IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Isolation, Characterization, Optimization Of Pigment Producing Bacteria And Use Of Biowaste As A Source For Pigment Production

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ABSTRACT

Pigment producing bacterium strain isolated from soil and water when characterized for morphological,physiological and biochemical parameters was identified accordingly. The isolated strain produced Red, Violet, Yellow and Fluorescent yellow pigmented bacteria on Nutrient agar medium.The optimum conditions for pigment production was determined at 30-37 degree Celsius for 7-10 days. From those isolates , maximum pigment production was noted. The selected Violet pigment producing bacteria and the optimum growth conditions were done by growing it on in different incubation, temperature, pH, Carbon source and Nitrogen sources. Biochemical tests and staining tests were performed accordingly. Antibacterial activity of pigment producing bacteria were noted against food borne pathogens. The pigment producing bacteria is used as a biocolerent in textiles, food industry etc we produced them on easily and cheaply available media. We produced them on easily and cheaply available media from bio-waste substances such as chicken feather&prawn carapaces, egg shell, and kitchen waste etc. We were able to produce pigment producing bacteria from those media accordingly

Key words- Pigment, antibacterial activity etc

INTRODUCTION

Colors provide attractive appearance to the marketable products such as food, textiles and pharmaceutical products. There are many artificial synthetic colors which have been widely used in several industries like food, cosmetics etc. These synthetic colors are expensive and hazardous. The adverse effect of synthetic colors have triggered intense research on natural color and dyes(***Reyes et al.,1996***).Recent research efforts have been made to replace synthetic pigments with natural pigments from plants, animals and ,microorganisms products. colors obtained from various different sources like fruits vegetables, minerals, microalgae and so forth.

Artificial or synthetic colors mostly used by the food processing and cosmetic industries are reliable and economical as compared to the natural colorants which are expensive, less stable, and possess lower intensity. Organizations like the World Health Organization (WHO), the U.S. Food and Drug Administration (FDA), and the European Food Standards Authority (EFSA) have recommended the safe dosage of artificial colors in food, drug and cosmetic items. Many synthetic colorants have been banned or being banned due to their hyper-allergenicity, carcinogenicity and other toxicological problems **.** These adverse effects of synthetic colors have made the scientific community skewed towards natural colors.

With the increasing awareness about the toxic effects of synthetic colors and consumer safety, there is an increasing interest in the development of colors from natural sources. Many research efforts have been made to replace synthetic pigments with natural pigments because nature is a rich source of colored pigment producing organisms including plants, animals and microorganisms. ***Tuli et al., 2015).*** The utilization of natural sources have been obtained since long time ago, pigments in foodstuff, dyestuff, cosmetic and their interest has increased due to the toxicity pharmaceutical manufacturing processes has been problems caused by the synthetic pigments. In this increasing in recent years because of harmful way the pigments from microbial sources are effects of synthetic pigments and their industrial good alternative. There are many artificial synthetic colorants, which have widely been used in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes, comprise various hazardous effects. There are many limitations of synthetic pigments. The precursors, used in the production process of synthetic pigment, have many carcinogenic hazardous effects on the workers. The wastes of the production process are also harmful. They are itself non-environment friendly and non-biodegradable. To counter these hazardous effects of synthetic colorants, there is worldwide interest in process development for the production of pigments from natural sources. Plants and microorganisms are the two major sources of natural pigments. Yet the natural pigments from plants also have drawbacks such as: instability against light, heat or adverse pH, low water solubility and are often non-availability throughout the year. Hence the microbial pigments are of great interest owing to the stability of the pigments produced and the availability of cultivation technology.

Pigment producing bacteria production is been widely produced from chicken feathers,crustaceans,eggshell,whey,pineapplewaste,ricebran,corns,baggasesetc. Fermentation is an inherently faster and more productive process as compared to other chemical processes so it is more

beneficial to use it for industrial production. Moreover, pigment production from microbial sources has gained attention owing to public sensitivity regarding "synthetic food additives." Microbes have also more versatility and productivity over higher forms of life in the industrial-scale production of natural pigments and dyes. Fermentation process has been increased by genetic engineering and further research for nontoxic microbial pigment can make quantum leaps in the economics of microbial pigments(***Abhishek Kumar et al,2015)***

MATERIALS AND METHODS

1.Sample collection

Sample collection was done from different natural resources. Soil samples were a collected from sea, river, estuaries etc.

2.Isolation of pigment producing bacteria

The samples which were serially diluted and plated on different agar plates. The agar plates which we used are Nutrient agar plates. . The samples were spread plated using sterile techniques incubated at 37^0C for 24-74 hours. After incubation, plates were observed . Then colony morphology and cultural characteristics of each plates were analysedFive different pigments like Red,Violet,Yellow,Orange and Fluorescent yellow were obtained and pure colonies of each pigment bacteria were streaked on agar plates.

Harvesting of pigment producing bacteria

Isolated colonies of identified cultures were suspended in nutrient broth in a conical flask and the flask was kept for incubation at 30^0C for 4-7 days in order to obtain maximum pigment production.

3.Screening of pigment producing bacteria

3.1Gram Staining- A method of staining used to distinguish and classify bacterial species into two large groups such as gram positive and gram negative.

3.2Motility test

Motility test is been performed whether the given microorganism is motile or non-motile.

3.3Biochemical tests

The bacterial isolates were characterized using biochemical test such as MR-VP tests, Indole test, Nitrate test, Citrate test, Oxidase and Catalase tests accordingly performed

4.Extraction of pigments from pigment producing bacteria

For screening of pigment producing bacteria, the isolated microorganism were streaked on Nutrient agar slants and incubated at 30^0 C for 7-10days.The organism produced maximum pigment production were selected for further study.

The pigment producing bacteria was harvested by centrifuging at 6000rpm for 20 minutes. Thensupernatants were discarded and the pellets were resuspended in ethanol. Then the mixture was vortexed and the suspension was centrifuged at 6000rpm for 10 minutes and supernatent was collected. Centrifugation was repeated till the pellet changes to colorless. After centrifugation,supernatants containing diffused pigments were filtered through Millipore membrane filter. The absorbance of filtrates was measured on UV visible Spectrophotometer in the range 350-750 nm. Then the filtrates were kept in water bath for ethanol evaporation. After evaporation of ethanol, dry pigment residues were resuspended in DMF (dimethylformamide) solvent. The solvent containing pigment was then used to evaluate its antimicrobial activity against human pathogens along with its control.

5.Antimicrobial activity

Antimicrobial activity of the pigments was tested by well-diffusion method. Antimicrobial activity refers to the process of killing of inhibiting the disease causing microoragnsims.Various antimicrobial agents are used for this purpose. Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. It is also known as Kirby-Bauer technique. Four food borne pathogens (*Escherichia coli, Salmonellatyphi, Staphylococcus aureus and Bacillus cereus*) were used against extracted pigment to evaluate its antimicrobial activity

6.Dye degrading ability test

The pigment producing bacterial isolates were tested for their ability to degrade dyes using Bushnell Hass broth. Biological dyes namely malachite green and methyl violet were used. The 24 hour old grown isolates were inoculated in Bushnell Hass broth containing 100ppm of respective dye and incubated at 37^0C for 48 hours. After the incubation period the tubes were centrifuged and the supernatant was subjected to colorimetric analysis

7.Optimization of cultural conditions for maximum growth

7.1Optimization of P^H

P^H of the selected media was adjusted ranging from P^H 4-8 with the help of P^H meter. The autoclaved liquid media was inoculated with the pigmented bacterial isolates and incubated at 37^oC.

7.2Optimization of incubation temperature

In order to determine the optimum temperature for maximum pigment production, the selected P^H of the media was adjusted and inoculated with pigmented bacterial isolates. The tubes were then incubated at different temperatures ranging from 4ºC to 37^OC. (4ºC, 20ºC, 25ºC, 30ºC, 37ºC)

7.3Effect of Carbon source-

Different carbon sources namely glucose,glycerol,sucrose,starch,lactose each at the rate of 1%were added in the media and their effect on the growth and pigment production was studied after incubation

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7.4Effect of Nitrogen source -Different nitrogen source viz. Peptone, Ammonium sulphate, Ammoniumchloride, Ammoniumsulphate and Beef extract were added in the media and their effect on the growth and pigment production was studied after incubation

Different nitrogen source viz. Peptone, Ammonium sulphate, Ammoniumchloride, Ammoniumsulphate and Beef extract were added in the media and their effect on the growth and pigment production was studied after incubation

8.Pigment production from bio-waste substrate.

Different industries related to the agricultural sector, poultry, fisheries etc. generate a lot of waste in the form of peels, seeds, whey, waste liquid, molasses, bagasse, feather, prawncarapace, fishscales, and egg shell and so on. The generated waste is not only biodegradable in nature but also rich in nutrient components (carbohydrates, proteins, fibers, minerals, vitamins etc.) depending upon source. In the present scenario researchers have shown a great interest in the processing of waste for fermentation processes in the development of value added products like microbial pigments. Utilization of waste not only eliminates the disposal problems but also solves the problem of environmental pollution. Moreover the development of fermentation derived food grade pigments needs high capital investment requirements in the terms of media components. Thus, effective use of cheaply available bio-waste residues for production of microbial pigments can make the process cost effective and environmental friendly. Thus we used here chicken feathers, prawn carapaces, egg shell, kitchen waste for pigment production.

RESULTS

Morphological characterization & Biochemical characterization

The selected Violet, Red, Yellow, Fluorescent Yellow and Orange pigment producing bacterial isolates were marked on the basis of their morphological and biochemical characteristics and the results are presented in Table 1, Table 2, Table 3 etc.

1.Gram staining

Table 1.1 Gram's staining of pigment producing bacteria

Pigment	Gram positive/negative	Shape
1.Red	Gram negative	Rod
2.Violet	Gram negative	Short rod
3.Orange	Gram positive	Short rod
4.Yellow	Gram positive	Cocci
5.Flourascent yellow	Gram negative	Rods

2. Motility

Table 2.1:Motility of pigment producing bacteria

Pigment	Motile/Non motile
1.Red	Motile
2.Violet	Motile
3.Orange	Non motile
4.Yellow	Non motile
5.Flourascent yellow	Motile

On observation Red and Violet pigment producing bacteria are motile and fluorescent yellow is slightly motile comparing with the others. The pigmented organism of Orange & Yellow were non motile

3. Colony morphology

Color	Size	Margin	Shape	Opacity	Consistency	Elevation
Red	3mm	entire	circular	opaque	smooth	convex
Violet	2mm	entire	circular	opaque	sticky	convex
Orange	1mm	entire	circular	opaque	sticky	Flat
Yellow	2mm	entire	circular	opaque	smooth	flat
Fl.Yellow	1mm	entire	circular	opaque	smooth	flat

Table3.1.Colony morphology of pigment producing bacteria

4. Biochemical Test

Biochemical test are done for the identification of the organism with the help of utilization of carbohydrates and sugar sources etc.

Table 4.1: IMVIC Test, Nitrate test, Catalase and Oxidase test

Pigment	Indole	Methyl Red	Vogues Proskauer	Citrate	Nitrate	Oxidase	Catalase	TSI
1.Red	-	+	-	-	-	+	+	-
2.Violet	-	-	-	+	+	+	+	-
3.Orange	-	-	-	-	-	-	+	-
4.Yellow	-	+	-	-	-	-	+	-
5.Flouras cent	-	-	-	-	-	+	+	-

5. Antimicrobial activity

. All the pigments showed antibacterial activity against the test pathogens. . (Table 5.1 shows the antibacterial activity of the extracted pigments against the test pathogen)

Microorganism	Red	Violet	Orange	Yellow	F.Yellow
Escherichia coli	20mm	14mm	11mm	9mm	13mm
Salmonella typhi	19mm	-	-	10mm	15mm
Staphylococcus aurues	12mm	20mm	-	-	13mm
Bacillus cereus	19mm	25mm	15mm	11mm	11mm

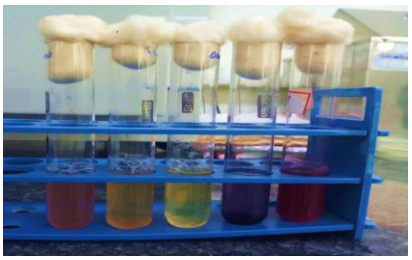


Fig .5.1 Pigments

According to Bergey's Manual, by observing the colony morphology, biochemical tests and antimicrobial activity the violet pigment producing bacterium is identified as ***Chromobacterium violaceum*.** *Chromobacterium violaceum* is a Gram negative, facultative anaerobic, non-sporing coccobacillus. It is motile with the help of a single flagellum which is located at the pole of the coccobacillus.Usually; there are one or two more flagella as well. It is a part of the normal flora of water and soil of tropical and sub-tropical regions of the world. It produces a natural antibiotic called violacein, which may be useful for the treatment of colon and other cancers. It grows readily on nutrient agar producing distinctive smooth low convex colonies with a dark violet metallic sheen(due to violacein production).Some strains of the bacteria which do not produce this pigment have also been reported.

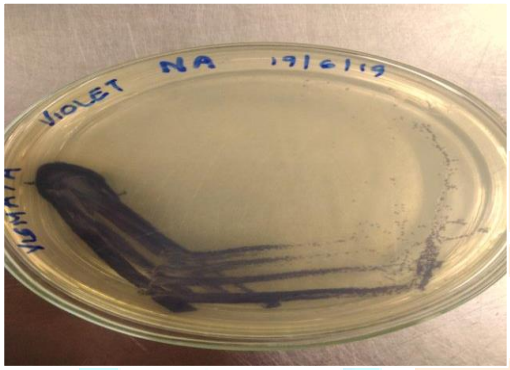


Fig.5.2.Chromobacterium violaceum

6.Dye degrading ability

Sample	OD Value	
	Malachite green	Methyl violet
Control	0.22	1.85
Red	0.04	1.53
Violet	0.06	1.50
Orange	0.03	1.54
Yellow	0.01	1.65
Fluorescent yellow	0.00	1.59

Table 6.1 Dye degrading ability of pigment producing bacteria

Pigment producing bacteria has got wide range of application in textile industry. Dye degrading ability helps to determine the amount by which the pigment producing can degrade the dye accordingly. In another study pigmented bacteria showed ability to degrade biological dyes. The degradation of dyes indicated that the bacterial isolates used the dye as a source of carbon which is studied using calorimetric analysis. Maximum decolorization was seen by isolate for malachite green *(Tejas et al., 2017).*Comparing with our study we got

maximum decolorization for Methyl violet. Application of Violet pigment from *Chromobacterium violaceum* in textile dying*(Chidambaram KVenil et al., 2016*

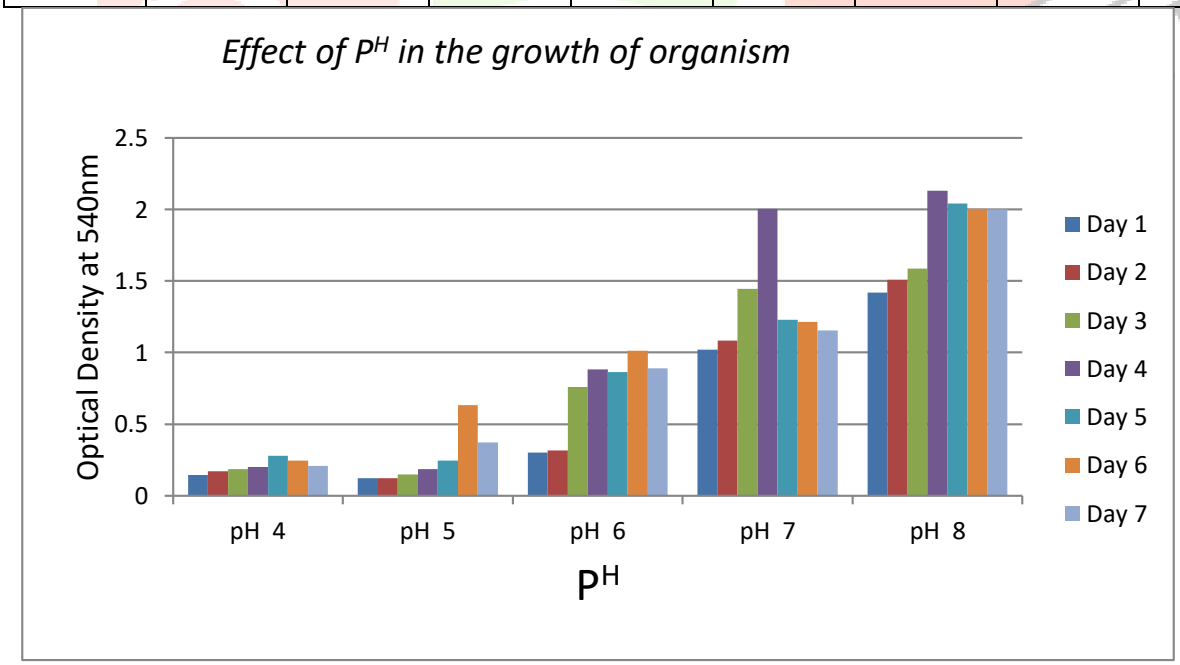
7. Optimizationof *Chromobacterium violaceum* on different growth conditions

7.1.Effect of P^H

The growth and type of pigment producing bacteria is affected largely by the P^H of the PCA broth medium in which the microorganisms grown.Slight changes in P^H can also alter the shade of color produced*(Goswami, G. et al.2010,).*The influence of P^H on the growth of pigment producing bacteria was studied at different P^H values ranging from 4-8.Shows that maximum growth of *Chromobacteriumviolaceum* was occurred at P^H

Table 7.1: Optimization at different P^H

Sample no	P^H	OD at 540 nm						
		Day1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	P^H4	0.145	0.170	0.188	0.200	0.279	0.244	0.208
2	P^H5	0.122	0.122	0.148	0.185	0.247	0.633	0.373
3	P^H6	0.300	0.317	0.758	0.884	0.865	1.014	0.889
4	P^H7	1.020	1.085	1.444	2.002	1.227	1.215	1.154
5	P^H8	1.420	1.507	1.585	2.131	2.039	2.002	2.001

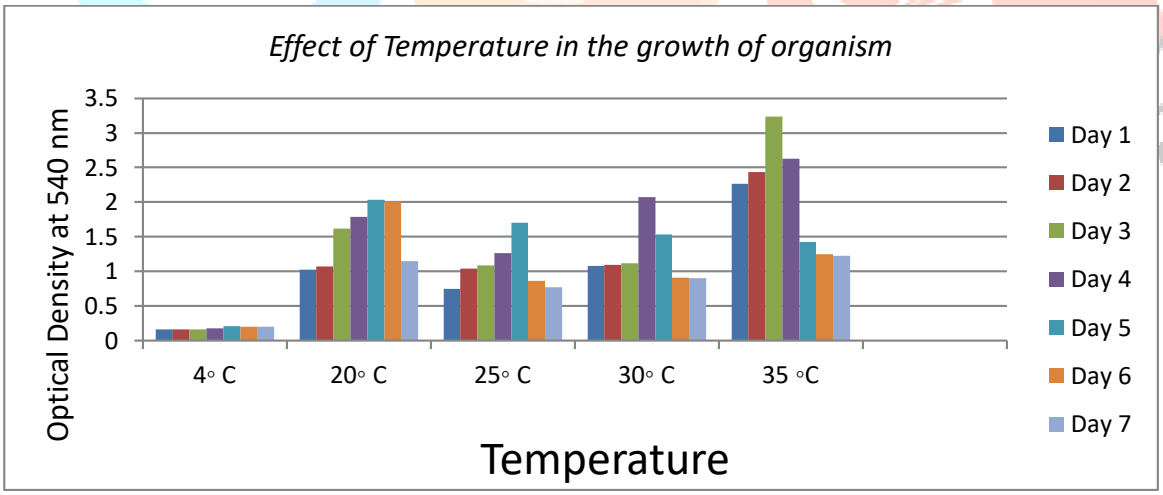


7.2. Effect of Incubation temperature

The biosynthesis of a pigment is significantly affected by the physiological parameter, temperature.In order to determine the effect of temperature on the growth production; the cells were incubated at 4, 20, 25, 30&37ºC, respectively.The incubation time was kept at the optimum 48-72 hours. Incubation temperature was found to be a critical parameter as it affected the growth of pigment producing organism. Maximum growth of *Chromobacterium violaceum* was occurred at the temperature 37ºC

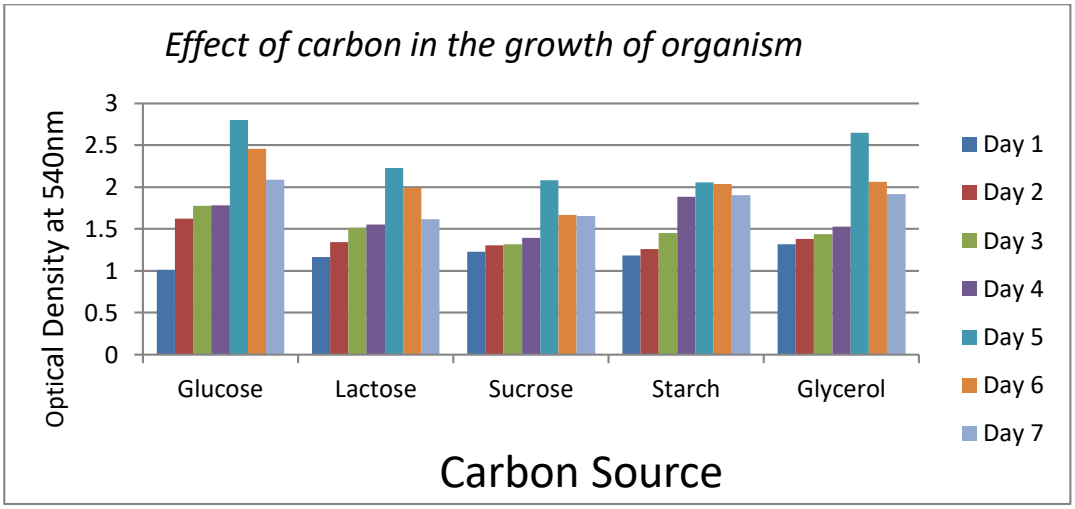
Table 7.2: Optimization at different temperature

Sample no	Temperature	OD at 540 nm						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	4ºC	0.157	0.158	0.164	0.175	0.204	0.202	0.200
2	20ºC	1.026	1.068	1.621	1.788	2.037	2.009	1.148
3	25ºC	0.749	1.041	1.088	1.261	1.704	0.865	0.771
4	30ºC	1.081	1.094	1.116	2.070	1.529	0.911	0.904
5	37ºC	2.266	2.436	3.233	2.625	1.426	1.251	1.225



7.3. Effect of Carbon source

When glycerol was used in the media, the growth of*Chromobacteriumviolaceum*was relatively reduced when compared to previous levels of production.The use of glucose as a source of carbon showed substantial increase in growth production when compared to glycerol. Sucrose,lactose and starch produced less growth. In our study the use of glucose as a source of carbon showed substantial increase in growth of *Chromobacterium violaceum*

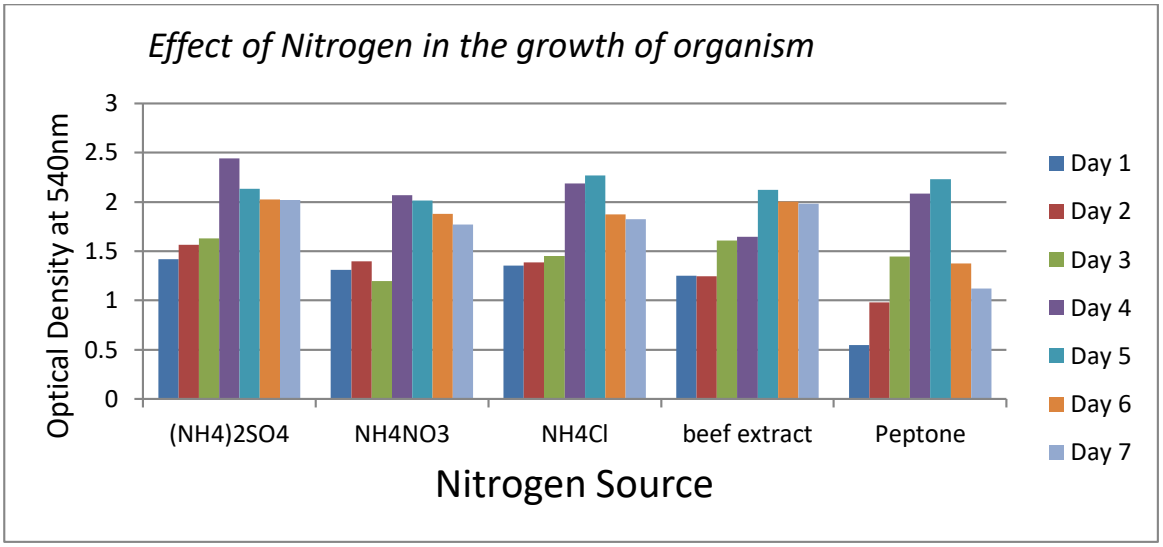


7.4. Effectof Nitrogen source

Five different nitrogen sources were used to study their effect on growth production.Peptone, Beefextract, Ammonium sulphate, Ammonium nitrate and Ammonium chloride were used as nitrogen sources separately in each trail at concentration of 0.5%in each media preparation.In our study highest growth of violet pigment producing bacteria was seen when Ammonium sulphate was used as a nitrogen source

Table 7.4. Optimization at different Nitrogen Source

Sample no	Nitrogen source	OD at 540nm						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	Ammonium sulfate	1.420	1.566	1.628	2.442	2.133	2.022	2.020
2	Ammonium nitrate	1.312	1.398	1.199	2.069	2.012	1.881	1.771
3	Ammonium chloride	1.352	1.385	1.453	2.185	2.267	1.873	1.822
4	Beef extract	1.252	1.248	1.606	1.647	2.120	2.003	1.979
5	Peptone	0.549	0.978	1.445	2.085	2.231	1.376	1.122



8. Pigment Production from Biowaste Substrate

Violet pigment was obtained from egg shell media. The media was kept for an incubation period of 7 days in order to obtain maximum pigment production. The pigment can be extracted from the media through centrifugation. Thereby we can produce pigments from easily and cheaply available media.

Use of waste chicken feathers as peptone for production of carotenoids in submerged culture of *Rhodotorula glutinis(Mesut Taskin et al., 2001).*Production and characterization of pigment producing bacteria such as Violacein by locally isolated *Chromobacterium violaceum* grown in Agricultural wastes. *(Wan Azlina Ahmad et al., 2012).*Recent studies on using liquid pineapple waste for the production violet pigment producing bacteria*(C.K Venil et al., 2013).*

Table.8.1. Pigment Production in Bio-waste Substrate

Substrate	Pigment production
Chicken feather& Prawn carapaces	negative
Prawn Carapaces	negative
Egg shell	positive
Kitchen waste	negative

Fig.8.1:Pigment Production in egg shell

CONCLUSION

Bacterial pigments have economic potential and industrial importance offering opportunities for application in textile, food, pharmaceuticals, cosmetics etc. But their current volume of production still has not attained the optimum level to meet the demand aroused due to the recent awareness for natural products. The current novel strategies like genetic engineering, molecular biology techniques and fermentation technologies are greatly contributing to higher production of bacterial pigments. For cost-competitive and higher production of bacterial pigments, these current processes of screening of new pigmented bacteria should continue in order to support the discovery and application of novel bacterial pigments that possesses high activities and useful properties from less expensive sources.

The *Chromobacterium violaceum* yield was observed to be regulated mainly by unconventional carbon source such as glycerol and sucrose while the most common carbon source i.e. lactose showed little impact on pigment production. Maximum increase in pigment yield was noticed with glucose supplementation. The nitrogen source also plays an important role in growth of pigment producing bacteria. Inorganic nitrogen sources had negligible effect while Ammonium sulphate showed maximum growth production. Incubation temperature is the most prominent physiological factor which regulated the pigmentation yield, with maximum growth obtained at 37^{o}C. P^{H}has an important role over other factors. Maximum growth of violet pigment producing organism was seen on P^{H}8. Over all the combination these three factors resulted in improving the yield, indicating the imperative role of growth parameters and their concentrations in regulation of metabolically mediated growth production in this isolate.

We were able to produce pigment producing bacteria from easily and cheaply available media substances such as bio-waste substrates. Submerged Solid Fermentation (SSF) from Egg shell media was able to show maximum pigment production accordingly

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