

Spectrophotometric techniques for the characterization of strains involved in the blue pigmentation of food: Preliminary results

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Abstract

Near infrared spectroscopy (NIRs) and ultraviolet visible spectroscopy (UV-vis) have been investigated as rapid techniques to characterize foodborne bacteria through the analysis of the spectra of whole cells or microbial suspensions. The use of spectra collected from broth cultures could be used as a fingerprint for strain classification using a combined polyphasic approach. The aim of this study was to evaluate the feasibility of NIRs and UV-vis for the characterization of blue strains belonging to the *Pseudomonas fluorescens* group. The bacteria were isolated from different food matrices, including some spoiled samples (blue discoloration). Eighty-one strains previously identified at the species level were grown in Minimal Bacterial Medium broth under standard conditions at 22°C. Two biological replicates were centrifuged in order to separate the bacterial cells from the extracellular products. Six aliquots per strain were analyzed on a small ring cup in transmittance mode (680-2500 nm, gap 2 nm). A subset of 39 strains was evaluated by UV-vis to determine changes in the spectral characteristics at 48 and 72 hours. Several chemometric approaches were tested to assess the performance of NIRs and UV-vis. According to the variable importance in projection (VIP), the 1892-2020 nm spectral region showed the highest level of discrimination between blue strains and others. Additional information was provided in the 680-886 and 1454-1768 nm regions (aromatic C-H bonds) and in the 2036-2134 nm region (fatty acids). Changes in UV-vis spectral data (at 48 and 72 hours) appear to indicate the presence of phenazine and catecholic compounds in extracellular products.

Introduction

Members of the *Pseudomonas fluorescens* group are involved in the deterioration of a wide variety of foods through the production of pigment, enzymes and other molecules involved in organoleptic decay (Andreani and Fasolato, 2017). This spoilage potential has been reported in a large number of well-publicized cases of several food lots being withdrawn from the market, especially cheese (e.g. blue mozzarella), resulting in economic losses and alarming consumers (Nogarol *et al.*, 2013; RASFF, 2010). This abnormal blue discoloration has been investigated by several authors (Andreani *et al.*, 2014; Caputo *et al.*, 2015; Nogarol *et al.*, 2013), but some peculiar aspects are still under debate. For instance, the chemical nature of the pigment is not well-defined, but recently matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis revealed that the blue pigment produced by colored *P. fluorescens* strains is probably an indigo-derivative molecule (Andreani *et al.*, 2015). Other observations have suggested the presence of indigoidine (Caputo *et al.*, 2015). Moreover, preliminary observations have shown that this dark blue compound is a diffusible pigment, while the final pigmented material is insoluble in water (Andreani *et al.*, 2014, 2015).

In the present study, the feasibility of two techniques, *i.e.* near infrared spectroscopy (NIRs) and ultraviolet visible spectroscopy (UV-vis), was investigated in order to characterize blue strains belonging to the *Pseudomonas fluorescens* group. Due to their ability to produce extracellular enzymes and release a wide range of secondary bioactive metabolites, *Pseudomonas fluorescens* is considered an efficient study model and an effective protein manufacturing factory (Son *et al.*, 2012). For these reasons, the use of spectra collected from broth cultures or whole cells could be used as a fingerprint for the classification of strains using a combined polyphasic approach.

In recent decades, spectrophotometric techniques have been investigated as alternative and rapid tools to identify and characterize microorganisms (Smith *et al.*, 2012). These methods usually do not require sample preparation or processing and are considered to be ecofriendly reagent-free analyses. Spectral data can be achieved from whole culture media, solid plates, purified bacterial cells, extracellular metabolites and several other matrices (Maquelin *et al.*, 2002; Marques *et al.*, 2015; Nakakimura *et al.*, 2012). The collection of spectral data

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allows for the creation of specific libraries that can be processed through multivariate analyses for the classification of foodborne pathogens, for their quantification in food matrices or for physiological studies (e.g. definition of sublethal injury cells) (Lin *et al.*, 2004; Nakakimura *et al.*, 2012; Smith *et al.*, 2012). The study of the most informative wavelength regions and band patterns could increase our understanding of blue pigment production in *P. fluorescens* strains.

Materials and Methods

Strains and cultural medium applied

A total of 81 strains (field and type strains) were analyzed, which had been previously identified at the species and strain level through a multilocus sequence typing (MLST) approach (Andreani *et al.*, 2014). Type strains and reference strains (n=18) were selected from the *P. fluorescens* group, while the field strains were collected from different foods such as mozzarella cheese, sushi, vegetables and other ready-to-eat foods. As described by Andreani *et al.* (2014), a monophyletic cluster called the *blue branch* has been defined by MLST analysis. From this genetic cluster, nine strains producing blue pigment and four

white strains were included in library construction.

The strains were plated on Pseudomonas Agar Base (CFC PAB; Oxoid Microbiology Products, Thermo Scientific) and incubated at 22°C for 24-48 h. Pure colonies were suspended in 10 mL of in MBM broth (0.7% K₂HPO₄, 0.3% KH₂PO₄, 0.05% trisodium citrate, 0.01% MgSO₄, 0.1% (NH₄)₂SO₄, 0.2% glucose) (Boles *et al.*, 2004) until pigment production was observed, as reported by Andreani *et al.* (2015). This medium was composed of salts with trisodium citrate and glucose as carbon sources and enhances the production of blue pigment. No amino acids or proteins were added, which could reduce the effects of interfering compounds present in commonly used growth media (Lin *et al.*, 2004). According to the preliminary growth curves, the culture broths were standardized in the range of 0.8-1 optical density (O.D.) at 600 nm. Two biological replicates were centrifuged (3000 rpm for 30 minutes) in order to separate the bacterial cells from the extracellular products. Supernatants and pellets were processed according to the spectrophotometric technique adopted for spectral library acquisition. In the experiments conducted in the UV-vis range the supernatants and cell pellets were re-suspended in physiological solution. For the NIRs analysis, only supernatants were used (Nakakimura *et al.*, 2012).

Near infrared spectroscopy

NIRs analysis was performed using a UNITY scientific SpectraStar 2500TW laboratory spectrophotometer. Six aliquots of supernatant per strain (200 µl) were analyzed on a small ring cup in transmittance mode (680-2500 nm) at room temperature. Reflectance data were recorded at 2 nm intervals and saved as log 1/R (where R is reflectance).

Methods based on latent variables were applied to investigate the data sets: Principal

Component Analysis (PCA) was useful as explorative system for the internal structure of the data, the samples were then categorized by Modified Partial Least Squares (MLPS). The Soft Independent Modeling of Class Analogies (SIMCA) approach was adopted to define the membership class using PCA in order to identify local models for subsequent cluster analysis (Alexandrakis *et al.*, 2008). This approach also allowed us to predict class membership in additional samples. The first exploratory approach evaluated all wavelengths (680-2500 nm whole spectrum) and the potential discriminating ability for the classes of interest. The classes analyzed were: i) blue pigment producing bacteria vs. all non-pigmented bacteria (Model 1; Table 1), ii) blue branch members vs. other *Pseudomonas* species (Model Blue Branch; Table 1) and iii) between genetically related blue strains and white strains included in the blue branch (Model Blue vs. White, Table 1). Discrimination was achieved on the basis of genetic clusters to understand whether spectroscopic reading of extracellular liquids could discriminate bacteria segregated on a genetic basis. Further discrimination was performed on the basis of alteration potential, thus creating four levels depending on the ability of these strains to produce extracellular enzymes involved in lipolysis or proteolysis as well as lecithinase. Activity was rated on a scale where 0 is zero activity and 4 is maximum production (according to the data reported by Andreani *et al.*, 2014). The accuracy of the classification was evaluated by cross-validation. The performance of each model was evaluated in terms of specificity (Sp) and sensitivity (Se). Further models were developed using the selected wavelengths based on the variable importance in projection score (VIP>1) criterion (Andersen and Bro, 2010). This index estimates the importance of each variable in the projection used in a model. A

variable with a VIP score >1 was considered important for the model, while under this threshold the wavelengths were considered to be less informative (Model 2 and Model 3; Table 1).

UV-visible spectroscopy

UV-visible spectroscopy was applied to evaluate pigment production in a subset of strains (n=48). Culture broths were examined at 48 h and 72 h, according to the visible production of blue pigment in MBM. As reported by Andreani *et al.* (2015), the pigment was visible when the O.D. reached 0.7 at 600 nm. Supernatants and re-suspended pellets were investigated only at 72h. Measurements were performed by a Multiskan GO Microplate Reader spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The analysis was carried out in 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) with a standardized inoculum (200 µL). O.D. 600 nm measurements were performed on each sample and the entire spectrum was acquired (200-1000 nm with a gap of 2 nm). In order to correct for possible absorbance interference from the broth, blank samples were also assessed.

Some mathematical pre-treatments were used, aiming to reduce the light scattering caused by the sample particles and to remove additional variation in the baseline shift. The standard normal variate and detrend (SNVD), smoothing, first and second derivatives were calculated using MATLAB data analysis software to determine the useful chemometric strategies. Partial least squares discriminant analysis (PLSDA) was performed using a multivariate approach in the calibration of white and blue data sets, and cross-validation was applied to test the accuracy of the model (Murray *et al.*, 2001). The models were evaluated considering the coefficient of determination for calibration (R²) and cross-validation (r²), and the corresponding standard error of calibration

Table 1. SIMCA models applied for the analysis of NIRs transmittance spectra.

Models	Pre-treatment	Range (nm)	Se	Sp	Informative wavelengths (nm)	Characteristic bands
Model 1	Raw	680-2500	0.61	0.99	680-956; 1004-1130; 1892-2020	-
Model 2	Raw	680-956; 1004-1130; 1892-2020	0.61	0.99	1892-2020	O-H stretch and deformation
Model 3	Raw	1892-2020	0.66	0.99	1892-1896; 1996-2020	R-NH ₂ ; C=O
Blue Branch	Raw	680-2500	0.93	1	1996-2020	O-H stretch and deformation; R-NH ₂ ; C=O
Blue vs. White	Raw	680-2500	0.89	0.89	680-886 1454-1768 2036-2134	fourth overtone C-H; third overtone C-H; stretch -CH ₃ ; aromatic C-H; first overtone O-H; aromatic C-H comb; first overtone -CONH- amides; CH ₂ fatty acids

(SEC) and cross-validation (SECV). Moreover, Se and Sp were also calculated. As previously reported, the VIP score was adopted for the interpretation of spectral variables.

Results and Discussion

Near infrared spectroscopy

In total, 1004 raw spectra were collected from the centrifuged supernatants. The spectra did not show specific peaks according to the different *Pseudomonas* species analyzed or peculiar shapes related to pigment production. This observation is in agreement with the work of Nakakimura *et al.* (2012), who similarly did not observe noticeable differences between spectra collected from bacterial species such as *Escherichia coli* and *Staphylococcus aureus*. Some differences in the absorbance and shape of spectra were observed in the 1880-2080 nm range (Figure 1A). This variability was mainly related to the different biological replicates and not associated with particular strains. The two main peaks (1450 and 1940 nm) were associated with water regions (*e.g.* first overtone band and O-H stretching modes) (Dubois *et al.*, 2005; Nakakimura *et al.*, 2012). The peak at 2300 nm was linked to fatty acid moieties (CH_2 bending/stretching combinations) (Dubois *et al.*, 2005). As a preliminary study, models were built without pre-treatment of the raw spectra (Table 1). The raw spectral information was not suitable to develop models for the classification of

Pseudomonas species or to categorize phenotypes. However, additional treatments of the spectral data allowed for classification. Only the results on the classification of blue strains are reported. To overcome multicollinearity problems, several mathematical applications were applied, and the chemometric approach reduced spectral complexity without altering the suitable information.

Model 1 investigated the possibility of classifying blue strains with respect to all the data sets. The application of the entire spectrum allowed for the classification of 87% of the blue strains. The VIP scores selected three main spectral regions (680-956 nm, 1004-1130 nm and 1892-2020 nm) for further analyses. Model 2 and Model 3 showed that the most informative region was the band related to the combination of O-H stretching and deformation (1892-2020 nm). This region provided an increase in classification performance; moreover, a similar region (1996-2020 nm) was selected according to VIP in the Blue Branch model. Interestingly, the spectral fingerprinting of supernatants collected from the same genetic cluster (Blue Branch) showed features useful for a clear discrimination (Table 1 and Figure 1B). These data suggest that the composition of extracellular metabolites produced by the blue branch strains (both white and blue) are different from those produced by the other strains. The last model (Table 1, Blue vs. White) was proposed to elucidate the fine differences between supernatants obtained from the blue strains and the white strains within the blue branch. Three main spectral regions were suitable for classification

purpose: 680-886 nm, 1454-1768 nm and 2036-2134 nm. The range from 680 to 886 nm includes some characteristic features, such as bands at 690-770 nm corresponding to the fourth overtone of C-H, those in the spectral region of 845-878 nm related to the third overtone of C-H stretching (Alexandaxis *et al.*, 2008) and the band located at 880 nm related to aromatic C-H bonds. The region from 1454 to 1768 nm is mainly associated with the first overtone of O-H (1450 nm), but other bands overlap in this region, such as the aromatic C-H combination band (1446 nm) and the first overtone of N-H (Dubois *et al.*, 2005). The bands at around 1632 and 1756 nm are putatively associated with the first overtone of -CONH- found in secondary and primary amides, *e.g.* DNA/RNA (Marques *et al.*, 2015). Moreover, a contribution from the fatty acid fraction was observed (1734-1765 nm; first overtone of CH_2). The region from 2036 to 2134 nm is close to the specific absorption region related to alkenes (CH_2 stretching and bending) and unsaturated fatty acids (Dubois *et al.*, 2005; Marques *et al.*, 2015). These data indicate that supernatants from closely related *white* and *blue* strains (ascribed to the blue branch cluster) were composed of a different array of molecules including fatty acids, aromatics and amides.

UV-visible spectroscopy

UV-visible spectroscopy was applied to study the broth cultures during the production of blue pigment. Table 2 shows the PLS-DA models developed in different matrices; SNVD was the only pre-treatment able to increase the calibration performance. In general, better classification (sensitivity and

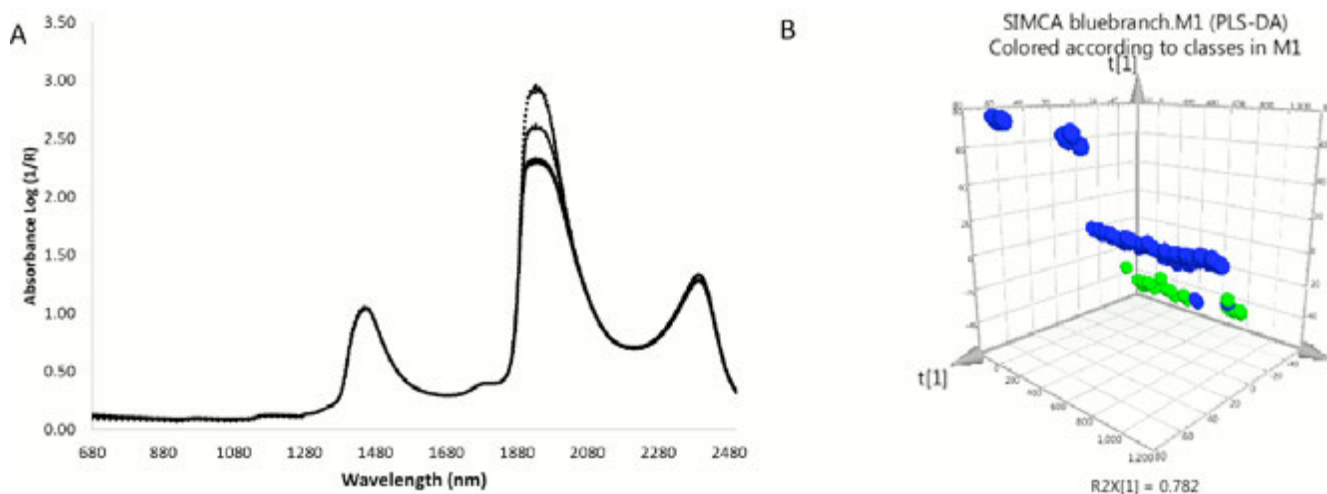


Figure 1. A) Near infrared spectra collected from blue and white strains; B) representation of the SIMCA model for blue branch raw spectral data (wavelength range 680-2480) nm; *Pseudomonas* ascribed to the blue branch are indicated in green.

specificity) was provided by spectra collected from broth cultures after 72 h of growth and from supernatants. These data suggest that extracellular metabolites play a key role in the differentiation between blue strains and other *Pseudomonas* strains.

Figure 2 shows three common spectra collected from blue strains in comparison with one spectrum from a white strain of *P. fluorescens*. Moreover, the reference spectrum of indigo (0.1 mg/ml; Sigma catalog number 229296) in MBM is also

reported. Gray bands show the VIP scores for each model; only values >1 are shown.

The most important features selected in the broth cultures at 48 h ranged between 432 to 692 nm. In this range, a specific absorption band at 600 nm is associated with the catechol groups of pyoverdines (Sánchez *et al.*, 2005). Pyoverdines are the major siderophores released by *Pseudomonas*; these molecules are yellow-green fluorescent pigments also known as fluorescein. This family of iron chelators is formed by three

different structural parts, where a quinoline chromophore group is bound to a peptide chain and to a dicarboxylic acid or a dicarboxylic amide (Tank and Saraf, 2010). The spectral information collected at 48 h suggested different behaviors regarding Fe³⁺ chelation in the broth cultures of blue strains with respect to the other *Pseudomonas* strains (Figure 2A).

After 72 h, other spectral regions were involved in the classification of broth cultures (Figure 2B). The classical

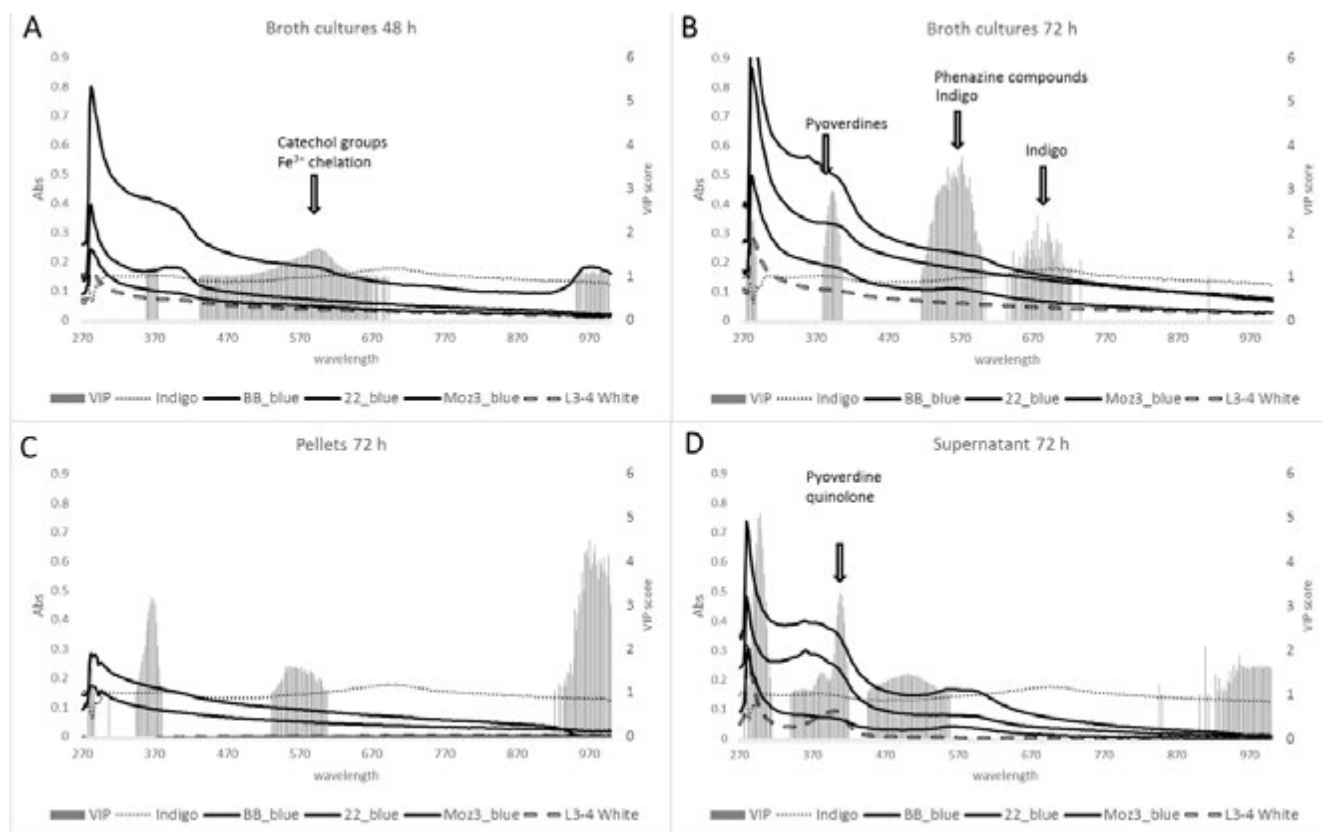


Figure 2. A) UV visible PLS models of broth cultures at 48 h; B) broth cultures at 72 h; C) pellets and D) supernatants. Dark lines represent the raw spectra of some blue strains and the dotted line represents the spectrum of a white strain. The absorption spectrum of commercial indigo is also shown. Gray bars indicate VIP scores > 1.

Table 2. UV visible spectra: performance of the PLS models.

Model	Pre-treatment	Se	Sp	R ²	RMSEC
Broth cultures 48 h	Raw	0.33	0.90	0.13	0.39
	SNV	0.78	0.73	0.21	0.37
Broth cultures 72 h	Raw	0.89	0.93	0.44	0.31
	SNV	0.78	0.93	0.40	0.32
Supernatants 72 h	Raw	0.80	0.93	0.60	0.27
	SNV	0.90	0.86	0.60	0.29
Pellets 72 h	Raw	0.63	0.96	0.22	0.36
	SNV	0.38	0.93	0.35	0.35

RMSEC: root mean squared error of calibration. Raw: raw spectrum; SNV: standard normal variate and detrend pre-treatment. R²: coefficient of determination in calibration. Se and Sp: sensitivity and specificity.

fingerprint of pyoverdine was related to the quinolone moiety (390 nm; Magro *et al.*, 2016). The wavelengths around 570 and 670 nm defined the different composition of the broth cultures, probably related to pigment production. Chromophore groups of certain pigments such as violacein or phenazine compounds show absorbance peaks in the region around 580 nm (Gibson *et al.*, 2009; Yuan *et al.*, 2008); moreover, a similar UV-vis spectrum of indigo produced by *Pseudomonas* sp. was described by Dua *et al.* (2014). The reference spectrum of a commercial indigo showed a peak at 696 nm (Figure 2). This spectral region was selected as informative by the VIP scores (Figure 2B). The peak of another cognate pigment, *i.e.* purified indigoidine, has been observed at 612 nm (Cude *et al.*, 2012).

According to the spectrum shape, the shift from 48 to the 72 h showed that the blue strains released more siderophores than their white counterparts did. The specific peak and shoulder at 570-580 nm could be considered as a specific marker for blue pigment. However, this fingerprint was present only after 72 h of growth, corresponding to the stationary phase, suggesting the release of a secondary metabolite.

This information was lost in the re-suspended pellets as shown in Figure 2C and as previously reported in Table 2. The data suggest that the microbial cell spectrum was not appropriate for this study. As observed in previous studies (Andreani *et al.*, 2014, 2015), the blue pigment is a diffusible compound that not tightly associated with cells. In the late stationary phase (at 120 h), dark aggregates are removed by centrifugation. This suggests complex behavior of this compound that has been described as insoluble in water (Andreani *et al.*, 2015).

The specific peak at 570 nm was maintained in the supernatant spectra collected at 72 h (Figure 2D). Moreover, the wavelengths corresponding to the quinolone absorption band were considered to be informative. Proteins also affect the first part of the spectrum with a specific peak at around 280 nm. Other additional information can be correlated to molecules containing aromatic moieties such as a pyochelin-like molecule or a secondary unidentified siderophore (310 nm) (Sokol *et al.*, 1992).

Overall considerations

These data are in agreement with the results described by Andreani and coworkers (2015) that reported an up-regulation of genes involved in iron uptake in blue strains. The bands defined by the NIRs analysis could be also associated with siderophore

molecules as described by the pyoverdine structure formed by 2,3-diamino-6,7-dihydroxyquinoline, a peptidic moiety and an acyl from a dicarboxylic acid or amide (Cézard *et al.*, 2015). However, the supernatants collected from the blue branch showed common spectral features that will require a more in-depth chemometric approach to define more descriptive wavelengths. Furthermore, the presence of aromatic molecules could be also correlated with pigment production. The presence of spectral regions related to fatty acids could be due to the presence of outer membrane vesicles that show specific peaks related to lipids in Fourier transform infrared (FTIR) spectroscopy (Carlsson, 2012).

Interestingly, after 48 h of growth, Fe³⁺ chelation was more evident in blue strains than in the overall population. It is likely that phenomenon highlights the variable efficiency of pyoverdine secreted by these strains and could reflect a difference in the structure of this iron chelator. To date, a wide number of strain-specific or species-specific pyoverdines have been described in *Pseudomonas* (Cézard *et al.*, 2015), but further characterization of this siderophore is required. After 72 hours, a larger amount of pyoverdine was released by the pigmented strains. This increase in siderophores was accompanied by the presence of additional chromophore molecules. The blue pigment is probably an indigoid molecule with a specific peak at 570 nm and the dye is released into the broth during the late phases of growth.

Conclusions

The feasibility of two different spectrophotometric techniques were investigated. NIRs and UV-visible spectra provided interesting information concerning the characterization and the production of blue pigment. Spectra are useful for strain classification regarding this peculiar spoilage activity. These libraries could be applied for further taxonomic studies; however, the dataset needs to be improved in order to increase the performance of identification. Blue strains therefore exhibit different behavior from other *Pseudomonas*. This phenomenon could imply a different competitiveness in the food environment. Moreover, new calibrations could be performed during challenge experiments with blue *Pseudomonas* to study the dynamics of pigment production in real food matrices (*e.g.* the governing liquid of mozzarella cheese).

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