PURIFICATION AND MOLECULAR STRUCTURE DETERMINATION OF FLEXIRUBINS FROM *CHRYSEOBACTERIA*

Jordan Krebs[1,2], Dave Sampsell[2], Dr. Chriss McDonald[2], Dr. Jeffrey Newman[1]

[1]Biology Department, Lycoming College, 700 College Place, Williamsport, PA 17701

[2]Chemistry Department, Lycoming College, 700 College Place, Williamsport, PA 17701

Abstract

The taxonomy of bacteria belonging to the *Flavobacteriaceae*, *Cytophagaceae,* and *Chitinophagaceae* families of the *Bacteroidetes* phylum has been widely debated and modified in the past few decades. The presence of flexirubins, a modestly studied class of pigments, has been used to differentiate related genera of the *Bacteroidetes* with the use of a simple KOH test. The purification and structural determination of flexirubins has only been completed for a few species: *Chitinophaga filiformis* Fx e1, *Flavobacterium sp.* strain C ½, *Flavobacterium sp.* strain Samoa, *Flavobacterium johnsoniae* Cy j1, and an unrelated sulfur-reducing bacterium, *Sulfurospirillium delayianium* 5175. These pigments have 5 possible structural variations; the variations cluster according to the genus of the species of origin.

In this study, the purification of these flexirubins from the genus *Chryseobacterium* has been optimized. An acetone extract of the raw pigments is prepared for reverse-phase based HPLC purification through a process that utilizes the characteristic shifts in color of the pigments due to pH changes. To date, a pool of up to 10 flexirubins has been purified from *C. oranimense.* UV-Vis data collected by the diode array detector on the HPLC shows that of the ten pigments, 4 peaks have a greater ratio. The major peak of the set has a λ_{max} of 452 nm. The elucidation of the chemical structures of these fractions is forthcoming. Obtaining the chemical structures of flexirubins of *Chryseobacteria* may better demonstrate the ability of such a study to differentiate closely related bacteria past traditional phenotypic, genetic, and biochemical approaches. Additional flexirubin structures from more diverse bacterial species may aid in determining the function and biosynthesis of these pigments. Any determined flexirubin structural differences should explain minor color variations of *Chryseobacterium* species when grown on TSBA.

Background

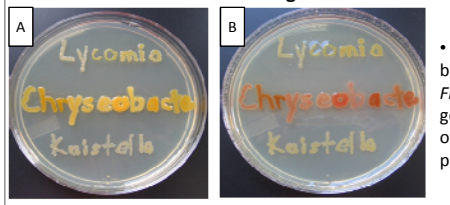
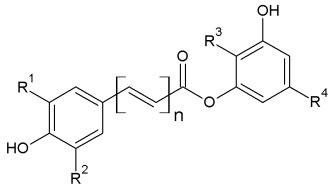


Figure 1: Color of Colonies of *Chryseobacterium*, *Lycomia*, and *Kaistella* species. *A.*, Color of Colonies Before 40% KOH Treatment. *B.*, Color of Colonies After 40% KOH Treatment.

- A key difference between the *Flavobacteriaceae* genera is yellow-orange pigmentation



R^1	H, Cl, CH_3
R^2	H, Cl
R^3	different length and branching alkyl chains
R^4	
n	6 to 8 polyene

Figure 2. General chemical structure and variation of a flexirubin. *A.*, General structure with R groups and table of determined structural variations.

- Purification and structural determination of flexirubins has only been completed for: *Chitinophaga filiformis* Fx e1, *Flavobacterium sp.* strain C ½, *Flavobacterium sp.* strain Samoa, *Flavobacterium johnsoniae* Cy j1, and an unrelated sulfur-reducing bacterium, *Sulfurospirillium delayianium* 5175.
- 32 pigments with 5 possible structural variations have been determined.

Methods

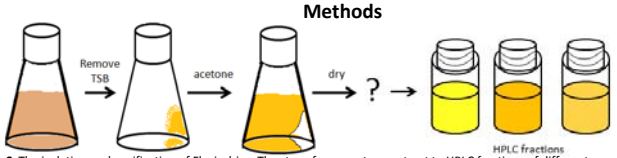


Figure 3. The isolation and purification of Flexirubins. The steps from acetone extract to HPLC fractions of different flexirubins as represented by "?" are the focus of this project.

- TSB grown cells are harvested by centrifugation, acetone is used to extract flexirubins and related molecules.
- Pre-HPLC and HPLC purification needs to be optimized

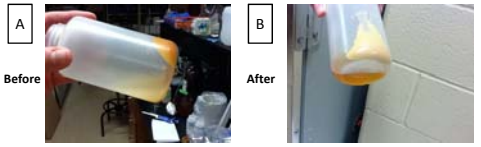


Figure 4. Cell Pellet and Solvent Before (*A.*) and After (*B.*) Acetone Extraction.

Results

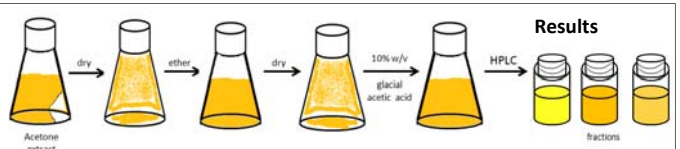


Figure 5. First pre-HPLC purification attempted. Ether was used to transfer the acetone extract and 10% glacial acetic acid was the HPLC sample solvent.

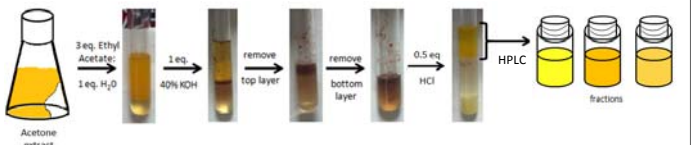


Figure 6. Second pre-HPLC purification attempted. This utilizes the reversible shift of the absorption maxima that occurs due to the addition of KOH and then HCl (this shift is seen between *Figures 1A* and *1B)*.

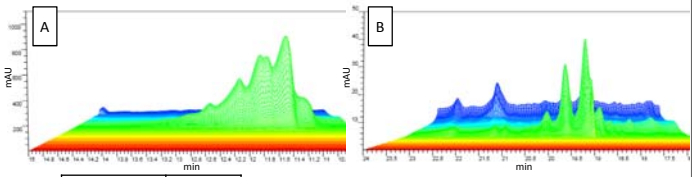


Figure 7. Spectra of optimized runs for Methods 1 (*A.*) and 2 (*B.*).

	Time (mins)	% MeOH
1	0 – 5	0
2	5 - 6	80
3	6 - 16	100
4	16 - 30	100

Table 1. Parameters of the optimization of Flexirubin purification (*below*) and Flex16Frac.M method (*right*).

			HPLC absorbance and separation*					
Method	Reagent System	Preparation Time	area of peak #3	λ_{max} (nm)**	number of peaks	separation over time (min)	injection volume	Method
1	1) Ether, 2) 10% w/v sample in glaicial acetic acid	2 hours	~16892.2*ŧ	452	~ 8	9.5 - 13.6	20 µL	Flex16Frac.M
2	1) 3 eq. ethyl acetate, 1 eq. H2O, 2) 1 eq. 40% KOH, 3) 0.5 eq. HCl	1 hour 20 mins	~18.87*ŧ	452	at least 10	16.4 - 23.8	20 µL	Flex16Frac.M
3	1% w/v Methanol	15 - 30 mins	~2769.9 *$	450	8 to 10	27.0 - 32.8	50 µL	Flex16Frac50_6.M
			N/A	450	2	36.4 - 37.5	50 µL	

* Represents most optimized HPLC separation for respective method. ** λ_{max} (nm) of #3 peak. *ŧ at 430 nm.*$ at 452 nm.

Results

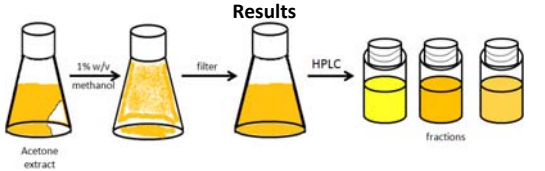
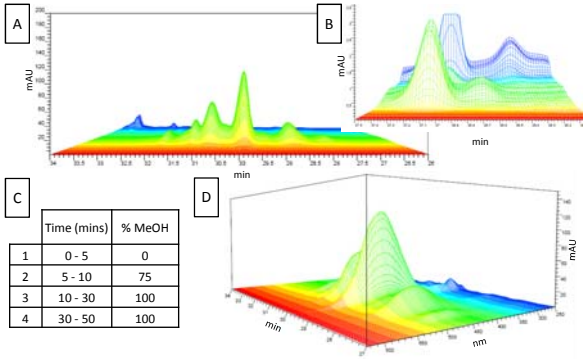


Figure 8. Second pre-HPLC purification attempted. A 1% w/v solution of acetone extract in methanol is made. This method was further optimized for Flexirubin purification.



	Time (mins)	% MeOH
1	0 - 5	0
2	5 - 10	75
3	10 - 30	100
4	30 - 50	100

Figure 9. Spectra of optimized runs for Methods 3 (*A.*, *B.*, and *D.*) and Flex16Frac50_6.M method HPLC Parameters (*C.*).

Conclusions

- The pre-HPLC and HPLC purification of flexirubins from *C. oranimense* has been optimized.
- The newest purification method (Method 3) is currently being used to purify *C. oranimense* flexirubins to enable structural elucidation utilizing 1-D and 2-D NMR, EI- and CI-MS, and IR.

Future Work

- Use Method 3 to purify and then determine the structures of flexirubins from *C. greenlandense*, *C. joostei*, *C. piperi*, and *C. shigense*.

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Acknowledgements

The author (JEK) wishes to thank Andrew Gale for technical assistance, along with Katherine Wellmon and Caitlin DeAngelo for helpful discussions. The author (JEK) wishes to thank the NIH Undergraduate Scholarship Program (UGSP) for financial support of his education. JEK is supported by a Haberberger Fellowship at Lycoming College. HPLC Instrument was purchased with funding from NSF award DBI-0960114 to JDN.