MORPHOLOGICAL, BIOCHEMICAL AND GROWTH CHARACTERISTICS OF *SERRATIA* STRAINS ISOLATED FROM SARDINE *(SARDINELLA LONGICEPS)*

V. NARAYANAN NAMBIAR, P. K. SURENDRAN & K. MAHADEVA IYER
Central Institute of Fisheries Technology, Ernakulam, Cochin-682011

Two aerobic, gram-negative asporogenous, red-pigmented, rod-shaped bacterial strains were isolated from oil sardine *(Sardinella longiceps)*. Their morphological, biochemical and growth characteristics are reported. The pigment was identified to be a prodigiosene. The strains were found to resemble *Serratia plymuthica*. Effect of temperature and certain carbohydrates on pigmentation was also studied. Iron was found to inhibit pigmentation, and mannitol or sorbitol removed such inhibition.

INTRODUCTION

During the course of our investigation on the bacterial flora of fresh sardines *(Sardinella longiceps)* caught off Cochin, sporadic occurrence of certain red pigmented bacterial colonies in isolation plates, was noticed. Strains from these colonies were isolated, purified and studied further. Their morphological and biochemical characteristics, growth habits as well as colour and nature of the pigment showed that they more or less ressembled *Serratia plymuthica* (Bergey's Manual, 1957). Yet, they differed in some of their biochemical characteristics from those of *S. plymuthica* and hence a detailed study of the strains and their pigments was carried out.

MATERIALS AND METHODS

Cultures

The media used for the isolation was Tryptone-Glucose-Beef extract Agar (TGA) having the following composition:

Tryptone (Difco), 5g., beef extract (Difco), 3g., glucose (Analar) 1g., Sodium chloride (Analar), 5g., agar, 15g., distilled water, 1 litre. pH was adjusted to 7.2 and the media sterilized at 121°C for 15 minutes.

The strains, designated as 'S12R' and 'S13R', were transferred to Nutrient Agar (NA) slants and Sea-Water Agar (SWA) slants and kept at room temperature (29 ± 1°C) for further studies.

MEDIA

For studying the pigmentation characteristics, the following basal medium was used. Peptone (Difco), 10g., beef extract (Difco), 3g., sodium chloride (Analar), 5g., agar, 15g. & distilled water, 1 litre. pH was adjusted to 7.0. To this basal medium was added the appropriate carbohydrates at 1% w/v level and/or ferrous or ferric salts to give 100 p p m of ferrous or ferric iron, as the case may be, before sterilization. The media was sterilized at 115°C for 20 minutes. The following carbohydrates were used: starch, glucose, mannitol, sorbitol and mannose. Ferric sulphate and ferrous sulphate were used as the iron salts.

MORPHOLOGICAL EXAMINATION

Motility was examined by the hanging drop method (Manual of Microbiological Methods, 1957) by using young cultures in Nutrient Broth (NB) incubated at room temperature for 18 hours. Gram reaction was studied by the Gram's method as modified by Hucker (Manual of Microbiological Methods, 1957) on young cultures grown both on NA and SWA slants (18 hours). The gram-stained cells were measured by means of a calibrated ocular micrometer.

BIOCHEMICAL CHARACTERISTICS

The ability of the strains to reduce nitrates, to produce indol from tryptone, to produce hydrogen sulphide from sulphates and cystine, to liquefy gelatin, to produce acetyl methyl carbinol from glucose, to ferment various sugars like glucose, lactose, sucrose, mannitol and maltose, and to utilize citrate as the sole source of carbon was studied by the standard methods (Salle, 1954). Starch hydrolysis was studied by the method of Mahadeva Iyer and Karthiayani, (1964). In all cases, unless otherwise specified, the incubation was at room temperature.

EXTRACTION AND CHARACTERIZATION OF PIGMENT

Cell suspensions of the two strains were spread over pre-set plates (6 Nos.) of starch agar or mannitol agar medium and incubated at room temperature for 72 to 96 hours for maximum pigmentation. The pigmented growth was washed with 150 ml. of sterile normal saline [0.85% w/v NaCl (Analar) in distilled water] into a conical flask (1 l. capacity) and 15g. of sodium hydroxide pellets were added with thorough constant shaking. The pink colour changed to orange yellow. After 2 hours, 150 ml. each of absolute alcohol (98% v/v) and petroleum ether (b. p. 40 - 60°C) were added and mixed well by thorough shaking. The lower alcohol-saline layer was drawn off and it was re-extracted with further small quantities of petroleum ether, until no more colour was removed. The petroleum fractions were mixed and the pigment was extracted with repeated additions of 85% (v/v) ethyl alcohol containing 1% (v/v) glacial acetic acid. Upon transfer to the alcohol, the pink colour returned. The alcohol layer was drawn off into another conical flask and was diluted with an equal amount of distilled water and added 2N aqueous sodium hydroxide until the colour changed to orange-yellow. This was extracted repeatedly with re-distilled chloroform and the chloroform extract was evaporated *in vacua* to dryness (with slight warming). The residue was dissolved in 85% (v/v) ethyl alcohol and the absorption spectrum

TABLE I

Morphological and biochemical characteristics of S12R, S13R and *S. plymuthica*.

Determination	Organism S12R	S13R	*S plymuthica**
Shape and Size	Short rods 0.5x0.7 to 0.8μ	Short rods 0.5x0.7 to 0.8μ	Rods. 0.6x1.5 to 1μ
Gram reaction	Negative	Negative	Negative
Motility	Motile	Motile	Motile
Growth in -			
i) Distilled water media	+	+	+
ii) Sea-water media	+	+	N. D.
Pigment	Red	Red	Red
Growth - at 37°C	Poor	Poor	No Growth
at 56°C	Nil	Nil	Nil
Optimum temp. of growth	30°C	30°C	30°C
Nitrate reduction	++(a)	+	+
Indol production	+	—	—
Acetyl methyl carbinol production	+	+	+
Hydrogen sulphide production			
i) From Cystine	+	+	+
ii) From sulphate	+	+	—
Citrate utilization	+	+	N. D.
Gelatin liquefaction	+	+	+
Starch hydrolysis	—	—	—
Sugar fermentation:			
1) Glucose	Acid & Gas	Acid & Gas	Acid & Gas
2) Lactose	Nil	Nil	Acid & Gas
3) Sucrose	Acid & Gas	Acid & Gas	Acid & Gas
4) Mannitol	Acid & Gas	Acid & Gas	Acid & Gas
5) Maltose	Acid & Gas	Acid & Gas	Acid & Gas
Sodium formate broth	Gas produced	Gas produced	Gas produced
Urea-dextrose-pot-chloride broth	Poor growth No pigment	Poor growth No pigment	Poor or no growth

* = Data taken from Bergey's Manual (1957).

(a)++ = Nitrate was reduced to nitrite first and subsequently to elementary nitrogen.

N. D. = Not described.

studied using Beckman Du Spectrophotometer for identification of the pigments.

Results and Discussion

Morphological and biochemical characteristics

Microscopic examination of the two strains showed typical gram-negative short rods (0.5 by 0.7 to 0.8 μ) arranged in pairs and short chains of four to six cells. Both the strains were motile and identical in morphology and all of the biochemical characteristics except production of indol. (Table I)

Colonies on NA and SWA (after 48 hours at room temperature) were small (2-3 mm.), smooth and round with entire margin. They were blood red on NA, starch agar (SA), TGA, Mannitol agar, Mannose agar and pink on SWA.

Good growth of both the strains were obtained on NA, SA, TGA, Mannitol agar and Tryptone-dextrose-starch agar (TDSA). Growth was poor on SWA. At 37°C there was comparatively less growth and no pigment production. There was no growth at 56°C. The optimum temperature for growth with production of pigment for both the strains was found to be 30°C.

Both the strains reduced nitrate to nitrite within 24 hours. Strain S12R further reduced nitrite to elementary nitrogen within 4 days, whereas strain S13R was not found to reduce nitrite further. Strain S12R produced indol from tryptone while S13R failed to produce indol. Both the strains produced acetyl-methyl-carbinol from glucose within 24 hours, produced hydrogen sulphide from cystine as well as from sulphate, utilized citrate as the sole source of carbon for growth, liquefied gelatin (with associate production of pigment), but did not hydrolyse starch. Both the strains produced acid and gas from glucose, sucrose, mannitol and maltose, but neither acid nor gas from lactose. Both the strains showed growth in sodium formate broth with gas production. Growth was very poor in urea-dextrose-potassium chloride broth and no pigment was produced. Both the strains produced ammonia and trimethylamine when grown in media containing peptone.

Pigmentation

In Nutrient broth (NB) there was visible growth after 18 hours, but no pigment was produced within 24 hours. Pigment production was observed only after good growth had been established (48 hours). On NA slants growth was visible after 3 hours but pigmentation was observed only after 18 hrs. This observation was similar to that of Williams (1973) who has reported that prodigiosene, being a secondary metabolite, is produced only in the late log. phase and stationary phase of growth. Pigment production on SWA slants was similar to that on NA slants. When the cultures were incubated at 37°C, though there was growth (poor growth when compared to the growth at room temperature), there was no pigment production even after incubation for 48 hours. But when these cultures grown at 37°C were subsequently incubated at room temperature, pigment production started within 18 hours. Similar findings have been reported by Williams *et al.* (1971). They have observed that *Serratia marcescens* when incubated at 38°C did not produce pigment, eventhough there was growth. When such cultures were subsequently incubated at 27°C, pigmentation ensued provided that the period of in-

cubation at 37°C did not exceed 36 to 48 hours depending upon the growth medium. This may be due to the temporary inhibition of the pigmentation process at 37°C, which is regenerated by subsequent incubation at room temperature.

Though pigment production was observed in NA and SWA, it was enhanced through the incorporation of carbohydrates into the media. Mannose, mannitol, starch and sorbitol enhanced pigmentation (though at varying degrees), whereas glucose suppressed pigmentation temporarily for 48 hours. (Table II)

TABLE II

Pigmentation of S12R and S13R on various carbohydrate media. *

Carbohydrate	Pigmentation after 24 hours S12R	S13R
Mannose	+++	+++
Glucose	—	—
Sorbitol	+	+++
Mannitol	+++	+++
No carbohydrate (control)	+	+

* = Carbohydrate at 1% (w/v) level added to basal media containing peptone 1%, Beef extract 0.3%, NaCl 0.5% in D. W. pH 7.0.
+++ = Excellent pigmentation.
+ = Poor pigmentation.
— = No pigmentation.
In all cases, growth of the bacteria was good.

When the strains were transferred three to four times through NA (or SWA), the property of pigmentation was lost and subsequent subculturing produced only non-pigmenting strains (which were identical in all other aspects with the original cultures). The loss of pigmentation may be due to the damage of the pigment synthesizing enzyme systems in the bacteria by some ingredients present only in trace amounts in the media. Studies have shown that the property of pigmentation could be regenerated when the non-pigmenting strains were transferred to a nutrient medium containing mannitol, sorbitol or mannose. Further studies revealed that iron was responsible for damaging the pigmentation system. Both ferric and ferrous iron could hinder pigmentation, ferric iron being a more potent inhibitor (Table-III). This poisoning of pigmentation system by iron could be removed and pigmentation regenerated by mannitol, sorbitol or mannose, mannitol being the most effective. (Table-IV)

TABLE III

Effect of iron on pigmentation.

Concentration of iron in the medium (ppm)		Pigmentation after 24 hours S12R	S13R
0	(NA slants) control.	++	++
10	(Fe ++)	+	+
	(Fe +++)	+	+
100	(Fe ++)	—	⊥
	(Fe +++)	—	—

The inoculum was highly pigmented (++) cells from nutrientmannitol agar slants. There was good growth in all the media but pigmentation was different.
++ = Very good pigmentation.
+ = Poor pigmentation.
⊥ = Very poor pigmentation.
— = No pigmentation.

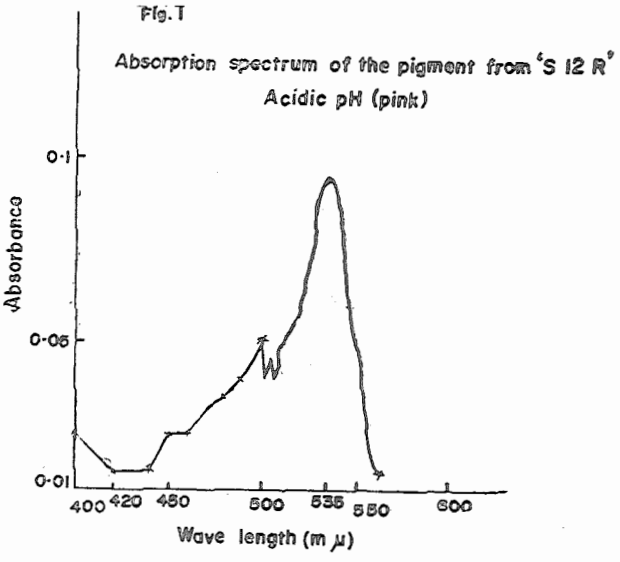
TABLE IV

Regeneration of pigment producing capacity of the bacteria by carbohydrates. *

Medium	Pigmentation after 24 hours S12R	S13R
Sea-water agar (SWA)	—	—
Nutrient agar (NA)	—	—
NA + glucose	—	—
NA+ starch	—	—
NA+ mannitol	+++	+++
NA + sorbitol	+++	+++
NA + mannose	++	++

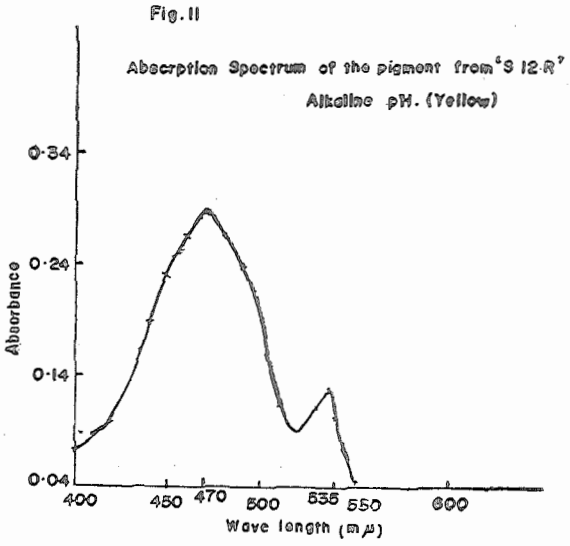
* Pigmentation was lost either by continuous subculturing on NA or SWA, by incubation at 37°C or by poisoning by iron salts.

+++ = Excellent pigmentation.
++ = Good pigmentation.
— = No pigmentation.

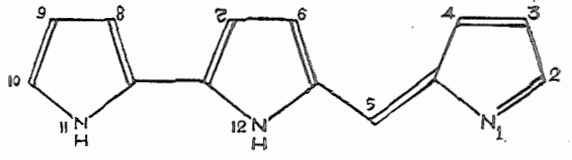


Characterization of pigment

The absorption spectrum of the pigment at two pH ranges (acidic and alkaline) gave absorption maxima at two different



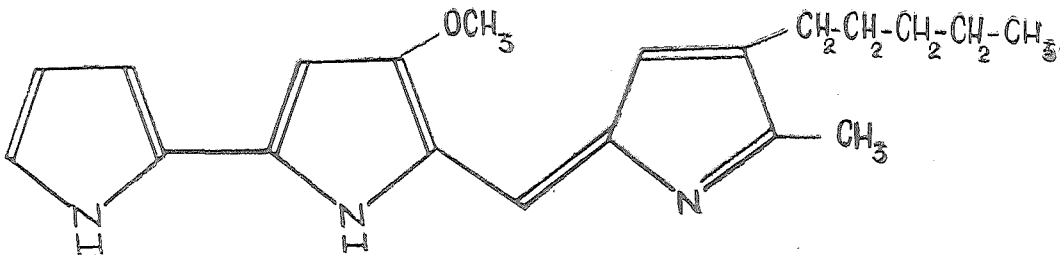
wave lengths. In the acidic pH (pink colour), the absorption maxima was at 535 nm, and in the alkaline pH (orange yellow), the absorption maxima was at 470 nm (Fig. 1 & Fig. 2). Chemical tests showed that the pigment was not a carotene (Skerman, 1959). The absorption maxima obtained were characteristic of the prodigiosene pigments (Skerman, 1959, Williams, 1973, Lewis & Corpe, 1964), having the following common nucleus: (Hearn *et al.* 1970).



Prodigiosene.

The pigment commonly known as 'prodigiosin' is a substituted prodigiosene – 2-methyl-3-amyl-6-methoxy prodigiosene – having the following chemical structure.

Based on the morphological and biochemical characteristics and the nature of the pigment produced by them, the two strains S12R and S13R could well



Prodigiosin
(2-methyl-3-amyl - 6-methoxy prodigiosene)

be placed in the genus *Serratia* of the family *Enterobacteriaceae*. (Bergey's Manual, 1957). Five different species of the genus *Serratia* have been described in Bergey's Manual, 1957 - *S. marcescens*, *S. indica*, *S. plymuthica*, *S. kiliensis* and *S. piscatorum*. Lewis and Corpe (1964) have described a marine strain, *S. marinorubra* which was originally described by ZoBell and Upham (1944). Lewis and Corpe (*loc. cit.*) have also isolated two marine redpigmented strains producing prodigiosin-like pigment, which they report not to belong to the genus *Serratia*. Both the strains which we have isolated could not be exactly grouped with any of these species, though they were more akin to *S. plymuthica* in most of their characteristics. The stricking differences between our strains and *S. plymuthica* were (1) The cells were smaller in size compared to those of *S. plymuthica*, (2) Our strains produced hydrogen sulphide from sulphates whereas *S. plymuthica* did not produce hydrogen sulphide from sulphates and (3) Our strains did not produce acid or gas from lactose while *S. plymuthica* produced acid and gas from lactose. Also, one of our strains, S12R produced indol from tryptone in which aspect this strain differed from both S13R and S. plymuthica. (Table I).

The strains isolated by Lewis and Corpe (*loc. cit.*) from marine sources were truly marine in that they invariably required sea-water or its constituents for growth. Though our strains were isolated from ocean-fresh oil sardines, they could very well grow in distilled water based media also. We have observed that sea-water or its constituents are not invariably necessary for the growth of majority of bacteria isolated from marine sources (unpublished). Hence the fact that our strains did not require sea-water or its constituents for growth is not an indication that they are non-marine.

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