**Optimization of Biopigment Production by Halophilic *Bacillus* sp. Using Agro-Industrial Wastes and Assessment of Its Antimicrobial Efficacy**

**Abstract**

Halophilic organisms are a type of extremophiles that survive in the environment with very high salt concentration. In the current study, brown pigment producing Bacillus sp was isolated from the salterns of Vemuladeevi, Andhra Pradesh, India. Further, it evaluate the potential of agro-industrial wastes of Carrot peels (CP), Pine apple peels (PP), Dry fish wastes (DFW) and Bakers waste (BW) as substrates for pigment production from *Bacillus* sp for reducing the production cost and examine its antimicrobial potential. The agro-industrial wastes of were used as the substrate. Among the four substrates tested, the pine apple peels has registered high amount of pigment (1.71±0.13OD Units/gram of dry fermented substrate) than others. For optimization, The production of pigment reached maximum at 37°C, basic pH 8, incubate in 120 rpm condition and 25% NaCl in all the agricultural substrates, but pine apple peels has registered the maximum compared to others in all above conditions. The result of antimicrobial activity was the pigment extract of *Bacillus* sp has registered the maximum inhibitory activity against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Vibrio cholera* and *Penicillium citrinum.* The results confirmed the feasibility of using Pine apple peels as a potential low cost growth medium for the large-scale cultivation of brown pigment using halophilic *Bacillus* sp.

**Keywords**: Biopigment, *Bacillus*sp, agro-industrial wastes, optimization, antimicrobial activity

**Introduction**

Food consumers have recently become more aware of the importance of human wellbeing, leading to the demand for high quality food preservatives.A natural food preservative like a coloring agent is safe for humans to consume, as it does not cause any deleterious effects to the human body, unlike synthetic coloring agents. There are plant-derived pigments (herbs and spices) and microbial pigments, with microbial pigments offering the greatest potential for improvement. Several distinct advantages of microorganisms include their short life cycle, low sensitivity to seasonal and climatic changes, ease of scaling as well as the ability to produce pigments whose color and shade vary depending on species, which are useful for a variety of applications from food to cosmetics. Hence, the identification of new alternatives microbes, the use of low-cost substrates, and the optimization of process parameters are the areas under focus towards economical pigment production in order to extend the application or to find alternative beneficial natural pigments for food industries1-3.

The term halophilic bacteria refer to organisms that can thrive under salty conditions, such as solar salterns, salt lakes, and salt mines. Compared to terrestrial organisms and their obligate microbes’ counterparts, halophiles exhibit diverse metabolic patterns4. Many reports have recently been published on halophilic bacteria's ability to produce pigments and their applications5-8.Studies have also shown that halophilic bacteria have several biotechnological potentials, such as pigments, exopolysaccharides, biopolymers, biosurfactants, compatible solutes, antioxidants, antimicrobial compounds, and antitumor agents9.

The high expense of synthetic growth media typically poses a barrier to the successful commercialization of bacterial pigments. In an effort to lower the cost of producing pigments, numerous research have been conducted to investigate the feasibility of employing less expensive growth media and agricultural waste to carry out this type of bioprocessing, which can also have a lower environmental impact10. Diverse economic activities produce significant amounts of agro-industrial and residential wastes; in recent years, biotechnology research has focused on using these residues as low-cost substrates to enable the development of microorganisms to produce value-added goods like pigments11-12. There are numerous techniques and approaches being developed that use a variety of less expensive substrates and wastes as substitute substrates for the manufacture of microbial pigments13.The main objective of this work was to evaluate the potential of low-cost agricultural products as substrates for brown pigment production from *Bacillus* sp for reducing the production cost and examine its antimicrobial potential.

**Materials and methods**

**Source of microorganism**

*Bacillus* sp. isolated from salt water, Vemuladeevi, Andhra Pradesh, India, and identified as a potential strain that produced brown pigment was used. This strain was maintained on Zobell marine agar medium (HiMedia, India). The organism was sub cultured at regular interval of 1 month and stored at 40C.

**Substrate preparation**

The agro-industrial wastes of Carrot peels (CP), Pine apple peels (PP), Dry fish wastes (DFW) and Bakers waste (BW) obtained from a local market. The obtained raw wastes were pulverised and utilised as substrates for screening bacterial isolates for pigment synthesis after being baked in an oven at 600C for 12 hours. 10 g of powder was soaked in water overnight, the excess water was drained, and the flasks were autoclaved at 121°C for 20 minutes. The flasks were allowed to cool before being infected with 10% of the inoculum and incubated at 28°C for 12 days. After the incubation period, the flasks were autoclaved for 30 minutes at 121°C. The fermented substrates were dried for 24 hours at 50°C. Dried substrates were pulverised and saved for later investigation.

**Pigment extraction and estimation**:

The final volume was made up to 50 ml after extracting 0.5 g of dried fermented substrate with 80% ethanol. The mixture was shaken continuously for one hour at a speed of 200 rpm. The supernatant was collected and centrifuged for 30 minutes at 5000 rpm to separate the pigments. The pigments were examined using a 475 nm absorbance measurement of pigment extract. Pigment yield was calculated as OD per gram (od/gdfs) of dry fermented matter at its maximum.

**Optimization for pigment production**

The optimization of growth conditions, particularly physical nutritional parameters is a prime importance in the development of any pigment production process owing to their impact on the economy and practicability of the process. Temperature has a substantial impact on pigment synthesis since microbes require an appropriate temperature to flourish and produce pigment. The pH of the growth medium is important in pigment production because it balances the medium's acidic and basic nature and creates a pleasant environment for microbial development. The selected bacterial isolates were inoculated into the agro-industrial wastes medium and incubated at different temperatures like 15°C, 20°C, 28°C, 37°C and 45°C, pH range of 5, 6, 7, 8, and 9, Agitation conditionkept at static, 30 rpm, 60 rpm and 120 rpm), NaCl concrange of 10%, 15%, 20%, 25% and 30% for about 7 days.After incubation, the absorbance (OD) of the produced pigments was analyzed using UV-Vis Spectrophotometer against a blank

**Antimicrobial potential of brown pigment**

**Plate assay**

Crude pigments were screened for antimicrobial activity against bacterial cultures of *E. coli,Salmonella typhi, Shigella flexneri, Staphylococcus aureus, Vibrio cholera* and fungal strains of *Aspergillus niger*, *Candida albicans, Fusarium solani, Mucor sp, Penicillium citrinum* by Kirby- Bauer disc diffusion method. The bacterial strains were maintained in Nutrient agar slants (Hi-media Laboratories Pvt. Ltd., Mumbai) at 4ºC and sub-cultured on a fresh nutrient broth 24 h prior to antibacterial test. The fungal strains were maintained in Sabouraud dextrose agar slants andsub-cultured on a fresh Sabouraud dextrose broth 24 h prior to antimicrobial test. Purified colonies of 18 to 24-hour old test cultures cultivated on their respective medium were used to prepare bacterial and fungal suspension in 0.9% saline solution and the turbidity was adjusted to 0.5 McFarland standards and the suspension was swabbed on Muller Hinton agar for bacteria and potato dextrose agar for fungi using a sterile cotton swab. Sterile discs (6 mm) prepared from Whatman filter paper No.1 impregnated with 20 µl crude pigment solution (at a final concentration of 2.5 mg/ml, 5 mg/ml and 10 mg/ml) were placed over the Muller Hinton agar and potato dextrose agar. Sterile filter paper discs soaked in ethanol served as negative controls. The diameter of the zone of inhibition was measured after 24 hours of incubation at 37°C for bacteria and 72 hrs for fungi with the help of zone meter provided in the commercial kit. The activity for each sample was carried out in triplicate and the results were expressed as mean ± standard deviation14.

**Minimum inhibitory concentration (MIC)**

Minimum inhibitory concentration of crude pigment was performed in Mueller Hinton broth for bacteria and Potato dextrose broth by Broth Dilution method15. The drug concentrations ranged from the crude pigment, 512, 256, 128, 64, 32, 16, 8, 4, 2and 1 mg/ml of standardized suspension (0.5% Macfarland turbidity) of the test organism was transferred into each tube. The control tube contained only organism and devoid of crude pigment. The culture tubes were incubated at 37°C for 24 h for bacteria and 72 hrs for fungi. The lowest concentrations which did not show any growth of tested organism after microscopic evaluation were determined as MIC. The assay was repeated three times.

**Minimum Bactericidal and Fungicidal Concentration (MBC/MFC)**

The MBC/MFC of the crude pigment were determined16 by plating a loopful of bacterial sample from each MIC assay tube with growth inhibition into freshly prepared MH broth and PD broth and the plates were incubated at 37°C for 24 hr. The MBC values were recorded as the lowest concentration of the extracts that did not permit any visible bacterial colony growth on the agar plate during the period of incubation. The assay was repeated three times.

**Results and discussion**

**Effect of agricultural waste type on pigment production after 12 days of incubation**

The aim of substrate selection was to evaluate the low-cost agricultural products that are most suitable for the maximum yield of brown pigment production. Carrot peels, Pine apple peels, Dry fish wastes and Bakers waste without any supplementation were used as substrates for cultivations of *Bacillus* sp. As shown in Table 1, the bacteria produced the highest yield of brown pigments when cultivated in Pine apple peels (1.71±0.13OD Units/gram of dry fermented substrate), followed by carrot peels (1.19±0.28od/gdfs), and dry fish wastes (1.18±0.21 od/gdfs), respectively, whereas the lowest yield of pigment production was observed from Bakers waste (0.83±0.19 od/gdfs). This observation is consistent with the findings of Gupta *et al*.17, who evaluated pigment extraction from various fruit wastes, including pomegranate, grape, lime, apple, and papaya. Among these, lime peel waste exhibited the highest pigment yield (1.65%) when extracted using a 1:1 mixture of hexane and acetone.

Table 1: **Effect of agricultural waste type on pigment production**

|  |  |  |
| --- | --- | --- |
| S.No. | Substrates | pigment  od/gdfs |
|  | CP | 1.19±0.28 |
|  | PP | 1.71±0.13 |
|  | DFW | 1.18±0.21 |
|  | BW | 0.83±0.19 |

Values represented mean ± SD

**Optimization parameters**

**Temperature**

The effect of temperature on brown pigment production by Bacillus sp was studied in Fig 1. The maximum pigment production was recorded at 37°C in PP (1.76 od/gdfs), CP (1.24±0.02 od/gdfs), and DFW (1.19±0.05 od/gdfs), whereas minimum amount of pigment was recorded at 15°C in BW (0.14±0.01 od/gdfs). Similarly, Venil *et al*.11 reported that Bacillus sp. showed increased production of brown pigments when incubated at 37°C, attributing it to the optimal activity of pigment-synthesizing enzymes at this temperature. Also Alka *et al*. 18 reported that the maximum pyocanin production from *Pseudomona aeruginosa* was recorded at 37°C

**pH**

Effect of varied pH on pigment production by *Bacillus* sp was studied (Fig 2). The production of pigment reached maximum at basic pH 8 in all the agricultural substrates, i.e.,PP (2.13±0.15 od/gdfs), CP (1.25±0.08 od/gdfs), and DFW (1.26±0.17od/gdfs) followed by pH 9 and 7. Minimum pigment production was recorded in pH 5 in BW substrates, (0.16±0.02 od/gdfs). Similarly, Asker and Ohta19, reported that canthaxanthin production was higher at pH 7.2 in *Halobacterium* sp. isolated from a salt farm in Alexandria, Egypt. Shatila et al20. reported that the growth and pigment production by *Exiguobacteriumaurantiacum* 144 was found to be highest at pH 7.0.

**Agitation condition**

The influence of agitation condition on pigment production by *Bacillus* sp was studied with different range of agitation condition (static to 120 rpm). The highest level of pigment production was achieved at 120 rpm in PP substrates (2.41±0.28 od/gdfs) followed by 60 rpm (1.65±0.24 od/gdfs) (Fig 3). Similarly, Khanafari et al.21examined that the *Halorubrums odomense* cultivated under shaking conditions at 120 rpm, it exhibited high growth and pigment production. Without shaking, little growth and no pigment production was observed. Masetto et al.22reported that agitation combined with aeration results in higher zeaxanthin accumulation in *Flavobacterium* sp.

**Nacl conc.**

The low level of pigment production by *Bacillus* sp in the concentration of NaCl was 10%, but pigment production was in maximum in the concentration was 25% (Fig 4). Similarly, Khanafari et al.21 found that the *Halorubrums odomense* produced high amount of pigment in 30% NaCl.

Fig1: Effect of temperature on pigment production Fig 2: Effect of pH on pigment production

Fig 3: Effect of Agitation on pigment production Fig 4: Effect of NaCl on pigment production

Fig 5: Antimicrobial activity of biopigment

**Antimicrobial activity of extracted pigment – Plate Assay**

Bacterial pigments have been long known to exhibit antimicrobial properties. Pigments such as carotenoids, melanins, flavins, quinones, monascins, violacein, and indigo have been reported as good antimicrobial agents23. As per the Fig 5, it was observed that the pigment extract of *Bacillus* sp has registered the maximum inhibitory activity against *Escherichia coli* (16.8±0.71 mm), *Salmonella typhi* (12.6±0.46 mm), *Staphylococcus aureus* (12.3±0.43 mm), and *Vibrio cholera* (11.0±0.51 mm)at the concentration of 10 mg/ml. Among five fungi tested, the *Aspergillus niger* (10.3±0.44 mm) and *Penicillium citrinum* (10.3±0.34 mm) were found most susceptible to the extracted pigment at the concentration of 10 mg/ml. The minimum amount of inhibitory activity was observed against*Candida albicans*(8.2±0.17) at 10 mg/ml concentration tested. The test concentration of 2.5 mg/ml showed the minimal antimicrobial activity against most of the pathogens. Similarly, Patki et al.24 examined that the yellow pigment extracted from bacterial isolates of mangrove soil (5 mg/ml) exhibited good antibacterial activity with maximum effect on *E. coli*(15.8mm) and *S. aureus*(16mm).

**MIC and MBC, MFC of biopigment**

The antimicrobial potency of brown bacterial pigment was quantitatively determined by the microdiluton method. Minimum inhibition concentration (MIC) values exerted by pigment are presented in Table 2. The pigment showed considerable antimicrobial activity against tested strains with MIC values ranging from 16 – 126 mg/mL. The present study showed that crude violet pigment has possessed low level of antifungal activity (>128 mg/ml) against *A. niger*, *C. albicans* and *Mucor* sp. Of interest, the pigment was most active against *E. coli (*>16 mg/ml) followed by *Salmonella typhi and Staph. aureus*with MIC value of 32 mg/ml. The violet pigment differs in its antibacterial potency against selective strains. MBC/MFC was defned as the lowest concentration of pigment that showed complete inhibition of colonies of microorganisms on agar plates. The *E. coli*showed MBC at 32 mg/ml whereas, for *A. niger*, *C. albicans* and *Mucor*sp were registered the MFC level of 128 mg/ml. Zhao et al.25investigated the orange pigment of *Monascus* has exhibited strong antibacterial activity against *E. coli*and the concentration of 2.5 mg/ml was the minimum inhibitory concentration against *E. coli.*

**Table 2: Minimum inhibition concentration (MIC) of biopigment**

|  |  |  |  |
| --- | --- | --- | --- |
| **S.No** | **Name of bacteria** | **MIC**  (mg/mL) | **MBC/MFC**  (mg/mL) |
| 1. | *E. coli* | >16 | >32 |
| 2. | *Salmonella typhi* | >32 | >64 |
| 3. | *Shigella flexneri* | >64 | >128 |
| 4. | *Staph. aureus* | >32 | >64 |
| 5. | *Vibrio cholerae* | >64 | >128 |
| 6 | *Aspergillus niger* | >128 | >256 |
| 7 | *Candida albicans* | >128 | >256 |
| 8 | *Fusarium solani* | >64 | >128 |
| 9 | *Mucor sp* | >128 | >256 |
| 10 | *P.citrinum* | >64 | >128 |

**CONCLUSION**

The use of Carrot peels (CP), Pine apple peels (PP), Dry fish wastes (DFW) and Bakers waste (BW) as substrate was cost effective and environmental friendly. From this study it was confirmed that the feasibility of using Pine apple peels as a potential low cost growth medium for the large-scale cultivation of brown pigment using halophilic *Bacillus* sp. Antimicrobial assays of crude pigment extract of 10 mg/ml exhibited highest inhibitory effect on *E.coli*, *S. typhi* and *S. aureus*. Therefore, it was suggested that these findings could inspire product developers in the food, cosmetic and pharmaceutical industries etc., to develop cost-effective natural colorants that would be more attractive.

**Disclaimer (Artificial intelligence)**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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