

Review Article

<https://doi.org/10.20546/ijcmas.2018.701.297>

Effect of Certain Compounds on the Pigmentation Characteristics of Some Bacterial Isolates

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ABSTRACT

Keywords

Pigmentation Characteristics, pigmented colonies

Article Info

Accepted:

16 December 2017

Available Online:

10 January 2018

Microorganisms produce various pigments like carotenoids, melanin's, flavins, monascins, violacein and indigo. Carotenoids are a group of bioactive compounds and are responsible for bright yellow, orange, red pigments of various plants, microorganisms and animals and are widely distributed in nature. These pigments have an important function to act as protective agents against oxidative damage. Recently carotenoids have attracted greater attention due to the beneficial role on human health. As pigments have been extensively evaluated over the years for its antibiotic, antigenic and antitumor effects, special consideration was made towards ability of chromogenic bacteria to produce pigmentation under certain chemical conditions.

Introduction

Many of the pigments are derivatives of certain groups of chemicals such as carotenoids, phenazine or pyrrolic dyes, azaquinones and anthocyanates

The microbial pigments are secondary metabolites, that is, they are compounds not found in all organisms. They are not derived from the normal blocks and metabolites of cells.

Some bacterial species produce and retain

water insoluble pigments intracellularly, thus causing the colonies to become colored (pigmented)

Some species which form pigmented colonies are

Serratia marcescens - Red
Staphylococcus aureus - Gold
Chromobacterium Violaceum - Violet
Flectobacillus major - Pink

Some colonies produce pigments that are water soluble for instance, *Pseudomonas*

aeruginosa forms a blue water-soluble pigment called pyocyanin. Some pigments are only sparingly water soluble and may precipitate in the medium, for example *Pseudomonas chlororaphis* forms a pigment called chlororaphin which accumulates in the form of green crystals around the colonies.

Certain water-soluble pigments are fluorescent in the agar medium around the colonies grows white or blue-green when exposed to ultraviolet light. For example, *P. aeruginosa* produce not only, the non-fluorescent pigment pyocyanin but also fluorescent pigment pyoverdine

For a bacterial strain to exhibit its characteristic pigmentation special media incubation temperatures or other conditions may be required. For instance, *Mycobacterium kansasii* forms a characteristic yellow pigment (b-carotene) only when the colonies are exposed to light (Table 1).

Pigmented forms are common among airborne inocula because the pigments, protect against the rays of the visible and near UV light ranges. Thus, on straw and dust and in other locations that are exposed to light, the ability to form such pigments confers a selective advantage over non-pigmented forms, which are more easily inactivated by light.

This protection against photodynamic damage was demonstrated in a study of a normally orange-colored, carotenoid-containing strain of halophilic bacterium and its colorless mutant. The bactericidal effect of visible light occurred under aerobic conditions and thus was apparently due to photo-oxidation

In photo-oxidation, several cellular pigments (flavins, cytochromes) apparently acts as catalysts. The carotenoids, localized in the cytoplasmic membrane, protect the sensitive regions of the cell from the effects of photo-

oxidation. Pigments produced by microorganisms are as follows

Pyocyanin is excreted by *Pseudomonas aeruginosa*

Iodine is excreted by *Chromobacterium iodinum*

Indigoidin is produced by several bacterium including *Pseudomonas indigofera*.

Antibiotic nature of pigment prodigiosin

Prodigiosin is the characteristic red pigment commonly produced by *Serratia marcescens*.

Kraft (1902) extracted the red prodigiosin from *Bacillus prodiosus*

Observations of antibiotic properties of pigmented cultures of *Serratia marcescens* were first reported in 1885, these early investigation (1949) described antagonistic effects both in-vitro and in-vivo of pigmented cultures against many bacterial and fungal species. Since that time the pigment has been evaluated for its antibiotic antigenic and antitumor effects

The antibiotic nature of prodigiosin and in spite of the importance many other antibiotics have served in current chemotherapy

The observations by Caujolle *et al.*, (1967) of malformations induced by DMSO were similar in few cases to those induced by the whole pigment and two of the pigment fractions, malformations of beak (parrot beak in prodigiosin c-2 and a-3) and anophthalmia of the left eye (c-2 and a-3)

Special consideration should be declared towards the inability of prodigiosin to inhibit *Staphylococcus aureus* at a concentration of 12.9µg in 0.03 ml DMSO, whereas EO4

demonstrated a positive zonal inhibition. Efimenko *et al.*, (1956) and Burgova *et al.*, (1957) reported that they found a fraction that possessed antibiotic activity, which appears to be different in many physical properties from our fractions. Both stated that this antibiotic fraction showed a red coloration, however in the present study the only fraction that came close to this description was the c-2 fraction which proved to be totally worthless as an antimicrobial agent in-vitro and was highly toxigenic during embryogenesis. The f-4 fraction from the pigment prodigiosin has been shown to be least embryotoxic and to inhibit the growth of several species of bacteria.

None of the discs charged with pigment fraction in petroleum ether, chloroform, acetone, ethanol and methanol exhibited any growth inhibition for all organisms tested. *Pseudomonas* spp was used only in the sensitivity studies incorporating the solvent DMSO. From the result it can be seen that PE-1, C-2 and A-3 in DMSO also failed to exhibit any in-vitro antimicrobial activity. Thus, eliminating these fractions as possible bactericidal substances, however prodigiosin, E-4 AND M-5 in DMSO showed definite growth inhibition for organisms tested including *Pseudomonas*. The comparable antibiotic properties between the whole pigment and E-4 fraction, least zonal inhibition occurred with M-5.

Treatment of soil with an aqueous, membrane filtered solution (3, 5 mg/ml) of the salt causes the soil microorganisms to fluoresce when examined with light from a mercury arc light source. The magnesium salt of 1-anilino-8-naphthalene sulfonic acid (Mg-Ans Nutritional biochemical, devaland, ohio) was reported to fluoresce upon binding to serum albumin (weber and Laurence, 1954).

Lepierre (1898) studied a fluorescent bacillus.

He found that the only common organic acid salts, which gave fluorescence when used with proper inorganic constituents, were citric, succinic. Oxy-glutanic and glutaric acid. He concluded that fluorescence depends, first upon on the dibasicity of the acid, second upon the presence of at least two ch₂ groups in the molecule.

Effect of certain compounds on pigmentation

Jordan (1899) and Sullivan (1905) stated that sulphates and phosphates regardless of the base, are the only inorganic elements necessary for the production of fluorescence.

Media were prepared containing 0.2 percent asparagine, with varying amounts of dipotassium phosphate and sulphates of sodium, potassium and ammonium, and also sulphuric acid. Control media were also prepared from each of the above media with the addition of 0.01 percent anhydrous magnesium chloride. Very little fluorescence was produced in any of the media without magnesium.

In some cases, the media containing the larger amounts of the sulphates gave a slight production of pigment. It was thought that these might contain a small amount of magnesium as an impurity.

In studying the media made from the different sulphates, it was found that the greater fluorescence was produced by the sample containing the sulphates of alkalis, gave little or no fluorescence, but in every case where magnesium chloride was added a good fluorescence was produced.

In order to prove definitely that the alkali sulphates will not suffice to produce fluorescence, it was decided to prepare a sulphate absolutely free from magnesium.

Ammonium hydroxide and sulphuric acid were mixed in proper proportion to form ammonium sulphate. A medium was made, using this pure sulphate with a pure sample of dipotassium phosphate and asparagine. The medium made with ammonium sulphate in place of magnesium sulphate will not produce fluorescence.

The media contained 0.2 percent asparagine, varying amounts of potassium phosphate from 0.0001 to 0.1 percent and ten different amounts (from 0.00001 to 0.5 percent) of the magnesium chloride or magnesium ammonium chloride. The media containing the chlorides free from sulphates, gave very little fluorescence. The samples of chlorides which contained the sulphate as impurity gave much more fluorescence especially when the larger amount of magnesium chloride was used. This indicates that the sulphate is necessary for pigment production.

Several sets of media were made with asparagine and magnesium sulphate, but no fluorescence was observed, when a phosphate was added, a good fluorescence was produced, it may be concluded, therefore, that in addition to organic constituent such as asparagine the medium must contain a phosphate, magnesium and sulphate.

In order to determine the amounts of different constituents which, when present in a medium, will produce the best fluorescence. Media were, therefore made with varying amounts and combinations of the three ingredients.

The fluorescence in the lower percentage of asparagine was about equally good.

The medium containing 0.3 percent was probably a little better. The pigment production was very good when the phosphate content was between 0.005 and 0.1 percent, and when the magnesium sulphate was

between 0.001 and 0.2 percent. The best and quickest pigment production was in the media containing 0.3 percent asparagine, 0.05 percent dipotassium phosphate and 0.05 percent magnesium sulphate.

In the light of the above, the following medium gives as the most satisfactory results for the production of fluorescence.

Magnesium sulphate anhydrous - 0.5g
Dipotassium phosphate anhydrous - 0.5g
Asparagine - 3.0g
Distilled water - 1000cc

Pigmentation of halophilic bacteria

Bacteria living in highly concentrated saline media have shown two physiological types with different levels of adaptation to high salinity, the moderate halophiles and the extreme halophiles (flanned, 1956, Larsen, 1962, Dundas, 1977).

Both groups are adapted to live in this type of environment. The first is quite heterogenous and includes bacteria that in general show a wide growth range with respect to salt, their optimum being usually around 1-2 M NaCl.

The moderate halophiles, which grow over a much wider range of salt concentration than the extreme halophiles, are not greatly different physiologically from fresh water or marine bacteria (Kushner, 1978).

The extreme halophiles, are by contrast, drastically modified. They all adapt their internal ion concentrations to the environment (Larsen, 1967) and their proteins have a strong acidic composition that makes their structures, salt dependent (Langi, 1974). In addition, the cell envelop is of acidic character and lacks peptidoglycan (Larsen, 1967). The salt range which permits growth, with few exceptions, has a lower limit around 2-3 M NaCl, and is

optimal at 4-5 M, most or all can grow in saturated NaCl (Larsen, 1967; Dundas, 1977) In nutrient rich batch cultures, the moderate halophiles show a faster growth rate than shown by the extreme halophiles, even at the higher NaCl concentrations preferred by the extreme types (Forsyth and Kushner, 1970, Matheson *et al.*, 1976).

Competition among halophilic bacteria can be studied more easily, since the extreme halophiles are strongly pigmented due to red or pink carotenoids whereas the moderate halophiles, with the exception of the red-pigmented *Spirochaeta halophila* (Greenberg and Canaleparola, 1976), are non-pigmented or show only brown or yellow pigments.

Pigmentation permits differentiation between colonies of the two groups and facilitates the use of mixed natural populations of the two groups of microorganisms

Temperature seems to be the dominant selective factor in the interval of 20-30 percent salt concentration, the moderate halophiles being favored by lower temperatures. This fact is not surprising considering the demonstrated thermophilism of extreme halophiles (Keradiopoulos and Halldorf, 1977). A fourth retinal-containing pigment in *Halobacterium halobium* cell membrane was examined by flash spectrophotometry.

The absorption maximum of this pigment was at about 480 NM. Flash light caused a photoreaction cycle. The photoreaction cycle involved at least two photo intermediates. The absorption maximum of the first one was at about 350nm and that of the second was at around 530nm. The spectral properties of this pigment and the content of the cell correlate with the sensitivity of photorepellent response to the light around 480 nm and suggest a name phoborhodopsin for this new pigment

Wild type *Halobacterium halobium* cells are

attracted to yellow red light (positive phototaxis) and repelled by near-UV/blue light (negative phototaxis). Retinal containing pigments function as the photoreceptors. Three rhodopsin like pigments are so far known in *H. halobium* membrane: Bacteriorhodopsin (br) Halorhodopsin (hr) and Sensory rhodopsin (sr)

Br (λ_{max} =568 nm and hr (λ_{max} =478nm) and show the phototaxis comparable to that of wild type strains.

Mutant (oni) which lacks the photo attractant response but shows an only photo repellent response whose sensitivity maximum is at around 480nm. It indicates that there is an additional photosystem other than the photosystem whose sensitivity maximum is around 370nm. Flash spectrophotometric data suggested that there was another retinal containing pigment (fourth rhodopsin like pigment) in cell membrane of *H. halobium*.

Role of tyrosine in pigmentation

Many species of bacteria and fungi have been reported to produce block of brown pigments from tyrosine (Ivins and Holmes, 1981, Barnett *et al.*, 1983, Wheeler, 1983; Vickers and Yu, 1984; Muller, 1985 Sadasivan and Neyro, 1985; Yoshimoto *et al.*, 1985) among them, melanin's have been extensively studied and are the best-known group of pigments that are synthesized from tyrosine.

Serratia marcescens produce a brown pigment when grown in minimal medium in the presence of tyrosine and high concentrations of copper (ii) ion.

The pigment was not related to the melanin pigments, but was similar to the pigment produced by the autoxidation and polymerization of 3,4-dihydroxy phenylacetate, which is synthesized in *S. marcescens* from tyrosine through the 3,4-

dihydroxyacetate catabolic pathway. Melanin's have been divided in to three classes, depending on the substrate used for pigment synthesis. Eumelanins are synthesized from tyrosine alone. In addition, cysteine is needed for phaeomelanin formation.

Allomelanins are derived from compounds such as acetate, catechol or 1,8-dihydroxynaphthalene (Alport and Buloch, 1958; Wheeler, 1983).

Among bacteria eumelanins have been demonstrated in actinomycetes (Kuster, 1976) and bacillus cereus (Aaronson and Vickers, 1965). Phacomelanin is produced by some vibrio strains (Pomerantz and murthy, 1974; Ivins and Holmes, 1981)

Some *S. marcescens* strains are able to produce different pigments. The best known prodigiosin is a linear tripyrrole attach to the cytoplasmic membrane (Vinas *et al.*, 1983; Parachuri and Harshey, 1987). Some strains of *S. marcescens* belonging to the biotype A 40 synthesise a rose coloured diffusible pigment pyrimine (Grimont and Grimont, 1984)

Tyrosinase is a copper containing enzyme which catalyzes the first reaction of the melanogenesis pathway.

Black or brown pigments other than the melanin's have also been reported in *Pseudomonas aeruginosa*, where the pigment is formed by homogenetic and excreted into the medium (Yabuuchi and Ahyama, 1972). Muller (1985) demonstrated that the excretion of the alpha-ketoacids of the aromatic amino acids in the *Morganella-Proteus-Providencia* group yielded blackish pigment in the presence of iron under alkaline conditions.

Influence of media composition on pigment formation

A mineral medium (gl) for *S. marcescens* was

prepared. Tyrosine was added to gl to give a final concentration of 1 g/l (gl medium). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added to various concentrations (from 0.8 μm to 8 mm). The effect of initial ph was studied from ph 5 to 9 and the influence of cysteine at concentrations of 1, 0.1, 0.01, 0.001 and 0 mg/ml was also investigated. Flasks with 95 ml of the medium were inoculated with 5 ml of an overnight culture grown in glt. Growth was monitored by following the od600 of the culture in spectrophotometer. Bacteria were removed by centrifugation and pigments production was measured in the supernatant fraction (OD 430) (Barnett *et al.*, 1983).

The main characteristics of the brown pigments produced by *S. marcescens* compared with those produced by the autoxidation of dl-dihydroxyphenyl alanine and 3,4-dihydroxyphenylacetate. The properties of the brown pigments are closer to the autoxidized 3,4-dihydroxyphenylacetate than the autoxidized dl-dihydroxyphenylalanine. The substrates of aromatic ring cleavage dioxygenases (catechol, 2,5-dihydroxy phenylactate, 3,4-dihydroxyphenylacetate and 3,4-dihydroxybenzoate) and dl-dihydroxyphenylalanine were able to produce brown pigments.

Many strains of *S. marcescens* have been reported to produce different pigments. The production of brown pigments by *S. marcescens* has been demonstrated. This occurs when tyrosine is added to the medium. Since copper (ii) is needed to obtain a good pigmentation and alkaline ph enhances it. Pigment enhancement resulting from copper addition happened at high copper concentrations and it is unlikely that a tyrosinase-type enzyme needs such high copper concentrations to be achieve.

Mutants unable to use tyrosine as sole energy and carbon source could only produce the

pigment if the pathway was blocked after the formation of 3,4-dihydroxyphenyl acetate. Many gram negative heterotrophic rods producing a violet pigment insoluble in water have been isolated. The physiology of these bacteria gave rise to several studies which resulted in their being classified within the genus, *Chromobacterium* (Cruess-Gallaghan and Gorman, 1935; Gilman, 1953; Leifson, 1956; Sneath, 1956a, 1956b; Eltinge, 1957)

Presence of spores or capsules, and gram staining were investigated using the techniques described by Buttiaux *et al.*, (1969). Flagellation was demonstrated by Rhodes method but because of instantaneous lysis occur with these strains in a non-saline medium, it was need to modify the method slightly according to a pattern describe before (Gauthier *et al.*, 1975).

Several media were used for studying the pigments. The best synthesis results have been obtained on solid Zobel's medium 2216 added with 1% soluble starch (w/v). The violet pigment was extracted with ethanol (90%) from separated by centrifugation (6000rpm, 4°C 15 min). Another pigment, straw yellow in color and insoluble in ethanol was extracted in the same way using petroleum ether. The pigments were studied by UV and visible spectrophotometry

The potential growth of the strains at various temperature, pH and salinities in Zobel's medium 2216, the bacteria appear to be psychrophilic and grow in a moderately saline medium (15 to 50%) i.e. with a salt content comparable with that of sea water. In addition, they require a neutral to alkaline pH to multiply and can usually tolerate a high alkalinity. Their old cultures become strongly alkaline.

The determination of the Na⁺ requirements of these strains show that their inability to grow in fresh water media is chiefly connected with the absence of that ion, their optimum Na⁺ concentration is about 0.4 M, all being more or less inhibited at concentrations higher than 0.6M. Violet types are more sensitive to low Na⁺ concentrations than *Alteromonas* species, since they cannot grow in the presence of less than 0.1 M Na⁺.

Bacteria of the genus, *Pseudomonas* may, under controlled nutritional conditions excrete water soluble fluorescent pigments in to the culture media. The ability to excrete the pigments is variable and depends on the composition of the growth medium.

Although all compounds essential for growth will certainly affect the production of the pigments. Various media have been defined for the evaluation of this important characteristic. Some require special ingredients (which if analyzed will be low in iron) and others require tedious and laborious removal by solvent extraction of the iron normally containing most ingredients of media.

The use of chelating agents and solvent extraction as suggested by Paton 101 is not only tedious and laborious, but if conducted with utmost efficiency will render the media iron free a nutritional condition permitting no growth of *Pseudomonas*.

Effect of iron on pigmentation

The principle factor, in addition to those essential for growth, which must be controlled to insure the excretion of fluorescent pigment by the genus *Pseudomonas* is the concentration of iron in the growth medium.

Table.1 Colony characterization of different pigmented isolates obtained from milk and air sample

Colony characteristics	colony			
	A	B	C	D
Size	2mm	3mm	pinpoint	1mm
Shape	circular	circular	circular	circular
Color	yellow	orange	cream	pink
Margin	entire	entire	entire	entire
Elevation	concave	concave	flat	concave
Surface	smooth	smooth	smooth	smooth
Opacity	opaque	opaque	opaque	opaque
Consistency	sticky	sticky	sticky	sticky
Grams nature	positive	negative	positive	positive
Morphology	Cocci	Short rods	Short rods	coccobacillary
Motility	--	--	--	--
Pigment solubility in water	++	++	++	++

Table.2 Biochemical characterization of the isolated Pigmented bacteria

pigmentation	amylase	catalase	gelatinase	urease
yellow	-	-	++	-
cream	++	++	++	++
pink	-	-	-	++
orange	++	-	++	+

Table.3 Ultraviolet spectrophotometric characterization of yellow pigmented isolate in presence of various compounds

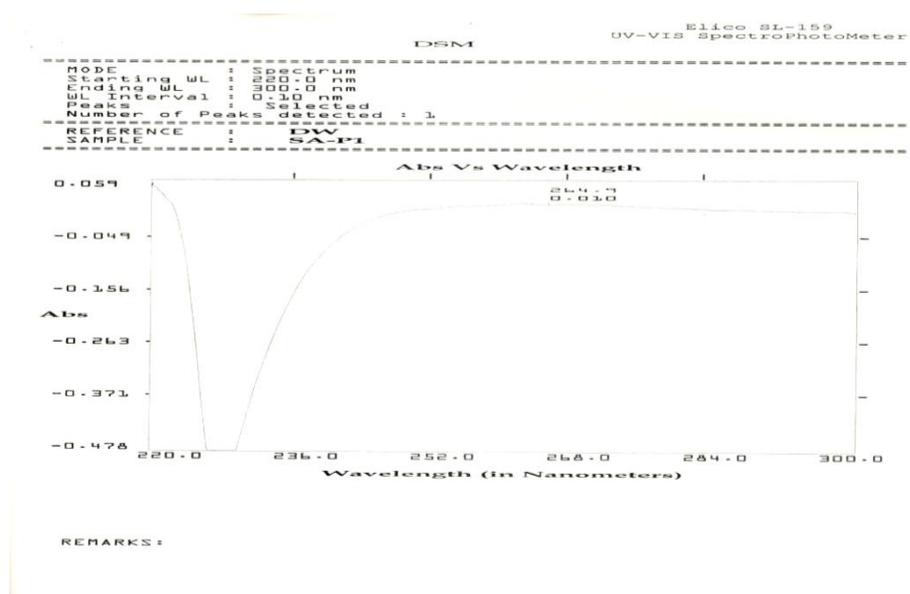
Compound used	Maximum absorption observed (nm)	Corresponding O.D
phosphate		
0.1%	264.9	0.010
0.05%	387.9	0.042
sulphate	No peak detected	
0.1%		
0.05%		
tyrosine		
0.1%		
0.05%		

Table.4 Ultraviolet spectrophotometric characterization of cream pigmented isolate in presence of various compounds

Compound used	Maximum absorption observed (nm)	Corresponding O. D
phosphate		
0.1%	207.5	1.822
0.05%	209.5	1.939
Sulphate		
0.1%	211.0	2.220
0.05%	209.5	2.006
tyrosine		
0.1%	210.0	1.990
0.05%	209.0	1.884

Table.5 Ultraviolet spectrophotometric characterization of orange pigmented isolate in presence of various compounds

Compound used	Maximum absorption observed (nm)	Corresponding O.D
phosphate		
0.1%	----	----
0.05%	207.5	1.842
Sulphate		
0.1%	209.4	2.078
0.05%	208.5	1.958
tyrosine		
0.1%	208.6	1.947
0.05%	214.0	2.298

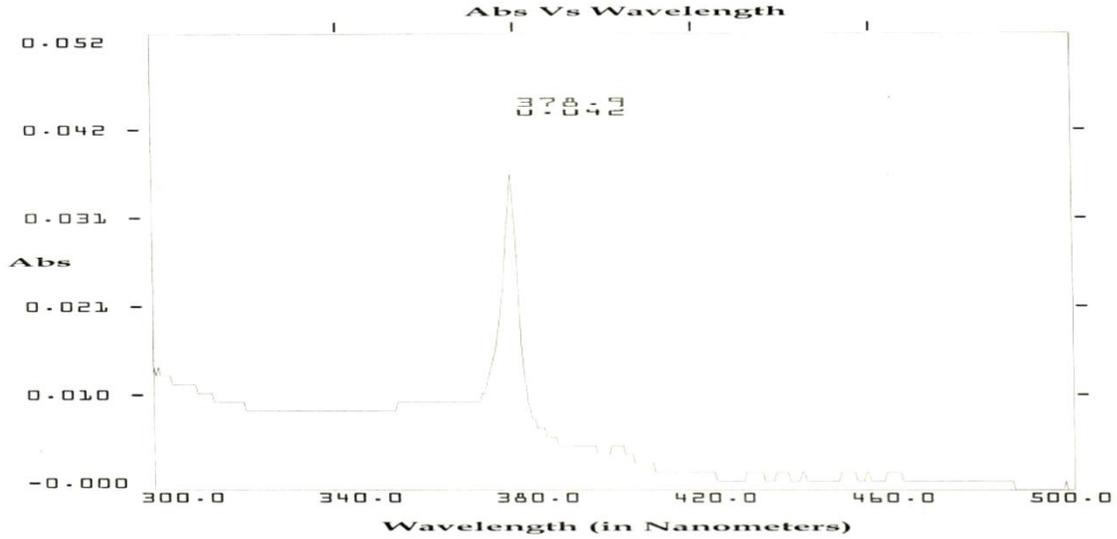


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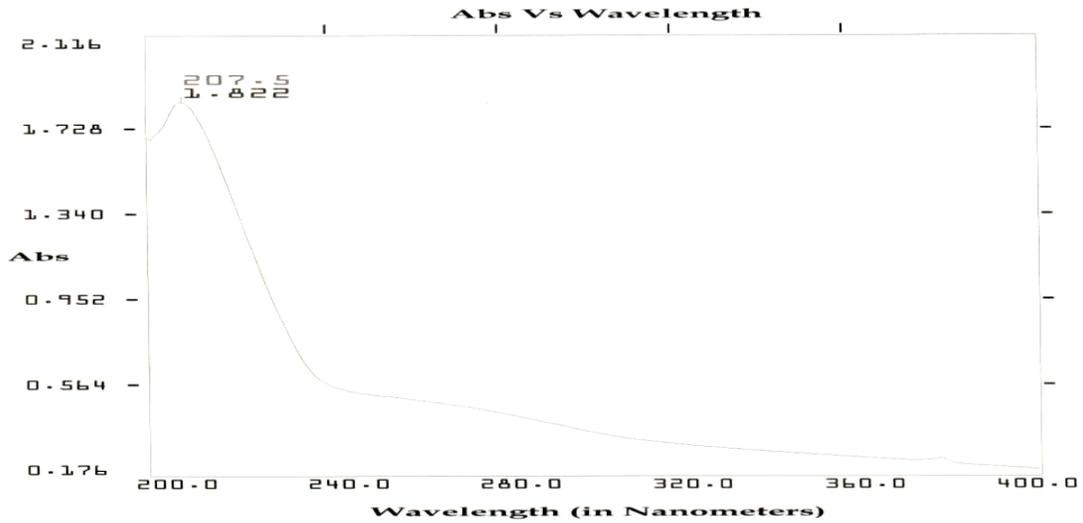


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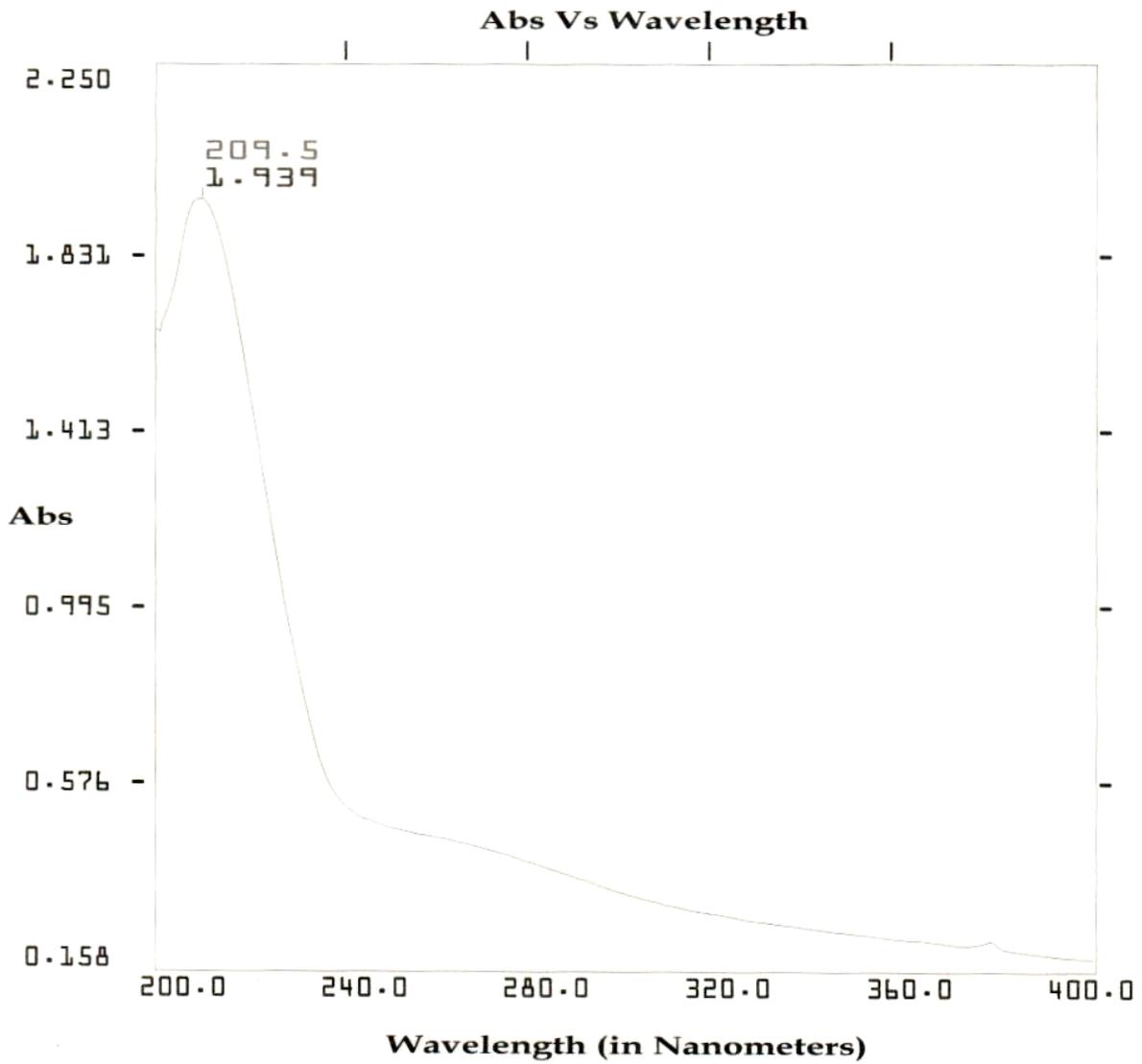
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Elico SL-159
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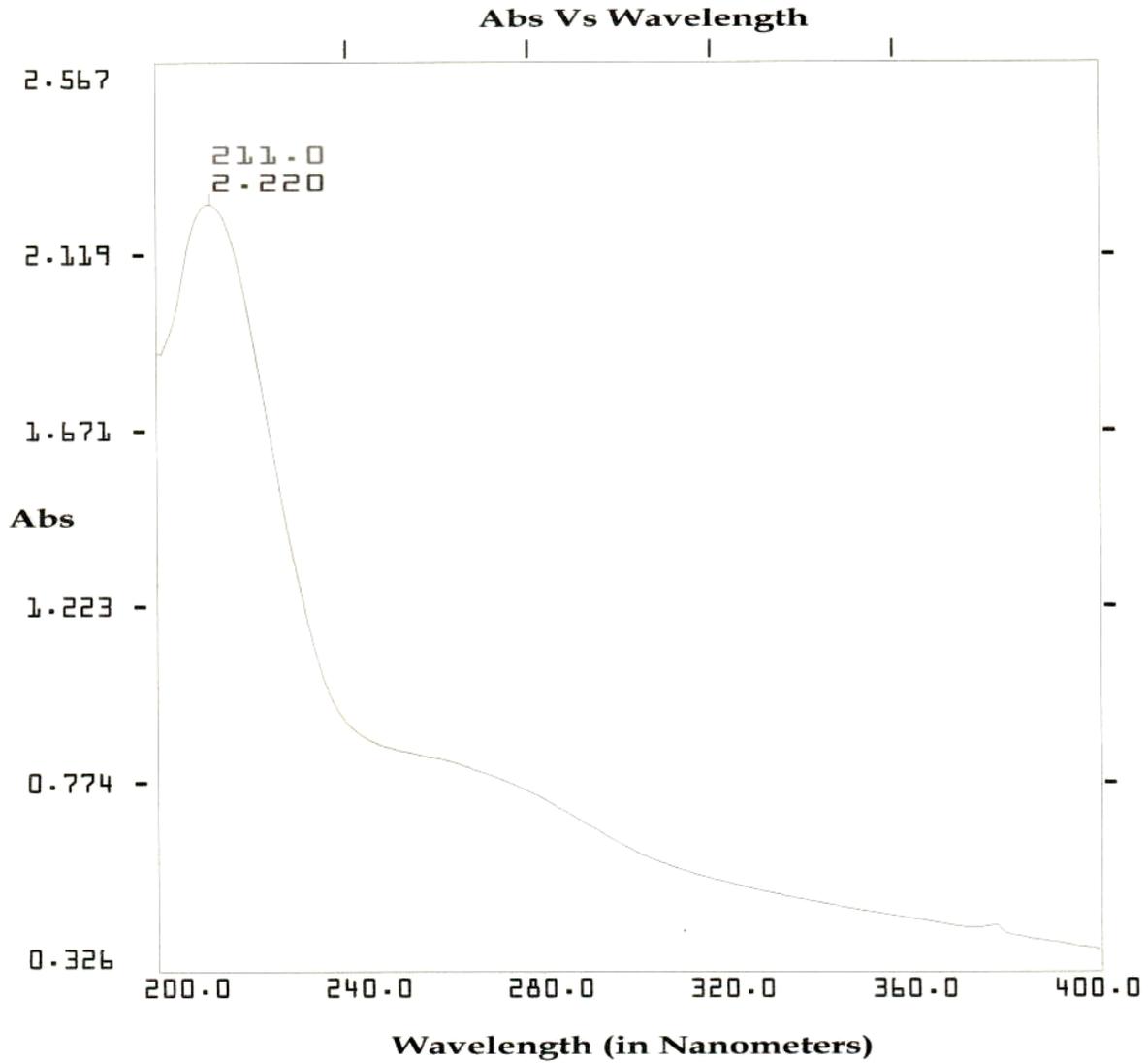
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Ellico SL-159
UV-VIS SpectroPhotoMeter

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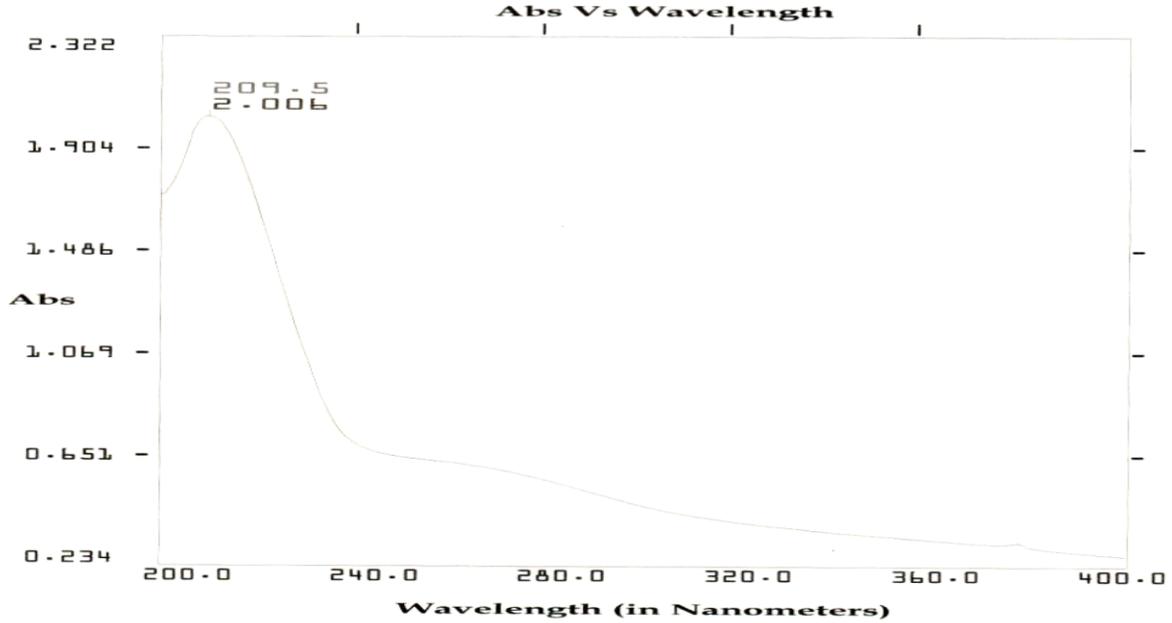


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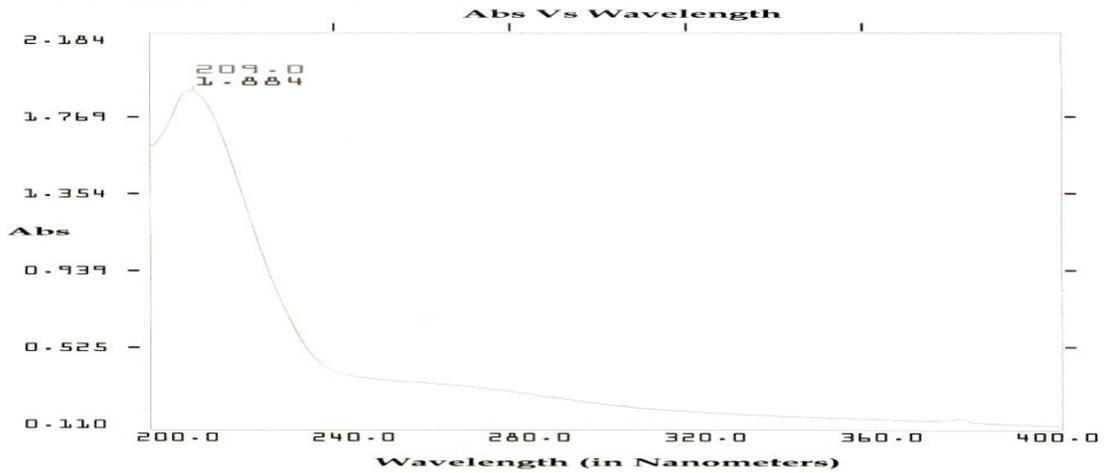


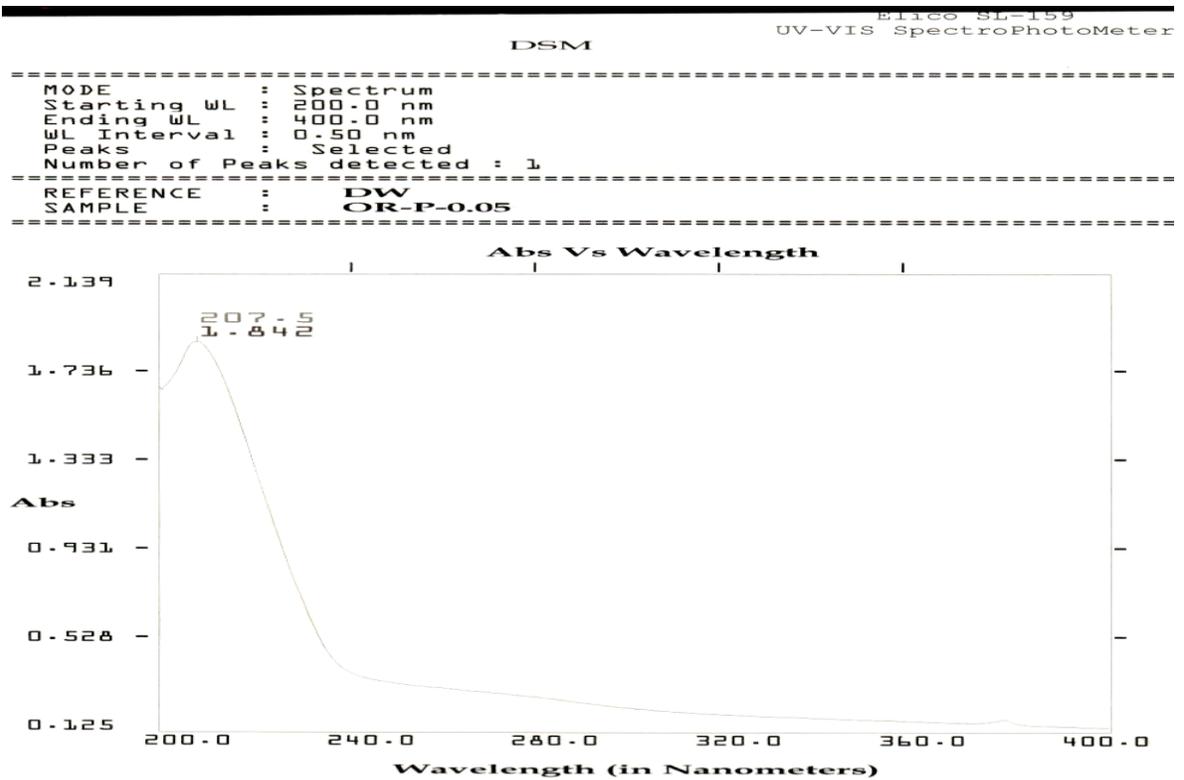
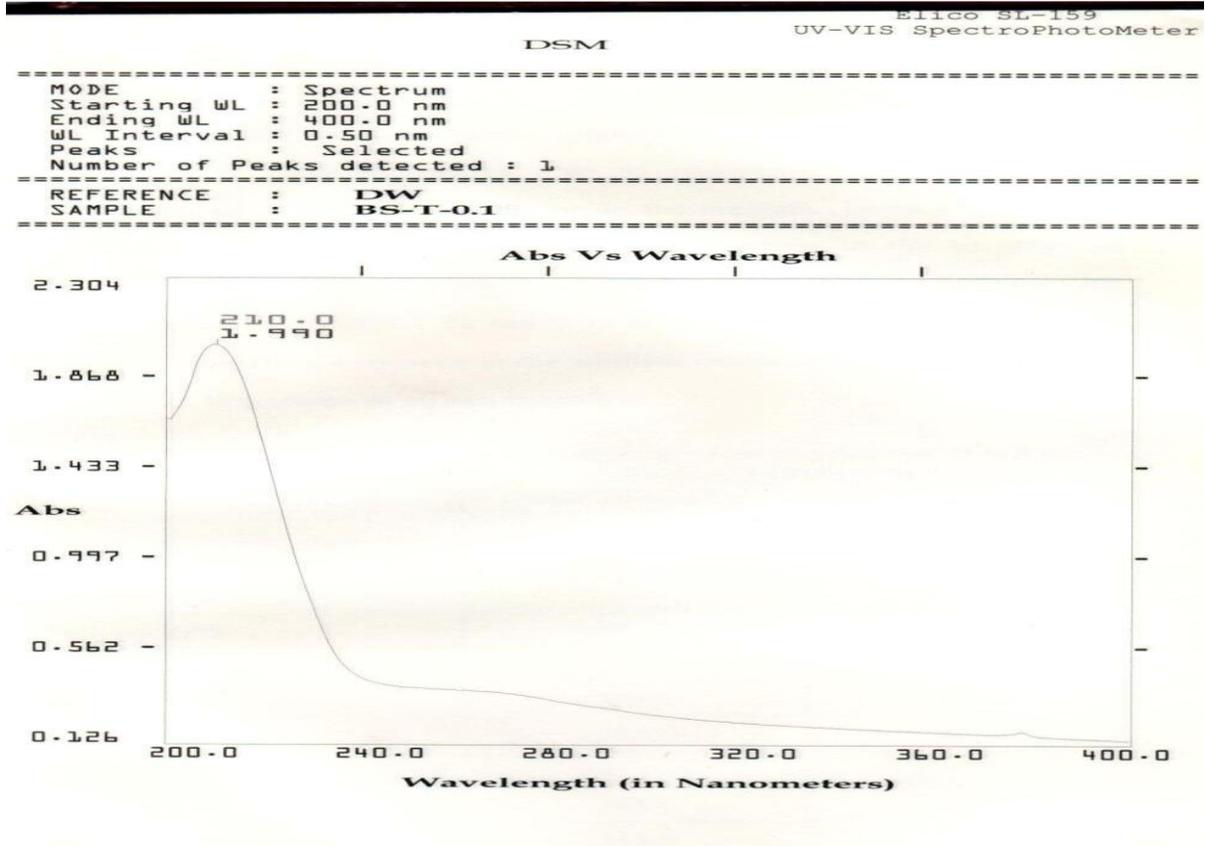
Ellico SL-159
UV-VIS SpectroPhotoMeter

DSM

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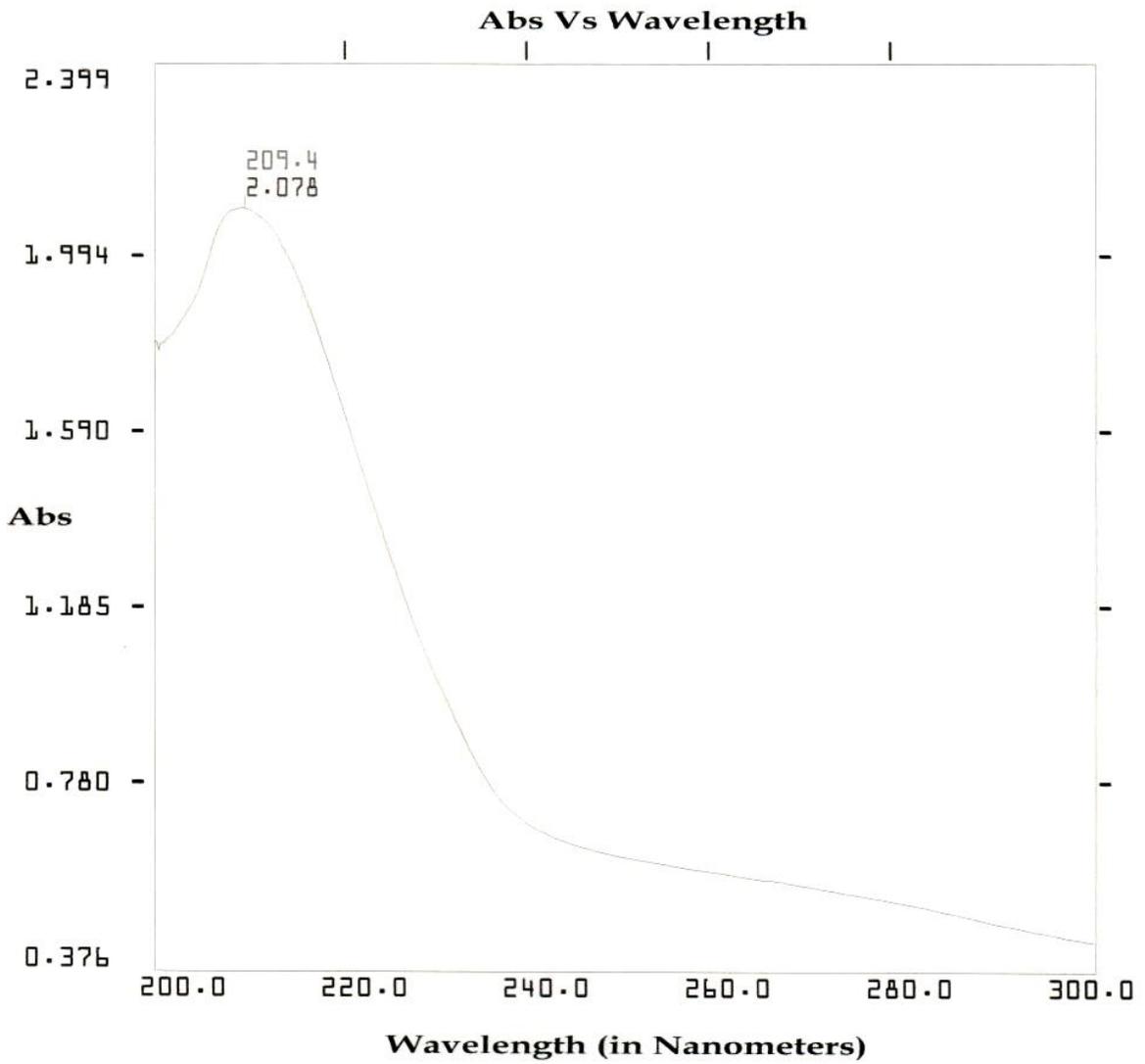




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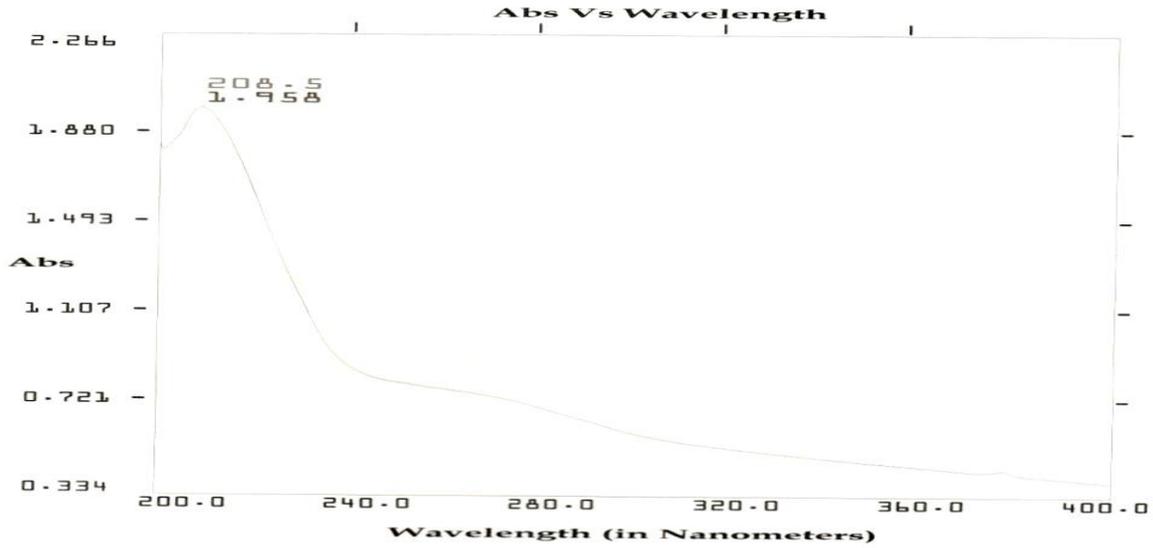
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Ellico SL-159
UV-VIS SpectroPhotoMeter
DSM

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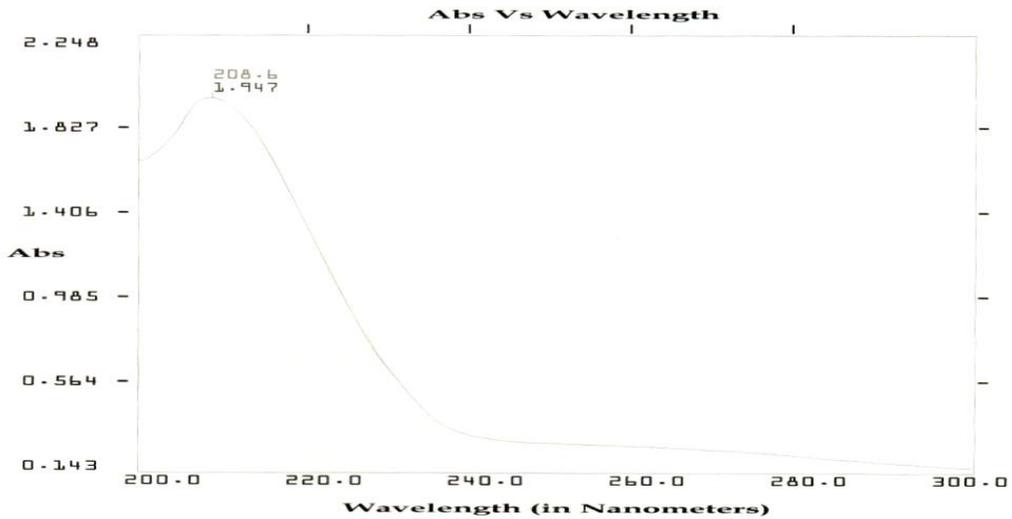
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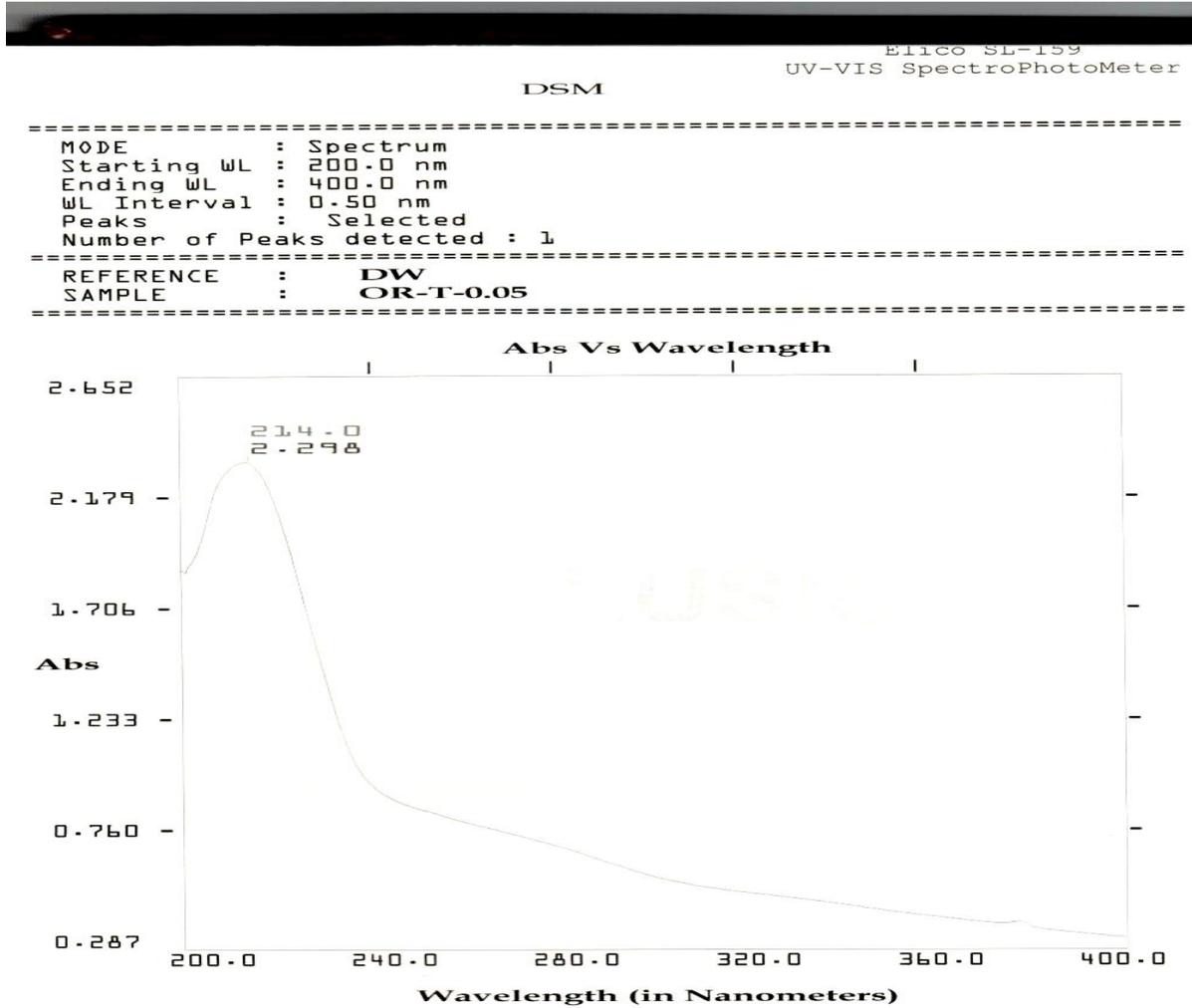


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It has been emphasized by Toffes and Moseley (1953) that the production of fluorescent pigment by a strain of *P. aeruginosa* is inversely related to the logarithm of the iron concentration of the medium. The presence of chelating agents can lower the concentration of iron. The metabolite iron is an absolute requirement for the synthesis of the cytochrome system enzymes.

The control of the availability of iron to bacteria using iron binding egg white protein conalbumin, appears to be an excellent method of assuring an efficient medium for use in the detection of fluorescent pigments.

When iron free conalbumin is present in the medium, the availability of iron is restricted.

With addition of 0.05% of phosphate in medium yellow pigmented colonies showed maximum pigmentation as compared to 0.1% of phosphate in the medium. No peaks were detected in presence of sulphate and tyrosine in the medium as shown in table 3.

In the presence of 0.05% of Phosphate in the medium the cream pigmented colonies show maximum pigmentation as compared to 0.1% of phosphate in the medium. These isolates also showed chromogenic characteristics in the presence of 0.1% and 0.05% of sulphate

and tyrosine in the medium. The maximum pigmentation was seen in 0.1% sulphate and 0.1% tyrosine as shown in table 4.

The maximum pigmentation of orange pigmented colonies was showed in 0.05% and no peak was detected in 0.1% phosphate in the medium. These pigmentation colonies also showed maximum pigmentation in the presence of other compounds such as sulphate and tyrosine with the concentration of 0.1% and 0.05% in the medium. The maximum pigmentation was seen in the medium containing 0.1% sulphate and 0.05% tyrosine as shown in the table 5.

It is concluded that the increase in the concentration of Phosphate decrease the pigmentation characteristics of cream, orange and yellow pigmented bacteria.

High concentration of sulphate 0.1% and high concentration of tyrosine 0.1% enhances pigmentation production in cream pigmented bacteria whereas high concentration of sulphate 0.1% and low concentration of tyrosine 0.05% shows good pigmentation characteristics.

As pigments have been extensively evaluated over the years for its antibiotic, antigenic and antitumor effects, special consideration was made towards ability of chromogenic bacteria to produce pigmentation under certain chemical conditions. It was observed that the chemicals in study markedly affect the pigmentation characteristics of the mentioned isolates

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How to cite this article:

Mohammed Abdul Aleem Qureshi, Saravanan Sankaran and Sowrirajan Subbaiah. 2018. Effect of Certain Compounds on the Pigmentation Characteristis of Some Bacterial Isolates. *Int.J.Curr.Microbiol.App.Sci.* 7(01): 2464-2483. doi: <https://doi.org/10.20546/ijcmas.2018.701.297>