigment not extracted
13	S in 0·25 N HCl in ethanol at 35°, U (3 min), then similar treatment of residue in methanol	+ + +	Much pigment not extracted
14	S in 0·25 N H_2SO_4 in methanol, U (3 min)	+ + +	Much pigment not extracted
15	S in 5% H_2SO_4 in ethanol, U (3 min)	+ + + +	Not much pigment left in residues
16	S in 2·5% H_2SO_4 in ethanol, U (2 min at 35°), repeat twice, resuspend residue in methanol, U (2 min at 35°), repeat twice, combine all extracts	+ + + + +	Bacterial remnants white

* S—Bacteria suspended. A—Autolysis. E—Extraction of pigment using. U—Ultrasonic treatment with temperature maintained at 20° unless otherwise stated.

Changes in Pigmentation with Time

Inoculating nutrient agar dishes with a three-day broth culture, bacteria were allowed to grow for varying periods of time at 25, 34 and 40°. The bacteria were harvested, the pigment extracted and the bacterial residue dried overnight at 54° and then at room temperature in a desiccator until a constant weight was obtained. An equal volume of water was added to the pigment extract and the carotenoids extracted several times with light petroleum (b.p. 60–80°), ether or a combination of both until recovery was complete (checked spectroscopically). The carotenoid extracts were combined, washed free of acid with distilled water, taken to dryness and redissolved in a standard volume (25 ml) of *n*-hexane. The optical density was determined at 434 mμ, the λ_{max} in hexane. The pigment solution was then taken to dryness and constant weight. A plot of optical density against the weight of the extract gave a straight line which did not pass through the origin. It was assumed that the extra weight was due to other lipids extracted along with the pigments. This value was subtracted from all pigment weights, thus allowing the expression of pigment in mg as shown in the figures. The results obtained are shown in Fig. 1.

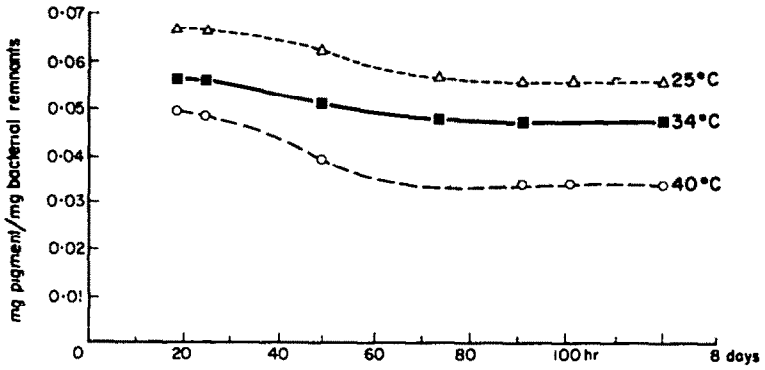

FIG. 1. PIGMENTATION OF *S. flava* WITH TEMPERATURE AND TIME.

Changes in Pigmentation with temperature

Nutrient agar dishes were inoculated with a three-day broth culture and allowed to grow for 72 hr at 8, 15, 20, 25, 34, 40 and 48°. After varying periods of time, the total pigment was extracted and the bacterial remnants dried as described above. The results are shown in curve (A) on Fig. 2.

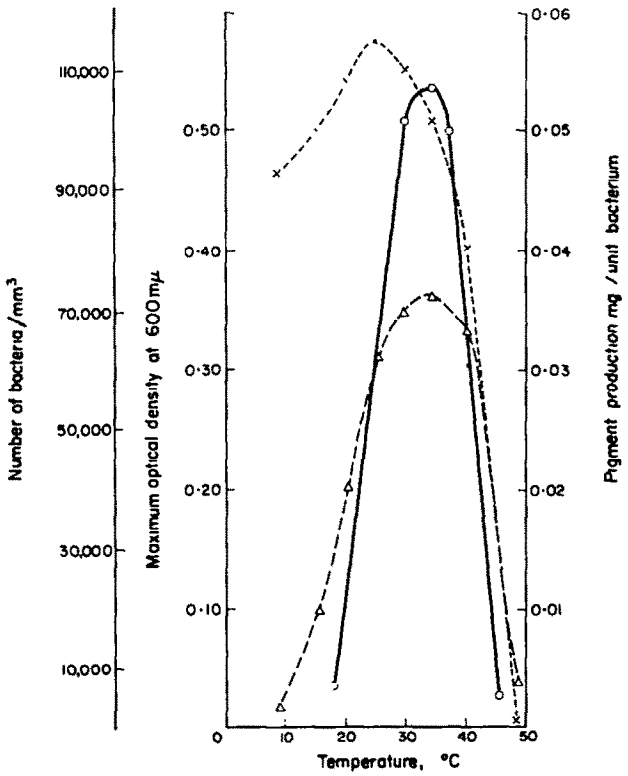

FIG. 2. RELATIONSHIPS OF GROWTH AND PIGMENTATION OF *S. flava* WITH TEMPERATURE.
Curve A: ×---×: Average pigment production for a three-day culture.
Curve B: △--△: Maximum optical density at 600 mμ.
Curve C: ○——○: Bacterial numbers.

Change in Growth with Time

An equal volume of a three-day broth culture was used to inoculate agar dishes and the bacteria were grown for given periods of time at 8, 15, 20, 25, 30, 34, 40 and 47°. At given intervals of time, a known area of the plate was removed from different parts of the dishes. The bacteria were washed off these areas with phosphate buffer, pH 7·2 (Oxoid buffered saline tablets). The volume in each case was made to 50 ml and the optical density (turbidity) of each suspension was determined at 600 mμ where the pigments have virtually no absorption. Figure 3 shows some of the results of the turbimetric readings plotted against time.

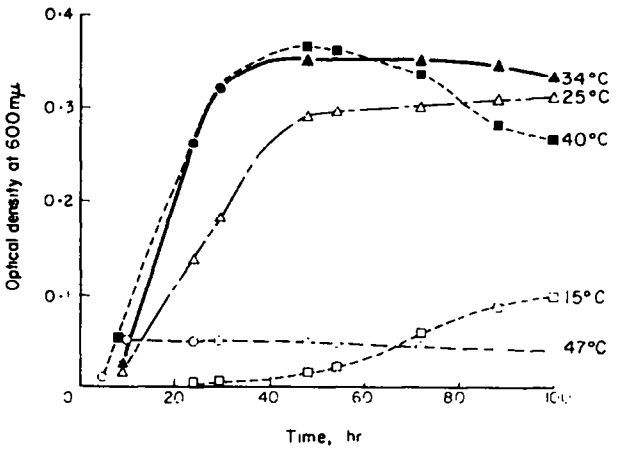

FIG. 3. BACTERIAL GROWTH WITH TIME AT VARIOUS TEMPERATURES.

Change in Growth with Temperature

If the optimum growth at any temperature is regarded as that point in time at which maximum numbers of bacteria are present, differences in growth with temperature can be plotted (Fig. 2B).

This curve was checked by a separate experiment carried out at 18, 30, 34, 37 and 46 in the following manner. Ten millilitre broths were inoculated with an equal volume of 24-hr broth culture (0·5 ml) and grown at the above temperatures. After certain time periods, 0·5 ml was taken from each, under sterile conditions. The sample was counted on a Neubeur haemocytometer (×400) using a white blood cell pipette for dilution. The diluting fluid was N saline containing 1% methylene blue. The maximum number appearing at each temperature is shown in Fig. 2C.

DISCUSSION

That some difficulty was experienced in devising a procedure which would effect a complete extraction of the carotenoids and leave the bacterial remnants white, may indicate that most of the pigment of *Sarcina flava* is not free, but bound by either chemical bonding or association with other cellular components. As ultrasonic treatment disintegrated the cells and the remnants were still pigmented, the pigment may therefore be associated with either the cell membrane or the cell wall. Saperstein and Starr,[6] working with *Sarcina lutea*, have

[6] S. SAPERSTEIN and M. P. STARR, *Biochem. Biophys. Acta* **16**, 482 (1955).

suggested that the carotenoids of chromatrophic bacteria are associated with the cell membrane, and Mathews and Sistrom[1] showed that the pigments of *Sarcina lutea* occur as sedimentable protein complexes.

The extraction procedure adopted involved the use of acid treatment which is known to cause isomerization in many cases. We have shown that the extracted carotenoids gave a characteristic green–blue colour with concentrated sulphuric acid,[7] but the strength of acid used for extraction did not have this effect. Changes in the peaks of the absorption spectra, due to *cis-trans* isomerization, were not observed, and using the standardized conditions (Table 1, N-16), we were always able to obtain reproducible results.

When the bacteria are cultured on plates, they are pigmented as soon as they are visible, except at 48° where no pigment develops. The results shown on Fig. 1 indicate that before 18 hr, pigmentation has reached a maximum and that after 75 hr, it drops to a steady value. This fall is presumably due to the establishment of an equilibrium between dead bacteria and those still alive, or to overcrowding of the individual colonies. The time taken to reach the maximum may depend on the age of the broth used for culturing.

The optimum temperature for pigmentation is 26° and, as seen in Fig. 2A, the amount of pigment produced falls steeply on each side of this temperature. It is worth noting that the optimum temperature for growth of *Sarcina flava* is quoted as 30–35°[8] and thus the lower temperature found for optimum pigmentation agrees with the statement of Goodwin,[5] quoted earlier.

The effect of temperature on growth shows an optimum temperature range of 30–35° (Fig. 2B and C), although the growth is relatively high over the range 30–40°. Virtually no growth occurred at 8 or 47° and outside the maximum temperature range, growth falls off steeply. Both methods used for this investigation produced similar results. Temperature, apart from apparently governing the amount of growth, also has an effect on the rate at which *Sarcina flava* divides. From Fig. 3, it is evident that the curves for 34 and 40° are reasonably similar except that at 40° the numbers fall off after 60 hr. The higher temperature probably causes an increased death rate. At 15°, growth is very slow and the bacteria are still increasing in number after 100 hr, although the overall growth is relatively small. At 47° not many bacteria multiply, but those which do complete their division within 10 hr, after which time there is a slow but steady decline in bacterial numbers up to 100 hr.

[7] T. W. Goodwin, *Modern Methods of Plant Analysis* (Edited by K. Paech and M. V. Tracey), p. 300, Springer-Verlag, Berlin (1955).

[8] R. S. Breed, E. G. D. Murray, and N. R. Smith, *Bergey's Manual of Determinative Bacteriology*, 7th ed., Williams and Wilkins Co., Baltimore (1957).