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Nostoxanthin Biosynthesis by *Sphingomonas* Species (COS14-R2): Isolation, Identification, and Optimization of Culture Conditions

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Abstract

Nostoxanthin, a yellow pigment, belongs to the xanthophyll group of carotenoids found in various species of bacteria and cyanobacteria. Several species of *Sphingomonas* can produce appropriate carotenoids for survive in various environments. This comprise nostoxanthin, a significant carotenoid. The study isolated the *Sphingomonas* species strain COS14-R2 from the *Cosmos bipinnatus* and identified it through the whole-genome sequence. The strain consists of a circular chromosome with a length of 3,677,457 base pairs.s ecThe genome consists of three carotenoid biosynthesis genes, specifically *crtB* (phytoene synthase), *crtI* (phytoene desaturase), and *crtY* (Lycopene beta-cyclase), which are involved in the synthesis of nostoxanthin. The strain has a circular, undulated colony morphology with a deep yellow color. It demonstrates optimal growth in liquid media at 25 to 35 °C and exhibits a high tolerance for pH levels between 5 and 11 and requires adequate quantities of carbon and nitrogen. We observed the highest concentration of nostoxanthin was recorded at 35 °C, pH of 7.5, glucose concentration of 40 g L^{-1}, and a yeast extract concentration of 5 g L^{-1} during dark incubation. The fed-batch fermentation process produced nostoxanthin at a concentration of 217.22 ± 9.60 mg L^{-1}, with a selectivity of 72.32% and a productivity of 2.59 g/L/h. The dry biomass extract was purified using column chromatography. The LC–MS/MS analysis of the purified fraction indicated that the molecular weight of nostoxanthin is 600.5098 m/z. The DPPH assay result of 75.5 ± 0.33% indicates nostoxanthin is highly effective in scavenging free radicals.

Introduction

Carotenoids are naturally occurring pigmented substances that determine the color of most fruits and vegetables, ranging from yellow to red. Plants and bacteria serve as alternate sources of natural carotenoids, a group of pigmented tetraterpenoids with diverse applications on the market [1]. Carotenoid pigments are classified as either carotenes or xanthophylls based on their chemical composition. Carotenoid consist solely of carbon and hydrogen atoms, but when oxygen atoms are added, they are converted into xanthophylls. The yellow to orange-red pigments function as protection against photo-oxidative damage. Bacteria role as antioxidants is a potential reason for their carotenoids production. Reactive oxygen species (ROS) can harm cells, but antioxidants may shield them from this damage. Regular cellular metabolism produces ROS, which can harm DNA, proteins, and lipids [2]. Carotenoids have the ability to eliminate reactive oxygen species, therefore reducing their harmful impact. Bacteria synthesize xanthophyll by internally scavenging reactive oxygen species and efficiently neutralizing radicals produced during irradiation [3, 4]. Astaxanthin is a highly potent xanthophyll pigment, higher than the potencies of β-carotene, α-carotene, and lutein. However, astaxanthin is considered to be highly safe, as no adverse effects have been recorded, even at high doses. Bacteria extract pigments such as lutein, zeaxanthin, and nostoxanthin, which are all the same. Additionally, adding two hydrogen groups to zeaxanthin produces the yellow pigment nostoxanthin. The pigments chemical composition demonstrates a significantly greater antioxidant capacity than zeaxanthin. However, the production of nostoxanthin by microorganisms is insignificant, and only a few *Sphingomonas* species have exhibited this ability [5]. The primary focus of commercial applications is to isolate, identify, and evaluate the metabolic characteristics of bacterial species obtained from nature. Whole-genome sequencing plays a crucial role in

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the identification of important secondary metabolites. Many secondary metabolites are organic compounds produced by specific gene clusters identified through comprehensive genome annotation. The genus *Sphingomonas* can produce carotenoids, including lycopene, β-carotene, zeaxanthin, caloxanthin, and nostoxanthin, to adapt to different environments [5–7]. In recent years, microorganisms have demonstrated considerable potential in biotechnological applications for the production of carotenoids. Researchers have conducted extensive studies to achieve large-scale industrial production of carotenoids. Large-scale extraction of carotenoids from plant sources is unfeasible due to economic, environmental, and logistical constraints. Bacterial fermentation presents a very efficient substitute source that offers significant advantages in extensive production, economic efficiency, and safety [8]. Consequently, there has been a quest for alternatives, including enhancing strains, establishing standardized fermentation conditions, and discovering and analyzing novel strains. Therefore, understanding the nutritional requirements of strains is essential for the growth and production of carotenoids in microorganisms. Additional research is required to analyze the composition of the culture media as a possible provider of carbon, nitrogen, and trace elements. Also, recent studies have focused on improving the process of carotenoid synthesis and specified the efficacy and economic viability of carotenoid production. In a batch fermentation procedure, *Rhodothermus marinus* used glucose as the only carbon source to achieve high levels of carotenoid and exopolysaccharide synthesis [9]. Carbon and nitrogen sources are essential for fermenting and synthesizing specific carotenoids. Moreover, the substrates physicochemical characteristics and processing parameters affect cellular growth and carotenoid profiles [10]. The strain identified through genome sequencing analysis shows the presence of carotenoid biosynthesis gene clusters. However, specific information about the metabolic pathway and gene clusters associated with this process has yet to be fully understood [11, 12]. Furthermore, *Sphingomonas* is considered to be safe, and certain strains of this bacterium have the potential to produce gellan gum, a type of polysaccharide commonly utilized in many food products to enhance their stability, gelling properties, and suspension [13, 14]. The study successfully achieved significant nostoxanthin production and aimed to evaluate the efficacy of eliminating free radicals.

Materials and Methods

Strains Isolation, Conservation and Characterization

The bacterial strain COS14-R2 obtained from the flower known as *Cosmos bipinnatus*. The flower was collected from Gimji-si, Jeollabuk-do, Republic of Korea. The strain morphology and color were identified using R2A agar (Difco, Maryland, USA). Subsequently, the samples were placed in a dark environment and incubated at 28 ± 1 °C for 7–14 days. The Gram staining procedure was conducted using an optical microscope (Leica DM2500, Germany). The stock culture was conserved using a 20% glycerol solution in R2A liquid media and stored at -80 ºC. Bacterial morphology was examined using scanning electron microscopy (SEM, Carl Zeiss Gemini SEM 300).

DNA Extraction and Genome Sequencing of COS14-R2

The pure bacterial strain COS14-R2 ($\sim 1 \times 10^9$) was used for DNA extraction (Fig. 1A). Genomic DNA was extracted through a Macrogen MG™ genomic DNA purification kit and quantified using a fluorescence-based approach. The concentration of pure genomic DNA measured by the NanoDrop spectrophotometer and Qubit fluorometer. At the same time, RNA integrity was assessed using an Agilent Technologies 2100 Bioanalyzer (Macrogen, Republic of Korea). For PacBio RS sequencing, 8 µg of genomic DNA was used to create a 20 kb library (Covaris Inc., Woburn, MA, USA) and purified with AMPure PB magnetic beads (Beckman Coulter Inc., Brea, CA, USA), resulting in an apparent size exceeding 40 kb. A total of 10 µL of the library was created utilizing the PacBio DNA Template Prep Kit 1.0 (designed for 3–10 kb). The subsequent steps are based on the PacBio sample Net-Shared protocol, accessible at http://pacificbiosciences.com/. Each sample was prepared using the Illumina methodology and sequenced utilizing the HiSeq™ 4000 platform (Illumina, San Diego, USA). The assembly was performed using the SMRT Portal (version 2.3) de novo assembler, and error correction was executed with Pilin (v1.21). The final step of annotating all relevant genomic characteristics on those Contigs can be executed using the rapid prokaryotic genome annotation pipeline (PROKKA). The 16S rRNA nucleotide sequence of strain COS14-R2, obtained from the whole-genome sequence, was compared for similarities against the reference genome database using NCBI microbial genome BLAST. The ten most prominent sequences from the search were chosen for pairwise comparison utilizing the EzBioCloud database. The phylogenetic tree was generated with maximum likelihood and neighbor-joining methods, employing a bootstrap value 1000 with MEGA7 software.

Culture Conditions and Carotenoid Pigment Production

The bacterial strain was cultivated in a 100 mL baffled Erlenmeyer flask containing 50 mL of media using an orbital

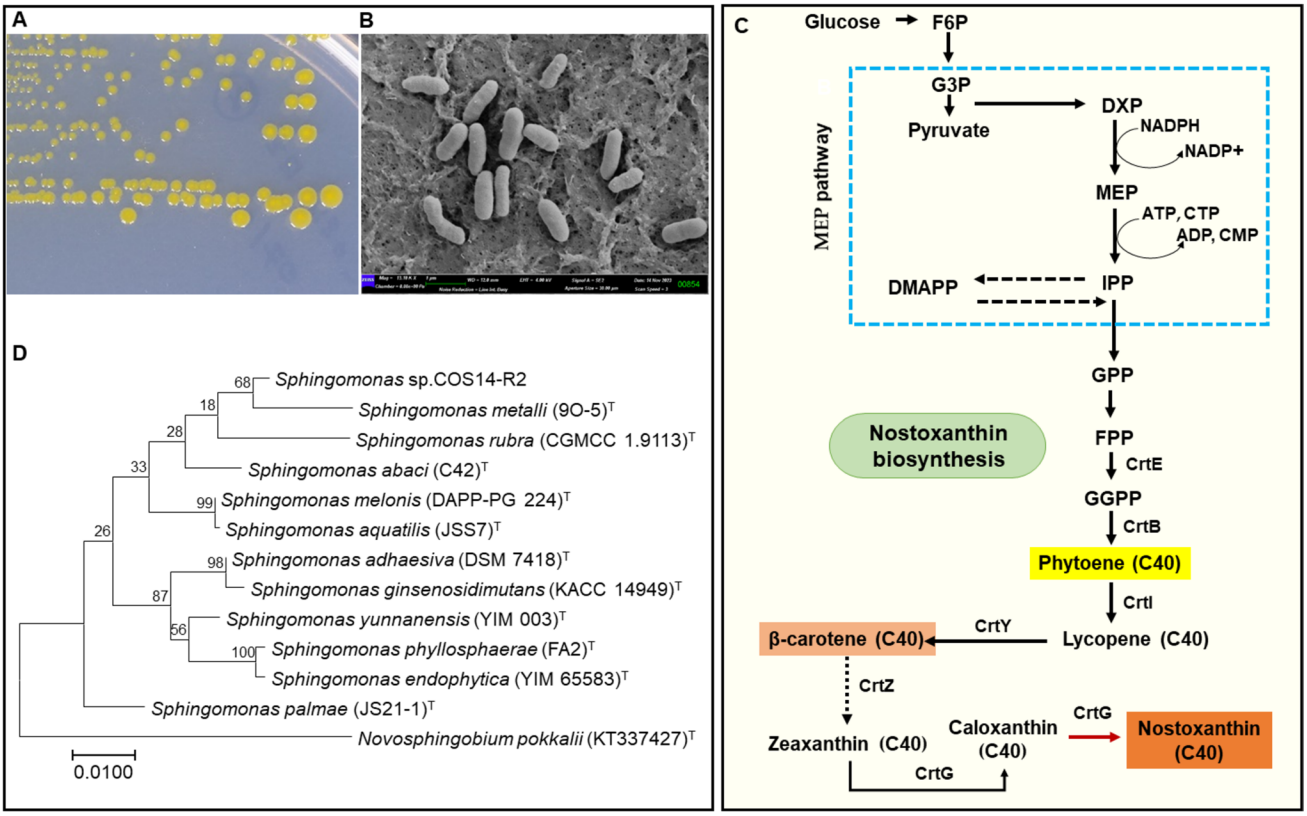

Fig. 1 Experimental strain morphology and nostoxanthin biosynthesis pathway. **A** Pure colonies of *Sphingomonas* sp. strain COS14-R2. **B** Scanning electron microscope image (SEM, × 10,000). **C** Simplified schematic representation of nostoxanthin biosynthetic pathway in *Sphingomonas* species. Presumptive pathways of carotenoid biosynthesis in strains of *Sphingomonas* is presented in a blue doted lined. Shorthand notations of enzymes are indicated in blue and red. β-carotene (whose biosynthesis is enabled by the presence of *CrtE*, *CrtB*, *CrtI*, and *CrtY*) appears to be the branch point for three different pathways that produce nostoxanthin (using *CrtG*), zeaxanthin (using *CrtZ*) and caloxanthin (*CrtG*). Abbreviations: F6P–Fructose 6-phosphate; G-3-P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-d-xylulose-5-phosphate; MEP, 2-C-methyl-d-erythritol-4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate, ATP–Adenosine triphosphate; ADP–Adenosine diphosphate; CTP–Cytidine monophosphate; CMP–Cytidine triphosphate. **D** Phylogenetic tree of strain COS14-R2 and related microorganisms created with the neighbor-joining method. Numbers indicate bootstrap values; T indicates type strain of a species

shaker set at 150 rpm. A defined quantity of inoculum (v/v) obtained from a culture in the exponential development stage is added to a new, sterile medium. To examine the influence of temperature on the production of yellow pigments, the incubation temperature was adjusted to different levels, ranging from 10 to 40 °C. Bacterial pigment synthesis has been enhanced to enable the adjustment of pH and the C/N ratio. To evaluate the influence of pH on pigment production, the pH levels of the culture medium were adjusted to cover a spectrum of values ranging from 5 to 11. The medium consisted of 40 g L^{-1} glucose, 5 g L^{-1} yeast extract, and 0.7 g L^{-1} $MgSO_4$ (SI. Table 1). The pH of the media was initially set to 7.5 before sterilization. Batch fermentation was conducted using a 300-mL medium in 100 and 1 L baffled Erlenmeyer flasks. The flasks were incubated at 35° C on a rotary shaker set at 150 rpm for 120 h. The culture was continuously irradiated by light-emitting diodes with four combinations of monochromatic narrowband light: red, green, blue, and white light (RGB 12 V, MANI LED, South Korea). Linear LED lights were positioned underneath the culture flask in every experiment. The study assessed growth rates, biomass generation, carotenoid levels, and concentration. During incubation, samples were collected at 48 and

Table 1 Genomic features of *Sphingomonas* species strain COS14-R2

Genome features	
Genome size (bp)	3,677,457
Total number of contigs	3
Genomic G + C content (%)	68.35
Coding sequences	3,375
Transfer-RNA genes	53
Ribosomal-RNA genes	6

72 h of growth. Centrifuged cells were collected and rinsed with distilled water. The dry weight (mg/L) of the cells was determined via freeze drying (FD8508, iLShin Biobase, The Netherlands).

Fed-Batch Fermentation

Fed-batch fermentation (Marado-PDA, CNS Co., Ltd., Korea) was carried out based on the batch fermentation, in duplicate, with an initial working volume of 2 L and a total volume of 2.5 L in the bioreactor. The following physical variables were controlled: pH 7.5 temperature, 35 °C; agitation speed, 200 rpm; and the dissolved oxygen concentration, 60%. Feeding solution was prepared with glucose (500 g/L) and MgSO4 (0.7 g/L) under a pH condition and fed for 72 h. The feeding strategies for the fed-batch process were based on the procedure described earlier [15, 16]. Glucose used as the sole carbon source for a fed-batch fermentation process was developed to obtain a high cell density culture with high nostoxanthin productivity and concentration.

Carotenoid Pigment Extraction and Quantification

The carotenoid content was removed from the recovered cells by employing acetone at 55° C and an ultrasonic bath (220 V and 60 Hz, JINWOO JAC Ultrasonic Device, Korea) until the cells were fully decolorized. Cells were collected from the fermented media of *Sphingomonas* sp. strain COS14-R2 at different time intervals. The yellow carotenoid pigment was isolated using centrifugation at 10,000 rpm, 20 min at 4 °C. The yellow supernatant was passed through a 0.2 µm nylon syringe filter (HENKE-JECT, Korea) and dried using a dust-free N_2 flush (TLS Technology, TLS HPS-1, Korea). The carotenoid concentration in a batch culture was measured using high-performance liquid chromatography (HPLC) with a UV–visible photodiode detector manufactured (Agilent, USA). The study was performed on a 250 × 4.6 mm D. S-3 m YMC carotenoid column at a wavelength of 470 nm. The mobile phases consist of a solvent mixture including ethyl acetate, methanol (95:5), ammonium acetate (0.4 g/L), and acetic acid (0.1%) in methanol. There are six distinct mobile phases. The flow rate was set at a constant value of 0.6 mL/min. The detection of carotenoids was carried out utilizing a diode array detector (DAD) within the wavelength range of 300 nm to 700 nm. The retention time (tR) of specific carotenoids on HPLC system is determined by calculating the standard (Sigma-Aldrich, Germany) xanthophyll, astaxanthin, zeaxanthin, and β-carotene retention time and establishing a linear relationship. The concentration of the solution ranged from 0.49 to 125 mg/mL. The total carotenoid content (TCC) was determined following the previously published methods [17, 18].

Column Chromatography

Silica gel column chromatography was used to purify the total carotenoid from COS14-R2. Silica gel (200–200 mesh) was activated at 110 °C for 4 h to remove the moisture using a wet packing column (600 mm × 500 mm). The column was equilibrated with petroleum ether and acetone (6:4, v/v). Prior to sample separation, the level of liquid was lowered to equal the stationary phase. Cell biomass was harvested from a fed-batch fermenter and washed twice using deionized water. The samples were freeze-dried and kept at – 80 °C before nostoxanthin extraction. Yellow colored freeze-dried biomass 5.33 g was soaked in acetone (1:10) in closed screw flask bottle. The extraction was conducted at a room temperature, with continuous magnetic stirring at a rate of 600 rpm. The subsequent extracts were dried in rotary evaporator (EYELA, Japan), One gram of sample was blended with silica gel, and loaded in the column. The elution was performed with a gradient of petroleum ether and acetone at a constant flow rate of 50 mL/min, and the fractions were screened using HPLC and UV. Based on the HPLC retention time (t_R) and UV absorption (λ_{max}) of specific carotenoids combined together. Totally 104 fractions were collected and similar characteristics fractions were concentrated by rotary evaporator. Further, the colored samples were air-dried in a dust-free N2 flesh (TLS HPS-1, Republic of Korea). Dry weight of the samples was determined after freeze drying. Each individual fractions were dissolved in methanol and filtered (0.22 µm) for further characterization. The retention time (tR) of specific carotenoids on a HPLC calculated by standard retention time and linear relationship. Four analytical standards of astaxanthin, lutein, zeaxanthin and β-carotene (Sigma-Aldrich, Germany) and F6 were dissolved in methanol at a concentration of 0.78–100 mg/mL (standard), 0.025–3.2 mg/mL (F6) and a standard calibration curve was constructed for quantification.

Chromatographic Analysis for Nostoxanthin Identification

The purity of F6 was assessed using thin-layer chromatography (TLC) compared to reference standards (1/10 mg/mL, zeaxanthin, astaxanthin, and β-carotene). Each fraction was applied to a TLC Silica Gel 60 F254 plate (Merck, Darmstadt, Germany). The plates were developed with petroleum ether, with a separation achieved using a mixture of acetone in a ratio of 6:4 (v/v). A distance of 7.8 cm was completed after 16 min of development at a temperature of 22 ºC. The plates were dried using a hair dryer and cool air. The values were recorded for both the samples and standards. The purified nostoxanthin fraction was identified and characterized by liquid chromatography coupled with MS. The freeze-dried F6 sample was suspended in methanol and underwent

water bath sonication. The known concentration of methanol solution was examined in an LC system (ACQUITY I-Class plus FTN) and coupled to the electron spray-ion trap mass spectrometer XEVO G2-XS QTof (Waters Corporation, Milford, MA) [16]. The purified fractions were examined using FT-IR spectroscopy (Perkin Elmer, Germany).

In vitro Free-Radical Scavenging Assay

The radical scavenging activity test was conducted using the methodology published by Xiao et al. [19]. Serial dilutions of the column-purified nostoxanthin (1 mg/mL) were made using ethanol as the solvent. Ascorbic acid and quercetin used as a standard (0.1 mg/mL). Each 1 mL portion of DPPH solution was promptly combined and thoroughly blended with various concentrations of nostoxanthin (ranging from 100 to 0.8 µg/mL) and ascorbic acid, quercetin evaluated with different concentration gradients.

Statistical Analysis

All experiments were performed in triplicate. The means and standard deviation of means were calculated by Microsoft Excel 2018 (Microsoft Office). Duncan's multiple range tests were used for conducting pairwise or individual (one-to-one) comparisons in the analysis of variance (ANOVA). A significance level of $p < 0.05$ was used to determine the individual differences. The statistical analyses were conducted using the IBM SPPS statistical program (Version 27, SPPS Inc., Chicago, USA).

Genome Sequence Accession Numbers

The genome sequence of strain COS14-R2 was deposited in the GenBank database under the accession number PRJNA1132764. The strain has also been deposited in Korean Agricultural Culture Collection (KACC) with the accession number KACC 23802. In addition, culture was deposited in the Korean Collection for Type Cultures (KCTC), and the accession number was KCTC 8638.

Results

Isolation and Identification of Yellow-Pigmented *Bacteria*

The COS14-R2 strain was obtained from the flower of *Cosmos bipinnatus* using experimental techniques. The isolate shows a prominent yellow pigmentation on R2A agar (Fig. 1A). The colonies displayed a circular morphology like a road, with a width ranging from 0.4 to 0.6 µm and a length ranging from 1 to 1.2 µm (Fig. 1B). The genome comprised a circular chromosome containing 3,677,457 nucleotide pairs. The sample has a G + C content of 68.35%. Additionally, three Contigs were found and the genome sequences were annotated using the EggNOG database (Fig. SI 1). In addition, the genome consists of 53 genes that encode transfer RNA and six genes that encode ribosomal RNA (Table 1). Analysis of the complete genome sequence reveals gene clusters that are responsible for carotenoid formation, such as *crtB* (phytoene synthase), *crtI* (phytoene desaturase), and *crtY* (Lycopene beta-cyclase) (Fig. 1C). The 16S rRNA gene sequence of COS14-R2 was obtained by comparing it with other sequences in the EzBioCloud database. The 16S rRNA sequences of *Sphingomonas* sp. and *S. abaci* showed a similarity of 98.15% and 97.87%, respectively. The phylogenetic relationship of 10 closely related strains of *Sphingomonas* species was determined using the MEGA software program. The COS14-R2 strain exhibits two distinct branches within the *Sphingomonas* genus, which are strongly supported by high bootstrap values (Fig. 1D).

Optimized Culture Conditions for Nostoxanthin Production

The strain COS14-R2 cultured in a minimum medium to evaluate the influence of varying temperatures on biomass and nostoxanthin production.. The highest cell density was recorded at 25 and 28 ºC temperatures, with related optical density (OD_{600}) values of 0.88 and 0.82, respectively. The highest biomass yield was observed at 28° C, with a value of 920 mg L^{-1}. However, there was a significant difference in the biomass yielded at various temperatures. The lowest biomass yield was recorded at 37 ºC (220 mg L^{-1}), while no growth or biomass yield was observed at 40 ºC (Fig. 2A). The significant difference highlights the importance of temperature regulation in the production of nostoxanthin, where nostoxanthin can be produced with a high efficiency of up to 92% at 35 ºC. However, the synthesis of zeaxanthin and β-carotene was limited. The maximum level of nostoxanthin production, which represents 89% of the total area, was seen after 72 h of incubation in darkness at a pH of 7.5 (Fig. 2C). In addition, the highest quantity of dry biomass was obtained at a pH of 7.5 (Fig. SI 2A, B).. The study utilized 13 distinct C: N settings.. These conditions significantly affected the synthesis of nostoxanthin (Fig. 3 and 2D). Even though further studies the light sources affect the biosynthesis of carotenoid in batch culture. Batch culture incubated at dark condition results in the highest carotenoid content, precisely 29.51 mg/g. The primary pigment, nostoxanthin, is abundant in dry cell biomass, with a concentration of 28.87 mg/g after 72 h (Fig. SI 4).

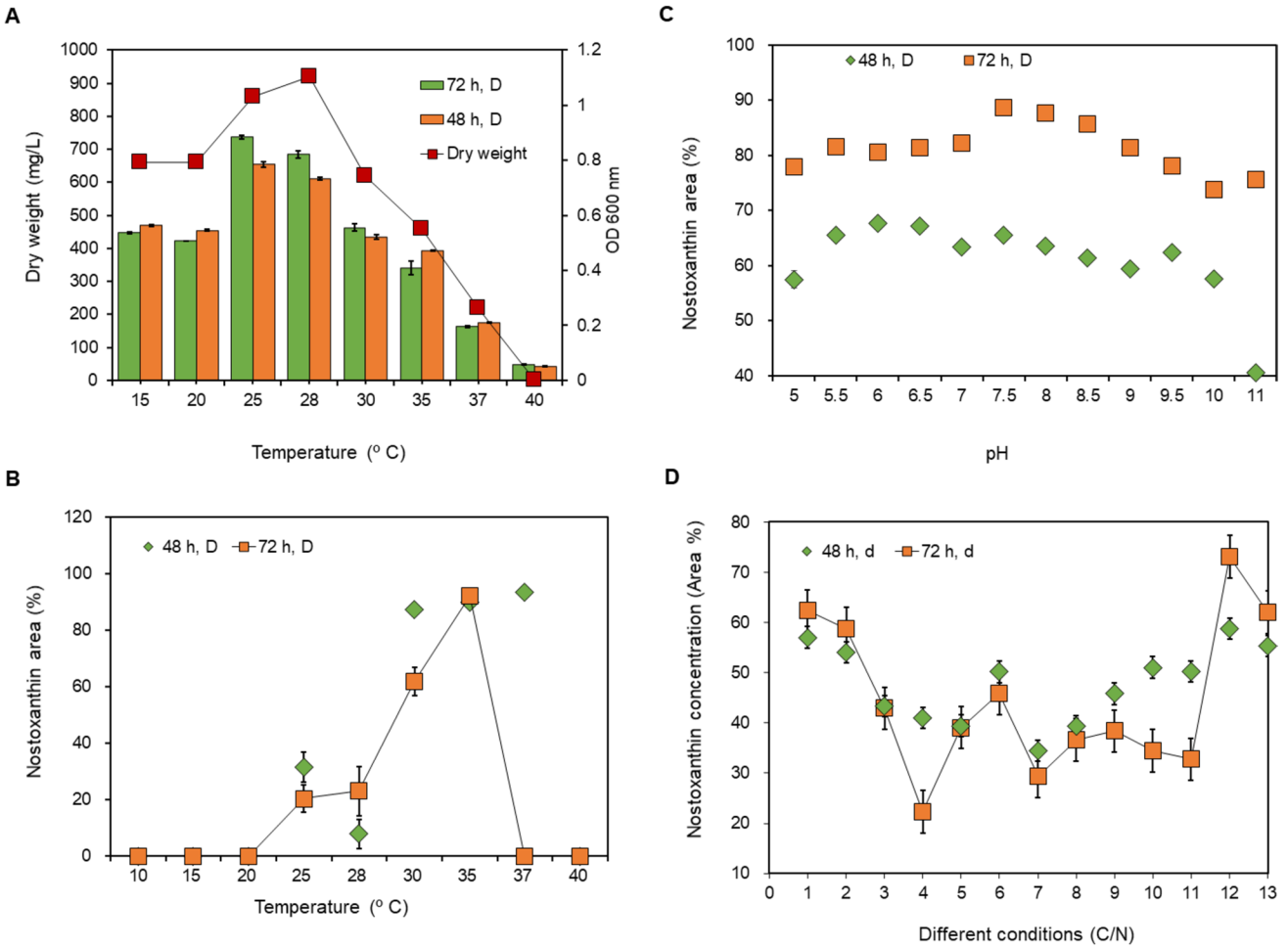

Fig. 2 Effect of different physiological parameters on bacterial growth and level of nostoxanthin production expressed in area percentage. **A** Incubated in different temperature, dry weight (left axis) and optical density (OD_{600}, right axis). **B** & **C** Effect of temperature and pH on nostoxanthin production in two different time intervals. **D** Production of nostoxanthin from different C: N ratios. Data shown correspond to values recorded after 48 and 72 h incubation, at 35 °C under continuous shaking at 200 rpm, being the means ± SD ($n = 2$)

Nostoxanthin Production by Fed-Batch Fermentation

The optimized culture conditions significantly enhanced the production of nostoxanthin during batch fermentation. The highest cell density was observed at 96 and 108 h, with optical density (OD600) values of 2.74 and 2.76, respectively (Fig. 3A). Notably, there was a significant increase in cell biomass was detected in the fed-batch culture after 48 h of incubation (Fig. 3B). The biomass produced varied from 2 to 10 g L^{-1}, dry cell weight (Fig. 3B). The highest dry biomass (10 g L^{-1}) and nostoxanthin production (217.22 ± 9.60 mg L^{-1}) were observed after 84 h.. The COS14-R2 strain synthesizes several carotenoids, with nostoxanthin being the most abundant carotenoid (Fig. 3C). In addition, the FBC extract contains specific carotenoids, including nostoxanthin (peak 1), lutein (peak 2), zeaxanthin (peak 3), and β-carotene (peak 4), which can be identified by standard carotenoids (Fig. 3D). The peak areas obtained from the HPLC analysis were determined by utilizing the standard peak retention time. However, the peak area at 11.327 min did not correspond to the values expected from the standards. In addition, a low concentration of lutein with a retention time (RT) of 12.478 min (4.47 ± 0.05 mg L^{-1}), zeaxanthin with an RT of 13.274 min (1.31 ± 0.01 mg L^{-1}), and β-carotene with an RT of 29.248 min (3.01 ± 0.03 mg L^{-1}) was observed after 84 h of incubation (Fig. 3C, D). The color in the FBC medium changed into an intense orange-yellow due to a significant accumulation of nostoxanthin and β-carotene (Fig. 3A).

Purification and Characterization of Nostoxanthin from *Sphingomonas* sp. Strain COS14-R2

The overall yield of the fractions (F1 to 10) is 13.01% from dried biomass (5.33 g). In addition, Table 2 displays the outcomes for yield, λmax, HPLC retention duration, and relative

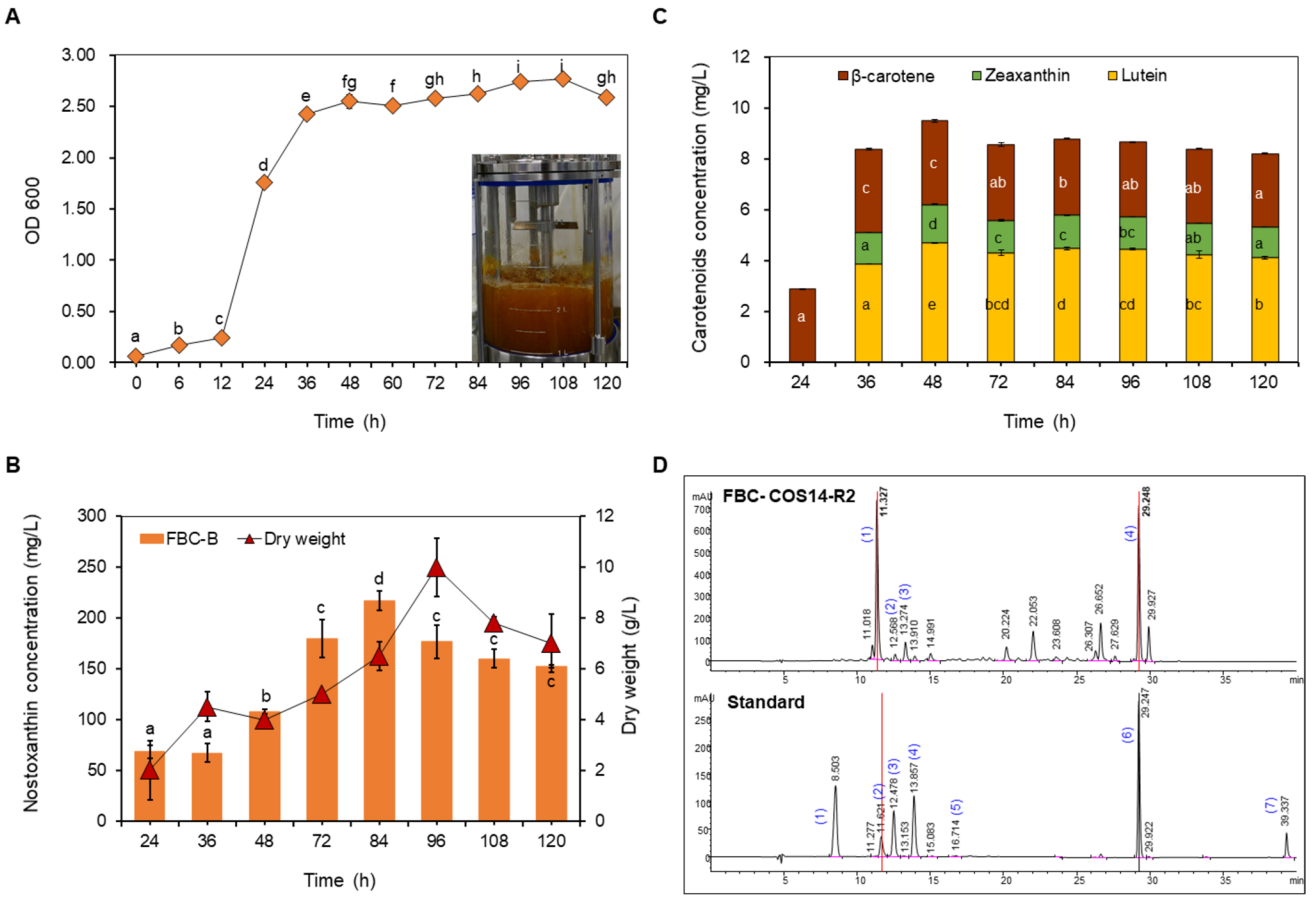

Fig. 3 The optimized culture conditions for the *Sphingomonas* sp. strain COS14-R2 and a fed-batch fermentation profile. **A** The growth curve of strain COS14-R2 in R2A media supplement with glucose (40 g L^{-1}) and yeast extract (5 g L^{-1}) and culture at 35 °C. Fed-batch fermenter vessel picture was capture at 84 h. **B** Specific carotenoid nostoxanthin concentration in fed-batch fermentation, the results represented in mean value ± SD ($n=2$), different alphabetic letters indicate significant difference $p<0.05$ by Duncan's multiple range test. Dry weight of the cell pellet was determined by difference after freeze drying (g L^{-1}). (C) Other carotenoid concentration of COS14-R2 extracted from different time intervals. Different letters on the bars indicate significant differences at $p<0.01$. (D). HPLC chromatograms of carotenoids extracted from COS14-R2 compared with commercial standard. FBC-COS14-R2: peak 1 nostoxanthin, peak 2 lutein, peak 3 zeaxanthin, peak 4 β-carotene of the *Sphingomonas* sp. strain COS14-R2 during 84 h of cultivation. Standard: peak 1 violaxanthin, peak 2 astaxanthin, peak 3 lutein, peak 4 zeaxanthin, peak 5 canthaxanthin, peak 6 β-carotene, peak 7 lutein. Numbers in parentheses indicate the peak numbers in Fig

purity percentage (Fig. SI 5). The fraction F6, obtained by isolation, yielded 16 mg (dry weight) and showed a high level of purity, with an area of 99%. The UV–visible spectrum of F6 from strain COS14-R2 shows the maximum absorption (λmax) at 450 and 480 nm (Fig. 4A). On HPLC single peaks was observed with retention times 11.378 min, as deciphered by comparison with commercial standards and no similarity were observed (Fig. 4B). The TLC elution system, consisting of a petroleum ether and acetone combination in a 6:4 (v/v) ratio, had the highest efficiency in separating pigments (Fig. 4C). The R_f value of nostoxanthin, determined to be 0.25, did not correspond to the R_f values of astaxanthin (8.1), zeaxanthin (7.5), and β-carotene (0.98). FT-IR and LC–MS/MS analyses were carried out to confirm the structural identification of nostoxanthin. The isolated nostoxanthin FT-IR spectra reveal prominent O–H stretching and reduction is observed in the broad and intense peak, which has a maximum between 3700 and 3100 cm^{-1}. The addition peeks were observed at around 2945 cm^{-1} and 2834 cm^{-1}, 1717–1621 cm^{-1}, 1450 cm^{-1}, and 1061–965 cm^{-1} (Fig. 4D). The peak 1717–1621 cm^{-1} correspondence to the carbonyl group and C = O peak. The presence of OH and C = O in each ionone ring explains some of the features of nostoxanthin, such as the ability to be esterified and a more polar nature. The purified nostoxanthin carotenoid was mixed with methanol and examined using positive-ion LC–MS/MS. The molecular weight formula of this nostoxanthin was determined to be $C_{40}H_{56}O_4$ using LC–MS/MS spectra data, specifically by analyzing the m/z 600.5098 signal (Fig. 4E). The mass spectra obtained

Table 2 Carotenoids fractions from *Sphingomonas* sp. strain COS14-R2 and their absorption maxima (λ_{max}) and HPLC retention time and area percentage (%). [a] Commercial standard are used as a references. [b] Fractions were eluted from column chromatography (petroleum ether: acetone). [c] Value between brackets indicate a shoulder in the spectrum. NA- reference compound not available

Carotenoid standards/Fractions	Yield of fractions (mg)	UV absorbance (λ_{max} nm)[c]	HPLC (RT. Min)	Relative area (%)
Astaxanthin[a]	NA	480	11.621	100
Lutein[a]	NA	(428), 444, 472	12.478	100
Zeaxanthin[a]	NA	450, 476	13.857	100
β-carotene[a]	NA	448, 478	29.247, 29.922	98.531, 1.466
F1	135	(428), 448, 478	14.117, 21.781, 26.018, 28.731, 29.378	8.754, 7.936, 11.333, 53.381, 14.053
F2	131	(424), 450, 474	11.399, 12.614	40.294, 51.946
F3	84	(426), 448, 474	10.732, 11.411, 12.63, 14.525	7.383, 79.735, 7.293, 5.589
F4	44	(420), 448, 474	10.734, 11.412, 14.529	9.178, 83.202, 7.620
F5	95	(420), 448, 474	10.731, 11.405, 14.525	17.219, 70.531, 12.250
F6	16	(424), 450, 480	11.378	99
F7	25	448, 474	10.74, 11.412	10.152, 82.045
F8	5	(418), 448, 474	10.739, 11.408	10.768, 78.164
F9	146	(418), 448, 474	10.745, 11.414	9.681, 82.167
F10	17	(418), 448, 474	10.746, 11.415	25.264, 65.523

using precursor ion m/z 600.5098 exhibited unique patterns of product ions indicating tetrahydroxy carotenoids and this patterns were identical to nostoxanthin. (Table 3).

In vitro Antioxidant Activity of Nostoxanthin Pigment

The pure nostoxanthin exhibits notable antioxidative properties, as evidenced by the DPPH assay ($75.49 \pm 0.3\%$) (Fig. 5A). The concentration of 50 µg/mL has a maximal free-radical scavenging activity of 75.49%. The IC_{50} value of nostoxanthin was found to be 30.97 µg/mL (Fig. 5B), whereas ascorbic acid and quercetin, which were used as standards, exhibited IC_{50} values of 12.14 and 7.79 µg/mL, respectively (Fig. 5C, D).

Discussion

Nostoxanthin, a naturally occurring pigment, can be found in some species of *Cyanobacteria* as well as specific genera of *Pseudomonas* and *Sphingomonas* [6, 11, 20, 21]. Furthermore, scientists have documented the presence of nostoxanthin in the marine bacterium *Erythrobacter flavus* [22]. Also, *Sphingomonas* sp. SG73, found in the Deep Sea sand, showed significant production of nostoxanthin. The sample exhibited a high purity level, with an HPLC area percentage of approximately 97%. A recent study identified several xanthophyll pigments in marine and freshwater creatures [23]. *Erythrobacter flavus*, a marine microbe, has been shown to synthesize sulfated xanthophylls, including nostoxanthin sulfate, zeaxanthin sulfate, and caloxanthin sulfate [22]. A yellow-pigmented marine bacterium, namely the *Erythrobacter* species SDW2 strain, was initially obtained from coastal saltwater on Jeju Island, Republic of Korea [24]. In addition, a strain of *S. jaspsi* that exhibited a yellow pigment was discovered in a freshwater sample from Misasa, Japan [25]. The present investigation, COS14-R2, was derived from *Prunus serrulata* Lindl. The colonies had a distinctive shape resembling a road and displayed a circular morphology. The investigation of the entire genome sequence reveals the existence of gene clusters responsible for carotenoid biosynthesis genes including *crtB*, *crtI*, and *crtY*.. The synthesis pathway of nostoxanthin has been proposed in *S. elodea*, and the *crtG* gene has been identified [5]. Jiang et al. suggested the production pathway of nostoxanthin for the recently identified *S. nostoxanthinifaciens* sp. [12]. The AntiSMASH annotation analysis revealed that the gene clusters *crtB, crtG, crtI, crtY*, and *crtZ* are responsible for the biosynthesis of nostoxanthin from the precursor geranylgeranyl diphosphate (GGPP) [26]. The carotenoid biosynthesis pathway begins with the creation of phytoene by combining two geranylgeranyl pyrophosphate molecules. Following that, lycopene undergoes cyclization, creating other carotenes, such as β-carotene. Xanthophyll carotenoids are formed through the oxygenation of carotenes. Jiang et al. (2023) reported, nostoxanthin is categorized as a carotenoid belonging to the xanthophyll group [12].

Although several bacterial species have been discovered to biosynthesis the nostoxanthin pigment, commercial manufacture has yet to be successful. *Sphingomonas* yellow-pigmented species are known for their abundant production

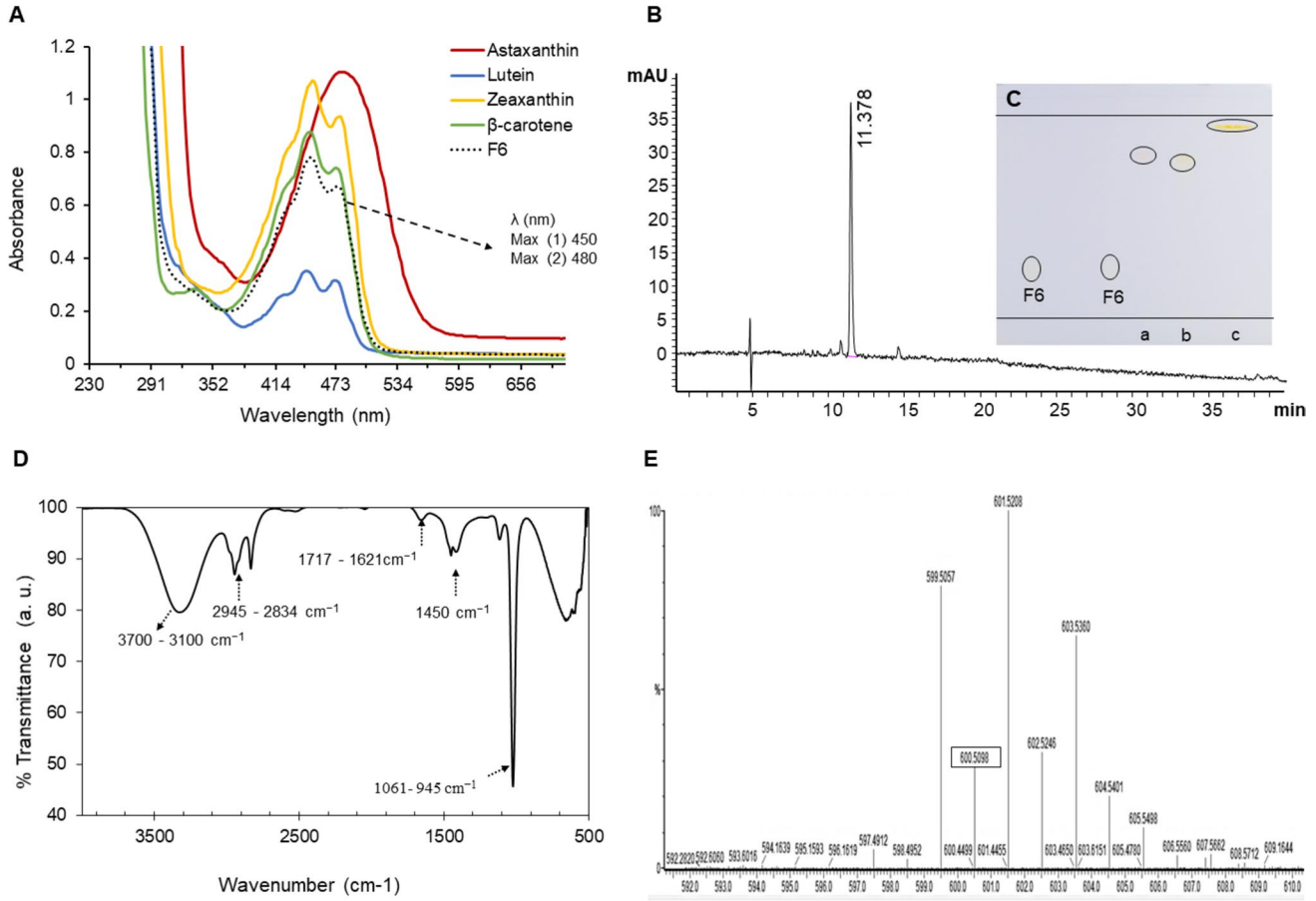

Fig. 4 Nostoxanthin purification and characterization. **A** UV–Vis spectra of purified fraction (F6, dotted line) showing the typical (two fingered) maxima of yellow carotenoid with maximum absorbance around 448–480 nm. The standard astaxanthin (red), zeaxanthin (yellow) and β-carotene (green) expressed in solid line. **B** HPLC elution profiles of purified nostoxanthin (F6 RT: 11.378 min) extracted from *Sphingomonas* sp. strain COS14-R2. **C** Thin-layer chromatography of purified nostoxanthin (F6), astaxanthin (**a**), zeaxanthin (**b**), β-carotene (**c**). **D** FT-IR spectrum (4000–500 cm^{-1}), and **E** LC–MS/MS spectrum of purified nostoxanthin carotenoid from COS14-R2 showing *m/z* 600.5098 and *m/z* 601.5208

of the carotenoid nostoxanthin. Kikukawa et al. found that *Sphingomonas* species have a remarkably high level of purity in nostoxanthin, estimated to exceed 97% [11]. The influence of cultural conditions on nostoxanthin synthesis has been evaluated by examining the effects of incubation temperature, initial pH, and carbon-to-nitrogen ratio (C/N) on production. Jiang et al. (2023) determined that the optimal temperature range for nostoxanthin synthesis in *Sphingomonas* species is 25 to 30 °C [12]. Notably, *Sphingomonas* strains were extracted from fouling membranes and were resistant to a broad spectrum of temperatures ranging from 8 to 42 °C [27]. The present study showed that the optimal conditions for achieving the maximum levels of nostoxanthin concentration and dry biomass yields were a temperature of 35 °C and a pH of 7.5. The pH level most favorable for the growth of the bacterium *S. nostoxanthinifaciens*, which produces nostoxanthin, was pH 8.0 [12]. Using carbon and nitrogen (C and N) sources in an appropriate medium is crucial for enhancing performance, as both sources are important factors. We achieved maximum concentration of carotenoid and biomass yield from 40 g L^{-1} and yeast extract 5 g L^{-1}, which had a significant impact on growth and nostoxanthin synthesis, respectively. The yield of biomass, generation of carotenoids, and accumulation of lipids in bacteria and yeast are influenced by organic carbon and nitrogen sources [28]. Jeong et al. (2022) utilized several carbon sources to produce xanthophylls, and they found that the presence of 5 g L^{-1} glucose significantly impacted the generation of yellow xanthophylls [24]. Various light sources influence on carotenogenesis. Iwai et al. (2008) reported that cyanobacteria generated β-carotene and caloxanthin in response to low light intensity [29]. Takano et al. (2005) observed that white light enhanced the synthesis of yellow pigment. In addition, they identified a specific cluster of genes accountable for the production of carotenoids in *Streptomyces* [30].

Table 3 List of strains produce nostoxanthin carotenoid and their characterization techniques

Species	Place	Source	Characterization techniques	References
Pseudomonas paucimobilis	USA	Culture collection	UV–Vis, TLC, HPLC, FD-MS, NMR	[6]
Sphingomonas sp.	Shizuoka, Japan	Deep sea sediment	UV–Vis, HPLC, LC/MS	[11]
S. nostoxanthinifaciens sp. *nov*	Jeju island in Korea	Korean fir (leaves)	UV–Vis, HPLC	[12]
Sphingobium sp.	Culture collection	Kunming Institute of Botany, China	UV–Vis, HPLC	[29]
Erythrobacter flavus	Karimun Jawa Island, Indonesia	*Acropora nasuta*	UV–Vis, HPLC, FT-IR, MS	[30]
Erythrobacter flavus	Karimunjawa Islands, Indonesia	Hard coral *Acropora nasuta*	UV–Vis, HPLC, FT-IR, MS/MS	[30]
Erythrobacter sp.	Jeju Island, Republic of Korea	Coastal seawater	UV–Vis, HPLC, TLC	[32]
Sphingomonas sp.	Kood Island, Thailand	*Tethya seychellensis*	UV Vis, HPLC	[44]
Xanthophyllomyces dendrorhous	Germany	Mutant strain	UV–Vis, HPLC, HR-ESI–MS	[45]
Nostoc commune	Japan	Culture collection	UV–Vis, TLC, HPLC, MS, NMR	[46]
Sphingomonas sp.(COS14-R2)	Republic of Korea	Flower	UV–Vis, TLC, Column chromatography, HPLC, TLC, LC–MS/MS	Present study

The color of the FBC medium transformed into an intense orange-yellow due to a significant accumulation of nostoxanthin and β-carotene. Nevertheless, the generation of nostoxanthin was maximum under the optimized culture conditions. Silva et al. (2004) discovered that the soil samples contained *Sphingomonas* sp. produced 1.7 mg dry biomass, among which β-carotene 29% and nostoxanthin 36% of total carotenoids [31]. The purified fraction F6 extracted from COS14-R2 exhibited λmax values at 450 and 480 nm in its UV–visible spectrum. The spectra closely matched those of nostoxanthin and indicated the presence of a β-β-carotene-type chronopher. A recent study detected the presence of nostoxanthin by observing a clear peak at wavelengths of 452 and 480 nm [11, 12]. However, the β-carotene levels at wavelengths 422, 450, and 476 nm nearly resemble those of nostoxanthin [32]. Pavelkova et al. (2020) found that the UV spectra closely calculated those of zeaxanthin, with λmax values at 422, 450, and 476 nm [33]. In addition, the purified fractions underwent HPLC analysis, which revealed that the retention time of 11.327 min corresponded to nostoxanthin. Previous studies have shown that nostoxanthin, obtained from *Sphingomonas*, was separated from complex xanthophyll mixtures using HPLC [5, 11]. FT-IR peak detected at around 2945 cm^{-1} and 2834 cm^{-1} corresponds to the stretching vibrations of C-H bonds. The presence of conjugated double bonds, either C=C or C=O, is indicated by the range of 1717–1621 cm^{-1}. The peak 1760–1665 cm^{-1} correspondence to carbonyl group and C=O peak from 1740 cm^{-1} to low frequency due to its conjugated system and mainly to the presence of hydrogen bonds in the dimer form [34]. The presence of OH and C=O in each ionone ring explains some of the feature of nostoxanthin, such as the ability to be esterified, a more polar nature and a high antioxidant capacity. The spectral band around 1450 cm^{-1} corresponds to the vibrations of CH_3 groups and CH_2 groups [35]. A clear peak can be seen in these spectra at around 1061–965 cm^{-1}, indicating the vibrations of C-O stretching in alcohols. This band is most likely a result of xanthophylls. Indeed, the presence of this band can be detected in the FT-IR spectra of the pure molecule [36]. The chemical formula of this carotenoid was identified as $C_{40}H_{56}O_4$ based on the ESI MS spectra data of m/z 601.5208 $[M=H]^+$ and m/z 600.5098 $[M^+]$. The mass spectra obtained using precursor ion m/z 600.5098 exhibited unique patterns of product ions indicating tetrahydroxy carotenoids. The production patterns were identical to nostoxanthin and primary products ions from carotenoids [11, 37]. The isolated nostoxanthin molecular mass was identified in *Sphingomonas* sp. SG73, which was isolated from marine sediment [11]. Nostoxanthin is a polyhydroxy derivative of β-carotene that is only present in some prokaryotes, particularly certain species of cyanobacteria and many bacterial species [29, 38–40]. The marine bacteria *Erythrobacter* sp. produced xanthophyll pigments at 263 mg/L and exhibited remarkable antioxidant activity [24]. *Sphingomonas* strain COS14-R2 can synthesize carotenoid pigments that are of economic significance. This strain accumulates substantial quantities of yellow carotenoid pigments in the fermentation broth, predominantly nostoxanthin. Furthermore, researchers have examined the antiradical capabilities of carotenoids by evaluating the energy required to break the carotenoid-hydrogen bond using the hydrogen atom transfer antiradical

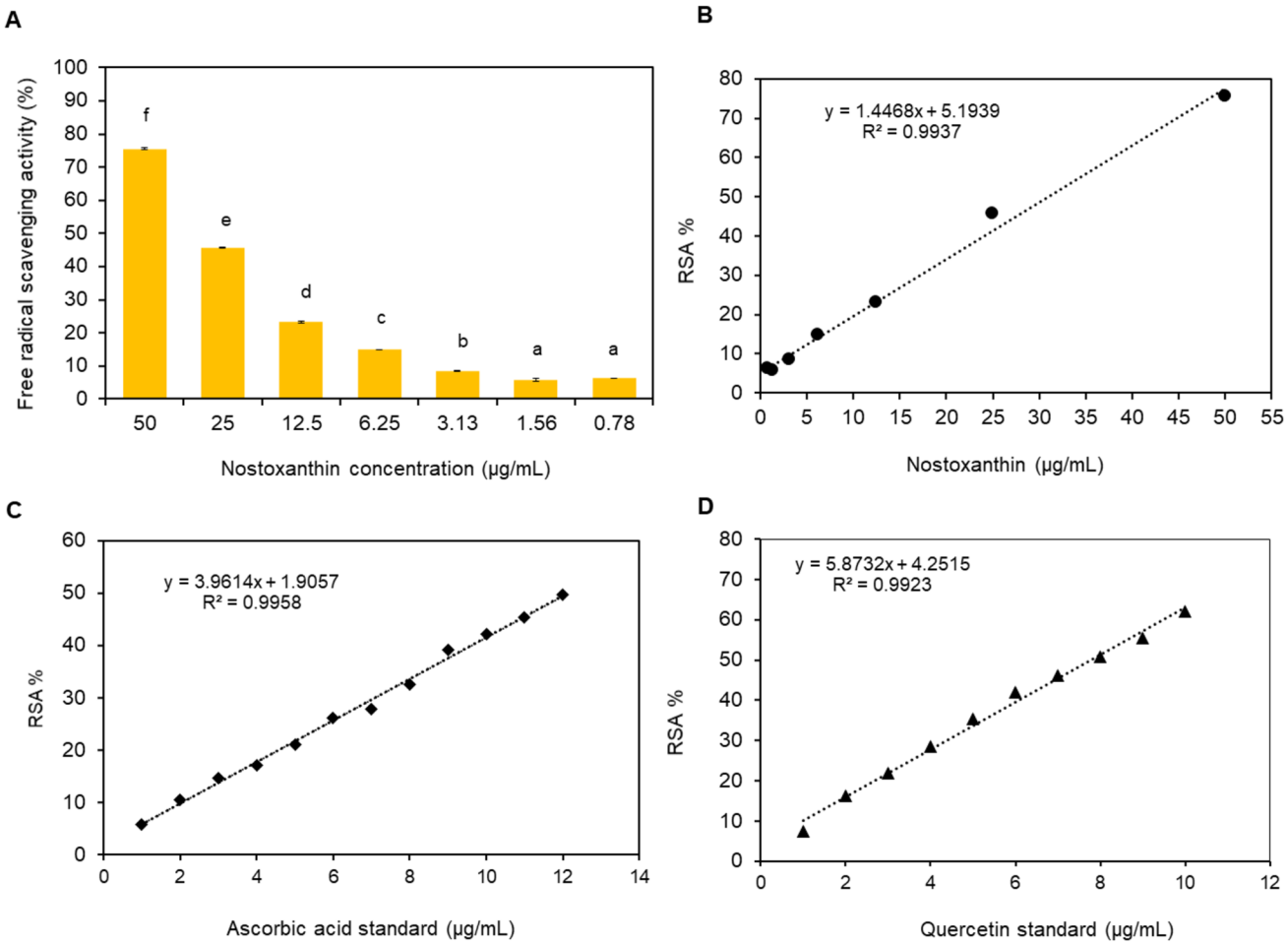

Fig. 5 Free radical scavenging activity using various concentrations of the nostoxanthin of the COS14-R2 strain for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. **A** Inhibition percentage of DPPH by different concentration of COS14-R2 nostoxanthin. **B** Standard curve of COS14-R2 nostoxanthin. **C** & **D** Standard curved of ascorbic acid and quercetin, respectively

mechanism [41]. Nostoxanthin, due to its increased number of double bonds and hydroxyl groups, demonstrates enhanced scavenging properties. Staphyloxanthin, obtained from some bacterial species, shows a noteworthy ability to scavenge DPPH radicals, with an IC_{50} value of 54.22 µg mL^{-1} [42]. Jeong et al. discovered that a xanthophyll pigment extracted from *Erythrobacter* species significantly eliminates radicals when administered at a concentration of 20 mg/L, with an IC_{50} value of 13.2 ± 0.4 µg mL^{-1} [27]. The findings indicate that the yellow nostoxanthin pigment generated by the *Sphingomonas* sp. strain COS14-R2 shows significant promise as a natural component with high potential for commercial use.

Conclusion

The use of microorganisms to synthesize carotenoids is becoming more common because of their eco-friendly traits, the accessibility of inexpensive substrates, the rapid production scaling up, and the ability to track processes and outcomes. Consequently, creating and improving microbial fermentation technologies is necessary to achieve sustainable industrial manufacturing of carotenoids. The global carotenoid market was valued at USD 1.90 billion in 2022, and it is anticipated to experience a compound annual growth rate (CAGR) of 4.5% from 2023 to 2032. The market is projected to reach a value of USD 2.95 billion by 2032 [43]. The optimization method precisely determines carotenoids concentration and growth parameters in the *Sphingomonas* sp. strain COS14-R2. As an alternative to glycerol, the strain capacity to use glucose as a carbon source is a significant plus. However, increasing the buildup of nostoxanthin to practical levels is crucial for making this commercially viable. This article presents a more straightforward approach to producing nostoxanthin by fed-batch fermentation and purification and the strain COS14-R2 also biosynthesis β-carotenoids, which are important types of carotenoids. Enhancing the characteristics of this strain

could result in a possible commercial production of nostoxanthin in the future. Many conjugated double bonds define the compounds structure, giving it its color and antioxidant characteristics. Nostoxanthin acts as an antioxidant, protecting cells against oxidative stress by reducing the harmful effects of free radicals. Its antioxidant properties make it a valuable subject of study in biotechnology for potential uses in health supplements, cosmetics, and food additives.

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Author Contributions JR, S-J K conceived the designed the study. S-J K data correction, investigation and visualization. JR involved in interpretation of the data and writing the manuscript. J-S. K and Y-J. K provided literature collection and contributed to visualization. All authors approved submission of the Manuscript.

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Data Availability The data that support the findings of the study are available on request from the corresponding author.

Code Availability Not applicable.

Declarations

Conflict of interest The Authors declare that they do not have any conflict of interests.

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for publication Not applicable.

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