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Solubility Study of Flexirubin Pigment Isolated from *Chryseobacterium artocarpi* CECT 8497 in Bio-Based Solvents

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Abstract

Flexirubin, a yellowish-orange pigment produced from *Chryseobacterium artocarpi* has gained increasing interest due to its natural colouring and pharmacological properties such as antioxidant, antibacterial and anticancer. However, the limitation of solubility of flexirubin in organic solvents becomes a major challenge for this pigment to be applied in industries diversely. Moreover, organic solvents might give negative impacts to environment, safety, and health. Therefore, application of green solvents to dissolve flexirubin pigment is needed to counteract these issues. In this study, bio-based solvents, which are D-limonene, ethyl acetate, ethanol, propanol, butanol were applied in the solubility study of flexirubin pigment and were compared with acetone as the positive control. These solvents were chosen due to their non-toxic and biodegradable properties. The flexirubin pigment was obtained from the cultivation of *Chryseobacterium artocarpi* CECT 8497 and extracted using acetone. The presence of flexirubin pigment was confirmed using Fourier transform infrared (FTIR) spectrophotometer and ultraviolet-visible (UV-Vis) spectrophotometer. Meanwhile, the appearance color of flexirubin was assessed using ColorFlex colorimeter. It was demonstrated that the polarity of solvents affects the efficiency to solubilize the flexirubin pigment. Therefore, it is vital to understand the solvent properties, especially the solvent polarity, in order to explain the solute-solvent interaction and solvatochromism phenomenon. The molar absorptivity coefficient obtained from UV-Vis absorption was calculated using Beer-Lambert law. The results demonstrate that the molar absorptivity coefficient of flexirubin increased from acetone, ethyl acetate, propanol, ethanol, D-limonene, and butanol with a range of 19 to 84 L mol^{-1} cm^{-1}. The hydrophobic structure of flexirubin pigment has caused it to deliver better solubility in non-polar solvents, D-limonene and butanol. Both solvents gave a slightly similar absorption with less polar acetone. In conclusion, this study confirms the feasibility of applying bio-based solvents as alternative solvents to expand the solubility potential of flexirubin pigment. This finding is expected to provide its usefulness as the potential bio-based solvent, especially in the pharmaceutical and food industries.

Keywords Flexirubin, bacterial pigment, solubility, bio-based solvents

1.0 INTRODUCTION

Bacteria is an example of a naturally occurring source of microbial pigments. Extensive studies conducted on bacterial pigments show that it has a lot of potential to be widely applied in various fields. The best comparisons made between bacterial pigment with other natural pigment sources are the low cost and large-scale pigment production, which increases its industrial applicability. Bacterial pigments come in various colors under the visible light range. Flexirubin pigment from *Chryseobacterium artocarpi* (*C. artocarpi*) is categorized under yellowish-orange bacterial pigment. Fewer studies were conducted on flexirubin

pigment compared to other yellow pigments, thus reducing its marketability rate. However, existing studies have demonstrated that flexirubin pigment from *C.artocarpi* can be used as a natural ink, antioxidant, and anticancer agents [1–3].

A previous study shows that the solubility of flexirubin pigment depends on the type of solvent used [4]. The efficiency of the solvents to dissolve solutes depends on their ability to form solute-solvent interactions without changing the pigment's structures [5, 6]. Flexirubin was observed to be only soluble in sodium hydroxide, sodium carbonate, and acetone, while slightly soluble in dimethyl sulfoxide (DMSO) and insoluble in water [7]. In addition, the solubility test done by Venil et al. (2014) [4] for flexirubin pigment from *C. artocarpi* demonstrated an efficient solubility of the pigment in acetone compared to other organic solvents such as ethanol, methanol, and ethyl acetate, benzene, chloroform, petroleum ether, and hexane. Different solubility of flexirubin pigment in water and solvents are attributed from its semi polar structure, which consists of polyenoic acid chromophore esterified with resorcinol carrying two hydrocarbon chains. However, acetone and DMSO can be threats to humans if exposed over a long period of time, primarily when used in high doses [8, 9].

Generally, acetone has been recognized as acute, chronic toxicity based on the toxicological profile report designed by the Agency for Toxic Substances and Disease Registry from the U.S Department of Health and Human Services. Furthermore, acetone can elevate pulse rate, vomiting, nausea, irritation in the eyes, nose, lung, and throat, and unconsciousness [10]. In the early research to study the effect of acetone towards humans, Satoh et al. (1996) [11] found that a long term exposure on shift workers that were exposed to acetone vapors and the symptoms recorded at the end of the duty period were tearing, eye irritation, and acetone odor. The most common symptoms observed in the workers after six months of acetone exposure were nausea, weight loss, faint feeling, and slow wound repairing [11]. With 4 hours of exposure to acetone (>12,000 ppm), it can cause dizziness, unsteadiness, unconsciousness, headache, and confusion. Meanwhile, acetone exposure in the range of 250-1000 ppm, 2500-8000 ppm, and 32,000-130,000 ppm would give irritation to the nose, eyes, and/or throat [12]. With all the cases reported related to the exposure of acetone vapor, it is highly recommended to replace the acetone with other safer solvents that can be used to dissolve flexirubin pigment from *C. artocapi*.

The combination of natural microbial pigment and the natural solvent is the most promising and practical approach and parallels with the worldwide trend in the seeking of eco-friendly and biodegradable properties in product manufacturing [1–3, 6, 7]. There has been a substantial increase in the current trend in employing the principle of green chemistry in the production of pigments for the applications as colorants and additives for food or drugs that are more environmentally friendly and biodegradable. For that matter, the solvents used in industries should be practically safe so that at the end of their usage, no hazardous waste will be produced. Green bio-based solvents such as bio-based alcohols and D-limonene are well-known green solvents that can be used to replace harmful traditional solvents and can be derived from renewable sources [13]. Therefore, ethanol, propanol, butanol, ethyl acetate, and D-limonene solvents were used in this study to investigate the effectiveness of solvents toward flexirubin pigment.

The D-limonene as solvent was included in this study because of its environmentally friendly properties with antifungal and antibacterial effects [14]. It is a terpenic (monocyclic terpene) compound that is made up of essential oil components of citrus (mandarins, oranges, grapefruits, limes) waste (CW) [15]. This solvent is widely applied in cosmetics, foods and beverages, and pharmaceuticals due to its biodegradable and antimicrobial properties that have attracted researchers' interest in applying D-limonene in their products [13,14]. It has been found that petroleum solvents, such as hexane or toluene can be substituted with D-limonene for natural products extraction [16]. With all the characteristics exhibited by D-limonene, it is suitable to be applied as an alternative solvent to extract the yellow flexirubin pigment from *C. artocarpi*.

This study aimed to find the safest and most effective bio-based solvent to enhance flexirubin pigment solubility and thus replace the acetone. The solvents ability to solubilize flexirubin pigment were analysed based on solvent properties, particularly the solvent polarity, solute-solvent interaction, and solvatochromism phenomenon.

2.0 METHODS

2.1 Cultivation and Extraction of Flexirubin Pigment

The cultivation and extraction of flexirubin pigment were prepared similar to the method by Venil et al. (2014) [7]. *Chryseobacterium artocarpi* CECT 8497 bacteria were cultivated in 500 mL of nutrient broth (NB) and incubated at 30^{o}C under 200 rpm shaking speed for 24 hours. The culture's optical density (OD) was measured at 600 nm using a UV-Vis spectrophotometer to determine the bacteria's metabolic activity of the growing cultures. A 500 mL of culture broth was further cultivated in a 4.5 L nutrient broth using a 7 L bioreactor for 24 hours. The resulting cultures were centrifuged at 10,000 rpm for 15 mins at 4^{o}C, forming layers of supernatant liquid and solid precipitates. The dark orange supernatant formed was removed via filtration while the yellowish recovered cell precipitate was extracted using 5% (v/v) of acetone. The mixture was treated by ultrasonication twice until the cell was completely bleached. The sonication process facilitated the cell rupture to release the flexirubin pigment located intracellularly inside the bacteria. The pigment was obtained after the cells were separated by centrifugation for 10,000 rpm for 5 min at 4^{o}C. The flexirubin pigment obtained was air dried and stored in a dark condition until further use.

2.2 Characterization of Flexirubin Pigment

The maximum absorption, λ_{max} of the flexirubin pigment was determined using the UV-Vis spectrophotometer with wavelength range from 300 to 800 nm. A 1 mg of flexirubin pigment was weighed and dissolved in acetone (5 mL) prior to the analysis.

Functional groups of flexirubin pigment were identified using the FTIR spectrophotometer. The scanning was done in the range of 4000 to 650 cm^{-1}, with a resolution of 2 cm^{-1}. To prepare the sample, a 1 mg of dried flexirubin pigment was finely ground with 200 mg KBr and then pressed under 0.414 bar to form a pallet.

The color of flexirubin pigment was analysed using a ColorFlex EZ colourimeter which is based on the CIELAB color system. The values of L*, a* and b* were used to calculate the chroma (C*) and hue angle. The L* indicates lightness from 0 (black) to 100 (white). Positive and negatives values of b* represent yellow and blue, respectively. Chroma denotes the colour saturation or purity. Values close to the centre at the same L* value indicate dull or grey colours, whereas values close to the circumference represent vivid or bright colours. Hue angle of 0 denotes red, whereas 90 is for yellow, 180 is for green, and 270 is for blue. The ColorFlex EZ colourimeter was calibrated prior to sample analysis by placing both black and white glasses with the shiny side against the reading port, consecutively. The sample was prepared according to Venil et al. (2014) [7]. A 1 mg of flexirubin pigment was weighed and dissolved in 10 mL of acetone. The sample was placed in the clean glass container and analyzed using ColorFlex EZ colorimeter. The L*, a* and b* values were recorded and the hue angle and chroma values were calculated using Equations 1 and 2.

$$\text{Chroma value} = \sqrt{[(a^2) + (b^2)]} \quad \textbf{Equation 1}$$

$$\text{Hue angle} = \tan^{-1}\left(\frac{b}{a}\right) \quad \textbf{Equation 2}$$

where
a = value which indicates green or red color
b = value which indicates yellow or blue color

2.2 Solubility Test of Flexirubin Pigment in Bio-Based Solvents

Dried flexirubin pigment weighed 0.05 g was added into 10 mL of different solvents; D-limonene, ethyl acetate, acetone, and ethanol. The solubilization was carried out using conventional stirring for one hour and left for 30 mins prior to UV-Vis characterization. The maximum absorption peak, λ_{max} was determined based on the scanning at wavelength from 300 to 800 nm using the UV-Vis spectrophotometer. Then, the solution was subjected to a serial dilution to obtain standard curve and molar absorption coefficient, ε of flexirubin pigment. The standard curve was plotted with absorbance on the y-axis and flexirubin concentration on the x-axis. The gradient was used to determine molar absorption coefficient of the flexirubin, based on the Beer Lambert's law (**Equation 3**). The units used were L mol^{-1} cm^{-1} and L mg^{-1} cm^{-1}.

$$A = \varepsilon . c . l \quad \textbf{Equation 3}$$

where
A = absorbance of the sample at maximum peak
ε = molar absorption coefficient of the flexirubin pigment
c = concentration of the flexirubin pigment
l = distance of light path

Furthermore, the effects of alkyl chain towards the maximum absorption of flexirubin pigment was studied using different types of alcohols based-solvents; ethanol, propanol, and butanol. The analysis was done as described above. The UV-Vis spectrum was used to study the solvatochromic effect that indicates the dissolution of flexirubin pigment in each solvent used.

3.0 RESULTS AND DISCUSSION

3.1 Production of Flexirubin Pigment

Flexirubin pigment was successfully extracted from cultivation of *Chryseobacterium artocarpi* CECT 8497. Acetone was used to extract the flexirubin pigment as it miscibles with the long unsaturated hydrocarbon chain of flexirubin pigment, separating the pigments from the cells. Ultrasonication was used to completely bleach the cells in acetone by rupturing the cell

wall of the bacteria for the extraction of flexirubin pigment. The sonication utilizes high vibrational energy (ultrasound) to destroy the cells, leaving no residual after the cells are ruptured.

3.2 Characterization of Flexirubin Pigment

3.2.1 Optical Properties

The flexirubin pigment exhibited a maximum absorption at 450 nm in acetone through observation by UV-Vis spectrophotometer (**Figure 1**), which indicates a yellowish-orange coloured pigment [17]. It is comparable to Aruldass et. al (2018) [18] review, where flexirubin-type pigments are yellow-orange in color. The presence of light-absorbing chromophore polyene (a long chain of conjugated double bonds), and phenolic hydroxyl groups (ω-phenyl octanoic acid) are the accountable constituents for the yellowish-orange hue produced by the flexirubin pigment. These chromophores are able to modify the position, intensity, and shape of absorption bands via the interactions among chromophores and solvents [19].

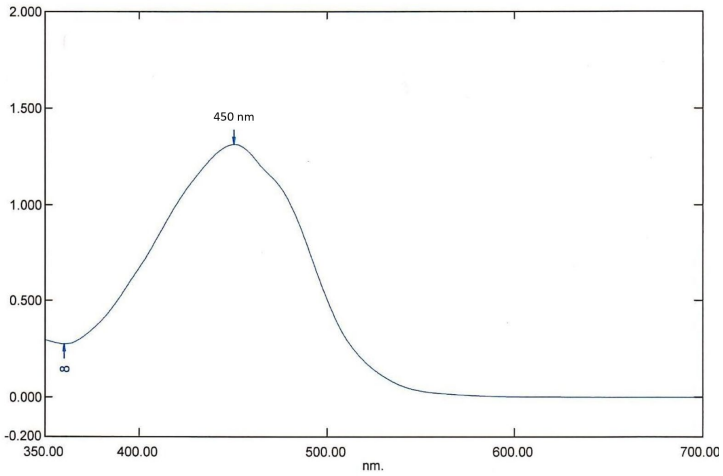

Figure 1 UV-Vis spectrum of flexirubin pigment.

The UV-Vis absorption depends on the microenvironment of the chromophore and solute-solvent interaction [18, 20, 21]. In polyene molecules, the electrons are delocalized throughout the system. Hence, the reaction mechanism of induced dipole moments in the adjacent molecules and dipole moment of the chromophore has been proposed to interact electrostatically as in **Figure 2**. As a result, the potential energy of the chromophore is diminished by the interaction energy of the dipole moments. As this reduction is more significant for the chromophore in its excited state than at the ground state, the transition energy for absorption and emission decreases, causing a bathochromic shift [23]. This shift is referred to as the red spectral range with a longer wavelength and lower energy [22, 23].

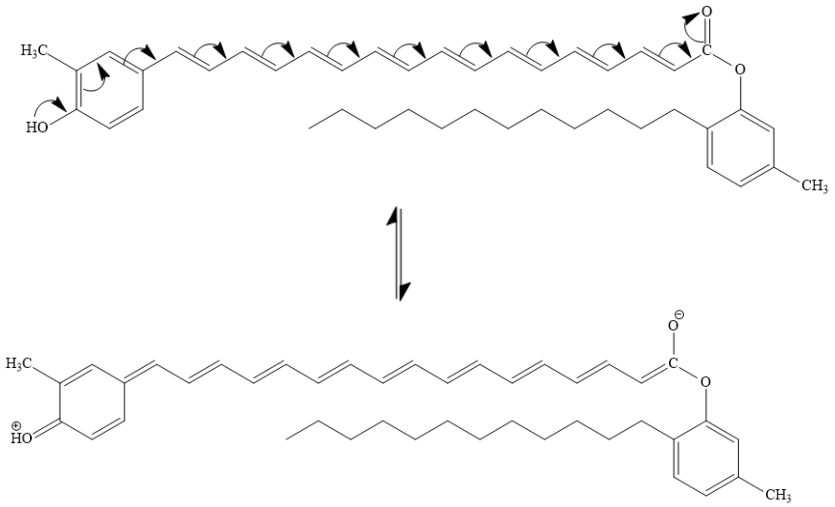

Figure 2 Proposed mechanism of electron delocalization at conjugated carbon-carbon double bond in flexirubin pigment.

3.2.2 Functional Groups

The FTIR analysis provides information about the structure of compounds by generating their functional groups. The FTIR spectrum for the flexirubin pigment is shown in **Figure 3**. From the spectrum, the peak that appeared at 3467 cm^{-1} was attributed to the hydroxyl group (OH) stretching. A solid and sharp rise observed at 1045 cm^{-1} demonstrated the presence of C–O stretching. Meanwhile, the peaks observed at the range of 2913–2995 cm^{-1} corresponded to the presence of C–H stretching. The peak attributed to C=C stretching was observed at 1656 cm^{-1} while the C– H bending appeared at 1421 cm^{-1}. The peaks formed are in agreement with the findings obtained by Venil et al. (2014) [7], which successfully confirmed the flexirubin-type of pigment.

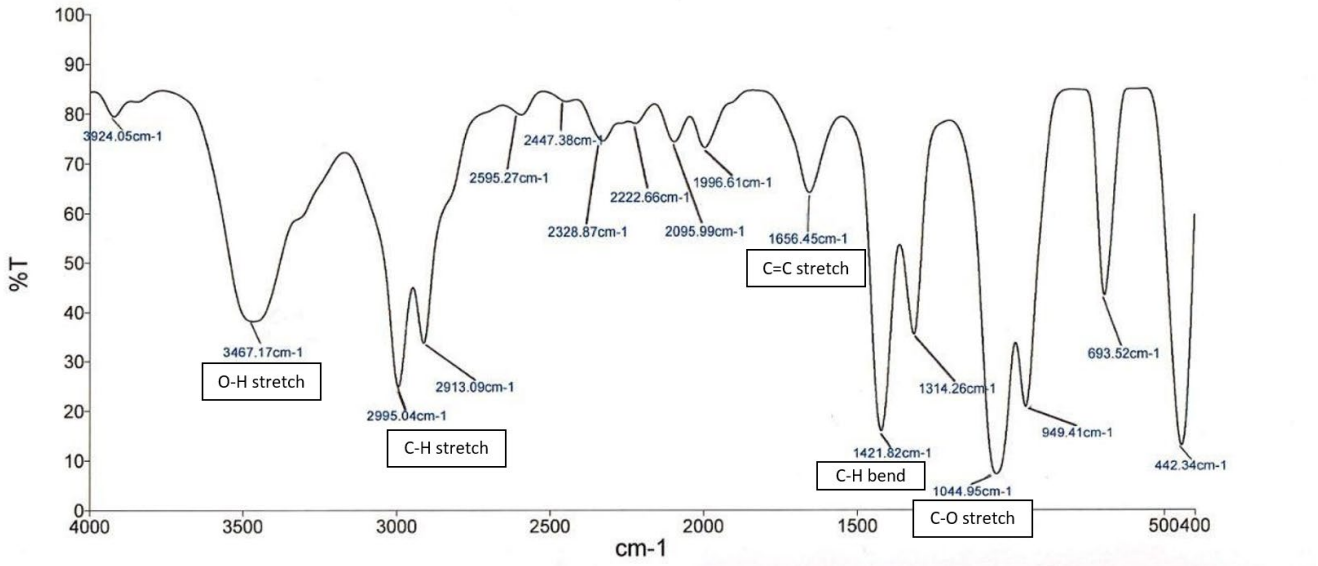

Figure 3 FTIR spectrum of flexirubin pigment.

3.2.3 Color Analysis using ColorFlex EZ Colourimeter

The colourimeter was used to further described the color strength and shades of flexirubin pigment. The colour analysis provides tristimulus values from the light absorbance measured specifically in the visible range. This allows a direct identification of flexirubin pigment color standards. The color measurement via CIELab was performed for flexirubin in acetone. The results were recorded and tabulated as shown in **Table 1**.

The L* value was 2.68, nearer to 0 (black) compared to 100 (white), indicating the darkness of pigment. Meanwhile, since a* = 0.17 and b* = 4.45 are positive, the flexirubin pigment appeared to be in the range of redness or less green color and yellowness or less blue color, respectively. The hue angle of flexirubin pigment is 87.81, which was calculated using the parameters obtained from Equation 2, indicating the color's location is between red and yellow. The bright yellowish-orange color of flexirubin pigment was proven with a chroma value of 4.45. In contrast, when the chroma value is detected close to the center of the color wheel, the pigment will be classified as a dull or pale yellow-colored pigment. This vivid yellowish-orange color may be due to the presence of concentrated flexirubin pigment from *Chryseobacterium artocarpi* CECT 8497. Overall, the parameters obtained are in a good agreement with the results obtained by previous studies.

Table 1: The L*, a*, b*, chroma, and hue values of flexirubin pigment in comparison with previous studies.

Tristimulus Values	CIELAB Colour Value		
	Experimental Value	Venil et al. (2014) [7]	Aruldass et al. (2016) [3]
L*	2.68	3.58	2.60
a*	0.17	-1.21	0.34
b*	4.45	5.41	3.45
Chroma	4.45	5.54	3.47
Hue	87.81	77.39	84.37

3.3 Solubility Study of Flexirubin Pigment in Bio-Based Solvents

3.3.1 Flexirubin Pigment in D-limonene, Ethyl Acetate, Acetone, and Ethanol

The UV-Vis spectra reveals the similar maximum absorption wavelength, λ_{max} for flexirubin pigments in different solvents, as shown in **Figure 4** and summarized in **Table 2**. All the maximum absorption wavelengths fall within the blue spectrum (449 - 451 nm), thus reflecting the complimentary yellowish-orange color of the pigment.

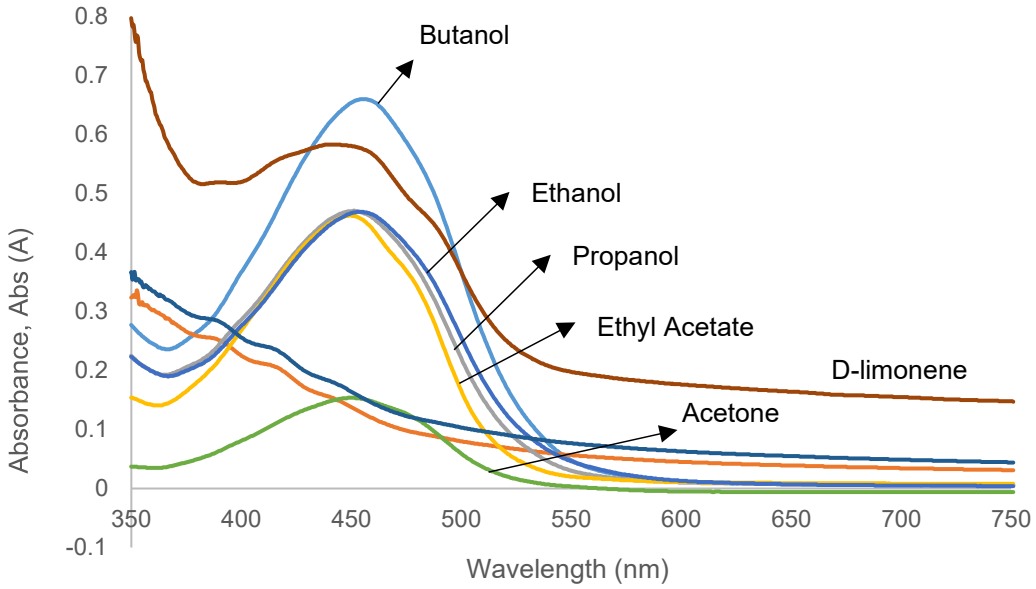

Figure 4: UV-Vis spectra of flexirubin pigment in different solvents; acetone, D-limonene, ethyl acetate, propanol, ethanol and butanol

The solvatochromism phenomenon took place when the flexirubin pigment as the solute is dissolved in different solvents. A positive solvatochromism shift occurred during the solubilization process of flexirubin pigment solute with the solvents, resulting in the bathochromic (red) shift. The red shift was due to the increasing polarity of solvents [25]. Ethanol demonstrated a longer absorption wavelength as it has higher polarity than acetone. The electronic transition changes simultaneously with the change in polarity of environment molecules. Electron transition in high solvent polarity typically shifts the $n \rightarrow \pi^*$ and $n \rightarrow \sigma^*$ bands to shorter wavelengths giving a hypsochromic effect due to the formation of hydrogen bonding. Meanwhile, $\pi \rightarrow \pi^*$ bands of polar compounds shift to longer wavelengths when the electron excites [21, 23]. In this transition, the excited state is more polar compared to the ground state. The flexirubin pigment that dissolved in acetone which is a polar aprotic solvent, absorbed UV-Vis light at 450.5 nm. Meanwhile, less polar or non-polar solvents such as D-limonene and ethyl acetate demonstrated a negative solvatochromism that resulted in a hypsochromic (blue) shift to a shorter wavelength [24, 25].

Table 2 UV-Vis absorption of flexirubin pigment in different bio-based solvents.

Solvent	Maximum Wavelength (nm)	Absorbance
D-limonene	449.50	0.553
Ethyl acetate	450.00	0.463
Acetone	450.50	0.154
Ethanol	451.00	0.471

3.3.2 Flexirubin Pigment in Alcohol-Based Solvents

In this section, flexirubin pigment in various alcohol-based solvents was analysed through the UV-Vis spectra. From **Table 3**, it can be seen that the increasing in number of carbons in each alcohol resulted in the absorption peak to slightly shifted to a longer wavelength. The increase in length of the alkyl chain will increase the hydrophobic and nonpolar region, thus reducing the solvent polarity. Different values of absorbance were based on the concentration and purity of flexirubin pigment. It was noted that based on Beer Lambert's law, the absorbance depends on the light-absorbing substance's concentration.

Table 3 UV-Vis absorption of flexirubin pigment in different alcohol-based solvents.

Solvent	Maximum Wavelength (nm)	Absorbance
Ethanol	451.00	0.471
Propanol	453.50	0.469
Butanol	455.50	0.660

However, the findings on the inclusion of bio-based alcohols to solubilize the production of flexirubin pigment from *Chryseobacterium artocarpi* CECT 8497 are preliminary, and further research is required. It is highly required to search for a low-cost, safe, and efficient strategy to increase the solubility of flexirubin pigment in order to improve the existing methods and provide an alternative solution that is harmless to humans.

3.3.3 Molar Absorption Coefficient of Flexirubin Pigment

The molar absorptivity property evaluates how strongly the pigment's chromophore absorbs light within the specified wavelength when mixed in a solvent with a known concentration [24]. Beer Lambert law was used to determine D-limonene, ethyl acetate, acetone, ethanol, propanol, and butanol molar absorptivity. Molar absorptivity in this study was used to explain the probability of the electronic transition that occurred inside the solvent and solute system.

Based on **Table 4**, the molar absorption coefficient of the flexirubin ranges from 19 to 84 L mol^{-1} cm^{-1} in the respective solvents. A high molar absorption coefficient was found in D-limonene and butanol. A high molar absorption coefficient is a potent indicator for the substantial tinctorial strength [24]. Hence, the flexirubin pigment color concentration was more intense in D-limonene and butanol compared to other solvents used. The finding demonstrates that flexirubin is stable in less polar solvents, which are D-limonene in general and butanol for the alcohol group, respectively. This may be due to the reason of low polarity possessed by D-limonene and butanol. Since the solvatochromism of both absorbance maxima correlated well with the relative polarities, the absorbance maxima of flexirubin in D-limonene and butanol can be correlated to the polarity of the solvent environment around each solute [25]. Flexirubin is a hydrophobic hydrocarbon, and hence it tends to dissolve well in less or non-polar solvents. The presence of ring structures and long saturated carbon chain in flexirubin favoured the long hydrocarbon chain of D-limonene and butanol, and hence, dissolved better in these two solvents.

Table 4 Molar absorption coefficient of flexirubin pigment in different bio-based solvents.

Solvent	Relative Polarity	Molar Absorption Coefficient	
		L mol^{-1} cm^{-1}	L mg^{-1} cm^{-1}
D-limonene	0.153	70.18	1.11×10^{-4}
Ethyl Acetate	0.228	58.76	9.26×10^{-5}
Acetone	0.355	19.54	3.08×10^{-5}
Ethanol	0.654	59.77	9.42×10^{-5}
Propanol	0.617	59.52	9.38×10^{-5}
Butanol	0.586	83.76	1.32×10^{-4}

Based on the findings, it can be concluded that it is possible to implement green chemistry principles in dissolving yellow flexirubin pigment from *Chryseobacterium artocarpi* CECT 8497 using a safe and biodegradable natural source of solvent. A bio-based solvent, D-limonene was chosen over butanol to avoid in the use of organic solvent in the application of natural colorants. Considering that, flexirubin pigment in D-limonene exhibited a significant absorbance intensity and gave nearly a similar wavelength of absorption of acetone. Hence, D-limonene is put forward to replace the long-used acetone in solubilizing flexirubin pigment. High molar absorptivity shown by this combination will improve the tinctorial quality and the performance of flexirubin pigment as colorant. The use of bio-based solvent will drastically reduce pollution and toxic industrial waste [26]. Since renewable sources have a better eco-toxicological profile, they can be used to substitute the harmful sources that will put human lives at risk, especially the workers involved in the product manufacturing processes.

4.0 CONCLUSIONS

In sum, this research discusses the possibility to use an alternative bio-based solvent to replace acetone for the solubility study of flexirubin pigment. Flexirubin is the predominant pigment extracted from *Chryseobacterium artocarpi* CECT 8497. The solvent selection was conducted because the use of safe solvents and harmless products are essential for industrial purposes. D-limonene and butanol were suggested to be the alternative choice of acetone since their absorption was comparable to that of

acetone. In fact, the flexirubin pigment from *Chryseobacterium artocarpi* CECT 8497 showed better solubility and stabilization in D-limonene and butanol compared to acetone and other bio-based solvents. They have a remarkable high absorbance intensity with a high molar absorption coefficient, which reflects a good tinctorial strength. The hydrophobic flexirubin favors the low polarity of D-limonene and butanol. The finding of solubility of flexirubin in bio-based solvents will contribute to various applications and encourage the development of a green solubilization process in designing safer products for human consumption.

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