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Flexirubin-type Pigment Production from *Chryseobacterium artocarpi* CECT 8497 and its Application as Natural Ink

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ABSTRACT

Interest and demand for bacterial pigments is growing due to rising awareness of toxicity of synthetic dyes. This study evaluated on the production of flexirubin-type pigment from *Chryseobacterium artocarpi* CECT 8497 using liquid pineapple waste in 5-L bioreactor and its application as environmental-friendly ink. Liquid pineapple waste supported bacterial growth and pigment production for *C. artocarpi* CECT 8497. The ink was successfully formulated with polyvinyl butyral and polyvinyl pyrrolidone giving a smooth texture. The functional groups of formulated ink identified using FTIR were OH, C-C and C-H. Flexirubin ink was stable during the entire storage period of 30 days at temperatures, ranging from 25 to 70°C, pH 1.0 to 11.0 and in the presence and absence of light. This is the first report on ink formulation of flexirubin-type pigment from *C. artocarpi* CECT 8497 and its potential application on plastic materials.

1. Introduction

Synthetic colorants are mostly used in food, textile, paper production, leather tanning and cosmetic industries; however, they are being banned due to their carcinogenicity, hyperallergenicity and toxicological issues [Venil et al., 2013]. Various limitations during preparation of synthetic dyes, such as utilization of strong acids, solvents, alkalis, heavy metals and high temperatures produces environmental unfriendly wastes which are harmful [Savvidis et al., 2013]. Sudan I, a synthetic azo dye that is widely used for making plastic, printing inks and waxes was found to be carcinogenic to liver and urinary bladder of mammals [Xu et al., 2010].

Recently, natural pigments are highly in demand as they are non-toxic to humans and environment, biodegradable and low in allergic reaction. Pigments from vegetables have been investigated for temporary of semi-permanent hair dyes in yak hair. Antraquinone, (alizarin-red pigment) from madder plant, anthocyanin (red pigment) from mulberry fruits, azulenes (blue pigment) from flower heads of chamomile, curcumin (yellow pigment) from turmeric were used as natural pigments hair dyes [Boga et al., 2013]. Water based ink jet ink for digital printing using natural pigments from plants was a novel approach in textile printing. Annato (orange pigment), cutch (brown), pomegranate fruit rind (yellow) and golden dock were used as colorant for cotton materials [Savvidis et al., 2013].

In line with this, it is well known that pigments from bacteria offer properties which have promising avenues for many applications. Yellow pigments, zeaxanthin from *Flavobacterium* [Alcantara and Sanchez, 1999] used as additive in poultry feed and carotenoids from *Streptomyces* sp. [Galaup et al., 2005] used as food colorant and feed additives. Pink-red pigment, astaxanthin from *Agrobacterium aurantiacum* [Yokoyama et al., 1994], *Paracoccus carotinifaceins* [Tsubokura et al., 1999] and *Halobacterium salinarium* [Calo et al., 1995] used as natural nutritional component in food supplement. Red pigment prodigiosin from *Vibrio* spp. was used to dye various fibers including wool, nylon, acrylics and silk [Alihosseini et al., 2008].

Although various applications of bacterial pigments have been investigated, there were no reports on the use of these pigments as printing ink. To date, most of printing inks were prepared using synthetic dyes and due to health and safety concern, natural pigments were considered as an alternative to these inks. Liu et al. [2015] reported on the pigment based ink for electronic inks which comprised of non-polar carrier fluid and pigment particles (carbon black) coated with metal oxide. There were reports on environment-friendly ink from plants sources for food packaging, painting and ink sprayed printing. Longmei [2012] reported on the application of ink derived from plant sources for colored drawing of food packaging. Green ink was prepared with red orchid, madder, comfrey, blue horse, indigo, Siong blue, Garcinia and pine smoke ink. The ink has high colority, fastness, strong water resistance, stable color and no blocking in painting head.

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Incidentally, flexirubin produced by *Chryseobacterium artocarpi* CECT 8497 was reported to show several biological activities such as treatment for chronic skin disease, eczema and gastric ulcers [Venil et al., 2016; Kim, 2013]. In view of flexirubin's potential commercial values, there is a demand for high-throughput and cost effective bioprocess for pigment production. The application of bacterial pigments as printing ink and its storage stability have not been reported before; hence the objectives of the present study were to produce flexirubin-type pigment from *C. artocarpi* CECT 8497 using liquid pineapple waste and to apply the pigments as printing ink.

2. Material and methods

2.1 Bacterium

C. artocarpi CECT 8497 isolated from an orchard at Universiti Teknologi Malaysia, Skudai, Malaysia was used in this study [Venil et al., 2014]. The culture was grown and maintained by regular subculturing in nutrient broth, NB (Merck, Germany; 8 g l^{-1}).

2.2 Production and extraction of flexirubin type pigment

C. artocarpi CECT 8497 was cultured in 5-L bioreactor and flexirubin-type pigment was extracted according to methods reported by Aruldass et al. [2016]. Briefly, starter culture of *C. artocarpi* CECT 8497, 500 ml (10%, v/v) was prepared and transferred into a 5-L bioreactor (Biotron, Korea) containing liquid pineapple waste (20%, v/v), L-tryptophan (125 g/L), KH_2PO_4 (12.5 g/L) and cultivated at 30 °C, agitation and aeration rate of 200 rpm and 2 L/min, respectively with initial pH of 7.0 for 24 h. Antifoam A (Sigma, Germany) was added during fermentation to reduce the foam formation.

The 24 h culture was centrifuged at 10,000 rpm for 15 min at 4 °C (Allegra™ 25R Centrifuge-Beckman Coulter™, California) and the supernatant was discarded. Intracellular yellowish-orange pigment was extracted from pellet by adding 5 % (v/v) acetone and ultrasonicating for 3 min to bleach the cells. Pigment was separated from the cells by centrifugation at 10,000 rpm for 5 min at 4 °C (Allegra™ 25R Centrifuge-Beckman Coulter™, California).

2.3 Preparation of ink

Polyvinyl butyral and polyvinyl pyrrolidone were used as resin for flexirubin ink formulation. The solvent used in this study was prepared by combining ethyl acetate (EA) with methyl ethyl ketone (MEK) at a ratio of 2 (EA): 1 (MEK). Varnish was prepared by adding resin (26 % of total production weight) to solvent and mixed well using magnetic stirrer. The viscosity of varnish was adjusted by adjusting the solvent mixture in order to get a smooth texture. Flexirubin type pigment (in acetone) was added to the varnish and mixed well for 30 minutes upon obtaining homogenous mixture and was subjected to characterization and stability analysis. The viscosity of formulated ink was measured using viscometer (Cole-Palmer, Germany) and expressed as centipoise.

2.4 Stability of flexirubin ink

2.4.1 Temperature

The effect of temperature on stability of flexirubin ink was evaluated for 30 days. Ink was kept at different temperatures ranging from 25, 30, 40, 50, 60 and 70 °C and the color change of ink was measured using color meter (ColorFlex EZ colorimeter, Hunter Associates Laboratory Inc., Virginia, United States).

2.4.2 Illumination

The formulated ink was kept in the presence and absence of light. L*a*b* values was measured using color meter ColorFlex EZ colorimeter, Hunter Associates Laboratory Inc., Virginia, United States) and was recorded at 0 day and subsequently after 1 month.

2.4.3 pH

The effect of pH on color stability of the ink was tested by adjusting pH from1.0 to 11.0 using hydrochloric acid, HCl (1 M and 0.1 M) and sodium hydroxide (NaOH) (1 M and 0.1 M). The color change of each solution was recorded and measured using color meter (ColorFlex EZ colorimeter, Hunter Associates Laboratory Inc., Virginia, United States).

2.5 Properties of ink and application on plastic material

The finished flexirubin ink was tested for its stability towards physical contact. The ink after being printed on a substrate must remain attached or should not display any major damage towards physical contact. The physical contact test included scratching using finger nail and stacked with other objects. The flexirubin ink was applied to plastic material printed as fruits to test the color intensity and properties of the ink as a finish product.

2.6 Characterization and color analysis of ink

Flexirubin ink was characterized using ATR analysis to determine the presence of flexirubin functional groups in prepared ink. 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_{ab}) 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 color. 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.

3 Result and discussion

3.1 Production and extraction of pigment

C. artocarpi CECT 8497 showed good adaptability to grow and produce flexirubin in liquid pineapple waste under controlled conditions. Yellowish-orange suspension was observed in 5-L bioreactor. Thus, liquid pineapple waste served as cheap and economical medium for replacing nutrient broth for flexirubin type pigment production by *C. artocarpi* CECT 8497. Addition of supplementations, such as L-tryptophan and KH_2PO_4 facilitate the growth of *C. artocarpi* CECT 8497 and pigment production. Dark yellowish-orange pigment was obtained after concentrated using rotary evaporator.

3.2 Preparation and characterization of flexirubin ink

Environmental friendly flexirubin ink was formulated by using polyvinyl butyral with polyvinylpyrrolidone composite, ethyl acetate and methyl ethyl ketone. Flexirubin ink formulation consists of resin polymer (7-8 %), solvent mix of ethyl acetate and methyl ethyl ketone (70 %), additives (3 %) and flexirubin type pigment (19-20 %). Flexirubin ink showed a smooth texture with less bubbles when applied to plastic material to evaluate the texture of ink qualitatively (Figure 1). The viscosity of flexirubin ink was 198.3 centipoise, where it indicates that ink is thick, flow slow and has strong intermolecular force between its molecules. Flexirubin ink may have straight and branched chains which make the ink to be more viscous. The viscosity of ink is essential for their different uses namely, fountain pens ink have low viscosity as they flow easily and ball point pens or high speed printing ink have high viscosity as the flow should be slower.

Polyvinyl butyral was chosen as resin because of its availability and ability to polymerize and bind homogeneously during the preparation of ink. Besides that, polyvinyl butryl is classified as low cost and tough polymeric material which used in wide range of industrial applications, including manufacturing of glass, metal, plastics and wood (El-Sherbiny et al., 2001). 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. The color of flexirubin ink was evaluated using color meter and the L* (2.35), a* (0.61) and b* (3.64) values were all positive indicating yellowness of the flexirubin type pigment. A hue angle of 80.48 indicates yellow color of the ink.

Flexirubin ink **Application ink on plastic material**

Fig 1 Flexirubin ink and application on plastic material. Smooth texture with minimal bubble was observed

Similarly, a report for biodegradable printing ink was found to comprise of poly(vinyl alcohol) (PVOH) (5–35 wt %), organic/inorganic pigment (5–80 wt %) and sol-vent (15–87 wt%) [Niaounakis, 2015]. On the other hand, a synthetic environmental friendly ink was formulated based on Pr_2MoO_6 doped with titanium and applied as surface coating and coloring of plastics. The formulated paint comprised of pigment, Pr_2MoO_6 doped with titanium (27 wt. %), resin, poly(methyl methacrylate) (68 wt. %), plasticizer (4 wt. %) and rheological agent, acralyn cold curing liquid (0.7 wt. %). A mixture of alcohol and water was used as solvent mix for the formulation (George, 2015).

In ATR analysis, functional groups present originally in flexirubin were also found in the flexirubin ink mixture. A broad absorption band at ν_{max} 3400 cm^{-1} (OH), medium absorption bands ranged from 2900-2950 cm^{-1} (C-H stretch) and weak absorption at 1650 cm^{-1} (C-C stretch) were detected. The absorption bands were similar to the flexirubin pigment reported values by Aruldass et al. [2016]. This shows that the formulation of flexirubin ink does not affect the chemical structure of the compound.

3.4 Stability of ink

3.4.1 Effect of temperature

Flexirubin ink showed good stability all the tested temperatures. The L*, a* and b* values were all positive indicating yellowness of the flexirubin type pigment. The lightness, L* value ranged from 1.6 to 2.3 at day zero for all temperatures, indicating the darkness of the formulated ink (Table 1). The flexirubin ink exhibited a range of hue angle from 63 to 68 ° indicating yellow color. The increase value of chroma with the increasing of day storage showed that flexirubin ink was getting darker. Flexirubin ink was found to be stable for a month as the hue angle falls within the range of yellow color. There were no drastic changes in L* values which varied from 1.6 (zero day) to 2.6 (30 days) for flexirubin ink, which means that there were not much color changes observed throughout the storage period.

Table 1 Effect of temperature on flexirubin ink

Temperature (°)	Zero day					One month				
	L*	a*	b*	Chroma value	Hue angle (°)	L*	a*	b*	Chroma value	Hue angle (°)
25	1.86	1.16	2.79	3.02	67.42	2.01	0.23	2.54	2.55	84.83
30	2.12	1.65	3.25	3.65	63.08	2.45	0.53	3.47	3.51	81.32
40	1.69	1.13	2.69	2.92	67.21	1.98	0.20	2.43	2.43	85.29
50	2.21	1.52	3.06	3.42	63.58	2.52	0.36	2.93	2.95	82.99
60	2.28	1.77	3.55	3.97	63.50	2.54	0.47	3.49	3.52	82.33
70	2.27	1.81	3.66	4.03	63.31	2.48	0.28	3.59	3.60	85.54

Chroma value: $[(a^2) + (b^2)]^{1/2}$
Hue angle : $\tan^{-1}$ (b/a)

3.4.2 Effect of illumination

The ink demonstrated good stability when exposed to light and kept in the dark. The hue angle values were 59.79 ° (light) and 60.20 ° (dark) at zero day and the values increased to 81.31 ° (light) and 80.40 ° (dark) on 30th day of exposure (Figure 2). This indicates that ink protects the color intensity of flexirubin from excitation of electron chromophore group to unstable short-lived excited state under the exposure of light. Less reactivity of flexirubin molecule towards undesirable chemical reaction such as photo-oxidation occurred [Ahmad et al., 2012]. Thus, it may be concluded that the flexirubin ink was stable in the absence and presence of fluorescence light.

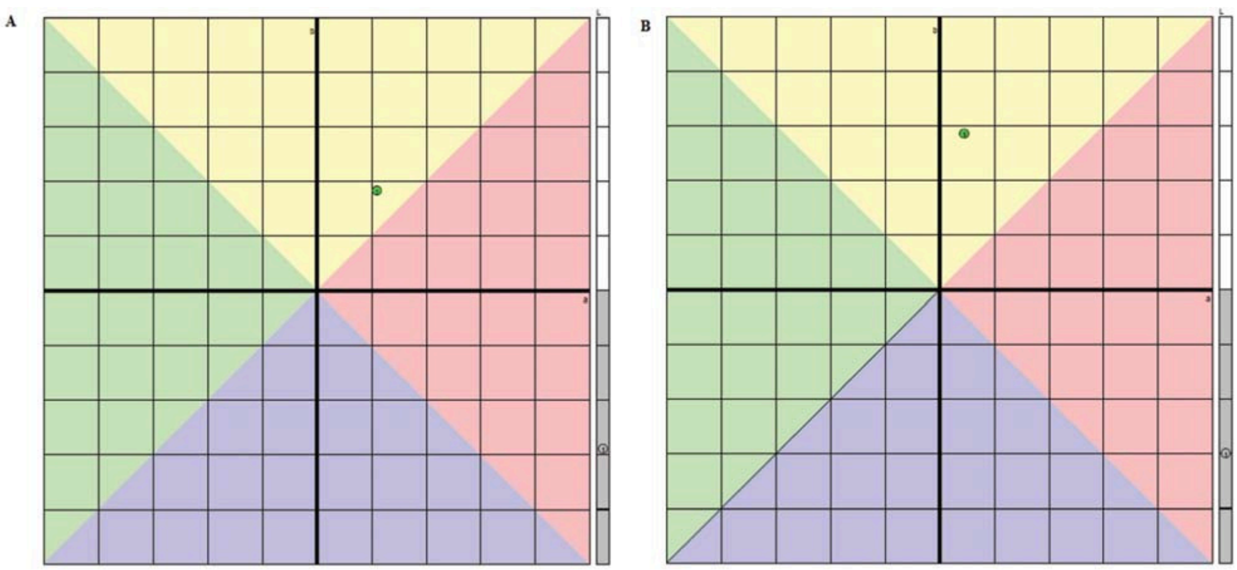

Fig 2 Effect of illumination on flexirubin ink (A) 2D plot on zero day stability (B) 2D plot on one month stability

3.4.3 Effect of pH

The hue angles of flexirubin ink were in the range of 70 to 80 ° and the color was stable from pH 1 to 11. There were no color changes observed in flexirubin ink when the pH was increased drastically to 11. This indicated that electronic pi system in the flexirubin ink structure was not destructed as the yellow color of the ink was retained. However, slight changes of the lightness, L* value of ink observed from 2.87 at pH 1 to 8.86 at pH 11 (Figure 3). This shows that darker ink was observed at extremely alkaline condition.

3.5 Properties of ink and its application on plastic material

The formulated flexirubin ink has thick consistency and showed good durability when being scratched and stacked with books or other objects. There was no peeling off or scratches observed on the dried ink, which indicates strong adhesion property of flexirubin ink on plastic material. The strong binding, flexibility and toughness properties of finished ink are due to the polymers which are present in the ink preparation. The polymer, polyvinyl butryl, consists of vinyl alcohol and vinyl butryl groups and they are hydrophilic and hydrophobic, respectively in nature. These groups act as adhesive and binders during the ink formulation [Kumar et al., 2016].

Flexirubin ink was applied to plastic substrate printed with shape of fruits. Since the ink has high viscosity, it able to be printed on the plastic material and showed good color intensity on the printed fruits and the color was dark. However, formation of bubbles was observed and the ink was unable to dry fast. Various methods can be carried out to reduce the bubble formation of ink, including addition of defoamers or antifoams. Natural pigments are less durable and much transparent at equal color intensity compared to synthetic pigment. However, flexirubin ink gives higher color intensity which indicates that this pigment is compatible to be used in formulations.

Conclusion

This study demonstrated the feasibility of liquid pineapple waste as growth medium and flexirubin production for *C. artocarpi* CECT 8497. It

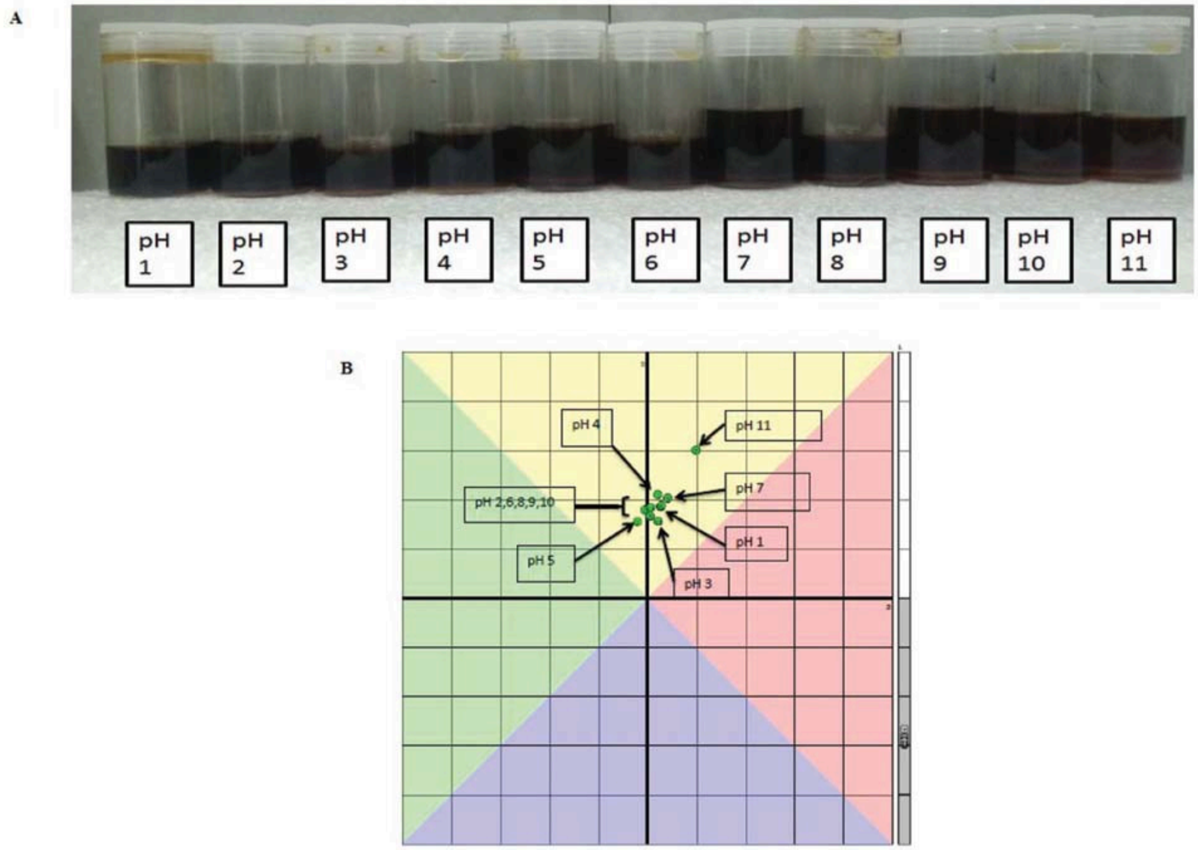

Fig 3 Effect of (A) pH on flexirubin ink (B) 2D plot on the pH stability

also demonstrated the potential application of flexirubin-type pigment as a printing ink colorant for plastic material, formulated using polyvinyl butyral with polyvinyl pyrrolidone, ethyl acetate, methyl ethyl ketone and additives. Results showed that flexirubin ink was stable at temperatures ranging from 25 to 70 °C and stable in the presence and absence of light for a month. The ink was extremely stable at pH ranging from 1.0 to 11.0. These results proved that flexirubin could be used as an effective natural colorant that would be beneficial in the ink printing industries.

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