ISOLATION, SCREENING AND OPTIMIZATION FOR PIGMENT PRODUCING BACTERIA

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❖ **Abstract :**

Pigments are the colored compound that are used to color material. They are found in two forms: Synthetic and Natural. Use of synthetic pigment in day-to-day life have increased the concern regarding the health issues in humans and environmental damage, but the use of natural pigment from various sources like plants, animals and microorganisms can be harmless to human being. Use of Microorganism for producing pigment can be very useful as they are obtained from various sources which can be natural reservoirs, for diverse microorganisms. Natural Pigment-producing bacteria are of increasing interest due to their potential applications in pharmaceuticals, cosmetics, textiles, and as natural alternatives to synthetic dyes. This study aimed to enrich, isolate and screen pigment-producing bacterial strains from diverse environmental samples including soil, water, and air. Initial screening was performed by enriching and then culturing samples on nutrient media and both pigmented and non-pigmented bacterial colonies were isolated. Further pigment producing colonies were selected based on colony coloration. Isolates exhibiting distinct pigmentation were subjected to further studies. Morphological and biochemical tests were conducted to identify and explore their potential to produce pigments and different biochemical activities. Preliminary results revealed a diverse range of pigmented bacteria with different colors like pink, yellow, orange, red. The study highlights the efficacy of selective enrichment and the importance of environmental sampling in discovering novel pigment-producing microbes. This research focus on to screen out and identify the bacteria capable to produce pigments from diverse sample from different geographical areas. From the pigment producing bacteria total three bacterial species were screen out and were further studied for their optimization characterization. These three isolates showed strong adaptability and productivity and pigment producing potential under the tested optimization parameters.

Keywords : **Bacterial Pigment, Optimization of Parameters**

❖ Introduction:

Pigments are the compound used to color material. Colors are the first parameter which our eye captures and evaluates after seeing an object that is fruit, plants, flowers, animals etc. Due to this most of the industries are investing in R&D for production of new colors. These colors were obtained from the synthetic chemicals. This use of colors has also led to increase in the use of synthetic dyes day by day. As use of this synthetic dyes are causing severe concern in the society (Barreto,et.,al, 2023). Concern has increased from human safety and environmental protection which has rekindled interest to obtain pigments from natural sources than synthetic dyes. Microbial pigments serve as great research importance, which are gaining demand globally (Fatima, M., et.al.,2022).
In recent times, industries have shifted towards using microorganisms as a source of many compounds like antifungal, enzymes, vitamins, antibacterial etc and the latest edition to this is use of pigments. In ancient times plants, animals, fruits were used as source for colors like leaves, nut shells, wild flowers, tree bark, Sea snails, moss, cochineal insects (Palanichamy, et.al.,2011), however non-microbial pigments limitations due to poor water solubility, seasonal growth, unstability in light, pH, heat whereas microbial pigment have more advantages as they are stable, easily produced, downstreaming process is easy, and they are water soluble (Fatima, M., et.al.,2022). Natural pigments are higher priced, require longer time and had complex process to produce. But the use of pigment from microbes provided a safe alternative to synthetic dyes. Use of chemical dyes have found to cause diseases like allergies, cancers etc (Palanichamy, et.al.,2011).

In ancient times, plants and animals were used as a source for extraction of colored compounds of industrially importance, but the process of its production has many drawbacks like it led to loss of valuable species, it was very expensive, had an ethical dilemma and have complicated extraction process. It also poses threat in the form of wastes like hazardous organic and inorganic compounds like toluidine, benzidine, toxic metal compounds like ammonia, alkali salts, lead, mercury, (Ram Naresh Bharagava, 2018., Recent Advances in Environmental Management) sewage etc which were laterly dumped in the environment (Palanichamy, et.al.,2011).

In recent year, interest in use of natural pigment has grown widely which are derived from microorganism and particularly from bacteria due to its biodegradability, eco-friendly nature, various application, no seasonal growth required. Unlike synthetic dyes, which pose various health allergies like formaldehyde, triclosan, zinc pyrithione can led to dermatitis (Armengol, E. S., et.al.,2022), respiratory trouble (Affat, S. S., et.al., 2021)like and environmental risks. Bacterial pigment offer a sustainable and non-toxic alternative for use in pharmaceutical, food, textile and cosmetic industries. Bacterial pigment production has various biological functions such as protection from ultraviolet radiation, stress. They also enhibit various activities like antibacterial, antifungal, antioxidant, anticancer, anti-inflammatory. These pigments are secondary metabolite which can be used for therapeutic development.

While considering the wide range of microbial pigments, the current study focuses on isolating and identifying bacterial species in the natural environment. In this present work, soil, water and air samples were collected from different geographical areas of Maharashtra were screened for the bacteria producing pigments. Optimization parameters were studied for different physical parameters to increase growth and pigment production. The variation in pH, temperature, salt concentration, inoculum size are required for pigment production. Microbes ability of pigment production can be increased or lost by different nutritional and cultural circumstances. It is crucial to maximize the growth and production of pigment by finding the suitable optimization combination of cultural conditions.

❖ Material and Methods

Sample Collection

Sampling for the pigmented bacterial isolates were done from various regions in Maharashtra state: Aurangabad, Nasik, Pune, Ahmednagar, Shrirampur, Jalna and Lonar from which soil, air and water samples were collected. From these samples bacteria producing pigment were isolated and the selected bacterial isolates were used for the optimization studies.

Isolation of pigment producing organism

1. Soil samples:

The soil samples were collected from Aurangabad, Nasik, Pune, Ahmednagar, Jalna and Lonar region of Maharashtra. The soil samples were collected with the sterile spatula and taken in sterile zip lock bag. The samples collected were kept at -4°C till the further use. The samples collected were inoculated in flask containing sterile Nutrient broth by adding 1gm of respective soil for enrichment and later the flask was incubated at 30°C for 48 hrs. After the incubation the broth were used for serial dilutions upto 10^{-9}. Serial dilutions (10^{-4} to 10^{-9}) were plated on sterile nutrient agar plates and plates were incubated at 30°C for 48 hrs. Bacteria producing pigmented colonies were then streaked on sterile Nutrient agar plates to obtain pure cultures (Brunelle, S.,2020).

2. Water Samples:

The water samples were collected from Aurangabad, Nasik, Ahmednagar, and Lonar region of Maharashtra. The water samples were collected in sterile screw cap bottles. The samples collected were kept at -4°C till the further use. The samples collected were inoculated in flask containing sterile Nutrient broth by adding 1ml of respective water sample for enrichment and later the flasks were incubated at 30°C for 48 hrs. After the incubation the broth were used for serial dilutions upto 10^{-9}. Serial dilutions (10^{-4} to 10^{-}

[9]) were plated on sterile nutrient agar plates and plates were incubated at 30°C for 48 hrs. Bacteria producing pigmented colonies were then streaked on sterile Nutrient agar plates to obtain pure cultures.

3. Air Samples:

The air samples were collected from Aurangabad, Nasik, Ahmednagar, and Lonar region of Maharashtra. For Air sampling sterile Nutrient agar plate were prepared which was later on exposed to air by the settling plate method. The plates were kept open for 15 mins in the open air. The plates exposed to air were incubated at 30°C for 48 hrs. Bacteria producing pigmented colonies were then streaked on sterile Nutrient agar plates to obtain pure cultures.

❖ Identification and Characterization of Pigment-Producing Bacterial Strains:

Pigment producing bacterial colony characterization was done from the Nutrient Agar plates which were previously incubated at 30°C. As per standard protocols The Colony characterization was done based on its, size, color, shape, margin, consistency, opacity, elevation and Gram's nature (Jackman, J. 2011).Further the biochemical and enzymatic tests were performed like Indole test (I), Methyl Red (MR) and Voges Proskauer (VP), Simmon's Citrate (C) (Powers, E. et.al.,1977), Nitrate reduction, Catalase, Gelatinase, Caseinase, Amylase (Cardoso, L., et.al.,2021) and carbohydrate fermentation test were carried out. Identification of isolated obtained in the pure form were characterized by its morphology, and microscopically by Gram's staining.

❖ Optimization Parameters :

The optimization of growth condition for particular physical parameters is very important in development of any pigment productions. Microorganisms require an optimal temperature to grow and produce pigment, therefore it is a key factor for pigment production. pH also plays a crucial role in pigment production by regulating the acidity and basicity of growth medium by creating a proper condition for growth. Along with temperature and pH other parameters like inoculum size, holding time and salt concentration were also studied. From the above isolates further only three isolates AG-12, GLY-7 and AGPA-10 were studied all other isolates were excluded due to their growth at high or low temperature, pH values.

1. Optimization of pH:

Flasks containing 50 ml nutrient broth were prepared. The pH of the flasks were adjusted at 3,5,7,9 and 11. The pH of the broth was adjusted by 1N HCL and 1N NaOH. The broth was sterilized at 121°C for 15 mins. Suspension of isolated cultures were prepared in 20 ml of sterile distilled water and adjusted to 1 unit optical density at 600nm and 1% of suspension were inoculated to respective flasks and later the flasks were kept for incubation at 30°C at 120 rpm. After incubation the O.D was taken at 600nm after 24 hrs (Srimathi, et.al.,2017) and biomass was calculated.

2. Optimization of Temperature :

Flasks containing 50 ml nutrient broth were prepared. The broth was sterilized at 121°C for 15 mins. Suspension of isolated culture were prepared in 20 ml of sterile distilled water and adjusted to 1 unit optical density at 600nm and 1% of isolated cultures were inoculated to respective flasks and the later flasks were kept for incubation at different temperature at 20°C, 25°C, 30°C, 35°C and 40°C. After incubation the O.D was taken at 600nm after 24hrs (Srimathi, et.al.,2017) and biomass was calculated.

3. Optimization of Biomass :

Flasks containing 50 ml nutrient broth were prepared. The broth was sterilized at 121°C for 15 mins. Suspension of isolated culture were prepared in 20 ml of distilled water and adjusted to 1 unit optical density at 600 nm and 1% of different concentration of isolated cultures were inoculated (0.5, 1, 1.5, 2 and 2.5 ml) to respective flasks and flasks were kept for incubation at 30°C at 120 rpm. After incubation the O.D was taken at 600 nm after 24 hrs (Fatima, M., et.al.,2022) and biomass was calculated.

4. Optimization of Incubation Time :

Flasks containing nutrient broth were prepared and sterilized at 121°C for 15 mins. 20ml of suspension from isolated culture were prepared in sterile distilled water and adjusted to 1 unit optical density at 600 nm and 1% suspension of isolated cultures were inoculated in the respective flasks of nutrient broth and later the flask were incubated at 30°C at 120 rpm. The OD of the broth was taken at 600 nm at different time interval like 10hrs, 20hrs, 30hrs, 40hrs and 50hrs (Chandran, et.al.,2014) and biomass was calculated.

5. Optimization of Salt Concentration:

Flask containing nutrient broth were prepared with different concentration of NaCl from 0.5 %, 1 %, 1.5 %, 2 % and 2.5% later the broth were sterilized at 121°C for 15 mins. 20 ml suspension was prepared from previously isolated cultures and adjusted to 1 unit optical density at 600 nm and 1% suspension of isolated culture was inoculated in the respective flasks containing nutrient broth and later the flasks were incubated at 30°C at 120 rpm. The OD of the broth was taken at 600 nm after 24 hrs (Bhatt et.al 2013) and biomass was calculated.

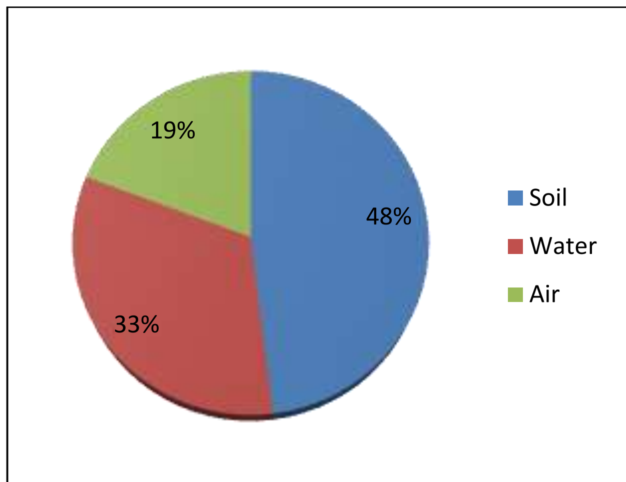
6. Optimization of Steady and Shaking Condition:

Flask containing nutrient broth were prepared and sterilized at 121°C for 15 mins. 20 ml suspension was prepared from previously isolated cultures and adjusted to 1 unit optical density at 600 nm and 1% suspension of isolated culture was inoculated in the respective flasks containing nutrient broth and later the flasks were incubated at 30°C at steady state condition and at shaking condition at 120 rpm for 24hrs. The OD of the broth was taken at 600 nm after 24 hrs and biomass was calculated.

❖ Results and Discussion:

1. Identification and Characterization:

The soil, water and air samples were collected from different regions of Maharashtra state. The collected samples were enriched in Nutrient broth and later transferred on Nutrient medium. 120 bacterial isolates were screened out from the samples taken (Figure 1). The isolates were studied for their morphological and cultural characteristics and showed 54% Gram positive cocci, 38% Gram positive rods and 8% Gram negative rods (Figure 3). Out of which 16% bacterial isolates showed pigment production (Figure 2). Different pigmentation were observed by total 120 isolates (Figure 4). From this twelve isolates were selected based on their pigment production during preliminary screening. These isolates showed intense and clearly visible pigmentation, suggesting higher pigment yield compared to other isolates. Pigment production was found to be stable and reproducible upon repeated sub-culturing, indicating genetic and phenotypic stability. The selected isolates also exhibited good growth characteristics and early pigment expression on Nutrient agar, making them suitable for optimization and large-scale production. These isolates were identified with the help of morphological and microscopical characteristics and biochemical tests. The identification was done using Bergey's Manual of Determinative Bacteriology upto genus level.
These twelve pigment-producing bacterial isolates were further studied. Three isolates were selected after optimization and preliminary characterization studies. These three isolates showed maximum growth and biomass under optimized conditions such as pH, Temperature, Inoculum size, and Salt concentration where as other bacterial isolates showed growth at acidic, alkaline conditions and required elevated temperature. The three cultures showed growth and pigment production during repeated experiments. Their biochemical characteristics are given in Table 1,enzyme activity (Table 2), Carbohydrate fermentation (Table 3) and Optimization parameter.The selected isolates demonstrated favorable physicochemical properties of the pigment, including intensity, stability, and ease of extraction.



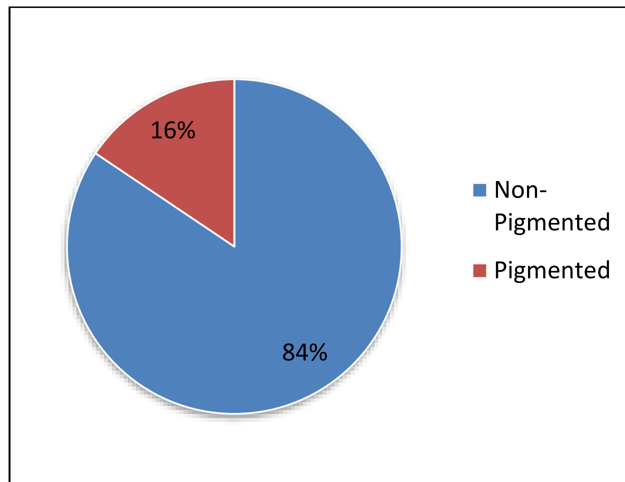
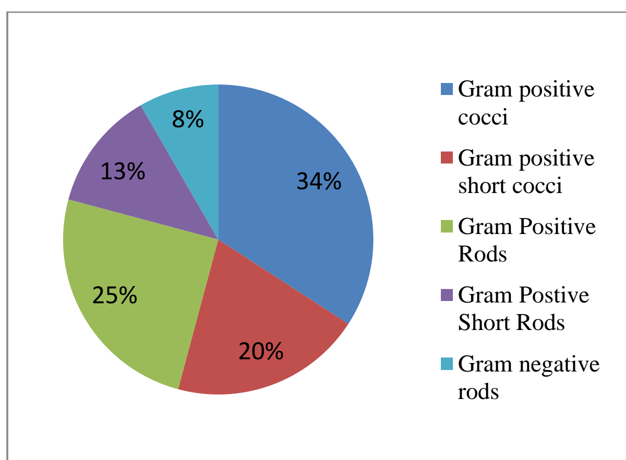


Figure 1: microbial isolates from distinct sampling site

Figure 2: occurance and distribution of isolates



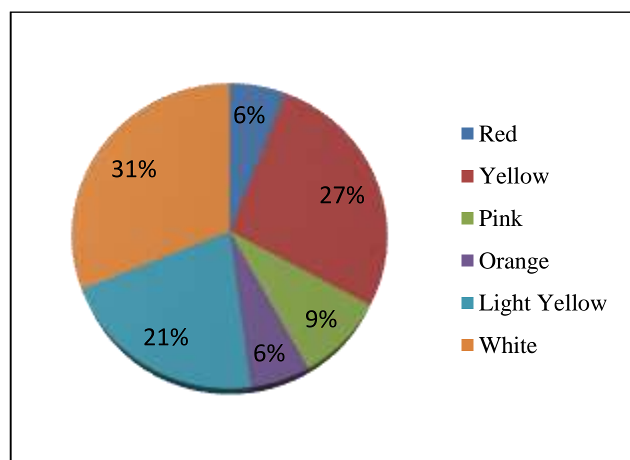


Figure 3: Percentage of Gram's and Morphology of Isolates

Figure 4: Pigment Coloration of Bacterial Isolates

Table 1: Biochemical studies of selected isolates:

	GLY-7	AG-12	AGPA-10
Indole	-	+	+
Methyl Red	-	-	-
Vogus Proskauer	+	+	+
Citrate	+	-	-
Nitrate Reduction	+	-	+

Table 2: Enzyme activity of selected isolates :

	GLY-7	AG-12	AGPA-10
Catalase	+	+	+
Gelatinase	-	-	-
Caseinase	**-**	-	+
Amylase	-	-	-

Positive (+) Negative (-)

Table 3 : Carbohydrate fermentation of selected isolates

	GLY-7	AG-12	AGPA-10
Glucose	+	+	++
Lactose	-	-	-
Dextrose	+	+	+
Maltose	+	+	++
Mannitol	+	-	+

Positive (+) **Negative (-) Acid+Gas Production** (++)

❖ Optimization Parameters:

Three selected bacterial isolates (AG-12, AGPA-10, and GLY-7) were subjected to systematic optimization studies to evaluate the influence of key physicochemical parameters on growth of the isolates. Growth of organism was quantified spectrophotometrically at the appropriate wavelength and cell density (OD_{600}). Growth was assessed under varying conditions of pH, Temperature, Inoculum size, Incubation time, Sodium Chloride concentration, Incubation time and Steady and Shaking condition. It was found that the production of pigment and growth were positively influenced by physicochemical parameters. It showed that change in different parameters like pH, Temperature, Inoculum Size and Salt Concentration showed variation in pigment production. Changes in environmental parameters significantly influenced both biomass accumulation and pigment yield, indicating that pigment production was closely associated with cellular growth. A gradual increase in growth was observed with incremental adjustment of each factor up to an optimal range, beyond which a decline was recorded (Muneefa, K. I., et.al., 2021).

GLY-7 (Figure5) shows maximum growth at pH 7 calculated in terms of O.D and biomass 0.102 in terms of wet weight (Figure 5.1), growth was observed at 30°C and biomass was 0.134 (Figure 5.2), 2% inoculum size gave maximum production 0.621 (Figure 5.3), 1% NaCl concentration was sufficient to show maximum biomass 0.658 (Figure 5.5) and incubation time of 30 hrs with biomass 1.103 (Figure 5.4).These findings are comparable to carotenoid-producing strains of *S.violaceoruber* reported by V. Palanichamy, et.al., (2011) which showed growth above pH 7.6 and temperatures between 28–30°C. In contrast, GLY-7 showed efficient growth and pigment production specifically at 30°C, eliminating the need for elevated temperatures and thereby reducing energy requirements in large-scale fermentation. Furthermore, GLY-7 maintained stable growth at only 1% NaCl, suggesting reduced osmotic stress and lower salt dependency compared to several moderately halotolerant carotenoid-producing bacteria. The ability to tolerate up to 2% inoculum before growth decline also indicates improved biomass productivity and better nutrient utilization efficiency.

Similarly, AG-12 (Figure 6) exhibited optimal growth and biomass at pH 7.0 (0.912) (Figure 6.1), 30°C (0.823) (Figure 6.2), 1.5% inoculum size (1.37) (Figure 6.3),1.5% NaCl concentration (0.912) (Figure 6.5) and Incubation time of 30 hrs produce biomass 1.09 (Figure 6.4). Ali Akbar et al. (2014) reported carotenoid production in species belonging to the genus *Micrococcus luteus*, with activity observed at pH 5 with temperature range 25°-40°C. Although low pH tolerance indicates acidic nature. The clearly defined optimal conditions observed for AG-12 suggest tighter metabolic regulation and improved pigment biosynthetic efficiency under neutral pH conditions. Moreover, its moderate salt tolerance (1.5% NaCl) supports potential application in slightly saline substrates and industrial media without requiring strict salinity control. Compared to previously described *Micrococcus luteus* strains, AG-12 demonstrates more precise growth optimization, which is advantageous for controlled fermentation processes.

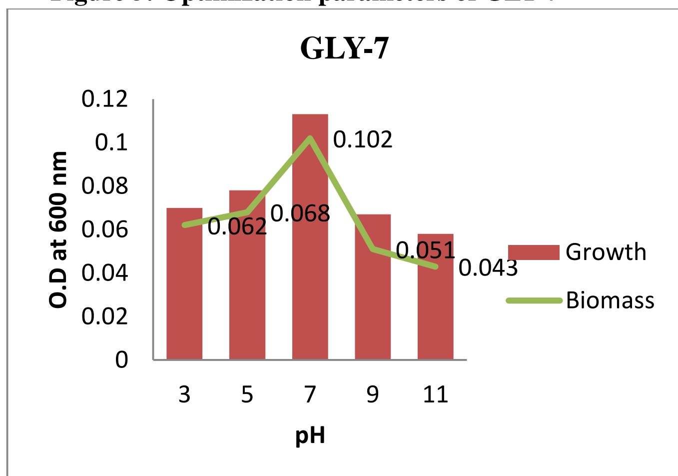
The isolate AGPA-10 (Figure 7) exhibited maximum growth at pH 7.0 (0.219) (Figure 7.1) and temperature of 30°C with biomass 0.267 (Figure 7.2), with an optimal inoculum size of 1% (1.11) (Figure 7.3), 1% NaCl concentration having biomass of 1.03 (Figure 7.5), and incubation time of 30 hrs with biomass 1.24 (Figure 7.4). The lower optimal inoculum requirement (1%) suggests efficient metabolic activation and biomass utilization, which is advantageous for large-scale fermentation systems as it reduces starter culture volume and overall production cost. Comparatively, Reddy et al. (2003) reported that *Kocuria polaris* grows between 5°C and 30°C, with optimum growth at 20°C, tolerates pH 7–12, and survives up to 2.9% NaCl. While *K. polaris* demonstrates psychrotolerant characteristics and broad pH tolerance, its optimal growth at 20°C indicates adaptation to colder environments rather than standard industrial fermentation temperatures. Most industrial bioprocesses are optimized around 28–37°C due to ease of temperature maintenance and compatibility with mesophilic production systems. In this regard, AGPA-10, with optimal growth at 30°C,

aligns more closely with conventional bioreactor operating conditions, eliminating the need for specialized low-temperature control. Although *K. polaris* tolerates higher NaCl concentrations (up to 2.9%), that is halotolerance where, AGPA-10 demonstrated stable growth at 1% NaCl with minimal variation across salt concentrations, indicating moderate halotolerance coupled with metabolic stability. Such stability under moderate salinity is advantageous in industrial settings where extreme salt conditions are generally avoided due to corrosion issues and increased osmotic stress costs. The shorter incubation time (30hrs) observed for AGPA-10 suggests faster growth and quicker metabolic adaptation compared to strains adapted to colder environments, such as *K. Polaris*.

All three isolates demonstrated significantly enhanced growth under shaking conditions compared to static incubation (Figure 8). Agitation improves oxygen transfer rate, nutrient diffusion, and environmental homogeneity. The pronounced increase in growth observed in the present isolates under shaking conditions suggests strong aerobic metabolism This characteristic further supports their suitability for industrial-scale submerged fermentation systems.

Overall, the optimization study demonstrates that growth in the selected isolates is significantly influenced by physicochemical factors, with neutral pH and mesophilic temperature (30°C) being universally favorable.

Figure 5: Optimization parameters of GLY-7



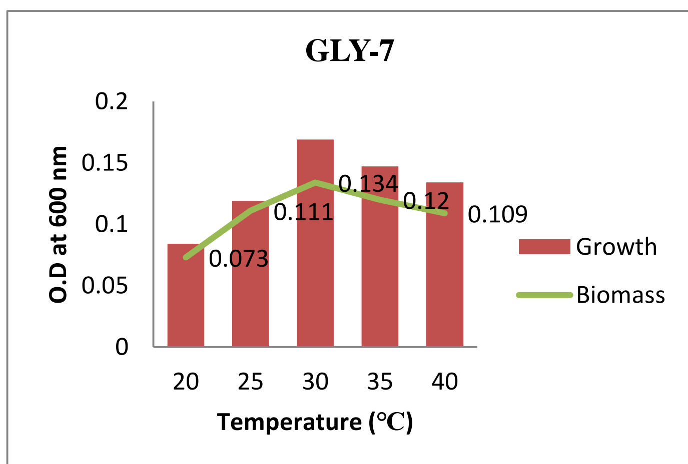
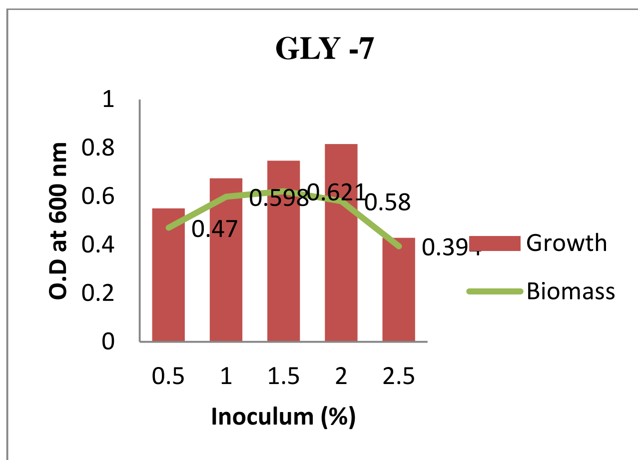


Figure 5.1 Effect of pH

Figure 5.2 Effect of Temperature



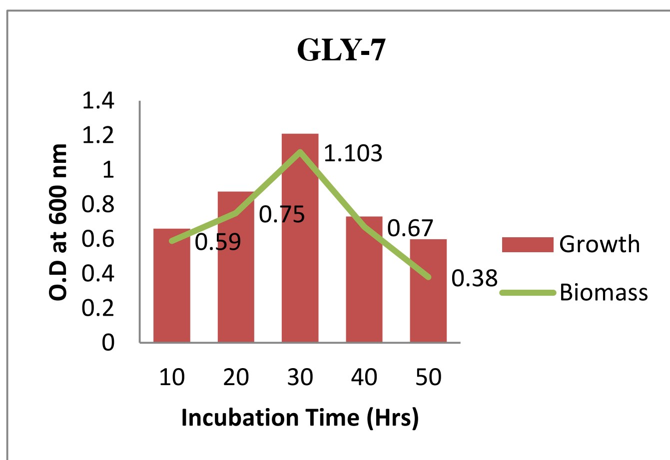


Figure 5.3 Effect of Inoculum

Figure 5.4 Effect of Incubation time

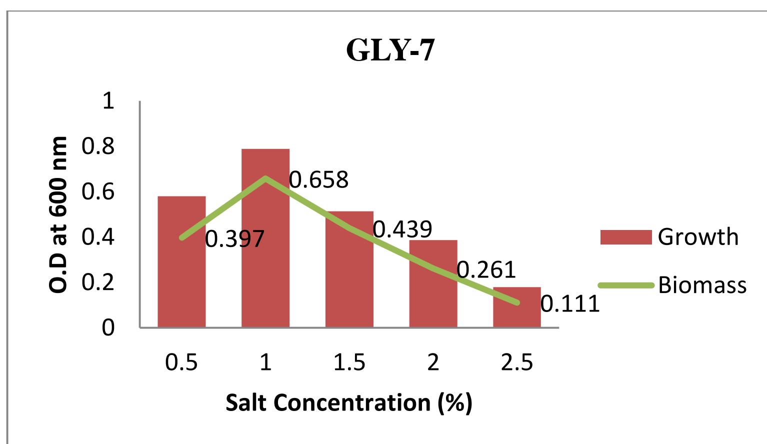
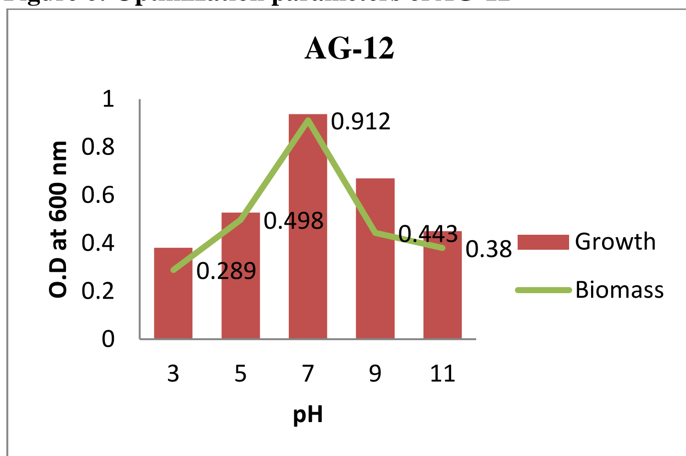


Figure 5.5 Effect of Salt Concentration

Figure 6: Optimization parameters of AG-12



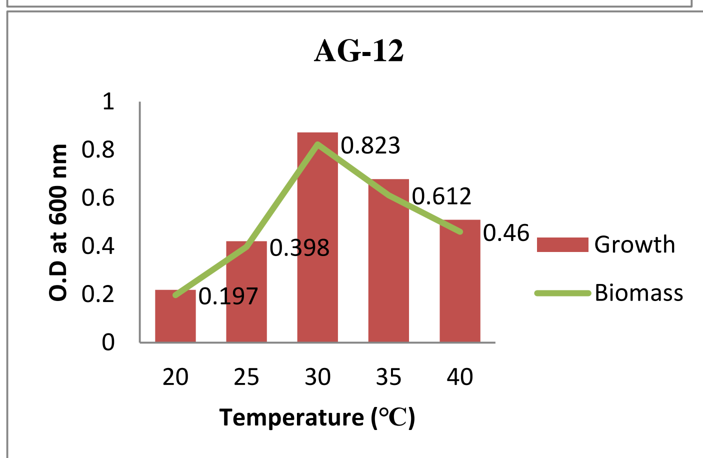
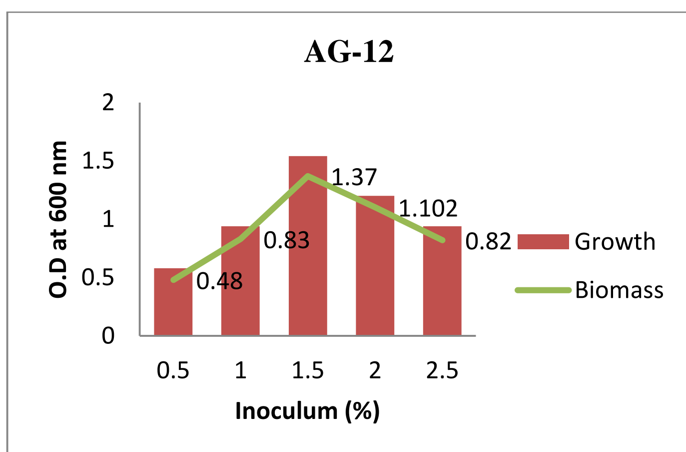


Figure 6.1 Effect of pH
Figure 6.2 Effect of Temperature



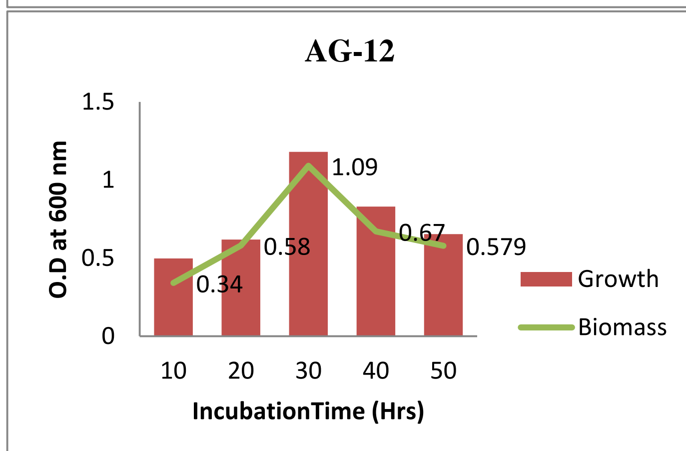


Figure 6.3 Effect of Inoculum
Figure 6.4 Effect of Incubation time

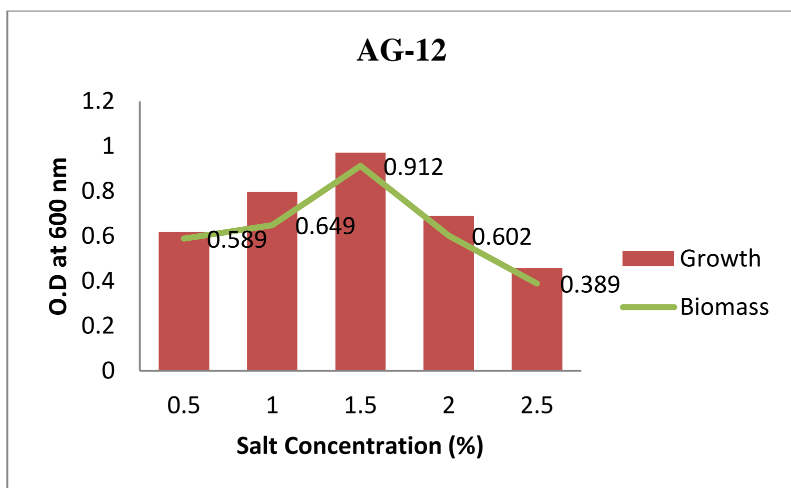


Figure 6.5 Effect of Salt Concentration

Figure 7: Optimization parameters of AGPA-10

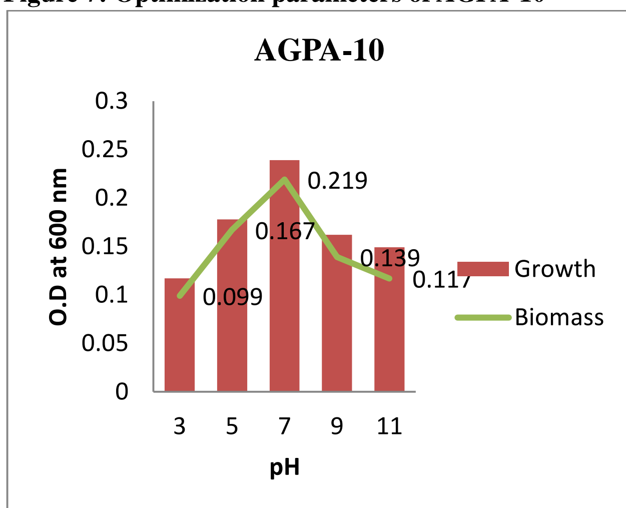


Figure 7.1 Effect of pH

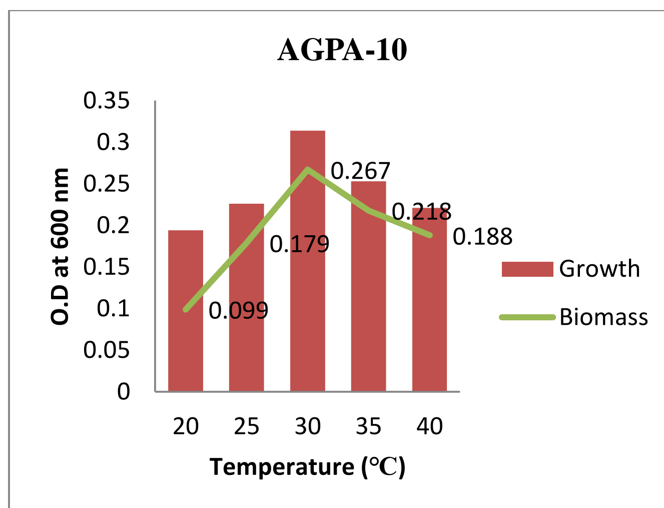


Figure 7.2 Effect of Temperature

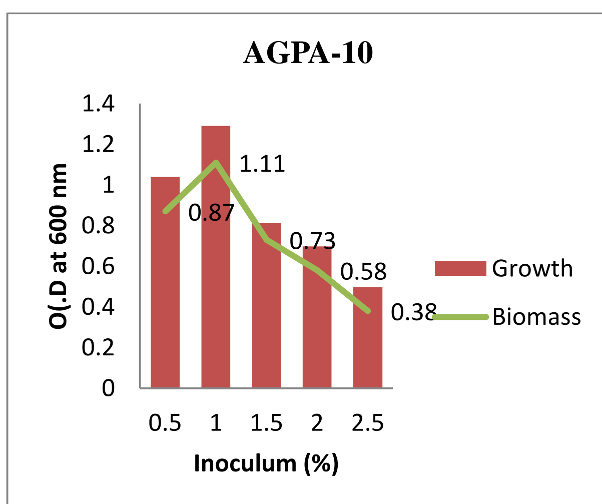


Figure 7.3 Effect of Inoculum

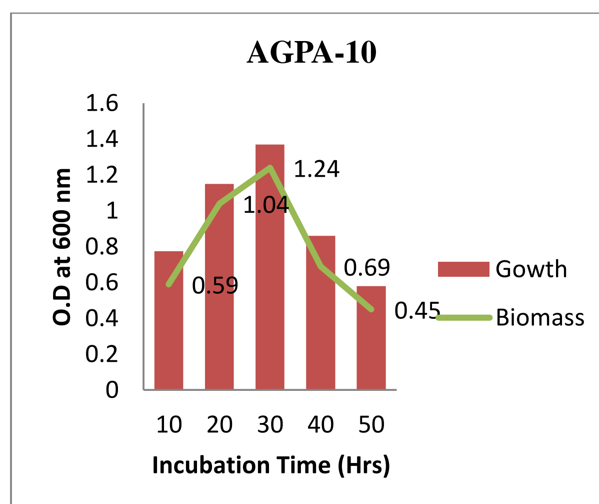


Figure 7.4 Effect of Incubation Time

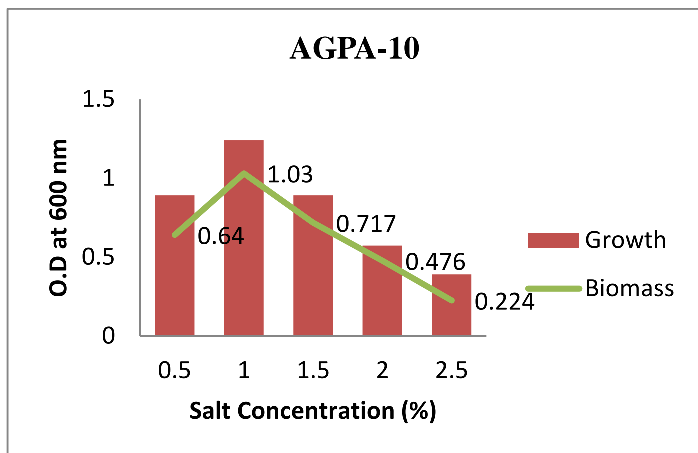
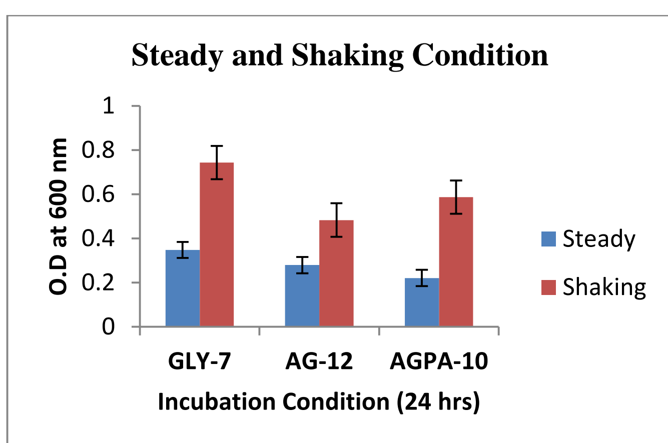


Figure 7.5 Effect of Salt Concentration

Figure 8: Optimization parameters of Steady and Shaking Condition



❖ Conclusion :

The present study focus on isolation and screening of pigment producing bacteria from diverse geographical regions from Maharashtra state. Three isolates were screened out which showed potential for pigment production and were subjected to systematic morphological and biochemical characterization and optimization parameters. The bacterial isolates GLY-7 showed maximum growth at pH 7.0, at temperature 30°C, 2% Inoculum size, and 1% NaCl concentration and incubation time of 30 hrs. AG-12 showed optimal growth at pH 7.0, 30°C, 1.5% Inoculum size, and 1.5% NaCl concentration and incubation time at 30 hrs. AGPA-10 showed maximum growth at pH 7.0 and 30°C, with a comparatively lower optimal inoculum size of 1% and 1% NaCl concentration and Incubation time at 30 hrs and all the three isolates showed maximum growth at shaking condition. The optimization parameters conducted showed GLY-7, AGPA-10 and AG-12 has strong adaptability and productivity, pigment producing potential under the tested parameters.

Overall, compared to previously reported carotenoid-producing bacteria, the present isolates demonstrate several industrial advantages: (i) efficient pigment production at ambient temperature (30°C), (ii) neutral pH optimization eliminating the need for pH adjustment, (iii) moderate salt tolerance suitable for diverse substrates, (iv) optimized inoculum requirements improving biomass efficiency, and (v) enhanced aeration response supporting scalable fermentation. These combined characteristics indicate superior industrial feasibility and cost-effectiveness, making GLY-7, AG-12, and AGPA-10 promising isolates for commercial carotenoid production.

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