

Physico-chemical Characterization of a Pink Red-like Pigments Produced by Five New Bacterial Soil Strains Identified as *Streptomyces coelicoflavus*

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Abstract Five new strains MFB11, MFB20, MFB21, MFB23 and MFB24 of actinomycetes showed an intracellular hydrophobic pink red-like pigment production. These pigments present similar physico-chemical characteristics with anthracycline antibiotics of prodigiosin family. Nevertheless, negative antibacterial assay, Thin-layer chromatography (TLC) and interaction with organic solvents analysis of these pigments revealed their difference from known anthracycline antibiotics. Morphological, biochemical and gene coding 16S RNA sequence analysis allowed identification of the producer strains as *Streptomyces coelicoflavus*; known to produce important aminoglycoside antibiotics and other bioactive compounds but not anthracyclines red-like pigments. The identification of the five strains and physico-chemical properties of the produced pink red-like pigments are presented in this report.

Keywords: *S. coelicoflavus*, red pigment, anthracycline, UV-Vis spectra, TLC, hydrophobe, thermosensitive, photosensitive

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1. Introduction

Natural bioactive compounds represent an important tool of inspiration in drug discovery design. Bacterial compounds used in human therapy and agriculture application hold the second place after those of plant [1,2]. In bacteria, screening for bioactive compounds leads mostly to Actinobacteria and particularly to the *Streptomyces* genus [3,4,5]. A genus which known by its high production of compounds with diverse chemical structures and bioactivities: antiviral, antibacterial, antifungal, anticancer, antidiabetic, anti-parasitic and immunosuppressive. These microbial metabolites produced by cell secondary metabolism could be classified as aminoglycosides, glycopeptides, peptides, tetracyclines, beta-lactams, macrolides, nucleoside, polyenes or anthracyclines [3]. Anthracyclines are a family of aromatic polyketide antibiotics with important cytotoxicity against human cancer cells [6]. The molecules consist of a variable aglycone skeleton and also variable sugar moieties, offering high range of structural diversity. These pigments are often yellow, orange, red, purple, pink or blue which are responsible of the harboring strain mycelium colors. Prodigiosin constitute a family of red pigmented anthracyclines initially isolated from the

unicellular gram negative bacteria *Serratia marcescens* [7]. Actinorhodine [8] and undecylprodigiosin [9] produced by *S. colicoolor* A2 strain with respectively red/blue and red color are ones of the anthracyclines found in *Streptomyces* genus. Other *Streptomyces* species has been reported to produce analogues of red pigments such as *S. peucetius*, Daunomycin and Doxorubicin [10]; *S. collinus*, Rubromycin [11]; *S. purpurascens*, Rhodomycin; *S. griseoviridis*, Roseophilin [12,13]. In this study, five new strains had been identified as *Streptomyces coelicoflavus* showed production of new pink red-like pigment anthracycline analogues. Strains identification and physico-chemical characterization of the pigments are presented in this report.

2. Material and Methods

2.1. Strains, Morphological and Biochemical Characteristics

Strains were isolated previously from soil samples [14]. Culture characteristics and phenotypic properties were determined after 2 weeks at 30°C using International Streptomyces Project (ISP) methodologies [15,16]. Observations of mycelia and spores chains were made by

light microscope (x1000). Standard biochemical assays were performed on mycelia grown in liquid ISP2 medium during 48 hours at 30 °C.

2.2. Amplification and Sequencing of 16S rDNA

Genomic DNA was prepared by kit as recommended (Promega). The 16S rRNA encoding gene amplification was performed by PCR using 27F and 1492R universal bacterial primers [17]. Amplicons were sequenced by ABI 3130 Analyzer automatic sequencer. The resulting sequences were assembled into a unique contig with DNA Baser Assembler software in the case of strain MFB11. The Basic Local Alignment Search Tool “BLASTn” (<https://blast.ncbi.nlm.nih.gov/>) was used for sequence and similarity searches in the GenBank database. Alignment of sequences was performed with Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

2.3. Preparation and Characterization of Red-like Pigment crud Extract

Erlenmeyer flasks containing 20 ml of ISP4 medium are inoculated with each strain and incubated 5 days in dark at 30°C in horizontal shaker. Mycelia were transferred to assay tube and extracted with 10 ml of ethanol by vigorous mixing at room temperature. Acid-base presumptive test was carried out by adding to 200 µl of ethanolic extracted pigment 10 µl of NaOH 1N (Base condition) or HCl 1N (Acid condition). The absorption spectrum of pigment extracts were determined by UV-Vis spectrophotometer. Concentrated pigment by fume hood air dry was analysed by thin-layer chromatography with silica gel G-60 F₂₅₄ (Merck). The solvent systems used consisted of (A) Petroleum ether: Ether (2:1), (B) Ether, (C) Methanol: Ethyl acetate: Chloroform (6:3:1), (D) Chloroform: Methanol (95: 5) and (E) chloroform.

The solvent containing chromatography tank was kept for 45 min for equilibration. The samples were spotted on silica gel plate; air dried and then dipped in the solvent system. When the solvent accomplished sufficient migration, TLC plate was removed and the retention factor (Rf) values were calculated.

2.4. Conditions of Conservation Effect on Red-like Pigment

The mycelia were extracted with 20 ml of ethanol. The ethanolic extracts were evaporated for obtained 12 ml for each extract and divided into 3 aliquots of 4 ml. Ethanol of 2 aliquots was evaporated and the solid extract was suspended in 4 ml of methanol or 4 ml of chloroform. The UV-Vis absorption spectrum of each extract was determined before conservation.

One millilitre of each ethanolic, methanolic and chloroformic extract was transferred in eppendorf tube and incubated during 30 days in 4 conditions: (i) room temperature in darkness, (ii) room temperature and exposed to light, (iii) 4 °C in darkness and (iv) -20 °C in darkness. After incubation, the UV-Vis absorption spectrum of each extract was determined.

2.5. In Vitro Antibacterial Assay

Pigments prepared from mycelia grown in ISP2 were screened for antibacterial activity by disk diffusion technique against clinical strains of Gram negative bacteria and Gram positive bacteria *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Micrococcus leutus* ATCC9341 and *Staphylococcus aureus* ATCC6538. Disks of 6 mm filter paper were loaded with 100 µl of ethanolic extracted pigment, dried and placed on previously inoculated Mueller Hinton agar containing plates with the test bacterium. After 24 h incubation at 37°C, bioactivity was determined by inhibition zone appearance (mm). Negative (T⁻) and positive (T⁺) controls were made as above by 100 µl ethanol and 40 µl of chlortetracycline hydrochloride (100 µg/ml) respectively.

3. Results and Discussion

3.1. Identification of the Strains

Aerial and substrate mycelia color phenotypes, biochemical characteristics, spores chains and sugars utilization are highly similar between the studied strains MFB11, MFB20, MFB21, MFB23 and MFB24 (Table 1). When compared to the JCM6918 *S. coelicoflavus* type strain [18,19], the appearance of substrate mycelia on ISP3 and ISP4 was pink and purple-red (or orange-red depending on strain) respectively for the studied strains and only sand yellow in the case of the type strain on the two media. The performed biochemical tests showed difference in citrate which was used by all the studied strains. Gelatin hydrolysis had been observed in few millimeters of the upper part of tube test in the cases of strains MFB11, MFB23 and MFB24. Indole production was noted in the case of strains MFB20 and MFB24. Most of the sugars tested as carbon source were commonly used by the strains studied and the JCM6918 type strain. Differences were situated in the case of xylose which was used by the strains studied but not the type strain. In the case of cellulose, strains MFB23 and MFB24 showed a very slight growth on ISP9 agar compared to negative control without sugar. Equally, very weak growth compared to negative control was obtained when strains MFB23 was grown on ISP9 agar with sucrose. In addition, the strains studied were able to use mannose, glycerol and (except MFB23) galactose; sugars with no indication found for the type strain in literature. These results showed that the strains studies present significant phenotypic similarity with the JCM6918 *S. coelicoflavus* type strain.

Sequences of 431 nucleotides (nt) contiguous downstream to 27F primer sequence, corresponding to the upstream of the 16S RNA coding gene had been obtained in the four strains MFB11, MFB20, MFB21 and MFB23. Alignment of the four strain sequences showed 100% identity and hence the four strains share the same 431 nt sequence. The BLAST search of this sequence revealed the presence of 2 sequences with 100% identity in data base. The first sequence with 100% coverage belonging to *S. coelicoflavus* strain NBRC 15399 (accession: AB184650.1) and the second with 96% coverage due to lack of the first 17 nt in this sequence belong to *S. coelicoflavus* type strain

JCM6918 (accession: AY999752.1). In this BLAST search, no other sequences showed 100% identity with the studied strains sequence. The four strains 431 nt sequence alignment with equivalent sequences of reported strains JCM6918 (AY999752.1), NBRC15399 (AB184650.1), USF6280 (AB548687.1), ZG0656 (EU201137.1) as *S. coelicoflavus* showed 100% identity. In the case of MFB11 strain, a sequence between 27F and 1492R primers of 1466 nucleotides was obtained. BLAST search indicated 100% identity only with NBRC 15399 *S. coelicoflavus* strain. Alignment of this MFB11 strain sequence with equivalent sequences of the reported *S. coelicoflavus* strains showed 100% identity with equivalent sequences (Table 2) of strains JCM6918 (AY999752.1), NBRC15399 (AB184650.1) and USF6280 (AB548687.1) but only 99.93% with that of ZG0656 (EU201137.1) due to the presence of one mutation T to C at position 1313 in this sequence. These results were consistent with phenotypic analysis and suggest strongly that the strains studied belong to the species *Streptomyces coelicoflavus*. This species had been reported to produce antioxidants [19], alpha-amylase inhibitors [20] and Alpha Glucosidase inhibitors [21].

Table 1. Morphological and biochemical characteristics of the studied strains compared to *S. coelicoflavus* type strain JCM6918.

Essaye	Strain					
	JCM6918	MFB11	MFB20	MFB21	MFB23	MFB24
Aerial on on ISP3	w-grey ^a	w-grey	w-grey	w-grey	grey	w-grey
Substrate on ISP3	S-yellow ^b	Pink	Pink	Pink	Pink	Pink
Aerial on ISP4	w-grey	Grey	Grey	Grey	Grey	Grey
Substrate on ISP4	S-yellow	P- red ^c	P- red	P- red	O-red ^d	P- red
Pigmentation	Nil	Nil	Nil	Nil	Nil	Nil
Spore chain	Spiral	straight	straight	straight	straight	straight
Indole production	-	-	+	-	-	+
Citrate utilization	-	+	+	+	+	+
Melanin formation	-	-	-	-	-	-
Gelatin hydrolysis	-	±	-	-	±	±
H ₂ S production	-	-	-	-	-	-
Casein hydrolysis	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+
Urease	+	+	+	+	+	+
Catalase	ND	+	+	+	+	+
Sugar utilization						
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Sucrose	-	-	-	-	±	-
Mannitol	+	+	+	+	+	+
Xylose	-	+	+	+	+	+
Arabinose	+	+	+	+	+	+
Cellulose	-	-	-	-	±	±

a: window grey, b: Sand yellow, c: Pourple red, d: Orange-red., ND: not determined in literature.

Table 2. Alignment percent identity matrix of MFB11 16S coding gene sequence with those of *S. coelicoflavus* reported strains.

N°	Strain	Length*	1	2	3	4	5
1	MFB11	1466	100.00				
2	JCM6918	1404	100.00	100.00			
3	NBRC15399	1480	100.00	100.00	100.00		
4	USF6280	1478	100.00	100.00	100.00	100.00	
5	ZG0656	1480	99.93	99.93	99.93	99.93	100.00

*: 16S rDNA nucleotides (nt) sequence length.

3.2. Color, Antibacterial, Spectral and TLC analysis of pigments

Extracted pigment of the five studied strains showed purple color at high concentration and pink at low concentration after dilution (Figure 1). UV-Vis spectra profiles of these ethanol crud extract showed a high similarity between the studied strains themselves (Figure 2) such as with prodigiosin and undecylprodigiosin [7,9]; exhibiting a characteristic peak located at nearby 534 nm (maximal wavelength: λ_{max}). Absorbance peaks at 260-270 nm and 350-491 nm were similar to that of Rhodomycin and analogues [22]. Other anthracycline such as Daunomycin present a maximal absorbance at 485 nm [23]. Moreover, prodigiosin acid-base presumptive test was positive for the pigments of the five strains. In an acid condition, pigment was pink and exhibited a sharp spectral peak at nearby 534 nm. While in basic condition the pigment turned yellow and possesses a spectral peak at nearby 458 nm for all strains. Figure 3 show precisely the case of strain MFB20. However, the shape of UV-Vis spectrum can present variation with shift of λ_{max} value according to the pigment concentration, sugar present in solution (result not shown) or solvent used (see below).

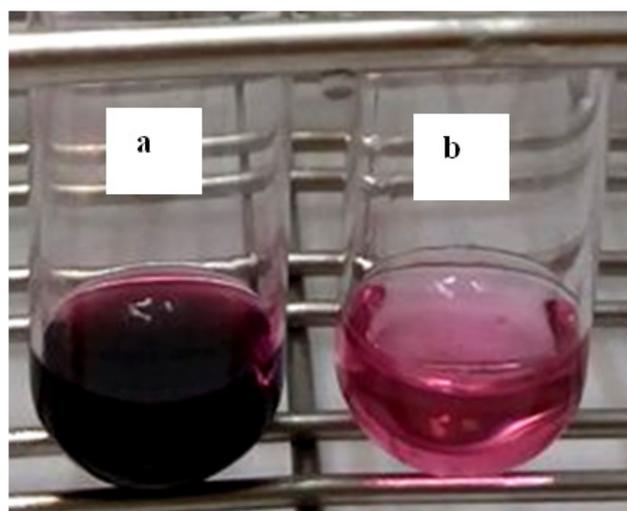


Figure 1. Ethanolic crud extract of pigment. a) Purple at high concentration. b) Pink at low concentration

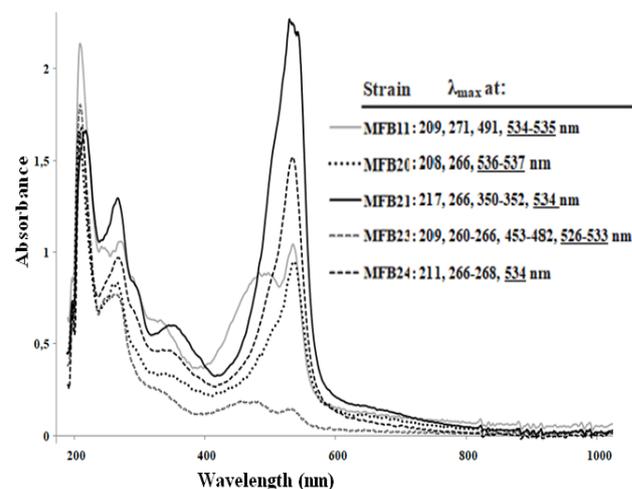


Figure 2. UV-Vis spectra of crud ethanolic pigment extract from strains mycelia with absorbance peaks values

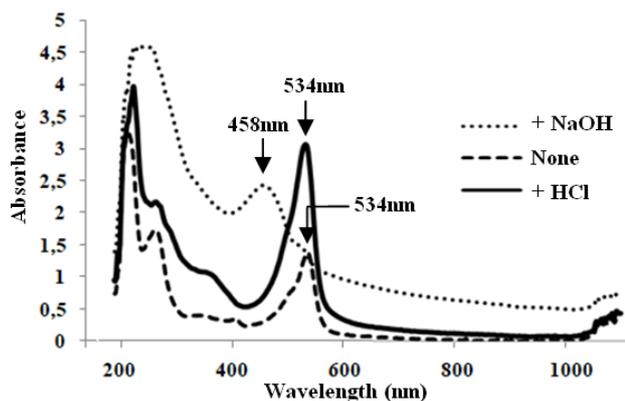


Figure 3. UV-Vis spectra of crude ethanolic pigment extract of MFB20 ethanolic extract in neutral, acid and base conditions

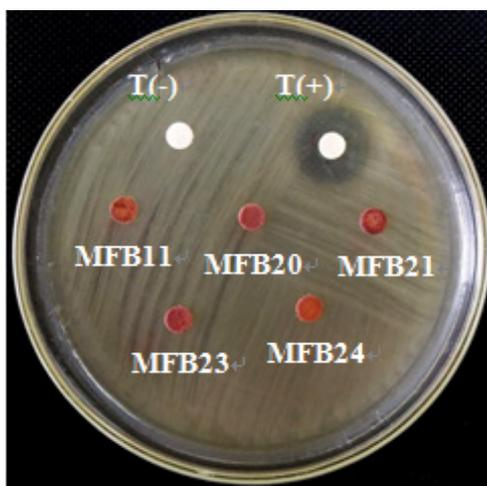


Figure 4. Antibacterial assay of different strain pigments

Against reported studies attributing antibacterial activity to anthracyclines [24,25] especially prodigiosin [26], antibacterial assay of the studied crude extract pigments was negative on both gram negative and positive test bacteria (Figure 4).

Results of TLC developed with solvent system E showed 5 bands (Table 3): (1) first pink band with an Rf values of 0.05 or 0.06 present in all strains but changed in MFB11 with (2) a second pink band whose Rf equal 0.26; (3) third band with Rf of 0.50 to 0.56 and (4) fourth pink-orange band with Rf of 0.63 to 0.66 present in all strains; and (5) a fifth yellow band with Rf of 0.83 present only in MFB11. These indicate similarity and diversity between the pigments in the studied strains. Three band Rf values were similar with bands obtained in *S. coelicolor* undecylprodigiosin [27]: 0.05 (purple), 0.54 (pink) and 0.83 (orange). However, study results indicate significant differences in bands number, colors and Rf values. The use of other reported solvent systems in *Serratia marcescens* prodigiosin TLC such as solvent system A [7], system C [28] and system D [29] had shown no similar results: only one band with Rf varying between 0.10 and 0.14 depending to the strains (Table 3) for solvent system A, one large band (Rf: 0.58) for solvent system C and two bands (Rf: 0.56 and 0.75) for solvent system D (Not shown). This comparative analysis showed that the studied pigments, even their similarity with anthracyclines of prodigiosin family, exhibit different TLC profiles and should contain structural differences.

Table 3. TLC Rf values and colors of bands in solvent systems A, B and E

Strain	A	B	E
MFB11	0.10 (Pink)	0.71 (Pink) 0.87 (Yellow)	0.26 (Pink) 0.56 (Pink) 0.65 (Pink orange) 0.83 (Yellow)
MFB20	0.12 (Pink)	0.73 (Pink)	0.05 (Pink) 0.51 (Pink) 0.65 (Pink orange)
MFB21	0.12 (Pink)	0.75 (Pink)	0.05 (Pink) 0.50 (Pink) 0.63 (Pink orange)
MFB23	0.14 (Pink)	0.08 (Pink) 0.73 (Pink)	0.05 (Pink) 0.56 (Pink) 0.66 (Pink orange)
MFB24	0.10 (Pink)	0.71 (Pink)	0.06 (Pink) 0.53 (Pink) 0.65 (Pink orange)

3.3. Hydrophobicity, Photosensitivity and Conservation of the Pigments

The pigments were not secreted in culture media and their extraction from mycelia grown in ISP4 can be made by organic solvents namely ethanol, chloroform, methanol or ethyl acetate. Total extraction of the pigment from mycelia was obtained only with ethanol. After ethanol evaporation, the extracted pigment is not water soluble in agreement with the case of prodigiosin [30] or aglycone moiety of other anthracyclines [31,32]. Curiously, part of it could acquire water soluble ability after air dry in fume hood as indicated for the case of strain MFB21 pigment (Figure 5) which showed 36% of soluble pigment after 24 h air dry. This new aspect of these pigments had no reported study in literature.

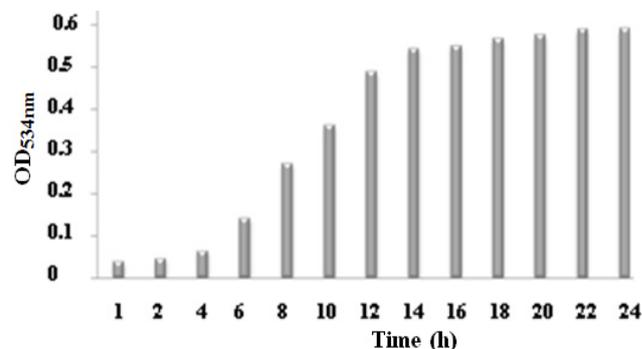


Figure 5. Solubility of MFB21 strain pigment after different periods of air dry

Otherwise, conservation test of the pigment was performed in ethanol, chloroform and methanol at four conditions. Absorbance of pigment at λ_{max} before incubation showed higher value in ethanol than methanol or chloroform for strain MFB11, in chloroform for strains MFB20 and MFB24 (Figure 6, first column). In the case of strain MFB21, OD values in ethanol, chloroform and methanol were approximately equivalent. Reference [30] showed that prodigiosin excited with light at wavelength of 535 nm fluoresced with maximal at 560–565 nm; connecting maximal absorbance to maximal fluorescence in the characteristic wavelength region of prodigiosin. Reference [33] reported for daunorubicin, idarubicin, pirarubicin, doxorubicin and derivatives that

fluorescence spectra showed increase of emission peak according to increase of solvent dielectric constant. The study result showed that is true for strains MFB21 and MFB24 pigments where OD in chloroform > OD ethanol > OD in methanol. In strains MFB11 and MFB20, absorbance of pigments in the solvent used had no correlation with solvent dielectric constant values.

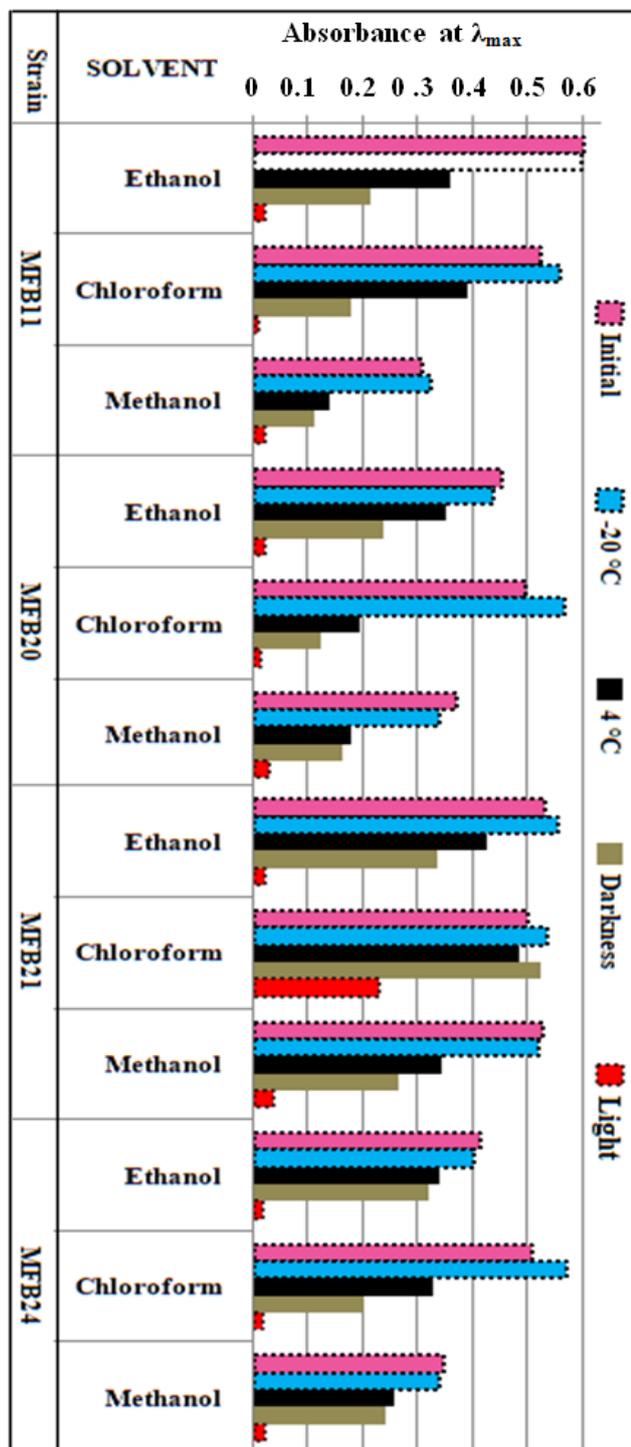


Figure 6. Absorbance at maximal wavelength (λ_{max}) of the pigments before (Initial) and after 30 days incubation at different conditions

After 30 days incubation at four conditions, results revealed mostly a large decrease of absorbance at the pigment specific λ_{max} . Drastic decrease of absorbance in pigment samples were noted under light exposition. This indicates the photosensitive property of the pigments

causing its molecular saturation or degradation. Incubation of the pigment at room temperature even in darkness condition leads to decrease of absorbance than at 4°C. These results are in agreement with anthracyclines studies indicating their photosensitivity and thermo-sensitivity [30,34]. The best temperature for conservation was obtained at -20°C. However, difference between solvent score after storage incubation was hard to highlight because difference in initial absorbance according to each solvent. The comparison need to be made only for each solvent to pigment initial absorbance. In this case all solvent were equivalent for storage at -20°C. However, for MFB-21 pigment in chloroform, incubation at room temperature was equivalent to that at 4°C and near to that at -20°C. Moreover, MFB21 pigment in chloroform was the most resistant to light effect and hence confirming that this MFB21 pigment in chloroform was the less thermo-sensitive and photosensitive than the other four strains pigments. These results indicate difference in interaction of each pigment with the solvents used.

4. Conclusion

Streptomyces coelicoflavus is a species known to produce important bioactive compounds but not anthracycline red-like pigments. This study highlight for the first time strains of *S. coelicoflavus* producing pink red-like pigments. Intracellular localization, photosensitivity and UV-Vis spectral characteristics of these pigments are highly consistent with those of hydrophobic anthracyclines and aglycone portion of water soluble ones. However, pigments negative antibacterial assay, TLC profiles (band's number, Rf and colors) and different interactions with solvents indicated specific diversity of the pigments produced by the studied strains. The five strains could be an important sources of new compounds or analogues of anthracyclines with possible interesting use in cancer chemotherapy. Next efforts on cancer cell antiproliferative effect and structural determination will be important steps to get more informative elements about these pigments.

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