Production of bacterial pigments in low cost medium and formulation of biodegradable ink

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Bacterial pigment production is an emerging field of research for its wide potential industrial applications. Prodigiosin (Serratia marcescens UTM1) and violacein (Chromobacterium violaceum UTM5) are such pigments which possess several biological properties and have gained increasing importance in industrial markets such as drugs, cosmetics, textile dyeing, etc. The present study demonstrates the use of low cost medium for growth of locally isolated red, violet pigment producing bacteria and their application as biodegradable ink on plastic materials. The natural inks were successfully formulated using polyvinyl butyral, ethyl acetate, methyl ethyl ketone, and applied on plastic materials. They were able to withstand heat up to 60°C and showed no damage to plastic material during physical contact. The hue and chroma values showed the formulated natural inks falls within the red and violet colour. The results have shown that the bacterial pigments act as natural colourants and have great potential as biodegradable inks.

Keywords: Chromobacterium violaceum, Dyes, Ink formulation, Natural colourants, Prodigiosin, Serratia marcescens, Textile dyeing, Violacein

The prolonged use of synthetic dyes has many concerns viz., allergy and toxicity to humans, hazardous waste generation, disposal related environmental issues and economic challenge. Awareness on safety and environmental protection have fostered fresh enthusiasm for natural colourants which accordingly reflected its annual growth rate @7% with a turnover of US $ 0.66 billion. The Global Industry Analyst reported that the demand for organic and natural colourants is expected to reach almost 10 million tons by 2017. Industrial production of natural colourants by microbial fermentation has several advantages such as easy propagation, faster fermentation, simple extraction and no seasonal variations. However, to increase the production of bacterial pigments from low cost substrate for economical viability is a challenging task.

The red pigment, prodigiosin from Serratia marcescens has a wide range of biological activities, such as antibacterial, antifungal and immunosuppressive agents. Similarly, the violet pigment, violacein from Chromobacterium violaceum has exhibited cytotoxic, antileishmanial, antiulcerogenic and antitumoral activities. Apart from pharmacological applications, the red and violet pigments are used in dyeing natural fibres. The market of natural colourants has grown intensely in the recent years for their increased use in more and more products, and thereby providing opportunity to explore bacteria as a sustainable source of natural colourants.

In this study, we explored the possibility of producing the red and violet pigments from Serratia marcescens UTM1 and Chromobacterium violaceum UTM5, respectively using low cost medium and incorporate the pigments in the biodegradable ink formulation. Evaluation on the performance of formulated biodegradable ink was also carried out.

Materials and Methods

The red pigment producing bacterium, Serratia marcescens UTM1 isolated from an oxidation pond located at Universiti Teknologi Malaysia, Johor, Malaysia and violet pigment producing bacterium i.e., Chromobacterium violaceum UTM5, isolated from the soil sample collected from the vicinity of a wastewater treatment plant in one oil refinery premise in Negeri Sembilan, Malaysia was used in this study. The culture was grown and maintained by regular sub-culturing in nutrient broth NB (8 g/L, Merck, Germany).
Preparation of low cost medium

Brown sugar medium, 10 % (v/v) (from stock solution 40 g L\(^{-1}\)) was used for culturing \textit{S. marcescens} UTM1\(^{12}\). Liquid pineapple waste (LPW) was obtained from Lee Pineapple Co. Ltd. located at Tampoi, Skudai, Johor Bahru, Malaysia. LPW was filtered using muslin cloth and centrifuged (AllegraTM 25R Centrifuge-Beckman CoulterTM, California) at 10000 rpm for 10 min at 4°C. The supernatant obtained was sterilized using 5% (v/v) of ethanol and the pH was adjusted to 7.0 using 1 M NaOH prior to use. About 10 % (v/v) LPW was used for culturing \textit{C. violaceum} UTM5\(^{13}\).

Production of red and violet pigments in low cost medium (5 L bioreactor)

Active cultures of \textit{S. marcescens} UTM1 and \textit{C. violaceum} UTM5 were prepared by inoculating a loopful of 24 h bacteria into a series of 250 mL Erlenmeyer flasks containing 62.5 mL NB followed by incubation at 25 30°C, respectively for 24 h in the dark under static condition. Similarly, the starter culture for \textit{S. marcescens} UTM1 and \textit{C. violaceum} UTM5 were cultivated in static condition by transferring 10 % (v/v) inoculum (active cultures) in a 2 L Erlenmeyer flask containing 500 mL NB supplemented with 200 mg L\(^{-1}\) and 150 mg L\(^{-1}\) L-tryptophan and grown at 25 30°C, respectively for 24 h in the dark. The starter cultures were then transferred into a 5 L bioreactor (Biotron, Korea) containing 4.5 L of 10% brown sugar and 10% (v/v) LPW for \textit{S. marcescens} UTM1 and \textit{C. violaceum} UTM5 followed by 24 h cultivation at 25 and 30°C, respectively, 200 rpm. The red and violet pigments were extracted using 1.25 L ethyl acetate and 300 mL acetone, respectively. The pigments were then concentrated using a rotary evaporator at 50°C (Büchi, Switzerland), air dried and used for further analysis.

Formulation of biodegradable ink

\textbf{Ink preparation}

Polymer, polyvinyl butyral was used as resin for ink formulation. Mix solvent was prepared by combining ethyl acetate (EA) with methyl ethyl ketone (MEK) at a ratio of 2 (EA): 1 (MEK). Varnish was prepared by combining resin (26% of total production weight) to mix solvent and additive (stearic acid) and mixed well using magnetic stirrer for 30 min. The viscosity of varnish was adjusted by adding mix solvent for smooth texture. The pigment were mixed into the varnish and stirred for 30 min to make a homogenous mixture.

\textbf{Properties of ink}

The finished ink was tested for its stability towards temperature and physical contact. The ink after being printed on a substrate must remain attached or should not display any major damage towards temperature and physical contact. The physical contact test included scratching by finger nail and stacked with other objects.

\textbf{Color analysis of pigment}

The values of L*, a* and b* were measured using a ColorFlex EZ colorimeter with the CIELAB colour system (Hunter Associates Laboratory Inc., Virginia, United States). These values were then used to calculate the chroma (C*) and hue angle (h\(_h\)) values. L* indicates lightness from 0 (black) to 100 (white). Positive and negatives values of b* represent yellow and blue, respectively. Chroma values denote the saturation or purity of the colour. Values close to the centre at the same L* value indicate dull or grey colours, whereas values near the circumference represent vivid or bright colours. Hue angle value denotes 0 for redness, 90 for yellowness, 180 for greenness, and 270 for blueness\(^{12,14}\). L*, a* and b* values of the red and violet pigments were compared with formulated red and violet inks.

\textbf{Results and Discussion}

\textbf{Red and violet pigment production and extraction}

Brown sugar and LPW are found to be suitable low cost medium for the production of red and violet pigment from \textit{S. marcescens} UTM1 and \textit{C. violaceum} UTM5, respectively. As reported by Aruldass \textit{et al.}\(^{13}\), presence of various compounds in brown sugar, namely furfural, furfuryl alcohol, methyl furan-3-carboxylate, 4-hydroxy-2,5-dimethyl-3(2H)-furanone and others may facilitate the growth of \textit{S. marcescens} UTM1 and red pigment production. High sugar contents, namely sucrose, glucose and fructose, monovalent (postassium) and divalent cations such as magnesium, calcium, and other elements including iron, manganese, zinc, copper, cadmium and sodium was found in LPW. These substances mimic as natural carbon and nitrogen sources for growth of \textit{C. violaceum} UTM5 and violet pigment production. Supplementation of tryptophan during fermentation acts as precursors which facilitated the growth and increased the yield of pigment of the bacterial strains. Although the market value for bacterial pigments are undefined well, pigment production in this study was cost effective as compared to the production using
synthetic medium, NB. Production of red pigment from *S. marcescens* UTM1 in 50 L bioreactor costs USD 59 in substrate NB; however, the same is reduced to USD 5.9 in agro-industrial based substrate. Similarly, a reduction in the cost of violet pigment production using LPW in 50 L bioreactor was observed from USD 281 in NB to USD 236 in LPW. Present study has demonstrated the feasibility of agro-industrial based substrates as low cost growth medium for bacterial pigment production. The pigments were concentrated using rotary evaporator and used for further analysis.

**Formulation of ink making**

Red and violet pigments were subjected for ink formulation that can be applied on plastic materials. The components that were used for ink formulation are pigments, mixed solvent (ethyl acetate and MEK), resin (poly(vinyl butyral-co-vinyl alcohol-co-vinyl acetate) or polyvinyl butyral) and additive (stearic acid) with the percentage of 19-20% (w/w), 70% (w/w), 7-8% (w/w) and 3% (w/w), respectively. Polyvinyl butyral was chosen as resin because of its availability and ability to polymerize and bind homogeneously during the preparation of ink. Besides, polyvinyl butyral is classified as low cost and tough polymeric material used in wide range of industrial applications, including manufacturing of glass, metal, plastics and wood. Solvent mix, consisting of ethyl acetate and methyl ethyl ketone was selected because both were highly volatile liquids and fast drying ability after applied onto a plastic surface. Stearic acid was chosen as additive as it has the advantage of good dye solubility, viscosity vs. temperature relationship and good compatible to a wide range of vehicle. The finished ink was observed to have a gluish structure with uniform colour upon formulation.

Niaounakis formulated biodegradable printing ink comprised of poly(vinyl alcohol), 5-35 wt%; pigment, 5–80 wt%; solvent, 15–87 wt%. According to Dunn, additives may be included for special performance features, if only small amount of additives (1-3 %) would change the fit for use performance.

**Application of pigmented ink on plastic material**

The formulated red and violet inks were applied to a plastic substrate printed with a shape of flower. The results were compared with the original flower picture as shown in Fig. 1. The inks showed good colour intensity when painted to the printed flower, although the ink colour was lighter. This is possibly due to the amount of pigment added. Also, natural pigments are less durable and much transparent at equal colour intensity compared to synthetic pigments. However, there was a bubble or foam formation after the ink dried. Disruption or displacement of the molecules from the surface needed to destroy these foams. Certain short-chain alcohols have been used to accomplish this. This problem can be resolved using chemical defoamers or antifoams. A defoamer is a compound that knocks down foam already present in the mixture (foam elimination). Besides ink application on plastic materials, polyvinyl butyral incorporated ink, namely alumina ink was printed on biaxially-oriented polyethylene terephthalate (BoPET) film and photographic paper and suggested to be used on electronic applications.

**Colour determination for bacterial pigment and formulated ink**

Colour can be described according to the Munsell colour space in terms of hue value and chroma. The colour is quantified by a colour order system developed in 1976 by the Commission Internationale de l’Eclairage (CIE), which uses 3 values, L*, a*, and b*. The CIE L* value is a measure of the lightness of an object, the CIE a* value is a measure of redness (positive value) or greenness (negative value), and the CIE b* value is a measure of yellowness (positive value) or blueness (negative value). While CIELAB uses Cartesian coordinates to calculate a colour in a colour space, CIELCH uses polar coordinates. This colour expression can be derived from CIELAB. The
L* defines lightness, C* specifies chroma and h° denotes hue angle, an angular measurement. The colour of both, pigment and its formulated ink, were determined by ColorFlex EZ and the result were shown in Table 1 and Fig. 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Colour co-ordinates</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>c*</th>
<th>h°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red pigment</td>
<td></td>
<td>1.51</td>
<td>2.12</td>
<td>-0.18</td>
<td>2.13</td>
<td>-4.85</td>
</tr>
<tr>
<td>Violet pigment</td>
<td></td>
<td>0.32</td>
<td>1.12</td>
<td>-1.09</td>
<td>1.56</td>
<td>-44.22</td>
</tr>
<tr>
<td>Formulated red ink</td>
<td></td>
<td>1.76</td>
<td>1.84</td>
<td>-0.21</td>
<td>1.85</td>
<td>-6.51</td>
</tr>
<tr>
<td>Formulated violet ink</td>
<td></td>
<td>1.15</td>
<td>0.84</td>
<td>-2.47</td>
<td>2.61</td>
<td>-71.22</td>
</tr>
</tbody>
</table>

Chroma (C*) = [(a*)² + (b*)²]½.
Hue angle (h°) = tan⁻¹ (b*/a*).

The CIELAB result obtained from the reading can be used to compare the colour between the pigment and its formulated ink. The expressions for these colour differences are ΔL*, Δa* and Δb*. Given ΔL*, Δa*, Δb*, the total difference or distance on the CIELAB diagram can be stated as a single value, known as ΔE*.

\[ ΔE*_{ab} = \left[ (ΔL^2) + (Δa^2) + (Δb^2) \right]^{1/2} \]

Using the equation for ΔL*Δa* and Δb*, the colour difference between red pigment and formulated red ink can be expressed as:

\[ ΔL* = 0.25, \ Δa* = -0.28, \ Δb* = -0.03 \]
\[ ΔE*_{ab} = [(0.25)^2 + (-0.28)^2 + (-0.03)^2] \]
\[ ΔE*_{ab} = 0.14 \]

Fig. 2 — Comparison of 2D plot (A) Red pigment vs. formulated red ink; and (B) Violet pigment vs. formulated violet ink
The total colour difference can be expressed as $\Delta E^* = 0.14$. On the $a^*$ axis, a reading of $-0.28$ indicates greener or less red. On the $b^*$ axis, a reading of $-0.03$ indicates bluer or less yellow. On the $L^*$ plane, the measurement difference of $+0.25$ shows that formulated red ink was lighter than extracted bacterial red pigment.

The colour difference between violet and formulated violet ink can be expressed as:

$\Delta L^* = +0.83$, $\Delta a^* = -0.28$, $\Delta b^* = -1.38$.

$\Delta E^{*}_{ab} = [(+0.83)^2 + (-0.28)^2 + (-1.38)^2]$

$\Delta E^{*}_{ab} = 2.67$

The total colour difference can be expressed as $\Delta E^* = 2.67$. On the $a^*$ axis, a reading of $-0.28$ indicates greener or less red. On the $b^*$ axis, a reading of $-1.38$ indicates bluer or less yellow. On the $L^*$ plane, the measurement difference of $+0.83$ shows that formulated violet ink was lighter than extracted violet pigment. These results suggest a simple geometrical arrangement of hue qualities derived using subjective scaling techniques where hues are scaled subjectively as proportions of the elementary hues yellow and red, red and blue, blue and green, and green and yellow. With the help of linear colorimetric transformations, both results can be expressed in CIE colour coordinates, and thus serve equally well for models about unique hues.

Sonication Effect of Finish Ink

Optimum benefits of a pigment are achieved when its total size is reduced to the primary particle size. The colour strength of a pigment depends on its exposed surface area, and the smaller the particle the higher the surface area and thus stronger the colour. Sonication, sound energy was used to agitate pigment particles in the solvent which eventually reduced the size of pigment to the minimum. Fig. 3 shows sonication results of the formulated red and violet inks. After being sonicated for 3 and 6 min, the ink showed much lighter colour when applied to plastic surface compared to non-sonicated ink.

Particle size is important for avoiding sedimentation, therefore small particles are preferred for obtaining stable inks; the reported values cover a broad range (5-50 µM), depending on the type of pigments and on the printing process. Ultrasonic energy can clean or homogenize materials, and also accelerate both physical and chemical reactions. Extra agitation attributed on the dyeing system by ultrasound would lead to dispersion (breaking up of micelles and high molecular weight aggregates into uniform dispersions) and degassing (expulsion of dissolved or entrapped gas or air molecules).

Ink performance test

Physical contact test (Scratching and Stacking)

In this study, the both inks were tested for its durability, i.e. when physical contact is applied, the ink should remain attached or no major damage occurred. Both inks showed good durability when scratched and stacked with books or other objects since the binder was similar. The formulated inks had strong adhesion or hardness on the plastic material as

![Fig. 3 — Effect of sonication (A) non-sonicated; (B) sonicated 3 min; and (C) sonicated 6 min](image-url)
there were no physical changes, namely peeling off or scratches observed on the dried inks. Vinyl alcohol and vinyl butryl groups that present in polyvinyl butyral were hydrophilic and hydrophobic, respectively in nature. The moieties behave as promoters of polymer adhesive and binders during the ink preparation. Thus, the formulated inks have strong binding, great flexibility and toughness, sturdy adhesion that could withstand to physical contacts.

**Heat test**

The printed ink must be able to withstand at different temperature in order to cope with various situation. Therefore, a heat test was carried out to the printed ink by placing into an oven for 5 min and incubated at 30, 40, 50 and 60°C, respectively. The printed red and violet ink were placed inside a beaker and left into oven for testing.

Since both inks have the same formulation, the performances for both were almost similar. Kuen-Shan et al., demonstrated an endothermic process for stearic acid at 67°C, which is its melting point. However, the unclear endothermic peak at low temperature indicated that the loss of water and volatile, and the relatively broad exothermic peak (350 to 510°C) was following the melting of polyvinyl butyral. However, the present results differ because of the change of percentage of composition that affected the physical properties of ink. Only small percentage of additive and resin, stearic acid and polyvinyl butyral, respectively were used in this study. Although formulated inks have stronger binder and adhesion properties, the percentage of additive and resin may not provide sufficient heat resistance properties to the formulated inks. Besides, bacterial pigments were reported to be sensitive to illumination and heat which make the colours of the inks fade.

**Conclusion**

There is an increasing interest involving bacteria as a possible alternative source of colourants in various applications. In this direction, biotechnology plays a crucial role for mass fermentation of bio-colourants. Most of the bacterial pigment production is still at the R&D stage. Hence, work on the bacterial pigments should be intensified especially in finding cheap and suitable growth medium which can reduce the cost and increase its applicability for industrial production.

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