Food Science and Applied Biotechnology, 2022, **5**(2), 199-210

UFT Academic Publishing House, Plovdiv

Food Science and Applied Biotechnology
e-ISSN: 2603-3380

Journal home page: www.ijfsab.com
https://doi.org/10.30721/fsab2022.v5.i2

OPEN ACCESS

Research Article

Isolation and characterization of a rare polar carotenoid 1'-OH-4-keto-ϒ-carotene from an indigenously isolated *Rhodococcus kroppenstedtii* MH715196

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Abstract

The catabolic diversity and biocatalytic potential in members of genus *Rhodococcus* makes it an ideal industrial workhouse for metabolite production. Despite their applicability, Rhodococci are least explored for carotenoids, located in their cell membrane. The goal of the present study was to identify the carotenoids of *Rhodococcus kroppenstedtii* MH715196. UV-Vis spectral data and molecular mass estimates showed that the principal carotenoid extracted from *R. kroppenstedtii* MH715196 was 1'-OH-4-keto-ϒ-carotene. Biosynthetic route of 1'-OH-4-keto-ϒ-carotene in *R. kroppenstedtii* MH715196 was postulated on the basis of molecular mass estimates in co-relation with putative carotenoid biosynthetic genes identified in the genome of the reference strains of *R. kroppenstedtii*. Three key enzymes, were then considered for phylogenetic analysis to establish the phylogenetic relationship across the *Rhodococcus* genus leading the way for carotenoid identification in other *Rhodococcus* species. The present study would be the first report on identification of a rare polar carotenoid from *R. kroppenstedtii* MH715196 which could be potentially explored as a food colorant in hydrophilic food matrices like jams, jellies, beverages etc.

Keywords: Carotenoids, Characterization, HR-LCMS, *Rhodococcus kroppenstedtii*

Abbreviations: NCBI – National Center for Biotechnology Information; HR-LCMS – High Resolution Liquid Chromatograph Mass Spectrometer; KEGG – Kyoto Encyclopaedia of Genes and Genomes; BLAST – Basic Local Alignment Search Tool

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Article history:
Received 12 May 2022
Reviewed 20 August 2022
Accepted 6 October 2022
Available on-line 13 October 2022

https://doi.org/10.30721/fsab2022.v5.i2.200

Introduction

The market for carotenoids has surged significantly over the years, mainly due to the increased usage of natural carotenoids as food colorants (Lourenço-Lopes et al., 2021) and nutraceuticals (Khalid et al., 2022). Commercially, carotenoids used as food color additives and supplements are generally obtained through chemical synthesis due to highly priced plant-based carotenoids (Saini and Keum, 2019). Although, chemical synthesis of carotenoids is an established process, its safety for human direct consumption is debated (Ye et al., 2008). Microbial carotenoids are valued and have recently gained popularity owing to their sustainability and cost effectiveness (Numan et al., 2018). Most of the naturally obtained carotenoids are hydrophobic in nature, which limits their use in hydrophilic food systems, hence there is need to explore new microbial species producing polar carotenoids.

Rhodococcus, an actinomycete genus that produces a variety of bioactive compounds, is one of the industrially important genera (Elsayed et al., 2017). Rhodococci are non-photosynthetic gram-positive bacteria with orange to red-colored colonies. The characteristic hue is due to the presence of carotenoids in their membranes, which help to prevent oxidative damage to the cells (Cappelletti et al., 2020). Although, certain actinobacteria have been reported to produce keto-carotenoids, Rhodococci are the only actinobacteria that can simultaneously produce both aromatic and keto-carotenoids from ϒ-carotene (Sandmann, 2021). In fact, very few genera, including *Rhodococcus*, are capable of producing monocyclic carotenoids that are ϒ-carotene derivatives, as a result of mono-cyclization activity of lycopene β-cyclase enzyme. These asymmetric carotenoids derived from ϒ-carotene, are of economic importance and difficult to obtain via chemical synthesis (Tao et al., 2004). Based on the carotenoids produced, Rhodococci are categorized as: β-carotene producers, ϒ-carotene producers and those that are neither β-carotene nor ϒ-carotene-like substance producer (Ichiyama et al 1989). According to the literature, carotenoids identified in the majority of *Rhodococcus* species are ϒ-carotene-like substance, but the specific structural identification is still unclear. Several reasons such as interference by lipophilic compounds like fatty acids (Mariutti and Mercadante, 2018) a lack of standard carotenoids and similarities in their UV-Vis absorption pattern and molecular masses makes identification of carotenoids a challenging task (Rivera et al., 2014). This alludes to a research gap in carotenoid characterization in *Rhodococcus* species.

Rhodococcus kroppenstedtii is a relatively unexplored *Rhodococcus* species that was documented by Mayilraj et al. (2006). Since then, few strains of *R kroppenstedtii* have been isolated from varied environment (Kulkarni et al., 2022, Lilwani et al., 2021, Madhukar 2021, Dhaouadi et al., 2020), but carotenoids characterization from none of the isolates has been reported so far. Currently, genomes of 8 reference strains of *R kroppenstedtii* are available in NCBI (Schoch et al 2020), however, the carotenoids from none of these strains are identified yet. Hence, the objective of the current study was to isolate and characterize the carotenoids from *R kroppenstedtii* MH715196 and to identify the probable genes involved in carotenogenesis. To achieve this, the carotenoid extracted from *R kroppenstedtii* MH715196 was characterized using HR-LCMS. Further, gene homology searches were performed in the genome of the reference strains of *R. kroppenstedtii*. The results of mass spectrum, gene homology search and the published pathways for the other *Rhodococcus* species (Osawa et al., 2011, Tao et al., 2006) were collated together to propose carotenogenic pathway in *R kroppenstedtii*. In addition, the evolutionary relationship was established between *R. kroppenstedtii* and other *Rhodococcus* and *Gordonia* species in terms of major carotenogenic proteins identified in the pathway. Thus, this work would be a valuable step in identification of novel high value polar carotenoids that can be explored as food colorants.

Materials and Methods

Chemicals All the chemicals employed for the present study were of molecular and analytical grade procured from Sisco Research Laboratories (SRL) Pvt. Ltd, Hi-Media Laboratories Pvt. Ltd, Mumbai, India.

Microorganism *Rhodococcus kroppenstedtii* (Accession No. MH715196) was isolated from a sediment sample of Rajapur hotspring in Ratnagiri, Maharashtra, India. Spectral scanning and

qualitative analysis of the orange pigment produced by this strain indicated the presence of carotenoids.The culture was stored at 4°C and sub-cultured on a regular basis in Tryptic Soy Agar (TSA) medium.

Culture conditions and Extraction of pigment 5% ^{v}/v culture (O.D $_{600}$=1.0) of *R. kroppenstedtii* MH715196 was inoculated in 25 mL optimized medium (Beef extract-20 g/L, $(NH_4)_2SO_4$- 2 g/L, $MgCl_2$-4 g/L, K_2HPO_4-2.5 g/L and Glycerol-8.75 g/L, pH-7.2) (Lilwani et al., 2021) in 100 mL Erlenmeyer flask and incubated in a rotary shaker for 96 h at 37°C with a shaking speed of 110 rpm. Carotenoids were extracted by solvent extraction method using ethanol (Kusmita et al., 2017) as a solvent. Culture broth (96 h old) was subjected to centrifugation at 4,500 rpm for 10 min. The cell pellet was recovered and washed twice with distilled water. For extraction of the pigment, the cell pellet was suspended in chilled ethanol and centrifuged as above. The supernatant containing the pigment was collected and the pellet was resuspended in ethanol, mixed and recentrifuged till a colorless pellet was obtained. The pooled pigment extract was refrigerated overnight and centrifuged for partial removal of fatty acids (Mariutti and Mercadante 2018). The extract was then purified by silica gel open column chromatography (Column: Glass, 30 x 1 cm, silica gel: 200-400 mesh size, Mobile Phase: Petroleum ether: Acetone (9:1), Flow rate: 1 mL/min) and the orange-colored fraction (λmax-470 nm) eluted from the column was analysed by HR-LCMS.

HR-LCMS Analysis Agilent Technologies 1290 Infinity UHPLC system and liquid chromatograph interfaced to a Mass Spectrometer (LC-MS) equipped with electro-spray ionization source and Hypersil GOLD column (C18, 100 x 2.1 mm, 3 micron) were used to examine the fraction obtained from the silica gel column. A 5.00 µL of the sample was injected (flow rate 0.3 mL/min) and operated in positive ionization mode from 150 to 1000 m/z. A TOF/Q-TOF mass spectrometer with two AJS ESI ion sources was employed for LC-MS detection. The tuning parameters used in the analysis were: gas temperature (250°C), gas flow (13 l/min), and nozzle voltage (1000 V). Running time for the LC was 30 min. Identification of major carotenoid from *R. kroppenstedtii* MH715196 was based on the UV-Vis spectrum and mass spectral characteristics.

***In silico* studies**

***In silico* analysis to propose putative carotenoid biosynthesis pathway** Genome Assembly and annotation report of *Rhodococcus kroppenstedtii* in NCBI (Schoch et al 2020) listed down eight candidates – two genome assemblies of strain DSM 44908 and six genome assemblies of strains BP-150, BP-284, BP-286, BP-289, 20200126096 and K5 respectively. Protein list of all the strains were retrieved. Gene homology searches of known carotenoid biosynthesis genes and the corresponding proteins in the genome assemblies of all the strains of *R. kroppenstedtii* helped to understand the biosynthetic origin of the orange pigment. The carotenoid biosynthesis pathway from the KEGG database (Altermann and Klaenhammer 2005) and the details of the enzymes involved from KEGG orthology (Mao et al., 2005) were mapped with the proteins identified in the genome assemblies of the reference strains of *R. kroppenstedtii* and carotenoid biosynthesis pathway of *R. kroppenstedtii* MH715196 was proposed.

Phylogeny studies Phylogenetic analysis was performed to showcase the relationship between the carotenoid biosynthesis proteins of *R. kroppenstedtii* and other species of *Rhodococcus*. An actinomycete genera *Gordonia* is closely related to genus *Rhodococcus* having same ancestral origin (Stackebrandt et al., 1997). Thus, few *Gordonia* species that are known to produce ϒ-carotene and its derivatives (Takaichi et al., 2008, Sowani et al., 2017) were also included for phylogeny analysis. Three proteins identified to be crucial for the carotenoid production in *R. kroppenstedtii;* lycopene β-cyclase (crtY), phytoene dehydrogenase related protein (crtO type) and hydroxyneurosporene synthase (crtC) were considered in this study.

The protein sequences of all three proteins from the available *Rhodococcus* and *Gordonia* species were retrieved from NCBI (Pruitt et al 2007). The taxonomic details of the various *Rhodococcus* genomes (Taxonomy ID: 1827) were accessed from NCBI-Taxonomic Browser (Federhen 2011) and all its species were listed. The sequences were collected by performing a BLAST (Johnson et al., 2008) search of respective query sequences against the *Rhodococcus* genome. Additionally, few of the sequences not listed in the BLAST results were obtained by manually querying the protein database.

The collected set of sequences were then subjected to multiple sequence alignment in Clustal Omega (Sievers et al., 2011). The alignment was performed with default parameters of mBed-like Clustering Guide-tree and iteration set to true and output order of sequences in ClustalΩ format. The phylogenetic trees were constructed for lycopene β-cyclase, phytoene dehydrogenase related protein and hydroxyneurosporene synthase proteins from diverse species of *Rhodococcus* and *Gordonia* in Mega 6.06 (Tamura et al., 2013) with Neighbor-Joining (NJ) method of distance-based tree building (Saitou and Nei 1987).

The phylogeny's reliability was tested using the Bootstrap resampling technique, which included 1000 replications (Stine 1989). Poisson Distance Correction was implemented as a substitution model considering uniform rates amongst all sites (Gu and Li 1998). Calculated evolutionary distances are represented in units of the number of amino acid substitutions per site. Essentially, a bootstrap consensus tree built from 1000 data replicates was presumed to accurately depict the evolutionary history of the taxa under study. Branches having partitions replicated in less than 50% bootstrap replicates were collapsed together in a consensus tree. Next to the branches are the percentage of replicated trees, in which the related taxa were clustered together in the bootstrap test with 1000 cycles. In case of lycopene β-cyclase 43 amino-acid sequences, for phytoene dehydrogenase related protein 40 amino-acid sequences and for hydroxyneurosporene synthase 14 amino-acid sequences were considered. Pairwise deletion option was implemented to eliminate the ambiguous positions from the sequence pairs. The final dataset provided a total of 457, 605 and 440 positions in the same order as the enzymes mentioned above.

Results and Discussion

HR-LCMS analysis The partially purified orange fraction obtained by silica gel open column chromatography showed absorption maxima of 470 nm with two shoulder peaks at 440 nm and 500 nm. The fraction was subjected to HR-LCMS analysis and identification of the carotenoid was confirmed with the help of molecular mass estimates (Fig. 1) obtained by positive ion ESI mass spectrum. By mass spectral analysis, the molecular mass of carotenoid was found to be 568.3. The product ion with m/z 551.3 is due to loss of water [M+H-18] typically obtained in carotenoids with hydroxyl group at allylic position (Rivera et al., 2014). In addition, the product ions with m/z 413.3 and 203.1 were also obtained. Of these, the product ion with m/z 203.1 is a characteristic feature of carotenoids with keto group present as a single substituent on the β-ionone ring. This ion is produced due to a cleavage between carbon 10 and 11 with the positive charge retained on the ketone moiety (van Breemen 2001). The product ion with m/z 413.3 could be due to loss of hydrocarbon fragment. The results of UV-Vis absorption and mass spectral characteristics are in accordance with the literature (Sowani et al., 2016) for a rare and polar ϒ-carotene derivative; 1'-OH-4-keto-ϒ-carotene. Thus, this is a first report on identification of a carotenoid from *R. kroppenstedtii* MH715196.

***In silico* studies**

***In silico* analysis to propose putative carotenoid biosynthesis pathway** In co-relation to mass spectral analysis, the gene homology searches in the 8 reference genome assemblies (two genome assemblies of strain DSM 44908 and six genome assemblies of strains BP-150, BP-284, BP-286, BP-289, 20200126096 and K5) of *R. kroppenstedtii* were performed. This led to the identification of genes and corresponding proteins related to carotenogenesis in *R. kroppenstedtii*. Five genes (CrtB, CrtI, CrtY, CrtO, CrtC) encoding homologues to enzymes in published carotenogenic pathways of other *Rhodococcus* species (Osawa et al., 2011, Tao et al., 2006) were identified in this investigation. Of these, CrtB and CrtY present on adjacent loci appears to form one cluster, whereas CrtO and CrtC could be forming another cluster sharing adjacent loci. The locus position of CrtY suggests that it could be present as an unclustered unit. These 5 homologues, however, could be identified only in the reference genome assembly of DSM44908 strain (Table 1).

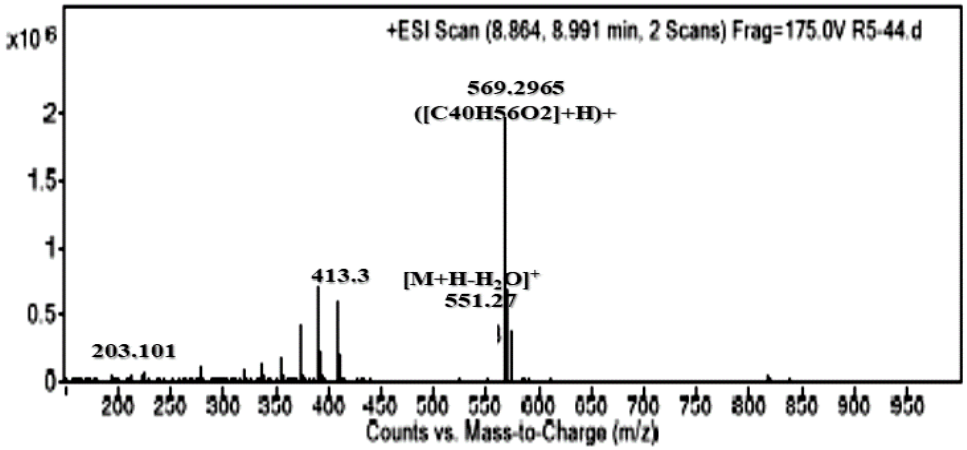

Figure 1. ESI-Mass spectrum of main carotenoid from *R. kroppenstedtii* MH715196

Table 1. Putative carotenoid biosynthesis genes and enzymes encoded by *R.kroppenstedtii*- DSM 44908

Gene	Biosample	Locus	Encoded Protein	Protein Accession ID
crt B	SAMN05444374 RefSeq-NZ_FOJN01000001.1	147833..148807	Phytoene Synthase (EC: 2.5.1.32)	WP_068100417.1
crt I	SAMN05444374 Refseq-NZ_FOJN01000001.1	144435..146003	Phytoene Desaturase (EC: 1.3.99.28/31)	WP_068361402.1
crt Y	SAMN05444374 Genbank-FOJN01000005.1	54233..55366	Lycopene beta-cyclase (EC: 5.5.1.19)	SFA48793.1
crt O	SAMN05444374 Genbank-FOJN01000018.1	32529..34181	Phytoene dehydrogenase related protein/ Beta-carotene ketolase (crtO type) (EC: 1.14.99.63)	SFA56921.1
crt C	SAMN05444374 Genbank-FOJN01000011.1	32415..33521	Hydroxyneurosporene synthase/ Carotene 1,2 hydratase (EC: 4.2.1.1.131)	SFA61577.1

In the other six genome assemblies (strains BP-150, BP-284, BP-286, BP-289, 20200126096 and K5), homologue of CrtC coding for enzyme hydroxyneurosporene synthase could not be found. This may be because many proteins have been listed as hypothetical/unidentified in these assemblies. Thus, the present work was predicated on the homologues identified in the genome assembly of *R. kroppenstedtii* DSM44908.

Based on the results of gene homology searches, a carotenogenic pathway (Fig. 2) has been postulated for *R. kroppenstedtii* MH715196 in this study. According to the pathway, condensation of 2 geranylgeranyl pyrophosphate results in pre-phytoene pyrophosphate that undergoes subsequent rearrangement in presence of phytoene synthase (CrtB) to form a 40C colorless hydrocarbon phytoene. Further, phytoene desaturase (CrtI) catalyzes 4 sequential steps of dehydrogenation

alternately on the either side of chromophore converting phytoene to lycopene via phytofluene, ζ-carotene and neurosporene. Lycopene formed, then undergoes mono-cyclization reaction to form monocyclic parental molecule ϒ-carotene which is catalysed by enzyme lycopene β-cyclase (CrtY).

A keto group is present on the β-ionone ring of ϒ-carotene, as indicated by a product ion with a m/z of 203.1 in the mass spectrum. In *R. kroppenstedtii* MH715196; this keto group is introduced by the activity of phytoene dehydrogenase related protein (CrtO type) resulting in formation of 4-keto-ϒ-carotene. However, it is suggested that in the other *Rhodococcus* species, this reaction is catalysed by β-carotene ketolase (CrtO type) to synthesize carotenes that are structurally similar to 4-keto-ϒ-carotene (Osawa et al., 2011, Tao et al., 2006)

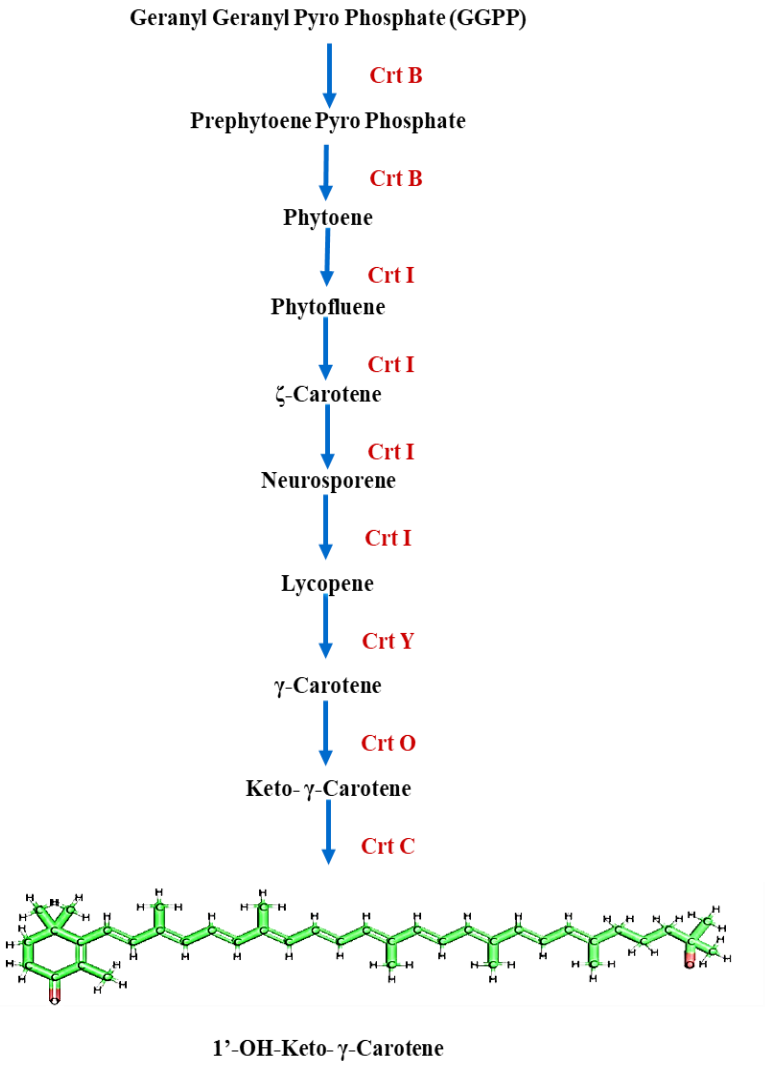

Figure 2. Proposed carotenogenesis pathway in *R.kroppenstedtii* MH715196. The enzymes that are encoded by putative genes identified in the *R.kroppenstedtii*- DSM 44908 reference genome (CrtB, CrtI, CrtY, CrtO, and CrtC) catalyze the synthesis of 1'-OH-4-keto-ϒ-carotene; where, Crt B=Phytoene synthase, Crt I=Phytoene desaturase, Crt Y=lycopene β-cyclase, Crt O=Phytoene dehydrogenase related protein and Crt C=hydroxyneurosporene synthase

Moreover, the product ion of m/z 551.3 in mass spectrum indicates the presence of hydroxyl group on the allylic end which is formed due to the activity of carotene 1,2 hydratase (CrtC) as proposed in

Rhodococcus sp CIP (Osawa et al 2011). However, in *R. kroppenstedtii*, a homologue of hydroxyneurosporene synthase has been proposed to catalyse the addition of water molecule at the *psi* end of 4-keto-ϒ-carotene leading to formation of 1'-OH-4-keto-ϒ-carotene. Despite the fact that the pathway is based on homologues of enzymes found in *R. kroppenstedtii* DSM44908, further studies need to be carried out to confirm the predicted genes and validate the pathway in *R. kroppenstedtii* MH715196.

Phylogeny studies. The phylogenetic trees for the three proteins (lycopene β-cyclase, phytoene dehydrogenase related protein and hydroxyneurosporene synthase) from various *Rhodococcus* and *Gordonia* species were built with Neighbor-Joining (NJ) method of distance-based tree building, which presented a systematic classification of species according to their amino acid sequence characteristics.

The phylogenetic tree for lycopene β-cyclase (Fig 3) showed a clustering pattern similar with regard to the whole genome of 50 *Rhodococcus* species as previously described by Sangal et al (2016).

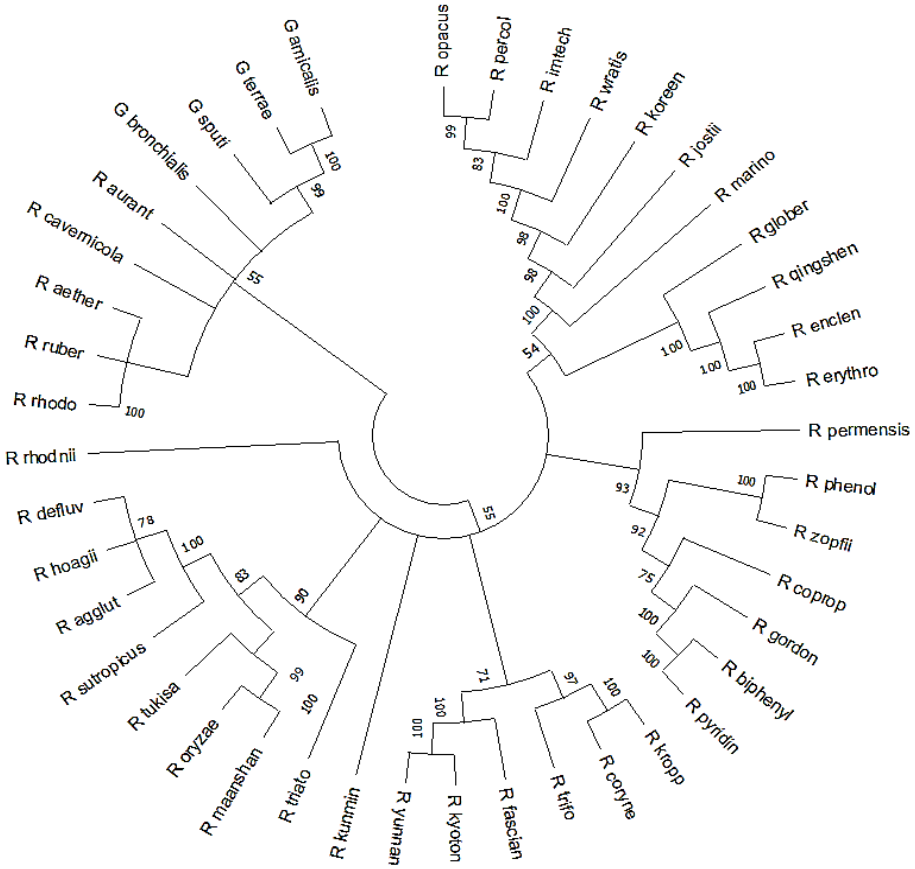

Figure 3. Phylogenetic tree for Lycopene beta-cyclase. Protein sequences from 43 different *Rhodococcus* species constructed by neighbour joining method. The percentage of replicated trees where related taxa are grouped together in the 1000 cycles bootstrap test is displayed next to the branches. The tree represents *R. kroppenstedtii* sharing a common clade with *R. corynebacterioids, R. trifolii, R. fascians, R kyotonensis* and *R. yunnanensis* with a clade strength of 71%. While *R. kroppenstedtii* and *R. corynebacterioids* shares a clade with 100% bootstrap value and *R. kroppenstedtii* and *R. trifolii* indicates a clade strength of 97% implying a strong similarity between them.

R. kroppenstedtii shared a common clade (71% clade strength) with *R. corynebacterioids, R. trifolii, R. fascians, R kyotonensis* and *R. yunnanensis.* Interestingly, the carotenoids from none of these

species of *Rhodococcus* have been characterized yet. The Clade strength of 100% between *R. kroppenstedtii* and *R. corynebacterioids*, whereas clade strength of 97% between *R. kroppenstedtii* and *R. trifolii* suggest a very close similarity which could help in characterizing the carotenoids from these species.

The results of a phylogenetic tree for phytoene dehydrogenase related protein supported the pathway suggested in this study. A distinct clading pattern was detected (Fig 4), while the clade strength of 100% between *R. kroppenstedtii* and *R. corynebacterioids* remained, as seen in the phylogenetic tree for lycopene-cyclase. In this tree, additional members sharing the clade strength of 95% were *R. fascians, R. percolates, R. opacus, R. erythropolis, R qingshengii, G.sputii and G. amicalis.* Of these, *R erythropolis* is reported to produce 4-keto-ϒ carotene (Tao et al., 2006) which is an intermediate for synthesis of 1'-OH-4-keto-ϒ-carotene pathway proposed in the present study. Also 95% similarity index with *G. amicalis* which is reported to produce 1'-OH-4-keto-ϒ-carotene (Sowani et al., 2016) supports the proposed pathway.

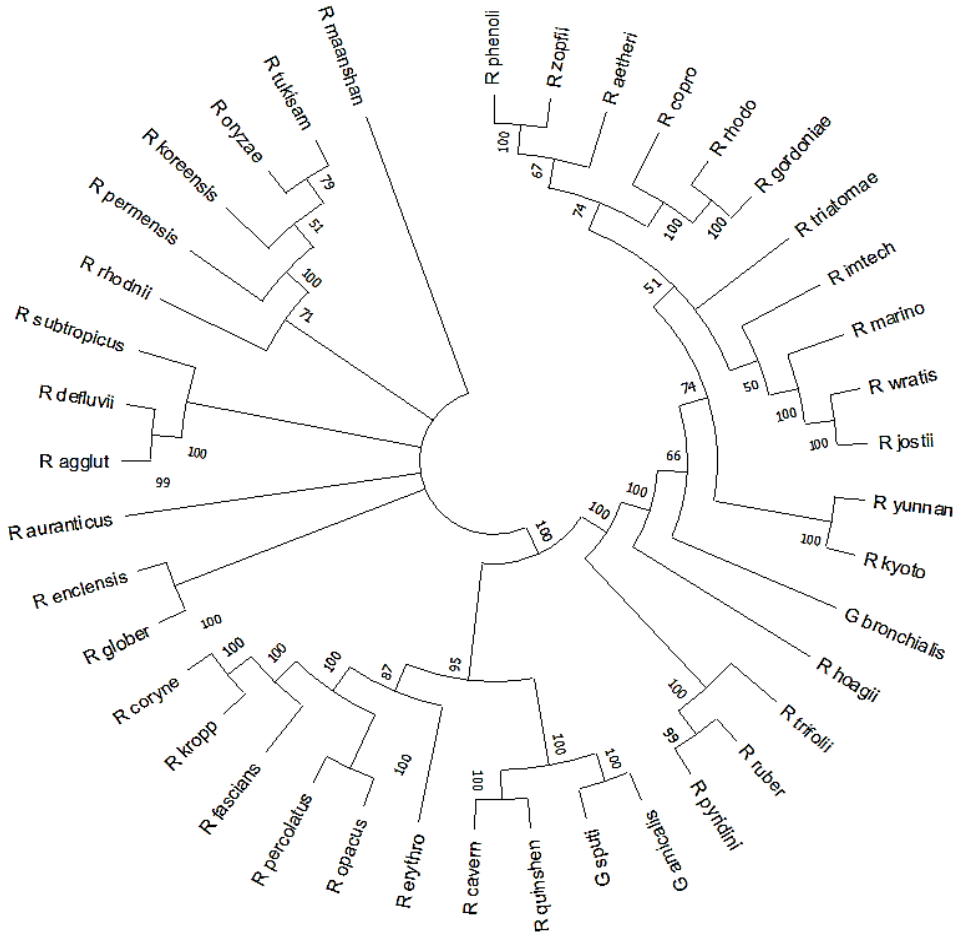

Figure 4. Phylogenetic tree for Phytoene dehydrogenase related protein (crtO type).
Protein sequences from 40 different *Rhodococcus* species constructed by neighbour joining method. The percentage of replicated trees where related taxa are grouped together in the 1000 cycles bootstrap test is displayed next to the branches. The tree represents *R. kroppenstedtii* and *R. corynebacterioids* sharing a clade with 100% bootstrap value. Whereas, *R. fascians, R. percolates, R. opacus, R. erythropolis, R qingshengii, G.sputii* and *G. amicalis* merged with this clade with 95% clade strength.

The phylogenetic tree for hydroxyneurosporene synthase (Fig 5) demonstrates inter-relationship between 12 *Rhodococcus* species and 2 *Gordonia* sp., notably *G. amicalis* sharing 97% of clade

strength with *R. kroppenstedtii*, which is in alignment with the proposed pathway. Also, among the other *Rhodococcus* species included in the tree, *R. rhodochrous* have been documented to produce ϒ-carotene derivatives with a hydroxy functional group (Takaichi et al., 1990), thus indicating that the postulated pathway could be correct. A significant degree of similarity between the two *Rhodococcus* species; *R. kroppenstedtii* and *R. corynebacterioids* in phyologeny studies, presents a possibility for identifying the latter's carotenoids.

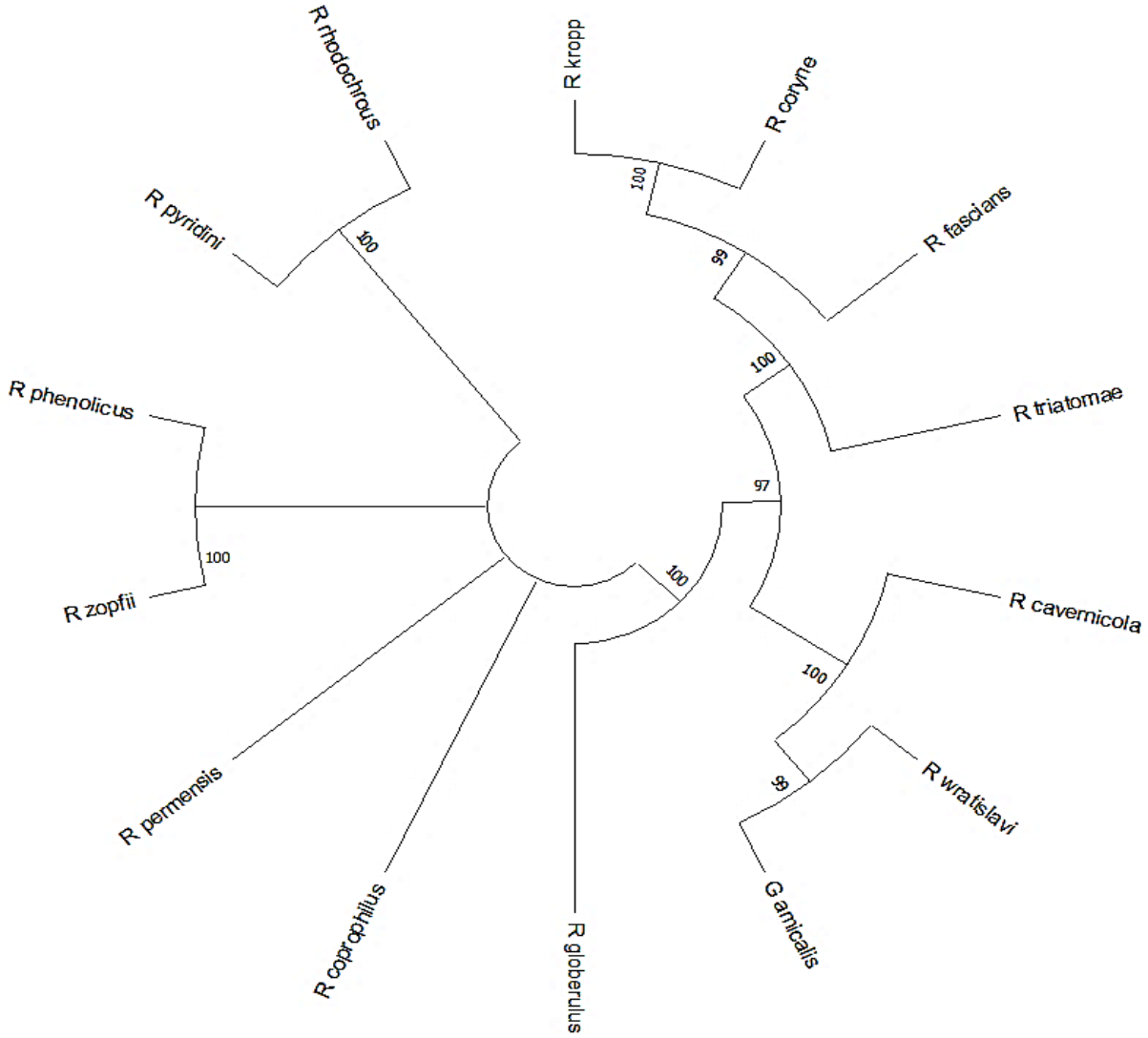

Figure 5. Phylogenetic tree for hydroxyneurosporene synthase. Protein sequences from 14 different *Rhodococcus* species constructed by neighbour joining method. The percentage of replicated trees where related taxa are grouped together in the 1000 cycles bootstrap test is displayed next to the branches. The tree represents *R. kroppenstedtii* and *R. corynebacterioids* sharing a clade with 100% bootstrap value. *R. fascians* and *R. triatomae* are seen to merge with them with strong clade strength. While *G. amicalis, R. wratislavi* and *R. cavernicola* has joined the clade with 97% bootstrap value

Conclusion

Carotenoids are hydrophobic tetra-terpenoid pigments ubiquitous in nature. They are primarily

employed for fortification or as restorative colorants in food products due to their wide range of clinical properties and bright yellow-red coloration. Because of their limited dispersibility in a hydrophilic food matrix, it is difficult to use them for varied applications. As a result, attempts to chemically synthesize hydrophilic carotenoids have been made recently. In the present investigation, a rare polar carotenoid; 1'-OH-4-keto-ϒ-carotene was identified from a novel *Rhodococcus kroppenstedtii* MH715196 that can be explored for its potential usage in food industry. Further, an attempt was made to propose biosynthetic pathway of 1'-OH-4-keto-ϒ-carotene based on gene homology searches in the reference genomes and comparison with published pathways for other rhodococci. Future research for verifying the predicted genes by knock-out mutant studies is suggested to validate the postulated pathway. The phylogenetic analysis revealed a very close similarity between *R. kroppenstedtii* and *R. corynebacterioids* with respect to carotenoid biosynthesis enzymes. Thus, this work could be used as a case study for exploratory research towards carotenoid characterization in novel/unexplored microorganisms.

Acknowledgement

Authors would like to acknowledge Sophisticated Analytical Instrument Facility-IIT Bombay for providing HR-LCMS facility for the present study.

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