



OBTAINING INFORMATION FROM A BIOSYNTHETIC PATHWAY USING MULTI-WAVELENGTH FLUORESCENCE SPECTROSCOPY AND CHEMOMETRIC TOOLS

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Abstract

In this work, the biosynthesis of an important compound produced by *C. violaceum* that presents broad spectrum of biological activities was monitored using three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy. In order to resolve the overlapping signals, the Multivariate Curve Resolution–Alternating Least Squares (MCR-ALS) and Parallel Factor Analysis (PARAFAC) methods were applied. With the new methodology, it was possible to identify and observe the dynamic behaviour of fluorophores involved in this biosynthetic pathway.

Keywords - EEM, Violacein, PARAFAC, MCR-ALS.

INTRODUCTION

Chromobacterium violaceum is a saprophytic Gram-negative β -proteobacterium first described at the end of the 19th century, which dominates a variety of ecosystems in tropical and subtropical regions of the world. It presents as a distinctive phenotypic characteristic, the production of a blue-violet pigment named violacein (Fig. 1) [1]. This pigment exhibit a broad spectrum of biological activities, including antibacterial and antiviral activities, as well as a cytotoxic effect against several tumor cell lines [2].

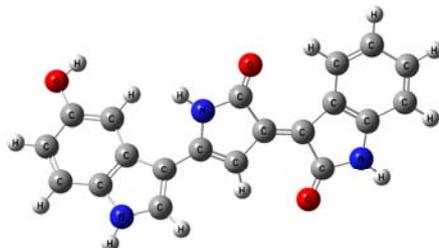


Fig. 1. Molecular structure of violacein.

Previous studies of its biosynthesis have shown that: i) the carbon skeleton of violacein is biosynthesized from two molecules of L-tryptophan [3,4]; ii) all nitrogen and hydrogen atoms are also provided exclusively by L-tryptophan [5]; iii) the oxygen atoms are obtained from molecular oxygen [5]; iv) five genes are involved in the biosynthesis, namely, *vioABCDE* [6-8].

Since all microorganisms contain natural intracellular fluorophores, such as amino acids, cofactors, enzymes and primary and secondary metabolites, the three-dimensional excitation-emission matrix fluorescence spectroscopy can be used to monitor these fluorophores showing a high specificity and sensitivity. In the case of biosynthesis of violacein, both the precursor (L-tryptophan) and the product (violacein) are fluorescent. It is expected that a more detailed understanding of this mechanism can be obtained when this technique is used to detect compounds that are consumed and produced within the bacteria.



The three-dimensional excitation-emission matrix fluorescence spectroscopy involves successive acquisition of emission spectra at multiple excitation wavelengths, thus creating an excitation-emission matrix (EEM) that provides a detailed map of the fluorescence properties of the sample over the range of scanned excitation and emission wavelength. However, fluorescence signals can be rather complex and, therefore, the analysis might become complicated owing to overlapping signals, instrumental background and scatter [9]. To solve these problems, the use of chemometric tools becomes necessary. Multivariate Curve Resolution – Alternating Least Squares (MCR-ALS) [10] and Parallel Factor Analysis (PARAFAC) [11] are powerful techniques for analyzing the data contained within EEMs, separating the fluorescence signal of the underlying fluorophores mathematically, in a similar way to chromatography.

In the present study, a simple procedure of monitoring a biosynthetic pathway using the EEMs measured off-line during *C. violaceum* cultivations in a bioreactor is proposed. MCR-ALS was used to decompose the pure components spectra allowing the identification and estimation of the concentration of fluorophores involved in this biosynthesis. The results are also compared with those previously obtained by PARAFAC.

EXPERIMENTAL

Organisms, culture media e cultivation conditions

Starting from a strain of *C. violaceum* CCT 3496, a colony was isolated, after culture by the streak plate method. This pure colony was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of sterilized culture medium (0.50% D-glucose, 0.50% bacteriologic peptone, 0.25% yeast extract and 0.03% L-tryptophan) and grown for 24 hours at 33 °C on an orbital shaker at 200 rpm. Afterwards, 10 mL of this, bacterial culture was transferred to a 1500 mL BioFlo bioreactor (New Brunswick Scientific) containing 1000 mL of sterilized culture medium and the parameters: temperature, agitation rate and air flow were adjusted (33 °C, 200 rpm and 1.0 L min⁻¹, respectively) and kept constants during all cultivation (approximately 36 h).

Sample collection

Every two hours, aliquots of 10 mL were withdrawn from the bioreactor with the aid of a glass syringe, injected into Falcon tubes of 15 mL and frozen. At a later stage, each aliquot was thawed in a thermostatic bath at 30 °C for 5 min and then centrifuged at 7000 rpm for 10 min. The supernatant was eliminated and 5 mL absolute ethanol was added to extract intracellular compounds. Then, it was centrifuged at 7000 rpm for 10 min for cell removal and the new supernatant was collected to subsequent analysis in the fluorimeter.

Multi-wavelength fluorescence spectroscopy

Fluorescence measurements were performed using a Varian Carry Eclipse fluorescence spectrophotometer. The EEMs spectra were collected with scanning emission spectra from 270 to 800 nm at 2 nm increments by varying the excitation wavelength from 250 to 620 nm at 5 nm increments. Excitation and emission slits widths were both maintained at 5 nm, and scanning speed was set at 9600 nm min⁻¹ for all measurements. A PMT detector voltage of 600 V was used.

RESULTS AND DISCUSSION

EEMs in the form of landscapes of samples collected during all cultivation are shown in Fig. 2. From the contour plot, it is possible to observe the dynamic behavior of fluorophores involved in the biosynthetic pathway. Additionally, the compound with maximum excitation between 260-290 nm and maximum emission from 330 to 360 nm, probably the L-tryptophan, is being consumed during the fermentation. This fact corroborates the idea that the L-tryptophan is consumed to form violacein.

In the application of MCR-ALS, the EEMs spectra were arranged in an augmented data matrix built with the 18 samples (each matrix consists of 266 emission spectra and 75 excitation spectra). Three



components were the number of chemical species chosen for the analysis of this data matrix. This value was estimated from previous results obtained by PARAFAC as well as initial estimates of excitation and emission spectra. MCR-ALS method was using non-negativity constrains in both spectral modes and a trilinearity constraint (equal shape and synchronization)

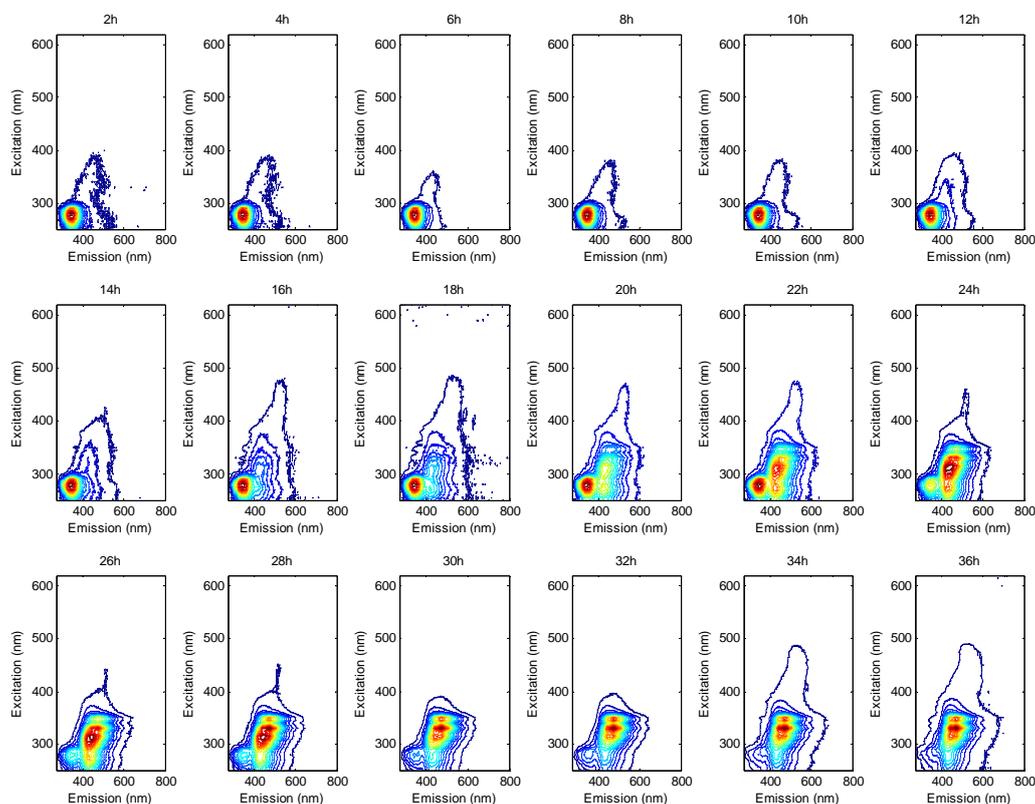


Fig. 2. Contour plot of the Excitation-Emission Matrices.

Fluorescence spectra obtained by MCR-ALS are shown in Figure 3. The observed emission at 350 nm when excited at 280 nm actually confirms the existence of tryptophan in the sample. The green curve (maximum emission at 430 nm when excited at 310 nm), refers to a product from tryptophan. The broad peaks centered at 340 nm in excitation and at 465 nm in emission were similar to pure spectra of NADH. Oxygen-incorporation from the molecular oxygen into violacein skeleton demonstrates the involvement of oxygenases in the biosynthesis that justifies the need of the cofactor NADH for production of violacein.

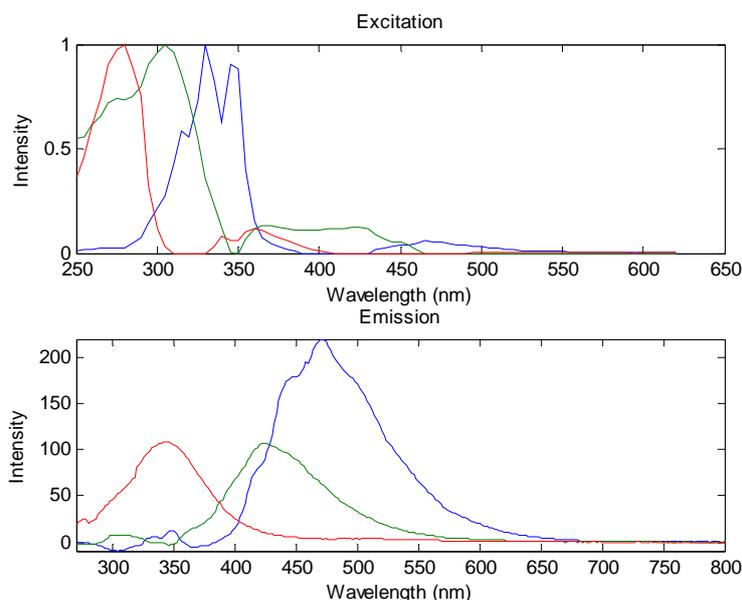


Fig. 3. Excitation and Emission spectra obtained by MCR-ALS decomposition.

CONCLUSION

The proposed methodology has enabled new and valuable insights into the biosynthetic pathway of violacein. More details about the conclusions derived from the application of PARAFAC and MCR-ALS methods in the investigation of the proposed system will be given in the presentation.

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