Appl Biochem Biotechnol (2009) 159:478–487
DOI 10.1007/s12010-008-8472-0

Raman Spectroscopy for Intracellular Monitoring of Carotenoid in *Blakeslea trispora*

Emmanouil H. Papaioannou · Maria Liakopoulou-Kyriakides · Dimitrios Christofilos · Ioannis Arvanitidis · Gerasimos Kourouklis

Received: 5 September 2008 / Accepted: 2 December 2008 /
Published online: 6 January 2009

Abstract In the present study, we explore the feasibility of Raman spectroscopy for intracellular monitoring of carotenoid in filamentous fungi *Blakeslea trispora*. Although carotenoid production from this fungus has been extensively studied through various chromatographic methods and ultraviolet-visible spectroscopy, no intracellular monitoring has been demonstrated until now. The intensity of the Raman spectrum, and more conveniently that of the strongest ν_1 carotenoid band at ~1,519 cm^{-1}, exhibits a good linear correlation with the carotenoid content of the sample as determined by high-performance liquid chromatography (HPLC) and ultraviolet-visible (UV-Vis) spectroscopy. Our results suggest that Raman spectroscopy can serve as an alternative method for the study and quantification of carotenoid in batch-mated submerged cultivations of *B. trispora* and similar organisms. Although not as accurate as HPLC, it allows a rapid sampling and analysis, avoiding the prolonged and tedious classical isolation procedures required for carotenoid determination by HPLC and UV-Vis spectroscopy.

Keywords β-carotene · Lycopene · Carotenoid · *Blakeslea trispora* · Raman spectroscopy · RP-HPLC-DAD

E. H. Papaioannou · M. Liakopoulou-Kyriakides (✉)
Section of Chemistry, School of Chemical Engineering, Faculty of Engineering,
Aristotle University of Thessaloniki, Thessaloniki 54124 Greece
e-mail: markyr@vergina.eng.auth.gr

D. Christofilos · I. Arvanitidis · G. Kourouklis
Physics Division, Faculty of Engineering, Aristotle University of Thessaloniki,
54124 Thessaloniki, Greece

I. Arvanitidis
Department of Applied Sciences, Technological Educational Institute of Thessaloniki,
57400 Sindos, Thessaloniki, Greece

Introduction

Carotenoids is one of the most important groups of natural pigments, with all-trans-β-carotene being the major hydrocarbon carotenoid possessing the higher vitamin A activity than all the others. It was isolated for the first time from carrots (where its name was derived from) in 1831 by Wackenroder, whereas its structure was elucidated in 1931 by Karrer [1]. The vitamin A activity of all-trans-β-carotene in combination with its color and antioxidant properties makes it very attractive in food and feed industry, as well as in medicine and cosmetic formulation [1].

Despite the availability of a variety of natural and synthetic carotenoids, only a few, including β-carotene and astaxanthin, are commercially produced by microbial cultivation [2]. Carotenoid biotechnological production is replacing the well-established chemical synthesis routes. This is due to a number of factors, including consumers' will for "all-natural" products and the continuous growth of the market [3]. Among the most efficient microorganisms in β-carotene production is the fungus *Blakeslea trispora*, which accumulates preferentially intracellular all-trans-β-carotene into lipid inclusions [4]. The studies about the production of all-trans-β-carotene with *B. trispora*, as well as its biosynthetic pathway, are numerous [5, 6]. UV spectroscopy [4, 7–9] and reverse-phase and normal-phase liquid chromatography procedures [5, 6, 10–14] have been used for quantifying the total carotenoid accumulation during or at the end of cultivation process after extraction using various organic solvents. Depending on the quantification method, the amount of detected carotenoids may be either simply attributed to β-carotene [7–9] or individual carotenoids may be expressed as a percentage of total carotenoids [2]. However, the development of reliable and fast methods for carotenoid determination in various media without or with minimal sample treatment prior to analysis still remains an important issue. Towards this direction, Raman spectroscopy could provide an alternative tool for the determination of the carotenoid content in the studied system.

The Raman effect is the exchange of momentum and energy between impinging light photons and the vibrational modes of matter through the inelastic scattering of light, and was first described by Raman [15]. In the case of carotenoid molecules, a resonance enhancement effect of the Raman signal was first described by Euler and Hellstrom [16]. According to Weesie et al. [17], the polyene carbon backbone of the molecule is responsible for the resonance. As the substitutions on the terminal rings have little effect on the Raman spectrum, the β-carotene and other C40 carotenoids spectra are very similar and cannot be easily distinguished. Raman spectra of wet biomass samples can be obtained, without major interference from water vibrations and the cell microenvironment, eliminating the problem of rapid carotenoid degradation when they are isolated from their natural microenvironment. The analytical potential of resonance Raman spectroscopy became obvious in 1970, when Gill et al. [18] detected lycopene and β-carotene in intact plant samples (carrot and tomato), as well as in carrot juice and tomato sauce. The resonance enhancement was sufficiently strong to overcome losses due to absorption of the exciting and scattered photons, as well as interference from fluorescence. The authors noted that small shifts in vibrational frequencies between the plant spectra and those of reference samples in *n*-hexane were probably due to solvent effects and suggested that this phenomenon could be probably used to probe the local environment. In addition, resonance Raman spectroscopy was demonstrated to be an accurate non-invasive technique for in vivo detection of carotenoids in skin. By means of selective excitation, lycopene and β-carotene can be determined and distinguished in the case of skin samples [19]. Finally, Raman spectroscopy has been also successfully used for online monitoring of the astaxanthin production, in the

case of single-cell yeast *Phaffia rhodozyma* [3], and for quantification of zeaxanthin production from bacterium *Flavobacterium multivorum* [20] as well as of carotenoids in tomatoes and related products [21, 22]. In the present work, Raman spectroscopy is employed for the first time in the quantification of total carotenoid produced in batch-mated submerged cultivations of filamentous fungus *B. trispora* in different media.

Experimental Section

Reagents and Solvents KH_2PO_4, $MgSO_4 \cdot 7H_2O$, casein hydrolysate, and yeast extract were purchased from Scharlau Chemicals S.A. Barcelona, Spain. L-asparagine was from Fluka Chemicals, India. Tween 80 and starch were obtained from J.T. Mallinckrodt Baker B.V., Deventer, Holland. Span 20, PDA, BHT, thiamin-HCl, all-trans β-carotene (type II synthetic), lycopene (from tomato), and linoleic acid were purchased from Sigma-Aldrich Inc. Chemicals Co, Steinheim, Germany. Corn Steep Liquor was from Tyte & Lyle, Thessaloniki, Greece. D-glucose was purchased from Riedel-deHaën, Seeize, Germany. Olive oil, soybean oil, and sunflower oil were obtained from local market. EtOH, reagent grade, was from Carlo Erba Reagents, Milano, Italy, and MeOH, high-performance liquid chromatography (HPLC) grade (LiChrosolv), from Merck KGaA, Darmstadt, Germany. Teflon membrane filters 0.2 μm, 13 mm, were obtained from Alltech Associates Inc, IL, USA.

Microorganism B. trispora ATCC 14271, mating type (+), and *B. trispora* ATCC 14272, mating type (−), were used.

Cultural Condition The strains were grown separately in Petri dishes with PDA for 3 days at 26 °C and used for the inoculation of the culture medium.

Cultivation Conditions The batch cultivation experiments were carried out in 500-ml Erlenmeyer flasks. The main broth consisted of (g/l): casein hydrolysate 2.0, yeast extract 1.0, L-asparagine 2.0, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, Span 20 10.0 and Tween 80 1.0, corn steep liquor 80.0, BHT 0.02, and thiamin-HCl 0.005. In media 1–3 (Table 1), 50 g/l D-glucose was added as carbon source while 2% *v*/*v* sunflower, olive oil, and equal-volume mixture of those oils with soybean oil were added as co-substrates, respectively. In the case of medium 4, 50 g/l starch was alternatively used as carbon source and 2% *v*/*v* linoleic acid as co-substrate.

Table 1 Nutrition media composition.

Medium number	Composition (g/l)[a]		Composition (% *v*/*v*)			
	D-glucose	Starch	Linoleic acid	Sunflower oil	Soybean oil	Olive oil
1	50	–	–	2	–	–
2				–		2
3				0.67	0.67	0.67
4	–	50	2	–	–	–

*All media contain also (g/l): corn steep liquor 80.0, Span 20 10.0, Tween 80 1.0, casein hydrolysate 2.0, yeast extract 1.0, L-asparagine 2.0, KH_2PO_4 1.5, $MgSO_4x7H_2O$ 0.5, BHT 0.02 and thiamin-HCl 0.005

Carotenoid Raman analysis The samples used in Raman experiments were withdrawn from culture broth after 39, 48, 60, 67, 72, 120, 192, or 240 h, filtered, and washed with distilled water several times until the filtrate became colorless. The filtration and washing procedures were adopted to avoid the direct observation of fungus into the submerged culture, as it forms hyphae clusters that are not homogeneous all over the culture. The samples were not subjected to further treatment in order to avoid carotenoid losses; they had a thickness of a few millimeters and a uniform macroscopic appearance, despite the fact that they consisted of dense fungal rod hyphae that could be discriminated by an optical microscope. Raman spectra were recorded in the back-scattering geometry by using a microscope-equipped triple monochromator (DILOR XY) combined with a Wright cryogenic CCD detector. The spectral width of the system was ~2.5 cm^{-1} at the 514.5-nm line of an Ar^+ laser used for excitation, whereas the laser power—measured before the ×10 standard microscope objective of the microscope—was ~0.1 mW. For each sample, spectra of 60 s and two accumulations from ten randomly selected positions on the sample were averaged. The same samples were consequently treated for determining their carotenoid contents by HPLC and UV-Vis spectroscopy and compared with the results from Raman spectra analysis.

RP-HPLC-DAD analysis of cellular carotenoids The carotenoids were extracted from fungus wet biomass after repeatable extractions with 3 ml EtOH, as previously reported (Papaioannou and Liakopoulou-Kyriakides, submitted for publication). The whole isolation procedure was carried out in diffuse daylight and the samples were kept at −20 °C to avoid oxidation or isomerization. All samples were filtered through a 0.45-μm membrane filter prior to analysis. The injection volume of the samples was 20 μl. A Vydac C18 201TP54, 5 μm, 250×4.6-mm column was used, with a flow rate of 1.5 ml/min and isocratic elution with MeOH. The system was equipped with a Q-Grad quaternary gradient pump of LabAlliance and a photodiode array detector (Finnigan Spectral System UV6000LP). Analysis was performed with the EZChrom Elite program. The identification of carotenoids was accomplished by comparing the retention times and absorption spectra with reference compounds and the characteristics of the absorption spectra previously reported [23–25].

UV determination of carotenoids Total carotenoid content was estimated spectrophotometrically at λ_{max}=450 nm by a ten-point standard calibration curve with reference β-carotene and expressed as milligram of total carotenoids produced per gram of dry biomass.

Results and Discussion

Indicative Raman spectra at the fingerprint spectral region of carotenoids from samples acquired during the cultures of *B. trispora* are illustrated in Fig. 1. The spectra presented refer to nutrition medium 1, while the other media exhibited quite similar Raman profiles with intensity variations depending on the carotenoid produced in the mycelium at the time of sampling. Three strong peaks appear at approximately 1,519, 1,156, and 1,005 cm^{-1}, whereas less intense peaks appear at 1,192, 1,212, and 960 cm^{-1}. Assignment of the most characteristic peaks can be made following the literature. In the case of crystalline β-carotene, the Raman spectrum is dominated by three main bands at 1,516, 1,156, and 1,007 cm^{-1} designated as ν_1, ν_2, and ν_3, respectively [26]. Normal-coordinate analysis has assigned these three bands predominantly to C=C in-phase stretching, C–C stretching, and

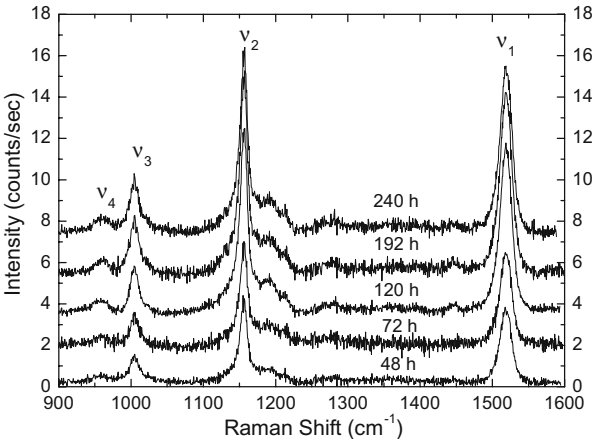

Fig. 1 Raman spectra of carotenoids in cells of filamentous fungus *B. trispora* in the case of nutrition medium 1 at various times of the cultivation process. The fluorescence background of the spectra was subtracted and the spectra were appropriately shifted for clarity

methyl in-plane rocking modes, respectively [27]. A weak band appearing at around 960 cm^{-1} in solution, the ν_4 band, is assigned to the C–H out-of-plane wagging.

The frequencies and the relative intensities of the modes and especially those of the ν_1 peak are crucially depending on the specific carotenoid and its environment. The frequency of the ν_1 peak decreases with increasing length of the conjugated central polyene chain due to electron–phonon coupling [28]. For example, its frequency is 1,528 cm^{-1} for β-carotene dissolved in acetone (nine conjugated double bonds) and shifts to 1,516 cm^{-1} for lycopene in the same solvent (11 conjugated double bonds) [29]. Nevertheless, all bands of β-carotene and lycopene were observed at the same spectral regions: 1,500–1,550, 1,150–1,170, and 1,000–1,020 cm^{-1} [30, 31]. In the solid state, the ν_1 band frequency decreases as well; in solid lycopene, the ν_1 band is located at 1,514 cm^{-1} and in solid β-carotene at 1,515 cm^{-1} [22]. Moreover, recent reports have also shown that the solution pressure and the solvent nature affect the Raman spectra of all-trans-β-carotene [32]. The lower frequency of the ν_1 band in CS_2 (1,521 cm^{-1}) as compared to that in *n*-hexane (1,526 cm^{-1}) is rooted [32], through the vibronic coupling of this mode, to the lower S_1 energy level and its alterations is induced by dispersive interactions for the case of non-polar solvents [33].

In our case, only one symmetric peak is resolved in the spectral region of the ν_1 band, at 1,519 cm^{-1}, for all media and sampling times. This is in contrast, for example, to the case of orange tomatoes where a two-peak structure appears with the higher intensity band near 1,520 cm^{-1} and a shoulder at 1,510 cm^{-1} due to higher amounts of β-carotene (1,520 cm^{-1}) in comparison to lycopene (1,510 cm^{-1}) [34]. Such a lower frequency shoulder is not evident in our spectra despite the fact that not only β-carotene (60% by HPLC estimation) but also γ-carotene and lycopene coexist. The frequency of the ν_1 peak of γ-carotene is expected to lie in between those of β-carotene and lycopene, as the former possesses ten conjugated double bonds with respect to nine and 11 conjugated double bonds of the latter two. Moreover, as the carotenoids are stored inside lipid inclusions [4], their corresponding ν_1 peak is expected to be shifted and broadened depending on their microenvironment. We conclude that the observed peak originates, in principle, from all carotenoids present, each contributing according to its concentration and its Raman cross section within its local environment.

Although it might be quite difficult to identify the individual carotenoids with any other method apart from HPLC, it is evident that the Raman signal is indicative of their total amount and can be used for quantification. This can be qualitatively demonstrated by the

plot of the ν_1 intensity evolution during culture in the case of medium 1 (Fig. 2a). These data are in line with our corresponding results (Fig. 2b) where classical methods (e.g., HPLC analysis) were used for carotenoid determination, showing that their concentration increases monotonically with time and reaches its maximum value after 8 days. In Fig. 3, the total carotenoid concentrations estimated by the UV spectroscopic measurements for all the studied samples are plotted against that obtained by the HPLC procedure. As it can be seen from this figure, the data distribution is linear (R^2>0.95), suggesting that both methods could be alternatively used quite well for the carotenoid quantification, independently from their sensitivity or accuracy. Note that the HPLC procedure employed here has a detection limit of about 7 ng/ml, while for the UV spectroscopic measurements this limit is estimated to be 0.3 μg/ml. Due to the higher sensitivity and accuracy of the HPLC, in the following, we have used the carotenoid concentrations estimated by this method in order to quantify the Raman data.

The intensity of the ν_1 band from the different cultures of *B. trispora* growing in nutrition media 1–4 versus HPLC-determined total carotenoid concentrations is presented in Fig. 4. The data distribution suggests a linear correlation between the ν_1 Raman integrated intensity and the total carotenoid concentration, at least within the probed concentration limits. Data can be fitted fairly well by a line {offset 119.9(4), slope 6.2(5),

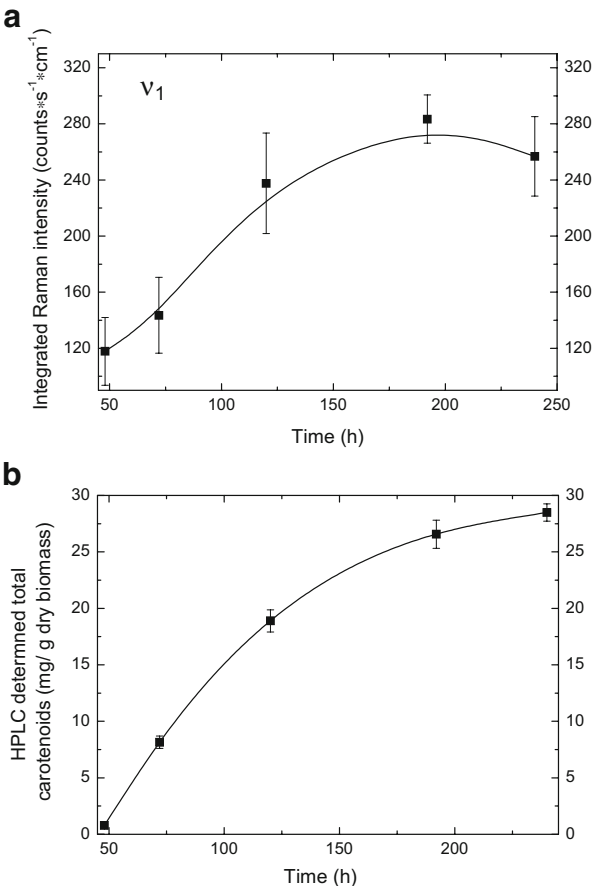

Fig. 2 **a** Time-dependent changes in the integrated intensity of the ν_1 Raman band during submerged cultures of *B. trispora* in medium 1. **b** Time evolution of the total carotenoid concentration in these samples as derived by the HPLC analysis

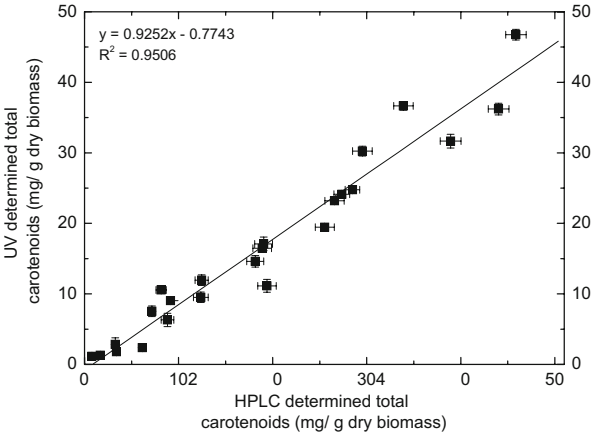

Fig. 3 Correlation between total carotenoids concentration as derived from UV spectroscopic measurements of various batches of *B. trispora* cultures and the HPLC analysis

R^2=0.9}. Linear correlations are also exhibited by the ν_2, ν_3, or ν_4 band intensities (not shown) as anticipated by their relation with that of the ν_1 band (*vide infra*). Error bars in the Raman intensity reflect the standard deviation from spectra recorded at ten randomly selected spots on the sample (as stated in the experimental section). This is the reason for their much larger values compared to the error bars of the HPLC or UV analysis. The intrinsic advantage of micro-Raman spectroscopy lies on the probing of very small sample volumes, reaching typically 1 μm in diameter or smaller, thus yielding information about local conditions. However, this is not necessarily an advantage when the average concentration is of interest, as the local density can vary even within the microorganism itself. With respect to the volume probed by micro-Raman spectroscopy, HPLC uses a significant sample quantity for the carotenoid determination and therefore local concentration variations are wiped out. In the case of micro-Raman, a complete two-dimensional spectral image of the sample would be ideally needed for a more precise carotenoid determination. On the other hand, carotenoids are far more stable in their natural environment, in contrast to the HPLC and UV methods where isomerization and/or oxidation during their isolation and the whole analysis procedure is due to cause errors [35]. Moreover, Raman measurements of carotenoids in their natural environment are much more straightforward; while the time duration in order to obtain a high-quality Raman spectrum is

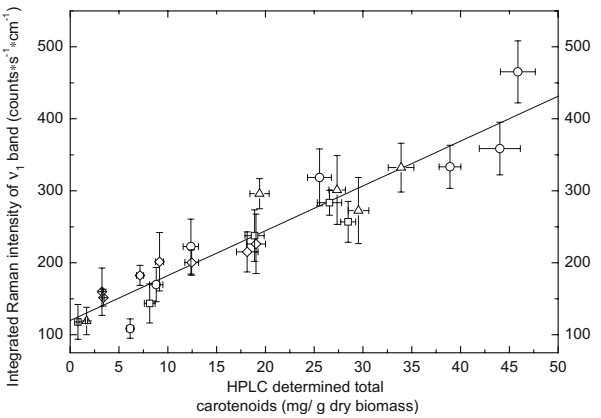

Fig. 4 Correlation between Raman ν_1 band integrated intensity from various batches of *B. trispora* cultures and carotenoid concentration (milligram per gram of mycelium dry weight), as derived from the HPLC analysis. The symbols used for the different media are 1: *squares*, 2: *triangles*, 3: *circles*, and 4: *diamonds*

only 2 min (20 min for ten measurements at different spots on the sample to have a good average picture), more than 5 h are needed for classical extraction procedures and HPLC analysis. Accuracy of Raman probing can be further improved by probing larger areas of the sample at the expense of time, while the latter can be reduced with higher laser power on the sample. Although the Raman intensity for the quantification of carotenoid content in different systems is unlikely to be comparable, as the local environment significantly affects their resonance Raman cross section, it would be feasible to compare results from similar systems. However, the Raman intensities in the literature cannot be directly exploited since they depend critically on the instrumentation used. In this respect, we have also measured the signal of the Raman peak at ~520 cm^{-1} of a (100) silicon wafer, which has an integrated intensity of 0.4 counts per second per centimeter with the experimental setup configuration used for the study of carotenoids in *B. trispora* cells (see the "Experimental Section"). This integrated intensity can be used as a normalization value for comparing Raman intensities originating from different instruments.

The other peaks present in the spectrum are also indicative of the abundance of the carotenoids. As it can be seen from Fig. 1, although the spectrum intensity increases with time, the overall spectral profile remains constant. This is better illustrated in Fig. 5 where the integrated intensities of the ν_2, ν_3, and ν_4 bands from all batches are plotted as a function to that of ν_1. All points are arranged in linear distributions which tend to cross close to the beginning of the axes, indicating that during the culture the relative intensities remain constant. Consequently, all peaks could be used, individually or as a whole, for the quantification of carotenoids. However, the ν_1 band is the most clearly observable as its integrated intensity is approximately 1.5 and 4.5 times larger than that of the ν_2 and the ν_3 bands, respectively. Moreover, as there are no other peaks in the vicinity of ν_1, it is quite straightforward to evaluate its integrated intensity by a single Lorentzian lineshape, rendering this peak the best choice for quantification purposes.

The Raman spectrum also yields information about different species and conformations throughout the cultivation process. In our case, the relative intensities and the spectral widths of the peaks did not change throughout the culture duration, indicating that the accumulation of carotenoids does not alter significantly their conformational arrangement and microenvironment or their relative percentage, in agreement with the HPLC analysis (Papaioannou and Liakopoulou-Kyriakides, submitted for publication). It is worth noting that the ν_4 band is of particular interest as it is forbidden for a purely planar molecule in the

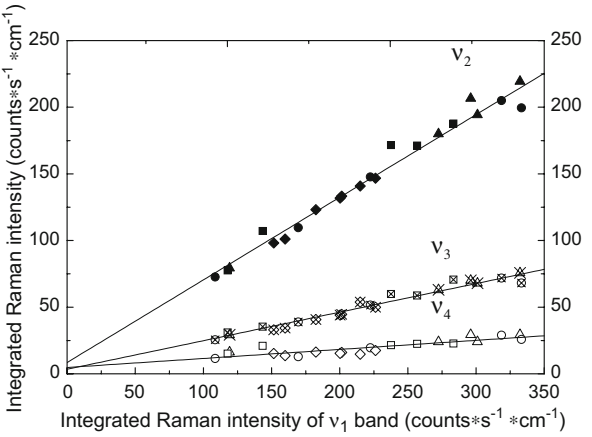

Fig. 5 Integrated intensity of the ν_2, ν_3, and ν_4 Raman bands plotted with respect to that of the ν_1 band. The symbols used for the different nutrition media are 1: *squares*, 2: *triangles*, 3: *circles*, and 4: *diamonds*. *Filled symbols* were used for ν_2, crossed for ν_3, and open for ν_4

transconfiguration. Its intensity is then sensitive in the distortion of the carotenoid molecule from its planar configuration yielding information about changes due to environment or isomerization. As data from all different batches line up well in Fig. 5, it is evident that the different nutrition media do not significantly interfere in the "quality" of the carotenoids produced by the microorganism.

Conclusion

The present work highlights the possibility of using Raman spectroscopy for the measurement of the total carotenoid concentration in filamentous fungus *B. trispora* cultures. HPLC analysis remains the only method that could discriminate among the different carotenoids, whereas UV spectroscopy gives only a raw carotenoid content estimation. Both these methods are applicable after carotenoid extraction from the fungus cells, a procedure that may inherently lead to estimation errors due to isomerization and/or oxidation during the process. The analysis of carotenoids with the use of Raman spectroscopy in *B. trispora* samples is fast, without intermediate calculations and, most importantly, carotenoids remain stable in the cells. In addition, the Raman intensity profile provides information about the evolution of different carotenoids and/or their interaction with their environment during the production process. In our case, it remains constant, indicating that the accumulation of carotenoids and the different nutrition media do not affect the "quality" of the carotenoid product.

Although a research-grade spectrometer system has been used for the presented ex situ analysis of samples acquired during the fermentation process, the miniaturization of today's analytical Raman setups and their flexibility (e.g., combination with fiber optics) can allow for in situ and rapid monitoring of carotenoid production content in similar organisms. Moreover, it can be applied in different, even aggressive, fermentation conditions as long as the acquired signal does not contain significant interferences from the fermentation environment (e.g., luminescence masking the Raman signal and/or "parasitic" Raman signal from other molecular species in the carotene's fingerprint spectral region). In this respect, working in near-resonance conditions is essential for fast signal collection and elimination of the out-of-resonance environment's spectral features with the precaution that low laser intensities are used to avoid any photo-degradation of the sample. The intensity of the Raman signal could be also used for a rough fast screening of similar new production organisms according to their efficiency in carotene production. However, as the signal can also depend on the exact carotene environment, which might be different for different microorganisms, it should be employed with caution and, eventually, a calibration with reference from HPLC measurements should be made for reliable quantitative results.

Acknowledgements The authors thank Professor Triantafyllos Roukas, Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki for *B. trispora* strains.

References

1. Simpson, K. L. (1992). In Hui Y.H (Eds), Encyclopedia of food science and technology: Carotenoids–vitamins part IV (pp.293–310, 2701–2712). New York: Wiley.
2. Lopez-Nieto, M., Costa, J., Peiro, E., Méndez, E., Rodriguez-Sálz, M., de la Fuente, J. L., et al. (2004). *Applied Microbiology and Biotechnology*, *66*, 153–159. doi:10.1007/s00253-004-1669-4.

3. Cannizzaro, C., Rhiel, M., Marison, I., & von Stockar, U. (2003). *Biotechnology and Bioengineering*, *83*, 668–680. doi:10.1002/bit.10698.
4. Tereshina, V. M., Memorskaya, A. S., & Feovilova, E. P. (2003). *Microbiology*, *72*, 448–454. doi:10.1023/A:1025096723803.
5. Candau, R., Bejarano, E. R., & Cerdá-Olmedo, E. (1991). *Proceedings of the National Academy of Sciences of the United States of America*, *88*, 4936–4940. doi:10.1073/pnas.88.11.4936.
6. Mehta, B. J., & Cerda-Olmedo, E. (1999). *Mycosci*, *40*, 307–310. doi:10.1007/BF02463967.
7. Mantzouridou, F., Roukas, T., Kotzekidou, P., & Liakopoulou, M. (2002). *Applied Biochemistry and Biotechnology*, *101*, 153–175. doi:10.1385/ABAB:101:2:153.
8. Mantzouridou, F., Roukas, T., & Kotzekidou, P. (2004). *Food Biotechnology*, *18*, 243–261. doi:10.1081/FBT-200040534.
9. Roukas, T., Mantzouridou, F., Boumpa, T., Vafiadou, A., & Goksungur, Y. (2003). *Food Biotechnology*, *17*, 203–215. doi:10.1081/FBT-120026342.
10. Anderson, R. F., Arnold, M., Nelson, G. E. N., & Ciegler, A. (1958). *Agricultural Food Chemistry*, *6*, 543–545. doi:10.1021/jf60089a009.
11. Mehta, B. J., Obraztsova, I. N., & Cerda-Olmedo, E. (2003). *Applied and Environmental Microbiology*, *69*, 4043–4048. doi:10.1128/AEM.69.7.4043-4048.2003.
12. Kim, S. W., Seo, W. T., & Park, Y. H. (1996). *Biotechnology Letters*, *18*, 1287–1290. doi:10.1007/BF00129956.
13. Kim, S. W., Seo, W. T., & Park, Y. H. (1997). *Journal of Fermentation and Bioengineering*, *84*, 330–332. doi:10.1016/S0922-338X(97)89253-7.
14. Kim, S. W., Seo, W. T., & Park, Y. H. (1997). *Biotechnology Letters*, *19*, 561–562.
15. Raman, C. V. (1928). *Nature*, *121*, 619. doi:10.1038/121619b0.
16. Euler, H. V., & Hellstrom, H. Z. (1932). *Physical Chemistry*, *15B*, 342–346.
17. Weesie, R. J., Merlin, J. C., Lugtenburg, J., Britton, G., Jansen, F. J. H. M., & Cornard, J. P. (1999). *Biospectroscopy*, *5*, 19–33. doi:10.1002/(SICI)1520-6343(1999)5:1<19::AID-BSPY4>3.0.CO;2-E.
18. Gill, D., Kilponen, R. G., & Rimai, L. (1970). *Nature*, *227*, 743–744. doi:10.1038/227743a0.
19. Darvin, M. E., Gersonde, I., Albrecht, H., Meinke, M., Sterry, W., & Lademann, J. (2006). *Laser Physics Letters*, *3*, 460–460. doi:10.1002/lapl.200610032.
20. Bhosale, P., Ermakov, I. V., Ermakova, M. R., Gellermann, W., & Bernstein, P. S. (2003). *Biotechnology Letters*, *25*, 1007–1011. doi:10.1023/A:1024199213519.
21. Baranska, M., Schultze, W., & Schulz, H. (2006). *Analytical Chemistry*, *78*, 8456–8461. doi:10.1021/ac061220j.
22. Bhosale, P., Ermakov, I. V., Ermakova, M. R., Gellermann, W., & Bernstein, P. S. (2004). *Journal of Agricultural and Food Chemistry*, *52*, 3281–3285. doi:10.1021/jf035345q.
23. Rodriguez-Amaya, D. B. (2001). *A guide to carotenoid analysis in foods* (pp. 14–22). OMNI Research.
24. Britton, G. (1995). In Britton G., Liaaen-Jensen S., Pfander H., *Carotenoids volume 1B: spectroscopy* (pp. 13–62). Wallingford: CABI.
25. Takaichi, S., & Ishidsu, J. I. (1992). *Methods in Enzymology*, *213*, 374–386. doi:10.1016/0076-6879(92)13139-O.
26. Bhosale, P., Ermakov, I. V., Ermakova, M. R., Gellermann, W., & Bernstein, P. (2004). *Journal of Agricultural and Food Chemistry*, *52*, 3281–3285. doi:10.1021/jf035345q.
27. Saito, S., & Tasumi, M. (1983). *Journal of Raman Spectroscopy*, *14*, 310–321. doi:10.1002/jrs.1250140504.
28. Schulz, H., Baranska, M., & Baranski, R. (2005). *Biopolymers*, *77*, 212–221. doi:10.1002/bip.20215.
29. Hoskins, L. C. (1981). *The Journal of Chemical Physics*, *74*, 882–885. doi:10.1063/1.441139.
30. Hata, T. R., Scholz, T. A., Ermakov, I. V., McClane, R. W., Khachik, F., Gellermann, W., et al. (2000). *The Journal of Investigative Dermatology*, *115*, 441–448. doi:10.1046/j.1523-1747.2000.00060.x.
31. Darvin, M. E., Gersonde, I., Meinke, M., Sterry, W., & Lademann, J. (2005). *Journal of Physics D*, *38*, 2696–2700. doi:10.1088/0022-3727/38/15/023.
32. Liu, W. L., Zheng, Z. R., Zhu, R. B., Liu, Z. G., Xu, D. P., Yu, H. M., et al. (2007). *The Journal of Physical Chemistry A*, *111*, 10044–10049. doi:10.1021/jp074048b.
33. Darvin, M. E., Gersonde, I., Meinke, M., Sterry, W., & Lademann, J. (2005). *Journal of Physics D*, *38*, 2696–2700. doi:10.1088/0022-3727/38/15/023.
34. Baranski, R., Baranska, M., & Schulz, H. (2005). *Planta*, *222*, 448–457. doi:10.1007/s00425-005-1566-9.
35. Papaioannou, E., Roukas, T., & Liakopoulou-Kyriakides, M. (2008). *Preparative Biochemistry & Biotechnology*, *38*, 246–256. doi:10.1080/10826060802164942.