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Published in:

The 7th International Symposium of Innovative Bio-Production Indonesia on Biotechnology and Bioengineering 2020

DOI:

[10.1088/1755-1315/762/1/012034](https://doi.org/10.1088/1755-1315/762/1/012034)

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Rahman, D. Y., Susilaningsih, D., & Van Der Maarel, M. J. E. C. (2021). Effect of light and preservatives on the stability of the phycocyanin from the extremophilic red microalgae *Cyanidioschyzon merolae*. In *The 7th International Symposium of Innovative Bio-Production Indonesia on Biotechnology and Bioengineering 2020* (IOP Conference Series: Earth and Environmental Science; Vol. 762, No. 1). IOP Publishing. <https://doi.org/10.1088/1755-1315/762/1/012034>

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To cite this article: D Y Rahman *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **762** 012034

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Effect of light and preservatives on the stability of the phycocyanin from the extremophilic red microalgae *Cyanidioschyzon merolae*

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Abstract. Synthetic dyes are replaced more and more in food products by natural pigments. A growing number of consumers are concerned with the potential health risk and behavior problems related to synthetic dyes. The phycocyanin of the cyanobacterium *Arthospira platensis* is the only natural blue pigment commercially available. The thermoacidophilic red microalgae *Cyanidioschyzon merolae* could provide an alternative phycocyanin source. As *C. merolae* grows at relatively high temperatures (45 to 56°C), the phycocyanin has a high thermostability even at relatively low pH. The stability of the *C. merolae* phycocyanin was determined for several products of relevant parameters. Average daylight (300-500 Lux) did not significantly affect the stability, while intense light (20,000 Lux) reduced the half-life to 35 hours. The preservatives such as glucose, sucrose, fructose, and sorbitol improved the stability of *C. merolae* phycocyanin considerably, with 20% glucose resulting in no loss of color at all. The results show that *C. merolae* phycocyanin can be used in various food products as a natural blue colorant.

1. Introduction

There is a growing interest in natural dyes for food, confectionery, and beverages as consumers become more skeptical about synthetic food colorants. Synthetic dyes are linked to adverse health effects and, in particular, correlated to behavior problems of children [1,2]. Plants and microalgae are promising candidates for finding new natural colorants as they contain a wide range of pigments to capture light. Indigo blue extracted from the plant *Indigofera tinctorial* is a classic example of a natural blue pigment used in dyeing textile [3]. However, the indigo blue insoluble in aqueous media, moderately soluble in triglycerides, make indigo blue challenging to apply in the food industry [4]. The carotenoid lycopene, extracted from tomato approved for use as a food colorant in both the United States and the European Union [5]. Very recently, a blue pigment extract of *Spirulina platensis* containing phycocyanin was approved by the FDA (\$ 73,530) for use in confectionery, ice creams, and several other food products. Microalgae is a potential source for blue pigment known as phycocyanin. Besides microalgae has higher growth rates and higher CO₂ fixation efficiency, the phycocyanin from microalgae can make as high as 25% of cell mass and can be used as a nutraceutical for both animal and human owing to its immunomodulatory and anti-cancer activity [6,7].

The extremophilic red microalgae *Cyanidioschyzon merolae*, a strict autotroph requiring light and carbon dioxide for growth, grows optimally at very low pH (0.5 to 3) and relatively high temperatures



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(up to 56°C) [8]. It produces relatively thermostable phycocyanin that can very easily extract from the biomass by osmotic shock with pure water [9]. Phycocyanin consists of a small apoprotein with a molecular weight of about 20 kDa [10] and a linear tetrapyrrole attached to apoprotein by thioester bonds. The absorption maximum of phycocyanin is 610–620 nm [11], giving it a bright blue color. Besides used as a food colorant, phycocyanin has nutritional and nutraceutical properties [12,13] and can even stimulate the immune system [12].

As *C. merolae* grows well at relatively high temperatures, the phycocyanin extracted from it is stable up to 82°C [9], in contrast to the phycocyanin *Spirulina* sp, which precipitates at a temperature above 65°C [7]. The stability of the phycocyanin solution depends on the aggregation state of the protein, further influenced by light, temperature, pH, and protein concentration [13]. Food, confectionery, and beverage products are composed of a wide range of components. It was shown that sugar and sugar alcohol sorbitol, often added as sweeteners/preservatives, stabilized *Spirulina* and *Nostoc* phycocyanin [14,15].

This study aimed to investigate which effect light and sugars have on the stability of phycocyanin extracted from *C. merolae* and get first indication of what type of food products this new phycocyanin might be used as a natural colorant.

2. Material and methods

2.1. Organism and growth conditions

Cyanidioschyzon merolae (NIES culture collection, Japan, strain 1332) was cultivated in 7.5 L photobioreactors with constants aeration and light (24 hours, approximately 100 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$) in Allen medium pH 2. Allen medium consist of 1.32 g L⁻¹ (NH₄)₂SO₄, 0.27 g L⁻¹ KH₂PO₄, 0.25 g L⁻¹ MgSO₄·7H₂O, 0.073 g L⁻¹ CaCl₂·2H₂O, 11 mg L⁻¹ FeCl₃, 2.8 mg L⁻¹ H₃BO₃, 1.8 mg L⁻¹ MnCl₂, 0.218 mg L⁻¹ ZnSO₄·7H₂O, 0.05 mg L⁻¹ CuSO₄, 0.023 mg L⁻¹ NH₄VO₃, and 0.024 mg L⁻¹ Na₂MoO₄·2H₂O. The medium pH was adjusted to 2.0 with 4 M H₂SO₄, and subsequently, it was autoclaved at 120°C for 20 min. The microalgae growth was analyzed by measuring optical density at 800 nm.

2.2. Extraction and quantification of phycocyanin

Cells containing phycocyanin were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The harvested cells were resuspended in ultrapure water, mixed well for 5 min and kept for at least 3 hours at room temperature. Cell debris was removed by centrifugation at 15,000 x g at 4°C for 15 min, and the blue supernatant containing the phycocyanin was transferred to a new tube. The crude extract was then subjected to ammonium sulfate precipitation (20-40%), followed by centrifugation [9]. The precipitate was dissolved in 50 mM citrate buffer (pH 5) at room temperature. The precipitate phycocyanin was dialyzed against 10 mM citrate buffer pH 5 to remove the ammonium sulfate. The concentration of phycocyanin was calculated using the following equation [16]:

$$\text{Phycocyanin (mg mL}^{-1}\text{)} = \frac{A_{620} - (0.474 \times A_{652})}{5.34} \quad (1)$$

Where A₆₂₀ is the absorption value at 620 nm, and A₆₅₂ is the absorption at 652 nm

2.3. Effect of light and preservatives on the phycocyanin stability

The light stability of phycocyanin was studied using a box cabinet equipped with LED light. The light intensity applied was normal light (as it was in the laboratory, equivalent to approx. 300-500 Lux), 10,000 Lux, and 20,000 Lux. The temperature inside the box was kept constant at 22°C. The effect of preservatives such as sugars and sugar alcohol on the phycocyanin stability was analyzed by adding a specific amount of preservatives to a 0.1 mg mL⁻¹ phycocyanin solution in 50 mM citrate buffer (pH 5). The following preservatives were used: glucose, sucrose, fructose, and sorbitol at 20% concentration. This mixture was incubated at room temperature (22 ± 2°C) and normal daylight conditions. The stability of phycocyanin at 20% and 50% glucose at higher temperatures (50 to 80°C)

was followed in time by incubating 0.1 mg mL⁻¹ phycocyanin in 50 mM citrate buffer pH 5 supplemented with 20% and 50% (w/v) glucose.

The change in the phycocyanin concentration in solution was monitored spectrophotometrically at regular intervals. Equation 1 was used to calculate the concentration. The remaining concentration of phycocyanin in solution was calculated using equation 2 [17]:

$$\%C_R = \frac{C_t}{C_0} \times 100 \quad (2)$$

Where C_t is phycocyanin concentration in time, and C_0 is the initial concentration of phycocyanin.

2.4. Degradation rate constants and half-life value

The degradation rate constant (k) of phycocyanin was calculated by regression of the experiments, data for time, phycocyanin concentration in time, and initial phycocyanin concentration. The equation used to calculate the rate constant was [18]

$$k = \frac{-\ln\left(\frac{C_t}{C_0}\right)}{t} \quad (3)$$

Where C_t is the concentration in time t , and C_0 is the initial concentration.

The half-life value ($t_{1/2}$), is the time it takes for the initial concentration of phycocyanin to reduce by 50%, was calculated using equation 4:

$$t_{1/2} = \frac{\ln 2}{k} \quad (4)$$

3. Result and discussion

3.1. Absorption spectra

Phycocyanin was extracted using ultrapure water from *C. merolae* cells grown in batch with light and air for 21 days. The extract was further purified by ammonium sulfate precipitation. The absorption spectra of the purified phycocyanin in ultrapure water, buffer pH 5, and buffer pH 7 in the range of 300 to 800 nm are shown in Fig. 1. A typical absorption maximum at 620 nm, characteristic for phycocyanin [19], was found for pH 5 and pH 7. The absorption maximum of phycocyanin of *S. platensis* varies slightly with pH; at pH 5 a maximum was found at 616 nm, while at pH 7 it was 620 nm [13]. The *C. merolae* phycocyanin extract showed the highest absorption at 620 nm at pH 5, which differs from *S. platensis*, having a maximum absorption at pH 7 [13,16]. At pH 5, phycocyanin predominantly exists as a hexamer, and it is believed that the hexameric form gives some protection facing denaturation [20]. The *C. merolae* phycocyanin extract obtained using ultrapure water, and ammonium sulfate precipitation is high quality, as reported before [9]. The purity index expressed that the absorption ratio at 624 nm and 280 nm (protein) is 9.9, considerably higher than that of *S. platensis* phycocyanin extract [13]. As Jespersen *et al.* concluded, phycocyanin extracts are not a well-defined product with varying compositions depending on the biological origin and production method [13].

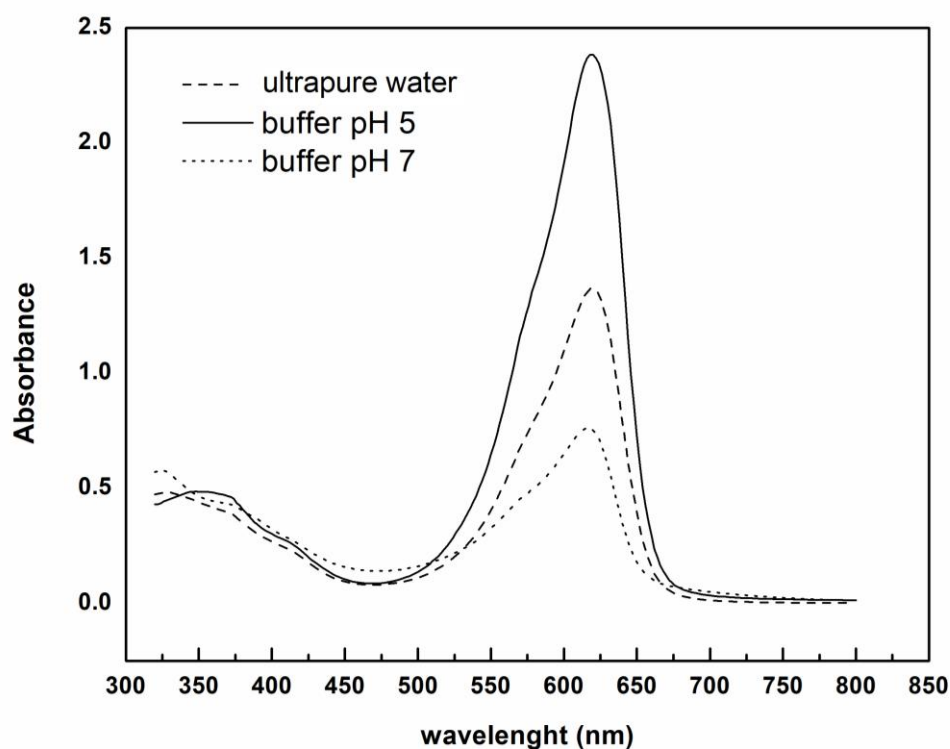


Figure 1. The spectrum of *C. merolae* phycocyanin in different solvents.

3.2. Light-accelerated degradation of phycocyanin

To determine the light sensitivity of *C. merolae* phycocyanin, the solution was exposed for up to 35 days with varying light intensities, ranging from daylight intensity (100–1000 Lux) to 20,000 Lux. As phycocyanin has a protein part, it makes phycocyanin sensitive to light. The light can lead to the photooxidation process, which has been recognized as a major lead to protein degradation. Phycocyanin has cysteine-cysteine binding, which cysteine is one protein that undergoes primary photooxidation [21]. The concentration of phycocyanin in the solution was monitored spectrophotometrically. Under normal daylight conditions, the phycocyanin was very stable at pH 5, with a half-life of 486.9 days (Table 1). At 10 or 20 times of the maximum daylight intensity, corresponding to 10,000 and 20,000 Lux, respectively, it was shown that the phycocyanin was degraded slightly (Table 1).

Table 1. Degradation rate constant (k) and half-life values ($t_{1/2}$) of *C. merolae* phycocyanin in ultrapure water, 50 mM buffer pH 5, and pH7 exposed to daylight, 10,000 Lux and 20,000 Lux.

Solution	daylight			10,000 Lux			20,000 Lux		
	k (day ⁻¹)	r^2	$t_{1/2}$ (day)	k (day ⁻¹)	r^2	$t_{1/2}$ (day)	k (hour ⁻¹)	r^2	$t_{1/2}$ (hour)
ultrapure water	5.3×10^{-3}	0.9897	130.2	7.6×10^{-2}	0.8605	9.1	3.1×10^{-2}	0.8561	22.4
50 mM buffer pH 5	1.4×10^{-3}	0.9632	486.9	2.2×10^{-2}	0.9000	31.1	1.9×10^{-2}	0.9709	35.1
50 mM buffer pH 7	1.7×10^{-2}	0.7048	39.9	6.6×10^{-2}	0.9030	10.5	6.2×10^{-2}	0.7438	11.1

Half-life values varying from 10 days (pH 7 and 10,000 Lux) to 35.1 days (pH 5 and 10,000 Lux) were found. However, the light accelerated degradation of phycocyanin has not been studied in much detail. Kohata *et al.* [22] investigated the light stability of phycocyanin extracted from the edible seaweed Nori. A rate constant of 4.5 h^{-1} for a first-order equation and 4.9 h^{-1} for a second-order equation was found when the Nori extract was exposed to 7800 Lux at pH 7. These values correspond to a half-life of 9.2 and 8.5 min, respectively. An estimation of the half-life of *S. platensis* phycocyanin was made from the light-accelerated degradation curves reported by Jespersen *et al.* [13]. The values deduced from these data were approximately 7.6 hours at pH 5 and 10.8 hours at pH 7. Very recently, Wu *et al.* [23] studied the light stability of phycocyanin extracted from *S. platensis*. The decrease in C_r values of extract exposed to 5400 Lux at pH 5 and pH 7 a half-life value of approximately 100 hours was calculated. The photodegradation can lead to changes in the primary, secondary, and even the tertiary structure of a protein, affecting long-term stability and bioactivity [21]. The high light stability of *C. merolae* phycocyanin makes it an interesting substitution to the synthetic blue colorant in those food products that are visible to the customer, such as candy, ice cream, and beverages.

3.3. Effect of sugar and sugar alcohol

Commonly used food sweeteners/preservatives such as glucose, sucrose, or sorbitol are known to improve the stability of phycocyanin from *S. platensis* [2,14,18] or *Nostoc* [15]. The sugars glucose, sucrose, fructose, and the sugar alcohol sorbitol at a 20% concentration improve the stability of *C. merolae* phycocyanin (Fig. 2).

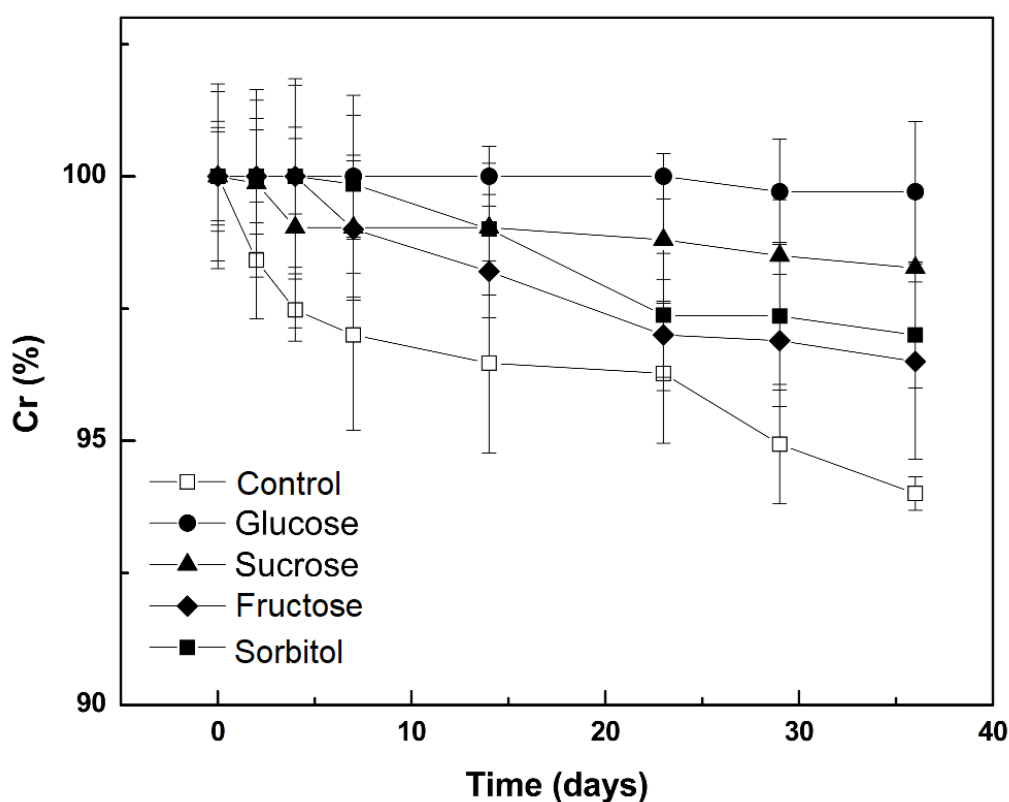


Figure 2. Effect of sugar at $22 \pm 2^\circ\text{C}$ on *C. merolae* phycocyanin

The C_r value of *C. merolae* phycocyanin in the presence of these sugars after 35 days at room temperature and pH 5 decreased only up to 3.5%. The half-life increased with a factor of 2 to 20 to over 8000 days in the case of glucose. In this study, the presence of glucose affected the stability of the phycocyanin more than other glucose. It could happen as the behavior of glucose is slightly different from other sugar or sugar alcohol assumed as it has the simplest structure and has a specific hydration effect [24]. It is considering the study of Back *et al.* [24] that glucose can increase the thermal stability of ovalbumin by about 7°C at 28% of glucose and 15.5% of glucose. The half-life of *Nostoc* phycocyanin in the presence of 0.08% sucrose at 25°C is considerably lower (70 days) than that of *C. merolae* phycocyanin exposed to a similar condition [15].

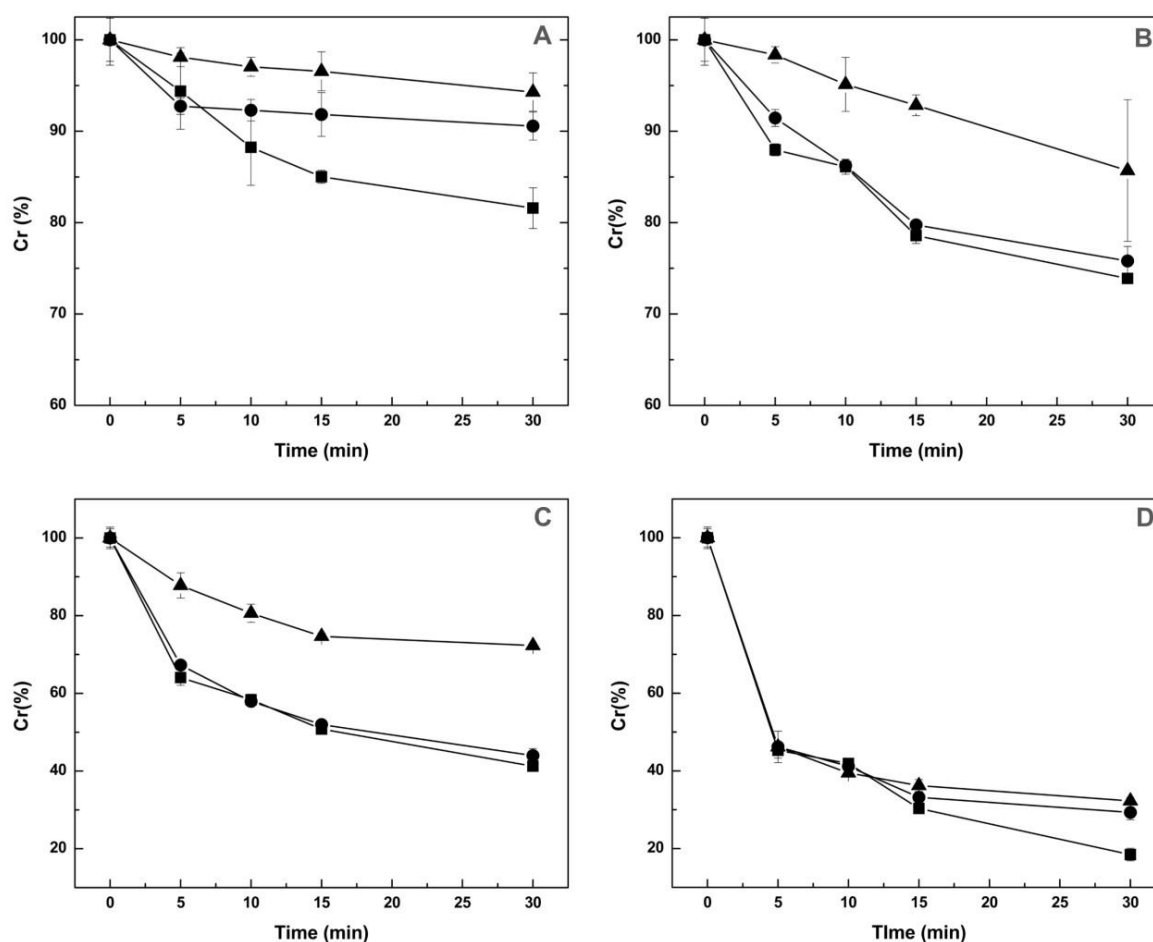


Figure 3. Effect of glucose at different temperatures on phycocyanin concentration: A. 50°C, B. 60°C, C. 70°C, and D. 80°C. Without glucose (close square), 20% glucose (close circle), and 50% (close triangle).

When exposed to a higher temperature of 50°C, 20% glucose still stabilized the phycocyanin (Fig. 3A). However, at a temperature of 60°C or 70°C, 20% glucose did not improve the stability, while 50% glucose still showed some positive effects (Fig. 3B and 3C). This result similar to the previous experiment of Martelli *et al.* [2]; the high concentration of sugar significantly increases the thermal stability of phycocyanin. Because phycocyanin is protein, which is extremely sensitive toward the light, pH, temperature, and oxygen, leading up to 90% loss in blue color [14]. The graphic clearly shows that the addition of a high concentration of glucose stabilizes phycocyanin to a small extent.

The C_r value at 60°C after 30 min range from 76% to 86% for 20 and 50% glucose, respectively. At 80°C, no stabilizing effect was found for 50% glucose (Fig. 3D). The half-life at elevated temperatures ranged from 17 min (20% glucose 80°C) to 354 min (50% glucose and 50°C). The control showed a half-life of 12 to 102 min for 80°C and 50°C (Table 2). Mishra *et al.* [14] found that a low concentration of sucrose (0.4%) did not significantly affect the stability of *S. platensis* phycocyanin kept at 5 or 35°C. *S. platensis* phycocyanin showed a 24% reduction in C_r value at 60°C when 20% glucose, sucrose, or sorbitol were added [25]. At 37 to 54% concentrations of glucose or sucrose and pH 7, the *S. platensis* phycocyanin had a half-life of 33 to 45 min [2], which is slightly higher than the half-life found for the *C. merolae* phycocyanin, incubated at pH 5 (Table 2).

Table 2. Degradation rate constant (k) and half-life values ($t_{1/2}$) of *C. merolae* phycocyanin in 50 mM citrate buffer (pH 5) with 20% or 50% glucose at different temperatures.

Temp. (°C)	control			20% of glucose			50% of glucose		
	k (day ⁻¹)	r ²	t _{1/2} (min)	k (day ⁻¹)	r ²	t _{1/2} (min)	k (hour ⁻¹)	r ²	t _{1/2} (min)
50	6.7 x 10 ⁻³	0.86	102.2	3.3 x 10 ⁻³	0.56	209.7	1.9 x 10 ⁻³	0.95	354.3
60	1.0 x 10 ⁻²	0.84	68.7	9.2 x 10 ⁻³	0.86	75.0	5.1 x 10 ⁻³	0.99	134.7
70	2.9 x 10 ⁻²	0.71	23.5	2.7 x 10 ⁻²	0.70	25.3	1.1 x 10 ⁻²	0.76	64.1
80	5.6 x 10 ⁻²	0.67	12.3	4.1 x 10 ⁻²	0.55	16.9	3.7 x 10 ⁻²	0.50	18.4

Sorbitol has a very similar effect on the stability of *S. platensis* phycocyanin as glucose or sucrose have the half-life at pH 5, and 62°C ranges from 58 min (20% sorbitol) to 193 min (50% sorbitol) [18]. So as with the *Nostoc* and *S. platensis* phycocyanin, the *C. merolae* phycocyanin is stabilized by sugar and sugar alcohol. Sorbitol has a half-life in a few minutes at higher temperatures to a few hours at elevated temperatures. As concluded by Martelli *et al.* [2], it is not the type of sugar but the concentration of sugar that is the critical factor in determining the stability of the phycocyanin at elevated temperatures. Sugars coat the protein part of the phycocyanin and thereby maintain the protein structure [25]. It has been suggested by Arakawa and Timasheff [26] that the stabilization of proteins by sugars is the result of the cohesive force of these sugars increases the surface tension of water. This, in turn, governs the preferential interaction of the proteins with solvent components in aqueous systems.

4. Conclusion

The phycocyanin of *C. merolae* is very stable at average daylight and slightly acidic condition, while intense light of 20,000 Lux at pH 5 reduced the half-life to 25 hours. The preservatives/sweeteners glucose, sucrose, fructose, and sorbitol improved the stability of phycocyanin from *C. merolae*; 20% glucose even stabilized the phycocyanin considerably, showing a half-life of 8,000 days. The *C. merolae* phycocyanin can be a good alternative to the *S. platensis* phycocyanin in the confectionery and beverages industry.

Acknowledgment

The works described in this paper was supported by the Ubbo Emmius Scholarship Program of the University of Groningen. We are grateful to Allen van Wijk for the technical assistance with the experiment. We are also grateful to the member of the Bioprocess Engineering of RC Biotechnology for the instrumentation support.

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