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Detection of carotenoids in psychrotrophic bacteria by spectroscopic approach

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ABSTRACT

The combination of Raman and Infrared spectroscopic signatures were used to find the different vibrational modes of individual carotenoid as their spectral fingerprint. Both have been previously demonstrated to be highly useful methodology for the identification and/or typing of microorganisms. In this study, we set out to evaluate whether these technologies could be applied to detect the presence of carotenoids in psychrotrophic bacterial isolates. FTIR and Raman spectra of four psychrotrophic bacteria viz. *Kocuria rosea*, *K. turfanensis*, *Sanguibacter suarezii* and *Planococcus maritimus* were examined during the investigation. FTIR spectra bands at 1653-1661cm^{-1} in different samples were assigned as part of chlorophyll, 1424-1426 cm^{-1} as -C-H- (CH_2) bending vibration from methylene of carotenoids or lycopene, 1366-1367 cm^{-1} band as the β-ionone ring of β-carotene due to the C-H, (–CH3) symmetrical bending. Interestingly, Raman spectra revealed intense Raman bands in the range of 1511-1530, 1153-1159 and 1003-1010 cm^{-1} representing bacterial carotenoids. We hypothesize the biosynthesis of carotenoid as adaptive strategy to cope up inhospitable cold environments of Leh and Ladakh. The strong, scattering bands by different isolates attributable to ν(C=C) phase stretching, ν(C-C) and δ(C-CH_3) methyl components systems, which could be probably membrane-associated C_{50} carotenoids. Their high intensities are due to resonance enhancement. It can be concluded that Raman spectroscopy is a sensitive and convenient detection tool for typing of the bacterial biomarkers with less time consumption.

Key words: pigments, carotenoids, Raman spectroscopy, FTIR, psychrotrophic bacteria

Introduction

The psychrophilic and psychrotrophic bacteria have evolved a complex range of adaptations to all of their cellular components, including their membranes, energy generating systems, protein synthesis machinery, biodegradative enzymes and the components responsible for nutrient uptake (Russel & Hammamoto, 1998). They produce a higher content of unsaturated, polyunsaturated and methyl-branched fatty acids, and/or a shorter acyl-chain length with a high proportion of cis-unsaturated double bonds and antesio-branched fatty acids. Besides, they have higher content of proteins with lipid head groups and non-polar carotenoid pigments (Salvino et al., 2006).

Carotenoid pigments represent the largest and most diverse class of natural products known to mankind. Till date over 700 structures have been reported from plants, algae, fungi and bacteria (Britton et al., 2004; Fons et al., 2011). Carotenoids belong to the category of tetraterpenoids with 40 carbon atoms, being built from four terpene units each containing 10 carbon atoms. Structurally, carotenoids take the form of a polyene hydrocarbon chain which is sometimes terminated by rings, and may or may not have additional oxygen atoms attached. Their colour, ranging from pale yellow through bright orange to deep red, is directly linked to their structure (Armstrong, 1997; Liu et al., 2005; Alija,

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2005; Rao & Rao, 2007).

Carotenoids are long strands of polyisoprenoid hydrocarbons found naturally occurring in microorganisms, plants and even animals. They are synthesized in a variety of bacteria, algae, fungi via non-mevalonate pathway in response to various environmental stresses and for protection against low wavelength radiation in exposed terrestrial habitats (Edwards et al., 2006a), whereas animals have to obtain them from food (Krinsky et al., 1994). Since a large number of Antarctic bacteria have been found to contain carotenoid type of pigments in their membrane, it has been speculated that these pigments may have a role in buffering membrane fluidity due to their ability to localize in the membrane as they interact with cell membranes and increase the rigidity of the membrane (Strand et al., 1997). These are lipid soluble class of molecules associated with the lipidic fractions sensitive to oxygen, heat and light (Higuera et al., 2004). However, these adaptive strategies do not seem to be widespread. Studies show more compact lipid head groups and decreased non-polar carotenoid pigment synthesis in some psychrophiles (Fong et al., 2001). A study on psychrophilic bacteria from Antarctica (*Sphingobacterium antarcticus* and *Micrococcus roseus*) showed that the synthesis of carotenoids was temperature dependent with an increase in the synthesis of polar carotenoids at low temperature of growth so as to stabilize the membrane (Chattopadhyay et al., 1997; Jaggandham et al., 2000).

Vibrational spectroscopic techniques viz FTIR (Fourier Transform infrared) and Raman spectroscopy have been applied to a variety of prokaryotic and eukaryotic cell types since beginning of 1990s. Use of these techniques owes the aim of discrimination, classification and identification of microorganisms. Structural details provided by Raman and infrared spectra are complementary and can be used to find different vibrational modes of each individual carotenoid.

Raman spectroscopy can provide detailed information on molecular vibrations, and has been successfully employed in many areas of investigation. Some of the earlier reports (Edwards et al., 2006b) demonstrated the survival strategies adopted by extremophilic bacteria by employing Raman spectroscopy through the identification of key biomolecular signatures of the protective chemicals synthesized by organisms in stressed environments. Carotenoids are also known as essential light sensitive molecules, excite very strong Raman intensities (900 - 1600 cm^{-1}) especially when resonantly excited in their π-› π* electronic absorption transition in visible (violet/green) wavelength because of the molecular structure of polyene molecule (Kollias et al., 1998; Tissa et al., 2000; Schulz et al., 2005). Three intense Raman scattering modes of carotenoids are defined as υ1- conjugated C=C stretching vibrations, υ_2- C-C vibrations coupled to C-CH_3 stretching or C-H in plane bending and υ3- CH_3 stretching modes (Schulz et al., 2005; Rimai et al., 1973; Tschirner et al., 2009), of these three, υ_1 band is the most diagnostic for carotenoid identity (Collins et al., 2011).

FTIR, on the other side, is a technique which is used to obtain an infrared spectrum, emission, photoconductivity of a solid, liquid or gas. FTIR that operates in the mid infrared region (4000-400 cm^{-1}) is a powerful tool for quantitative analysis of fats, oils and palm carotene (Moh et al., 1999). The IR bands of the characteristic functional groups (CH_3, CH_2, C=C, C=O, OH, *etc.*) can be assigned when possible. Some special functional groups such as C=C=C, 'cross epoxides', *etc.*, which cannot be easily identified by ^{1}H-NMR methods, can be detected in the FT-IR spectra (Lorand et al., 2002).

The present study is aimed to investigate the potential of vibrational spectroscopy (IR and Raman spectroscopy) to detect the presence of carotenoids biosynthesized by psychrotrophic bacteria as a potential biomarker from cold environments of Leh and Ladakh, India.

Material and Methods

Organisms and growth conditions

Psychrotrophic bacteria were isolated from the soil samples collected from Leh and Ladakh which are situated at an altitude of 11,562 and 19,700 ft., respectively in Himalayas. The molecular characterization validated the isolates as *Sanguibacter suarezii* KK6 (accession no. JN255745), *Kocuria turfanensis* KK7 (accession no. JN255748), *Kocuria rosea* KK12 (accession no. JN638047) and *Planococcus maritimus* KK21 (accession no. JN638058). These were cultured in Antarctic Bacteria Medium (ABM) containing peptone (0.5% w/v) and yeast extract (0.2% w/v) with continuous shaking in an incubator at 10°C as described by Shivaji et al. (2005). Cultures were incubated on a rocking platform up to two weeks.

Sample preparation for FTIR analysis

The growth medium was carefully decanted from the adherent bacterial cells. After a gentle wash step with 15 ml Phosphate Buffer Saline (PBS) (140 mM NaCl, 2.7 mM KCl,

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10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), the cells were collected from the flasks, transferred in 10 ml fresh PBS and centrifuged for 30 min at 4000 g.

After aspiration of the supernatant, the cells were resuspended in 15 ml autoclaved milli-Q water and centrifuged for 30 min at 4000 g. Harvested bacterial cells were then freeze dried (lyophilized), sealed with paraffin wax and stored at 4°C. Prior to FTIR analysis the resulting lyophilized cell pellets were transferred to 15 ml 95% methanol (HPLC grade) in sterile Eppendorf tubes and gently sonicated in an ice water bath (two 30 sec. bursts at 17 W). Samples were allowed to extract for 12 h at -20°C. After extraction, the supernatant was collected by centrifugation (5000 rpm for 5 min at 4°C). If the sample still appeared to be cloudy the centrifugation step was repeated.

The FTIR spectra were obtained with portable exoscan attenuated total reflectance FTIR (A2 technologies) with omnic software and an internal reflection accessory made of Composite Zinc Selenide. The pigmented extracts of samples were measured without any preparation, directly on the ZnSe ATR (Attenuated Total Reflection) crystal in the registered spectrum of 3000-800 cm^{-1}.

Sample preparation for Raman analysis

Sample preparation for Raman analysis was same as done for FTIR sample preparation up to aspiration step. After aspiration of the supernatant, the cells were resuspended in 1.5 ml autoclaved milli-Q H_2O by using a 2 ml syringe equipped with a 21G needle. Subsequently, the suspension was transferred to a 2 ml Eppendorf tube, followed by centrifugation for 10 min at 12,000 g in an Eppendorf centrifuge. The supernatant was removed and the cell pellets were stored at –80°C until use. Samples were thawed at room temperature and centrifuged for 1 min at 12,000 g. Next, the supernatant was removed and the wet pellet was transferred to a one cavity fused silica microscope slide (Hellma Benelux). Samples were allowed to dry for 20 min at 35°C. Raman spectra of each isolate were measured in independent run.

Resonance Raman spectroscopy

A Renishaw InVia Reflex Raman microprobe spectrometer with a multi-wavelength facility was used to assess the effect of excitation wavelength on the recording of spectra from the psychrotrophic isolates. The collection optics is based on a Leica DMLM microscope. A refractive glass 50X microscopic objective lens was used to focus the laser onto a 2 μm spot to collect the backscattered radiation. Approximately 5-10% of 300 mW laser light of 785 nm (diode laser) with integration time of 20 seconds was used to excite the sample on measurement compartment, which holds the fused silica slide with the bacterial samples. The spectrometer was calibrated according to the manufacturer's guidelines. The scan ranges were 200–2000 cm^{-1} in the fingerprint region of resonance-enhanced Raman spectra obtained from carotenoids.

Results and Discussion

To assess whether FTIR and Raman spectroscopy can be used as typing methods, psychrotrophic bacterial isolates were individually examined. Level of homogeneity was too high in the spectra of all the isolates, indicating a strong similarity in molecular composition of carotenoids. During the study the data from Raman and Infrared spectroscopy was analyzed to find out the different vibrational modes of each individual carotenoid. These two types of spectroscopy incorporate vibrational transitions that occur during the scattering of light by molecules in Raman, and the absorption of infrared radiation by the molecules in Infrared absorption. At room temperature, almost all molecules are in their lowest vibrational energy levels. This is referred to the ground state and has a quantum number, v=0. Many fundamental vibrational transitions can be studied by using Raman and Infrared spectroscopy. On the contrary, these types of spectroscopy have different selection rules. Raman active modes involve a change of polarizability while IR active modes involve a change of dipole moment.

Beta carotene was taken as standard and sample was scanned in the registered FTIR spectrum of 3000-800 cm^{-1}. The characteristic peaks identified in the fingerprint zone are represented in Figure 1. In *Planococcus maritimus* KK21 first intense peaks in pigment fingerprint area are at 1654.01, 1425.17, 1157.85, 1107.72, 1021.09 and 701.53 cm^{-1}, in *Kocuria turfanensis* KK7 and *K. rosea* KK12 fingerprint peaks are at 1655.47and 1653.31, 1426.03 and 1426.18, 1367.82 and 1366.31, 1315.24 and 1315.00, 1158.35 and 1158.25, 1107.81 and 1107.59, 1054.58 and 1055.95, 1023.29 and 1021.41, respectively.

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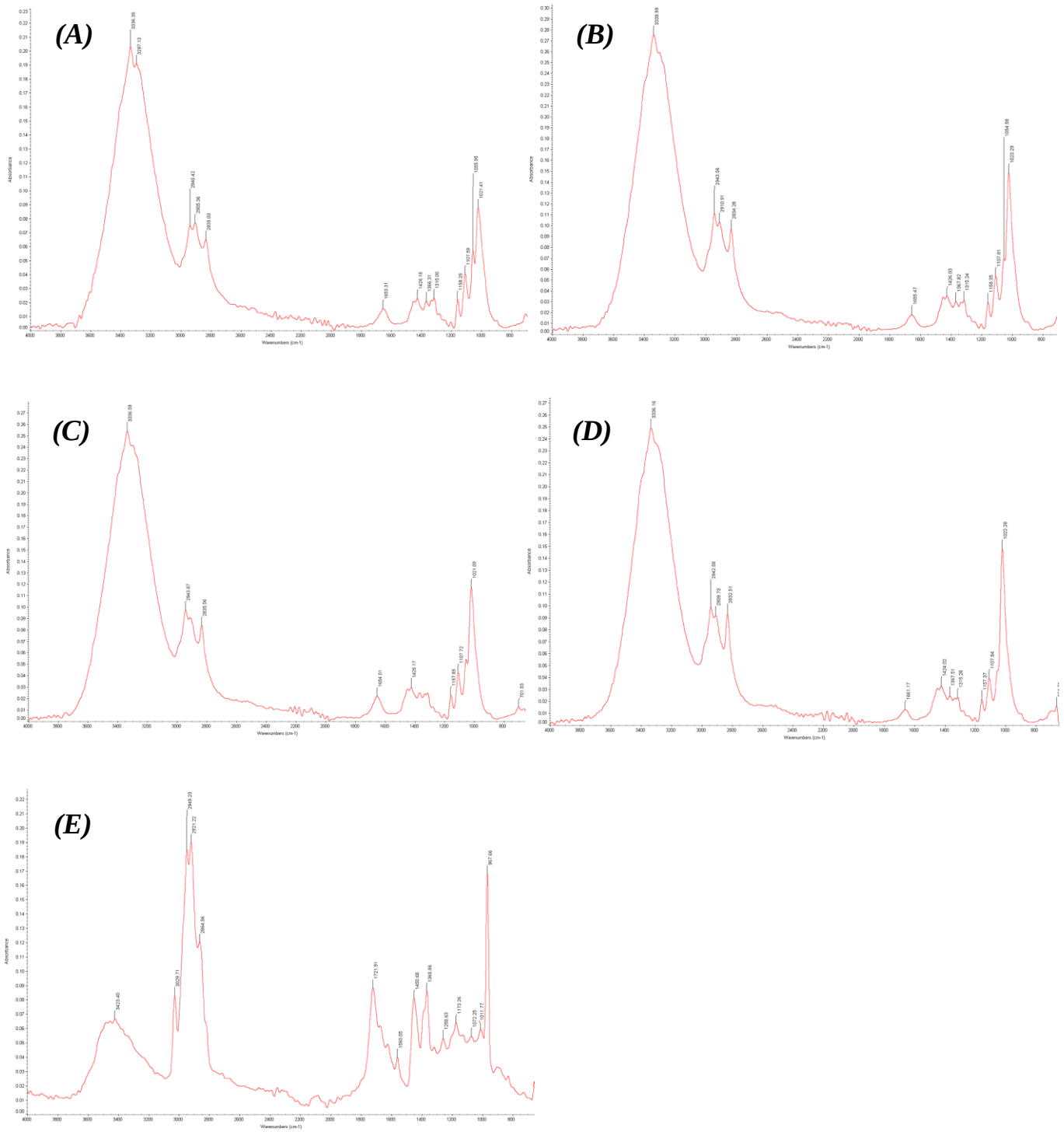

Figure 1. *FTIR fingerprint region (800-3000 cm^{-1}) and identification of carotenoids signals: (**A**) Kocuria rosea, (**B**) Kocuria turfanensis, (**C**) Planococcus maritimus, (**D**) Sanguibacter suarezii (**E**) beta-carotene (reference standard).*

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Yellow pigmented bacterial strain of *Sanguibacter suarezii* KK6 however gave fingerprint peaks at 1661.17, 1424.02, 1367.51, 1315.28, 1157.37, 1107.84, 1022.28 cm^{-1} as depicted in Figure 1. Peaks at 1661, 1653, 1654 and 1655cm^{-1} are caused by chlorophyll and protein content (Tavitian et al., 1986; Parlog, 2011; Konwar & Baruah, 2011). The next representative band at approximately 1425, 1426 and 1424 cm^{-1} appears due to the bending vibration of methylene $–CH_2$ (scissoring) which is also seen in beta carotene standard fingerprint peak at 1450.68 cm^{-1} and can be assigned to lycopene pigments (Parlog, 2011), while peak between 1366-1367 cm^{-1} in *S. suarezii* KK6, *K. turfanensis* KK7 and *K. rosea* KK12 are attributed to the β-ionone ring of beta-carotene due to the C-H, ($–CH_3$) symmetrical bending (Abdul et al., 2010; Parlog, 2011) and also observed in standard beta carotene fingerprint region. Last, peak observed at 701.53 and 670.85 cm^{-1} in *P. maritimus* KK21 and *K. rosea* KK12, respectively is probably due to the methylene CH_2 rocking band from a chain.

Infrared spectroscopy was used to obtain a signature about the outer structure of the carotenoids. There are many distinct features in the infrared spectra. The analysis of Infrared spectra was much more difficult as compared to Raman spectroscopy. One factor that affected the classification process was oxidation. When analyzing the literature for β-carotene spectrum, vibration at approximately 1149 cm^{-1} is attributed to C-O stretching, as it is around 1157 or 1158cm^{-1} in our samples. This is most likely due to oxidation. Vibrational peaks at around 1121, 1122, 1123 cm^{-1} in different pigment extracts show deformation in polyene chain, same as reported earlier by Yuan (2012) where he found polyene ring deformation at 1025cm^{-1}. Not much research has been done on detection of bacterial (psychrotrophic) carotenoids employing FTIR (ATR) spectroscopy, hence for better reliability and confirmation of carotenoids in psychrotrophic bacteria we opted for Reflex Raman spectroscopy.

Interestingly, the spectra from most of the isolates showed range of three regions with strong signal intensities. The Raman spectrum (Figure 2) of strain KK6 with yellow colour produced Raman spectra at 1530, 1159 and 1006 cm^{-1}, KK21 with orange colouration showed strong bands at 1519, 1158 and 1006 cm^{-1}, whereas KK7 and KK12 which had pinkish orange tint gave strong Raman bands at 1511, 1153 and 1003 cm^{-1} (weak bands at 1448, 1286 and 954 cm^{-1}) and 1512, 1154, 1004 cm^{-1} (weak bands at 1284 and 957 cm^{-1}), respectively. Raman spectra giving weak signal intensities in the range of 1003-1010 cm^{-1} are due to C-CH_3 (υ_3) rocking motions of the methyl group, very strong Raman signals in the band range of 1153-1159 $cm^{-1}$are C-C phase stretching of conjugated backbone of carotenoid structure and strong signal intensities between 1511-1530 cm^{-1} in different isolates is C=C phase stretching of the same conjugated backbone of carotenoid structure. The results obtained during the study corroborated with Shibata et al. (1986) who recorded strong scattering bands of *Bacillus megaterium* at 1515 cm^{-1} and 1157 cm^{-1} and concluded them as conjugated double bond systems probably membrane associated carotenoids despite of high dipicolinic acid content in *Bacillus* endospores. Raman laser beam of 785 nm has been used earlier by Buijtels et al. (2008) for the identification of several *Mycobacteria* spp. which gave peak intensities at 1150 and 1520 cm^{-1}. Earlier work on Raman typing of bacteria has demonstrated that these parts of spectrum represent bacterial carotenoids (Kirschner et al., 2001; Scholtes-Timmerman et al., 2009; Maquelin et al., 2009). Raman bands in the same band spectrum range of carotenoids were interpreted earlier as the strategies adopted by extremophilic colonies from mars to ensure their survival and degradation of organic material to carbon, as they recorded Raman bands of degraded organic material also (Edwards et al., 2006a; 2007). In previous reports it has been depicted that in cyanobacteria the scytonemins are exclusively produced as a UV radiation protectant biomolecule (Edwards et al. 2006a; 2007). In all the isolates the carotenoids (which were recorded by Raman spectroscopy) are hence concluded as survival strategy in stressed environments and are responsible for the yellow-orange pigmentation in bacterial colonies.

Different excitation wavelengths in Raman microspectrscopy can be used, as we used 785 nm diode laser to identify biomarkers in different microorganisms. Heraud & Beardall (2000) demonstrated the presence of ß-carotene and chlorophyll a in individual cells of *Dunaliella tertiolecta*. In healthy and starved colonies of algae *Chlorella sorokiniana* and *Neochloris oleoabundans* carotenoids, chlorophylls and triglycerides were detected using confocal Raman microscopy at 532 nm (Huang et al., 2009). Raman spectroscopy has already been proved as a feasible technique for the detection of various organic compounds and fossilized microorganisms in rock samples (Wynn-Williams & Edwards 2000; Edwards et al., 2005; Marshall et al., 2007; Vitek et al., 2009).

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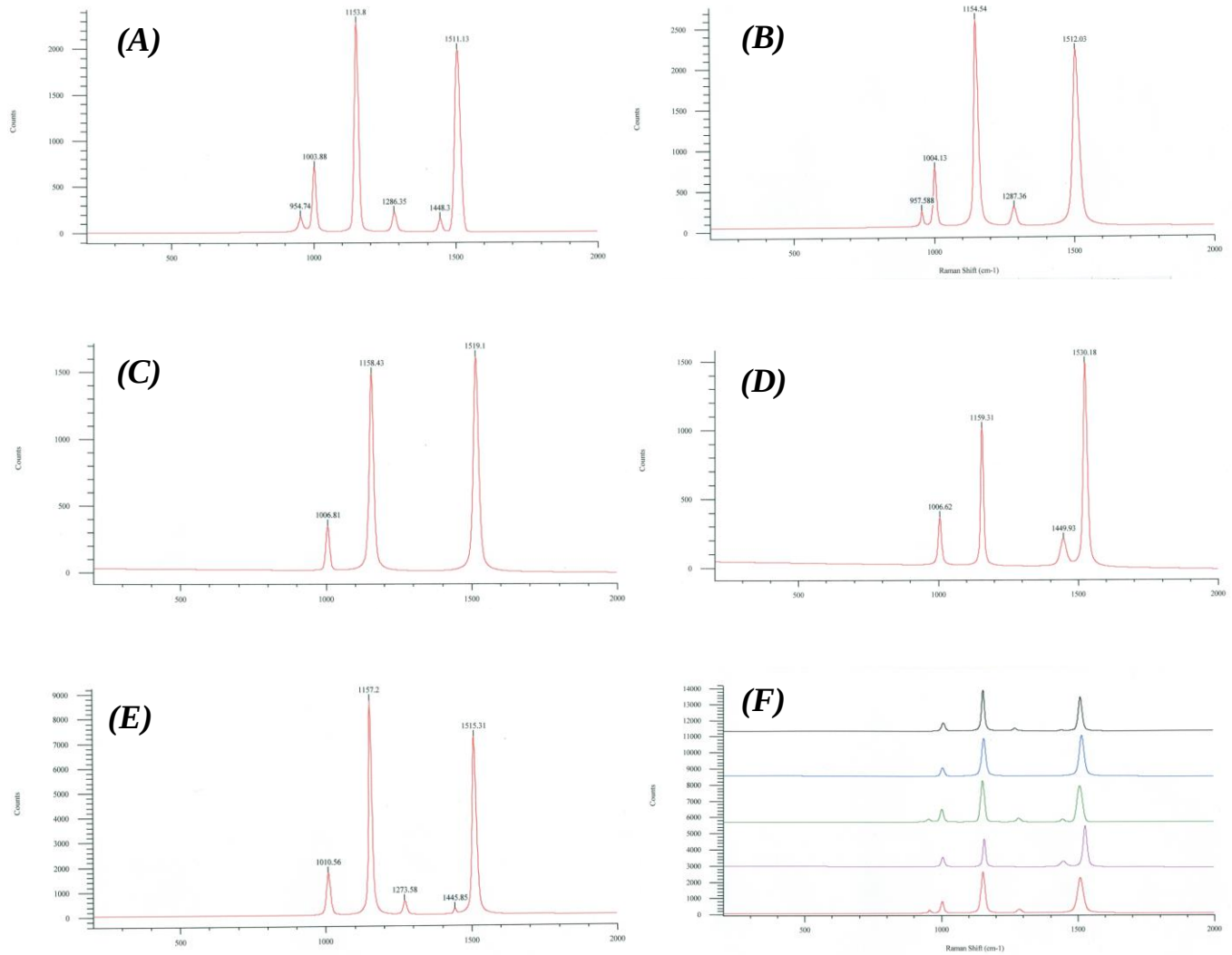

Figure 1. *Raman spectra of psychrotrophic bacterial isolates and identification of carotenoids signals:* ***(A)*** *Kocuria rosea,* ***(B)*** *Kocuria turfanensis,* ***(C)*** *Planococcus maritimus,* ***(D)*** *Sanguibacter suarezii,* ***(E)*** *Beta- carotene and* ***(F)*** *Raman spectra of all the bacterial samples compared with standard (Beta-carotene), having a relatively high carotenoid content with low signal intensity spectral bands in the range 1003-1010 cm^{-1} due to C-CH_3 (υ) rocking motions of the methyl group, very strong Raman signals in the band range of 1153-1159 cm^{-1} are C-C phase and strong signal intensities between 1511-1530 cm^{-1} in different isolates is C=C phase stretching of the same conjugated backbone of carotenoids structure.*

Nevertheless, to the best of our knowledge, this is first time that the presence of carotenoids in psychrotrophic strains of *Kocuria*, *Planococcus* and *Sanguibacte*r has been reported employing vibrational spectroscopy. Pigmentation in these psychrotrophic isolates is responsible for the yellow-orange shade of bacterial colonies and can be concluded as key biomarker feature as survival strategy in cold environment of Leh and Ladakh, India.

Conclusion

The experimental data collected during the study show that FTIR and Raman spectroscopy can be employed as a fundamental next-generation instrument for the characterization and detection of biomarkers. It can be concluded from the results that Raman spectroscopy holds great promise as a rapid, accurate and easy to use alternative

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for the identification of carotenoids in psychrotrophic species (in our case) when only small sample amount is available in contrast to HPLC which is often expensive, tedious and prohibitively requires large samples. Vibrational spectroscopy can be successfully used as a rapid, alternative to the expensive chromatographic techniques, providing significant data for sample classification.

Both Raman spectroscopy and Infrared spectroscopy proved effective in analyzing the full fingerprint of carotenoids. IR Spectroscopy provided information about the outer structures, whereas Raman provided with a signature of the inner structure with minimal information about the outer structure. The combined use of Raman and Infrared spectroscopy is a great way to determine a full spectral fingerprint of carotenoids. The combined data acquisition needs to be improved and will be studied in future research.

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