Identification of the wild type bacterium *Paracoccus* sp. LL1 as a promising β-carotene cell factory

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ABSTRACT

Carotenoids are biomolecules naturally produced as secondary metabolites. The scientific interest in microbial carotenoids production is gaining more attraction because of their economic sustainability and cost-effectiveness. This study aimed to define the *Paracoccus* sp. LL1 carotenoids and fatty acids quali-quantitative profiles under controlled fermentation processes carried out at two different temperatures. Moreover, the whole genome of the selected strain LL1 has been sequenced and analyzed, allowing us to identify the gene clusters involved in carotenoid biosynthesis. The fatty acid profile detected and the genome sequencing allowed to rename the strain investigated *Paracoccus marcusii* strain LL1. Stearic and vaccenic fatty acids have been detected in the highest percentage as the main cellular membrane fatty acids characteristic of the strain investigated.

Whereas, twelve different carotenoids produced by the bacterium investigated have been identified. Among these the most produced was β-carotene, which reached up a final concentration of 0.35 ± 0.01 mg g^{-1} of dry biomass.

Furthermore, *Paracoccus* sp. LL1 biomass extract was investigated for antibacterial activity against selected strains.

This study allowed pointing out the great potential of the wild type bacterium *Paracoccus marcusii* strain LL1 as a promising β-carotene producer, representing an interesting alternative for natural carotenoids production.

1. Introduction

Carotenoids are biomolecules naturally produced by plants, algae, fungi, and bacteria as secondary metabolites (López et al., 2023). Due to their antioxidant, prooxidant actions, modulation of signaling pathways, antimicrobial and anti-inflammatory properties (Amengual, 2019; Bernabeu et al., 2023; Bhatt & Patel, 2020; Britton, 2020; Meléndez-Martínez, 2019; Morelli & Rodriguez-Concepcion, 2023), carotenoids production is gaining more attraction for several profitable industrial sectors, such as textile, food and feed, pharmaceutical, nutraceutical, and cosmetics (Barreto et al., 2023; Numan et al., 2018; Ram et al., 2020). The interest regarding the food sector is direct both for food supplement as well as for food smart packaging production. In this sense the antimicrobial and antioxidant effects recognized to carotenoids are

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attracting the attention for innovative smart packaging development, based on the employment of biomolecules (Yu et al., 2023; Mussagy, Oliveira, et al., 2024; Gonçalves de Oliveira et al., 2024).

The international market size for carotenoids, referred to food and nutraceutical supplements, was 2.0 billion dollars in 2022 and it is expected to reach 2.7 billion dollars by 2027, showing a compound annual growth rate (CAGR) of 5.7%, and the 32% of this market is represented by β-carotene (The Global Market for Carotenoids, 2024).

Currently the 80%–90% of carotenoids are produced by chemical synthesis, mainly by applying the Grignard coupling and Wittig condensation methods (Bogacz-Radomska & Harasym, 2018; Singh & Sambyal, 2022). It has been reported that synthetic carotenoids contribute to the generation of toxic waste, mainly represented by chemicals that can negatively affect both the environment and the human health (Joshi et al., 2023). This boosted scientific interest to find potential alternatives. Plants and algae have been investigated a lot for their ability of carotenoids production (Ram et al., 2020). On the other hand, plant/algae-derived carotenoids are costly, need long cultivation times, are strictly dependent on climatic conditions, have lower yield consistency, and often necessitate extensive land and water resources (Lopez et al., 2023). Nowadays, the scientific interest in microbial carotenoids production, mainly as intracellular inclusion due to their lipophilic characteristics (Mussagy et al., 2019) is increasing because of their economic sustainability and cost-effectiveness (Ram et al., 2020). The microorganisms capability to grow rapidly in inexpensive media, with no seasonal restrictions, the wide variety of carotenoids produced, followed by the opportunity to change the molecules production according to the medium composition, are all advantages of microorganisms over vegetables as natural carotenoids sources (Narsing Rao et al., 2017).

Microalgae, fungi, yeast, and bacteria have been investigated for carotenoids production, from the most known β-carotene, torulene, lutein and fucoxanthin to the less common torularhodin, canthaxanthin and astaxanthin (Afroz et al., 2023; Joshi et al., 2023; Mussagy et al., 2019; Papapostolou et al., 2023). Moreover, nowadays the interest on microbial rare carotenoids, such as bacterioruberin, adonixanthin and adonirubin, is attracting the scientists' interest due to their similarity in bioactivity with astaxanthin (Iwata et al., 2018; Hirakida et al., 2022; Mussagy, Oliveira, et al., 2024 a; Mussagy et al., 2024, 2024a, 2024a, 2024b, 2024b; Mussagy, Oliveira, et al., 2024 b).

Microbial carotenoids production has been reported to be affected by stressful environmental conditions such as variation in nutrient composition, temperature, salinity, dissolved oxygen percentage, light and pH (Barreto et al., 2023; Mata-Gómez et al., 2014; Ram et al., 2020; Xiaomei et al., 2022).

Screening of new microbial species for these molecules of biotechnological interest represents the starting point for the evaluation of their industrial application (Vargas-Sinisterra et al., 2021). Moreover it is important to address the research on wild type or mutant strains, since genetic engineering techniques are not allowed by the legislation regarding the industrial use of genetically modified organisms (GMO) in many countries, especially in feed/food sectors (Yaderets et al., 2023).

Bacteria offer several advantages, in comparison with the other carotenoids-producing microorganisms, thanks to their short life cycles, metabolic adaptability, and the easy techniques of propagation (Barreto et al., 2023). Moreover, in comparison to microalgae, fungi and yeast, characterized by more rigid and complex cell walls, that often required physical pretreatment or organic solvents mixture for pigment extraction, bacterial cells lysis is much simpler, resulting in easier extraction procedures (Papapostolou et al., 2023). These characteristics are attracting the biotechnological interest towards these microorganism, and in particular toward the employment of wild type strains. The need to identify new microbial species, especially among bacteria, as carotenoids producers is strongly encouraged for the identification of new sustainable solutions to be improved from a biotechnological point of view, for meeting future market requirements (Raita et al., 2023; Saubenova et al., 2024).

Among the bacterial source of carotenoids, members of the genus *Paracoccus* sp., such as *P. carotinifaciens*, *P. marcusii*, *P. zeaxanthinifaciens* and *P. haeundaensis,* have been shown to produce these important biomolecules, and are protected by intellectual property, confirming the possibility to be employed at industrial scale, being this a fundamental requisite for patentability (Chelliah & Nidamangala, 2005; Hayashi, 2019; Hirasawa & Tsubokura, 2006; Hirschberg & Harker, 1999; Kim & Kumar, 2018; Kim & Lee, 2004; Osanjo et al., 2009; Tetsuhisa et al., 2008). These bacteria are considered interesting cell-factories for carotenoids production, mainly astaxanthin, zeaxanthin and total carotenoids, suitable for the food and feed industries (Chougle et al., 2012; Joshi & Singhal, 2016; Pyter et al., 2022; Sajilata et al., 2010). The most representative example is given by *P. carotinifaciens,* produced at industrial scale and commercialized as Panaferd®, approved by the European Food Safety Authority (EFSA) for its application as feed additive for salmon and trout (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2010).

The genus *Paracoccus* sp., Gram-negative, catalase positive, oxidase-positive bacteria, belongs to the *Alphaproteobacteria* (Osanjo et al., 2009). To date the genus *Paracoccus* contained 106 recognized species (https://lpsn.dsmz.de/genus/paracoccus). These bacteria are naturally present in different environments such as soil, sediment, lake, activated sludge, fish and sea water (Kämpfer et al., 2019; Lee et al., 2011; Roh et al., 2009; Yoon et al., 2019).

Among the *Paracoccus* sp. applied for carotenoids production, the wild type strain LL1 (KP288668) has been isolated by Sawant et al. (2015) from Lonar lake in India and it has been identified as potential single cell factory for polyhydroxyalkanoates and total carotenoids (Khomlaem et al., 2020; Kumar, Jun, & Kim, 2018; Kumar & Kim, 2019; Muhammad et al., 2020). In our knowledge, the complete carotenoids profile of this promising bacterium was not characterized, as well as the LL1 specie. Considering that the carotenoids profile characterization represents a suitable pipeline for the identification of target applications for these important biomolecules, as pharmaceutical, nutraceutical or food and feed supplements, this study aimed to define for the first time the *Paracoccus* sp. LL1 carotenoids quali-quantitative profile. Moreover, in order to give a bacterial specie identification, the cell membrane fatty acids profile was evaluated as chemotaxonomic parameter and the whole genome of *Paracoccus* sp. Strain LL1 has been sequenced and analyzed to better identify its taxonomic, and the biosynthetic genes involved in carotenoids production.

With this purpose, the best reproducible *Paracoccus* sp. LL1 growth conditions, selected from literature (Harker et al., 1998; Khomlaem et al., 2021; Kumar, Jun, & Kim, 2018; Sawant et al., 2015), have been applied for the strain characterization. Finally, since the antimicrobial activity of these molecules against different pathogens of clinical interest, mainly regarding food born poisons, is not yet been reported in a quantitative way (Vargas-Sinisterra & Ramírez-Castrillón, 2021), the *Paracoccus* LL1 biomass extract was also tested for its antibacterial activity.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The wild type *Paracoccus* sp. LL1 (from National Center for Biotechnology Information (NCBI) GenBank Accession No. KP288668) was maintained in Luria-Bertani (LB) agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L agar), at 4 °C (Fig. 1S from the Supplementary Material).

Seed cultures were prepared by transferring a full loop into 25 mL LB broth in 250 mL conical flasks and kept at 180 rpm (orbital shaker, FALC), for 24 h at the respective temperature, 24 and 30 °C, used for the growth experiments. The seed cultures have been used for preculture cultivation, at the same conditions reported above.
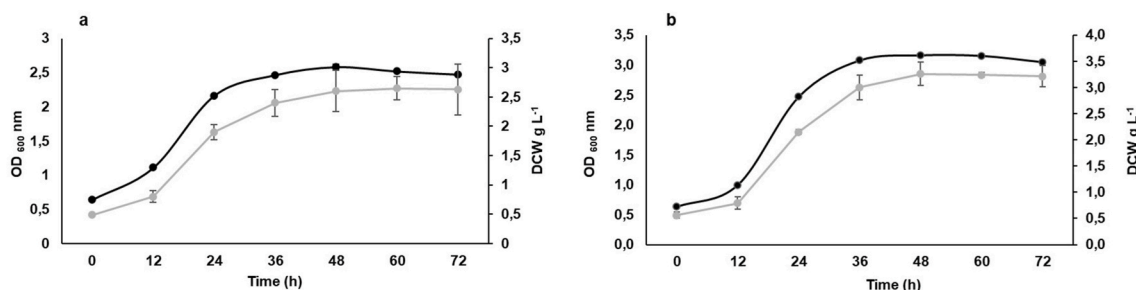
Fig. 1. Optical density (OD) (black line) and dry cell weight (DCW) (grey line) detected for *Paracoccus* sp. LL1 fermentation processes carried out at 24 °C (a) and 30 °C (b).

2.2. Batch fermenter cultivation

Batch cultivation of the wild type *Paracoccus* sp. LL1 was carried out in 5 L fermenter (Biostat Biotech B, Sartorius Stedim Biotech, Goettingen, Germany), using a working volume of 3.5 L of minimal salts medium supplemented with 20 g/L of glucose, and an inoculum size of 10% (v/v) (Khomlaem et al., 2021; Kumar, Jun, & Kim, 2018). Minimal medium composition was: 2 g/L of KH_2PO_4, 25 g/L of NaCl, 2 g/L of $(NH_4)_2SO_4$, 8 g/L of K_2HPO_4, 6 g/L of yeast extract, 0.5 g/L of tryptone, 2 g/L of sodium citrate, 2 g/L of $MgSO_4 \times 7H_2O$, 9 g/L of $Na_2HPO_4 \times 12H_2O$, 20 mg/L of $CaCl_2 \times 2H_2O$, and 1 mL/L of trace element, represented by: 4.98 g/L $FeSO_4 \times 7H_2O$, 0.44 g/L $ZnCl_2 \times 7H_2O$, 0.78 g/L $CuSO_4 \times 5H_2O$, 0.24 g/L $Na_2MoO_4 \times 2H_2O$ and 0.81 g/L $MnSO_4 \times 4H_2O$ (Khomlaem et al., 2020).

Fermentation parameters, selected from previous literature, with the exception of dissolved oxygen (DO), since no reproducible/exact values were reported, were: air flow 2.5 L/min, rpm 300, dissolved oxygen (DO) maintained at 20% by an automatic control of rpm and air flow, pH controlled at 7.5 ± 0.2 by using 4 N HCl and 4 N NaOH. Two different temperatures have been tested, 24 °C and 30 °C (Fig. 2S from the Supplementary Material). The lower and higher temperatures have been selected among the optimum growth temperatures of the bacterium, according to literature and confirmed by parallel growing tests contextually carried out (data non reported) (Harker et al., 1998; Khomlaem et al., 2020; Khomlaem et al., 2021; Kumar, Jun, & Kim, 2018; Kumar & Kim, 2019; Muhammad et al., 2020; Sawant et al., 2015).

Fermentation processes were carried out for 72 h. Samples were collected aseptically from the reaction vessel, centrifuged at 10,000 rpm for 10 min at 10 °C (centrifuge 4-16 KS, Sigma, Germany). Cell pellets were washed with 0.9% NaCl and frozen at −20 °C for freeze drying prior to analyses.

2.3. Cell growth and dry cell weight determination

Samples, collected twice per day, were investigated for the cell growth curve and dry cell weight determination. Culture growth was evaluated by measuring the optical density (OD) at 600 nm (UV2700, Shimadzu), whereas the dry cell weight (DCW) has been quantified as follow: 10 mL of culture samples were centrifuged at 10,000 rpm for 10 min at 10 °C (centrifuge 4-16 KS, Sigma, Germany). NaCl (0.9%) sterile solution was used for washing the cell pellets to rid of medium residues, recovered by centrifugation once more, and dried at 80 °C (Heating oven, FD240, Binder) until constant weight was reached.

The specific growth rate (μ, h^{-1}) has been calculated according to the following equation (Eq. 1):

$$\mu = \frac{\ln OD2 - \ln OD1}{t2 - t1} \qquad \text{Eq. 1}$$

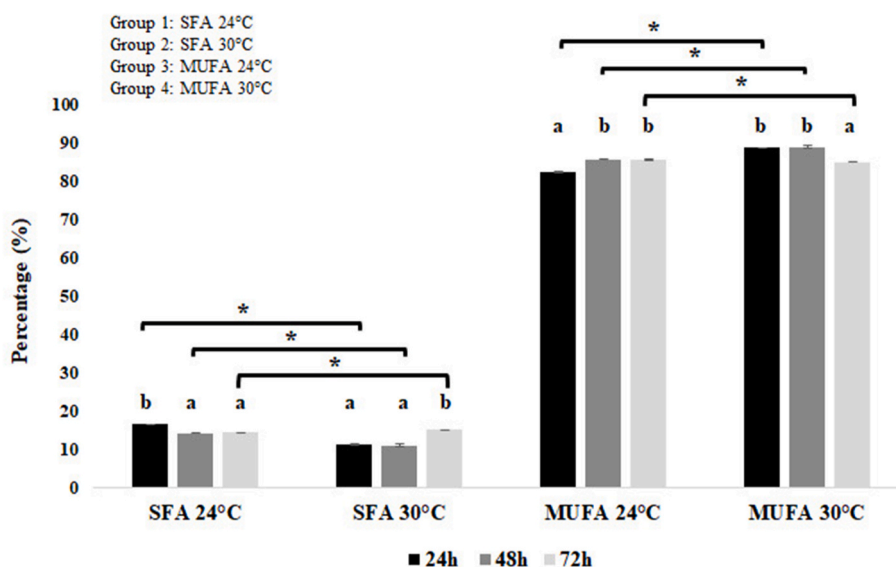Where μ was calculated on the Δ optical density and Δ time ratio.

Fig. 2. SFAs and MUFAs percentage detected during the fermentation processes (24 h black, 48 h grey, 72 h light grey) carried out at 24 and 30 °C. Groups 1 and 2 indicate the SFAs percentage detected at 24 °C and 30 °C respectively, whereas groups 3 and 4 indicate MUFAs percentage detected at 24 °C and 30 °C respectively. Different letters above the columns indicate significant differences in the same group (P < 0.05 by Kruskall–Wallis). Significant differences between groups are indicated with asterisk (P < 0.05 by Mann–Whitney *U* test).

2.4. Intracellular and extracellular carotenoids extraction

Intracellular carotenoids extraction was carried out according to Hagaggi and Abdul-Raouf (2023) with slight modification. *Paracoccus* sp. LL1 freeze-dried biomass was suspended and soaked in methanol in a solvent/pellets ratio of 2:1 (v/w). The samples were wrapped with aluminum foil for light protection, stored in darkness for 3 h, and vortexed for 30 s every hour. The extract was centrifuged at 10 °C for 10 min at 10,000 rpm for removing residual cells, and the supernatant was filtered through a 0.45 μm syringe filter. The biomass residue was re-extracted, repeating the process with fresh methanol until cells bleaching. The final methanol used for the extraction procedure ranged from 1 to 1.2 mL, depending on the initial biomass. The solvent was removed by using a gentle stream of nitrogen and the dry extract was dissolved in Methanol:Methyl tert-butyl ether (MeOH:MTBE) (1:1 *v/v*). Extracellular carotenoids were extracted using ethyl acetate according to Khomlaemet al. (2021).

2.5. Analytical conditions for the HPLC-PDA-MS analysis for carotenoid identification

HPLC analyses were carried out on a Shimadzu (Kyoto, Japan) Nexera X2 instrument coupled to an LCMS-2020 spectrometer via an atmospheric pressure chemical ionization (APCI) source operating in both positive and negative ionization mode. Chromatographic separation, UV–vis and MS acquisitions were carried out by using the operating conditions described in a previous work (Spadaro et al., 2024).

Carotenoids identification was accomplished by comparing retention and spectral data (both UV and MS spectra) of the detected peaks with those reported in the literature. Relative quantification was carried out for all carotenoids, while absolute quantification was performed for β-carotene using a standard calibration curve, built in the linear range 0.05–1 mg mL^{-1}, at six concentration levels (five replicates each). In both cases peak integration was performed for photodiode array (PDA) chromatograms extracted at 450 nm.

2.6. GC analyses of FAMEs

Fifty mg of freeze-dried bacterial culture underwent a dual-stage derivatization protocol to obtain fatty acid methyl esters (FAMEs) derivatives (section Derivatization procedure for FAMEs analyses of Supplementary Material). GC-MS analysis was performed on a GCMS-QP2020 NX system (Shimadzu, Duisburg, Germany). The split injection was carried using an AOC-20i autosampler (Shimadzu) equipped with a 10 μL syringe (Shimadzu). The gas chromatograph was equipped with a split/splitless injector (280 °C) and an inlet liner, split/splitless type, straight FocusLiner™ design (95 mm × 5.0 mm OD × 3.4 mm ID, volume 810 μL) (wool packed) (Merck Life Science). A GC capillary column of 30 m × 0.25 mm ID coated with a 0.20 μm film of SLB-IL60 (Merck Life Science) was used for FAMEs separation. Chromatographic conditions were applied according to previous work (Ramesh et al., 2024). The peak attribution was carried out evaluating two different identification criteria: mass spectral matching and linear retention index (LRI) correspondence. A homolog series of carbon saturated FAMEs (C4-C24, Merck Life Science) was used to determine LRI values. A commercial database, namely LIPIDS GC-MS Library (version 1.0, Shimadzu) was used.

The quantification of FAMEs was performed on a Nexis GC-2030 system (Shimadzu) equipped with a flame ionization detector (FID) and an AOC-20i autosampler. Split/spitless injector, inlet liner, GC column, carrier gas, linear velocity and temperature program were the same as described above for the GC-MS analyses. FID temperature was thermostated at 280 °C (sampling rate 40 ms). Carrier gas from the column entered the FID detector and was mixed with hydrogen combustion gas (40 mL min^{-1}) and air (400 mL min^{-1}). All the samples were injected in triplicate for a major data precision. Quantitative data were expressed in percentage (%) terms (area normalization) as a means of three replicates ± standard deviation (SD).

2.7. GC-FTIR analysis of FAMEs

GC-FTIR system consisted of a Nexis GC-2030 (Shimadzu) and of a Fourier transform infrared (FTIR) DiscovIR (Spectra Analysis Instrument Inc., Marlborough, USA) employing a solid deposition interface. Separation of the analytes was achieved using similar GC conditions as described in section 2.6. The exit analytical column flow was delivered to the FTIR interface by means of an uncoated capillary having dimensions of 0.25 m × 0.20 mm ID. The injection volume of sample was of 1.0 μL (split ratio 1:10) at an injector temperature of 280 °C. FTIR parameters were: 50 °C disk temperature, 3 mm min^{-1}, disk speed. IR data were acquired, processed and visualized using the Thermo Galactic GRAMS/AI software (version 9.3, Thermo Fisher Scientific, Waltham, MA, USA). Identification was attained by means of library search program using a first derivative correlation algorithm (spectral ID, Thermo Fisher Scientific). Compounds were identified by searching experimental IR spectra in a lab-constructed IR spectral database of lipids, namely LIPIDS GC-FTIR. Library search Quality match score expressed by the spectral ID software in 1 to 0 scale (where 1 = minimum similarity and 0 = maximum similarity) were converted into 1%–100% units (where 100 = maximum similarity), by applying the formula previously reported by Salerno et al., (Salerno et al., 2020).

2.8. Whole genome sequencing (WGS) of Paracoccus sp. LL1

Paracoccus sp. LL1 whole genome sequencing was carried out by using total genomic DNA extracted from approximately 100 mg (wet weight) of *Paracoccus* sp. LL1 biomass, obtained by cultivation at 30 °C, 200 rpm overnight. An i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, Inc, Korea) was used for the total genomic DNA extraction, according to the manufacturer's procedures. A DNA sequencing library was supplemented with 100 ng of gDNA. After being broken up by an enzymatic process, the gDNA was purified using magnetic beads. The fragmented DNA was ligated to an adaptor index. The quality and quantity of the indexed libraries were measured using the Agilent Technologies 2100 Bioanalyzer with a DNA 1000 chip and Qubit Fluorometer and pooled in equimolar amounts. The genome of *Paracoccus* sp. LL1 was sequenced at Macrogen (Daejeon, Korea) using a combination of the PACBIO Sequel II system and Illumina sequencing platform. The HGAP assembler (v3.0) with PacBio reads only was applied for *de novo* assembly. Error correction of contig bases with Illumina reads was then performed using Trimmomatic (v0.38) and Pilon (v1.21). Annotation of the assembled genome and gene prediction were carried out by using the Prokka v.1.14.6 software tool (Seemann, 2014).

The species of *Paracoccus* sp. LL1 was identified using 16S rRNA gene analysis. The 16S rRNA gene sequence was obtained from the whole-genome sequence and reference 16S rRNA gene sequences of *Paracoccus* genus were obtained from the NCBI database (USA), which were imported into MEGA11 software (Kumar, Jun, & Kim, 2018). The 16S rRNA gene sequences were selected and aligned using ClustalW. Then, the phylogenetic tree was then constructed by the Neighbour-joining method with a bootstrap value of 1000 using MEGA11 software.

2.9. Agar spot test for antimicrobial activity assay

The indicator microorganisms selected for the initial screening of the activity of *Paracoccus* sp. LL1 extract activity included *Salmonella enterica serovar* Typhimurium LT2 (DSMZ18522; Braunschweig, Germany), *Listeria monocytogenes* OH, *L. monocytogenes* CAL, *L. monocytogenes* SA and *L. innocua* 1770, as well as *Pseudomonas putida* WSC358, *Ps. Putida* KT2240 and *Ps. Fluorescens* BF13. *Listeria* strains were obtained from the CREA-ZA (Research Centre for Animal Production and Aquaculture - Lodi, Italy), while the *Pseudomonas* strains were

provided by Prof. Livia Leoni of Roma Tre University, Rome.

The bacteria cultures were routinely propagated in tryptone soy broth (TSB; Oxoid, Basingstoke, UK) under optimal conditions (aerobiosis, 30 °C for the *Listeria* and *Pseudomonas* strains and 37 °C for the *Salmonella* strains).

The spot-on-agar assay entailed the application of 3 μL of each extract, (50 mg/mL),obtained as previously describe in section 2.4 and resuspended in phosphate-buffered saline (PBS)onto tryptone soy agar (TSA, 1.2%; Oxoid) plates that had been previously inoculated of $1 \cdot 10^6$ CFU/mL indicator strains in the exponential growth phase (Leinberger et al., 2021). Subsequently, the plates were incubated for 18 h, and the zones of microbial growth inhibition (radii halos) around the spots were measured in millimeters (mm), in accordance with the methodology described by Balouiri et al. (2016). Furthermore, to corroborate the efficacy of the test, 3 μL of kanamycin at concentration of 50 mg/mL (Sigma-Aldrich) was added to the plates as positive control, while plates without carotenoids extract were used as negative control. To minimize degradation, the samples were protected from light and analyzed as soon as possible following preparation.

2.10. Statistical analysis

Experimental data are presented as the means ± standard deviation of three replicate measurements for each sample. The statistical analyses were carried out by using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL, USA). Statistically significant differences have been performed by applying the non-parametric tests, Kruskall–Wallis and Mann–Whitney U. For each variable examined, statistical significance was accepted at the level of $p < 0.05$.

3. Results and discussion

3.1. Microbial growth determination

The OD (Optical Density) and DCW (Dry Cell Weight) changing for different temperatures is shown in Fig. 1.

For both the fermentations carried out at 24 °C and 30 °C the highest growth rate was estimated at 0.05 h^{-1}.

The exponential growth phase started after 10–12 h and was held until 36–40 h before reaching the steady-state, for both the temperature tested (Fig. 1). The stationary phase held until 72 h. The final DCW reached up by *Paracoccus* sp. LL1, was 2.6 ± 0.4 g/L and 3.2 ± 0.2 g/L at 24 °C and 30 °C respectively, according to previous results reported by Kim and Kumar (2018).

According to previous literature, in *Paracoccus* sp. LL1 the temperature increase was resulting in a simultaneous biomass increasing (Chougle & Singhal, 2012; Raita et al., 2023).

3.2. Fatty acids profile detected during fermentation processes

A total of 18 FAME compounds were identified and quantified in the analyzed samples evaluated at 24 and 30 °C after 24 h, 48 h and 72 h, as reported in Table 1. The peak assignment was established using an approach based on the use of two different identification criteria named MS similarity matching and LRI correspondence. However, in the case of positional isomers, fragmentation patterns produced undistinguishable MS spectra, and similar LRI values made the identification process difficult. For instance, by searching into the mass spectral database it was not possible to assign the correct identity of the most abundant compound in the lipid fraction (retention time 46.8 min in GC-MS chromatogram of Fig. 3 S Supplementary Material). In fact, two different candidates arose: methyl cis-12-octadecenoate (Me. 18:1ω6-Z) and methyl cis-11-octadecenoate (Me. 18:1ω7-Z). MS similarity matching values of 95 and 94% were obtained for methyl cis-11-octadecenoate and methyl cis-12-octadecenoate, respectively, while LRIs were 1820 for methyl cis-11-octadecenoate and 1826 for methyl cis-12-octadecenoate,

thus the peak identification was compromised. To avoid mistaken identification, GC-FTIR technique was employed due to its capability to univocally identify the positional isomers. In detail, the infrared spectra recorded from the solid spot allowed to confidently identify the most abundant component of the lipid fraction as methyl cis-11-octadecenoate. Search of the FTIR spectra in dedicated database gave a quality match factor (QMF) of 98% as illustrated in Fig. 4 S (Supplementary Material). Noticeably the second hit listed obtained a QMF of 61% (below the 90% QMF value imposed as limit for confident discrimination). Furthermore, methyl cis-12-octadecenoate ranked in 42nd place with a QFM of 18%.

The main fatty acids detected at 24 °C were represented by: trans-5-dodecenoic acid (C12:1ω7-E), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9-Z) and cis-vaccenic acid (C18:1ω7-Z). During the fermentation these fatty acids concentrations, except for cis-vaccenic acid (C18:1ω7-Z), were significantly affected by the process ($p < 0.05$). In fact, palmitic acid (and stearic acid % values were decreasing from 1.10% ± 0.01% and 15.11% ± 0.01% down to 0.55% ± 0.01% and 13.17% ± 0.00%, respectively, at the end of the process. On the contrary trans-5-dodecenoic acid and oleic acid concentrations increased during the fermentation process, starting from 1.89% ± 0.03% and 0.92% ± 0.01% up to 2.69% ± 0.02% and 2.00% ± 0.08%, respectively. The main fatty acids detected in LL1 biomass when the fermentation process was carried out at 30 °C were represented by: trans-5-dodecenoic acid, palmitic acid, stearic acid, and cis-vaccenic acid. Their concentrations, excepting for palmitic acid, were significantly affected by the fermentation process ($p < 0.05$).

Trans-5-dodecenoic acid level was increasing from 0.62% ± 0.02% up to 1.03% ± 0.01% at the end of the process. The same behavior was observed for stearic acid that increased from 9.48% ± 0.03% up to 13.18% ± 0.06%. On the contrary the cis-vaccenic acid concentration was decreasing during the fermentation process from 87.42% ± 0.13% down to 83.04% ± 0.06%.

These results agree with the well-established bacterial homeoviscous adaptation response, characterized by the fatty acids membrane cell composition adjustment according to temperature changes (Bramkamp, 2022; Chwastek et al., 2020). Bacteria adapt their membrane by increasing the unsaturated fatty acids when the growth temperature decreases (Mansilla & de Mendoza, 2016). In fact, comparing the SFAs and MUFAs percentage, it was possible to notice an opposite trend when fermentations were carried out at different temperatures (Fig. 2). When the fermentation temperature was set at 24 °C SFAs concentration was decreasing from 16.54% ± 0.02%–14.38% ± 0.00%, followed by an increasing in MUFAs percentage from 83.42% ± 0.03%–85.56% ± 0.00%. On the contrary, when the process was carried out at 30 °C, SFAs increased from 11.20% ± 0.08%–14.98% ± 0.04%, whereas the poly-unsaturated fatty acids (PUFAs) showed an opposite trend, decreasing from 88.77% ± 0.08% down to 84.99% ± 0.03% at the end of the process. This trend is ascribable to the common responses of Gram-negative bacteria to the growth temperature decreasing, resulting in an increase in the percentage of unsaturated fatty acid (Suutari & Laakso, 1994; Zhang & Rock, 2008), for the correct membrane fluidity preservation (Hassan et al., 2020; Lee et al., 2024).

The cell membrane fatty acids composition characterization represents a suitable technique for bacteria identification, and it is applied as a chemotaxonomic parameter in order to distinguish closely related species (Cody et al., 2015; da Costa et al., 2011; Tindall et al., 2010). Comparing the main fatty acid identified in this work with previous studies it was possible to observe the lack of 3-hydroxydecanoic acid (C10:0 3-OH) in *Paracoccus* sp. LL1, while it was detected in *P. caemi, P. denitrificans, P. homiensis, P. sphaerophysae, P. ravus, P. versutus,* and *P. halophilus* in a percentage ranging between 7.5 and 2.0 (Lee et al., 2011; McGinnis et al., 2015; Yoon et al., 2019). On the other hand, the other predominant fatty acids identified in LL1, trans-5-dodecenoic acid, palmitic acid, stearic acid, oleic acid and cis-vaccenic acid, were consistent with those reported for other *Paracoccus* species previously

Table 1

Total fatty acids (FAs) composition detected in *Paracoccus* sp. LL1 by GC-MS and GC-FID analyses during fermentation at 24 °C and 30 °C after 24h, 48h and 72h, along with H Statistic and Asymptotic Significance values. Abbreviation: MS sim: mass spectral similarity, LRI exp: experimental linear retention index; LRI ref: reference linear retention index; FTIR Sim: IR spectral similarity Fatty acids was also grouped into different chemical classes as follows: SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

FAME	MS Sim	LRI exp	LRI ref	FTIR Sim	24 h_24 °C	48 h_24 °C	72 h_24 °C	H Statistic	Asymptotic Significance	24 h_30 °C	48 h_30 °C	72 h_30 °C	H Statistic	Asymptotic Significance
trans-5-Dodecenoic acid - C12:1ω7-(E)[a]	95	1197	–	–	1.89 ± 0.03^a	2.37 ± 0.05^b	2.69 ± 0.02^c	7.261	**0.027**	0.62 ± 0.02^a	0.83 ± 0.06^b	1.03 ± 0.01^c	7.200	**0.027**
Lauric acid - C12:0	91	1197	1200	*n.d.*	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	5.600	0.061	0.56 ± 0.02^c	0.02 ± 0.00^b	tr ^{a}	5.513	**0.023**
Myristic acid - C14:0	91	1398	1400	*n.d.*	0.04 ± 0.00^c	0.02 ± 0.00^b	0.01 ± 0.00^a	8.000	**0.018**	0.22 ± 0.01^b	0.02 ± 0.00^a	0.01 ± 0.00^a	7.784	**0.020**
cis-Myristoleic acid - C14:1ω5-(Z)	92	1408	1414	*n.d.*	0.02 ± 0.00^b	0.01 ± 0.00^a	0.02 ± 0.00^b	8.000	**0.018**	0.01 ± 0.00	tr	tr	4.343	0.114
Palmitic acid - C16:0	96	1600	1600	92	1.10 ± 0.01^b	0.69 ± 0.02^a	0.55 ± 0.01^a	7.513	**0.023**	0.74 ± 0.02	0.73 ± 0.06	1.64 ± 0.11	5.468	0.065
cis-Palmitoleinic acid - C16:1ω9-(Z)	91	1607	1603	*n.d.*	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	2.000	0.368	0.01 ± 0.00^a	0.01 ± 0.00^a	0.02 ± 0.00^b	8.000	**0.018**
cis-Palmitoleic acid - C16:1ω7-(Z)	93	1619	1616	91	0.25 ± 0.01^b	0.27 ± 0.01^b	0.22 ± 0.01^a	7.714	**0.021**	0.19 ± 0.01^a	0.18 ± 0.01^a	0.35 ± 0.02^b	6.058	**0.048**
Margaric acid -C17:0	95	1702	1702	92	0.18 ± 0.00^a	0.28 ± 0.01^b	0.44 ± 0.03^c	7.784	**0.020**	0.16 ± 0.00^b	0.10 ± 0.00^a	0.11 ± 0.00^a	6.889	**0.032**
trans-10-Heptadecenoic acid - C17:1ω7-(E)	96	1713	1713	*n.d.*	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	5.778	0.056	0.01 ± 0.00	tr	tr	4.343	0.114
cis-10-Heptadecenoic acid - C17:1ω7-(Z)	92	1724	1719	*n.d.*	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	4.571	0.102	0.01 ± 0.00	tr	tr	4.343	0.114
Stearic acid - C18:0	96	1803	1800	94	15.11 ± 0.06^b	13.04 ± 0.02^a	13.17 ± 0.09^a	7.513	**0.023**	9.48 ± 0.11^a	10.04 ± 0.32^a	13.18 ± 0.06^b	7.200	**0.027**
Oleic acid - C18:1ω9-(Z)	95	1818	1810	92	0.92 ± 0.01^a	1.81 ± 0.08^b	2.00 ± 0.08^b	7.448	**0.024**	0.38 ± 0.03^b	0.10 ± 0.00^a	0.27 ± 0.03^b	7.261	**0.027**
cis-Vaccenic acid -C18:1ω7-(Z)	93	1826	1820	98	80.09 ± 0.19	81.09 ± 0.17	80.41 ± 0.09	6.200	0.051	87.42 ± 0.13^b	87.72 ± 0.51^b	83.04 ± 0.26^a	5.956	**0.049**
Nonadecanoic acid - C19:0	91	1903	1900	*n.d.*	0.05 ± 0.00^a	0.06 ± 0.00^b	0.13 ± 0.02^b	7.086	**0.029**	0.03 ± 0.00^b	0.02 ± 0.00^a	0.02 ± 0.00^a	7.714	**0.021**
9,10-methylene-Octadecanoic acid - 9, 10-methylene C18:0	93	1916	1611	*n.d.*	0.03 ± 0.00^a	0.02 ± 0.00^a	0.05 ± 0.00^b	7.784	**0.020**	0.03 ± 0.00^b	0.02 ± 0.00^a	0.02 ± 0.00^a	6.171	**0.046**
trans-12-Octadecenoic acid - C18:1ω16-(E)	93	1951	1945	*n.d.*	0.08 ± 0.03^a	0.17 ± 0.01^b	0.10 ± 0.01^a	7.714	**0.021**	0.01 ± 0.00^a	0.02 ± 0.00^a	0.04 ± 0.01^b	6.889	**0.032**
cis-13-Eicosenoic acid - C20:1ω7-(Z)	98	2022	2015	*n.d.*	0.14 ± 0.01^b	0.08 ± 0.00^a	0.08 ± 0.00^a	6.889	**0.032**	0.13 ± 0.01^a	0.17 ± 0.03^{ab}	0.23 ± 0.02^b	7.119	**0.028**
trans-8, trans-10-Octadecadienoic acid - C18:2ω8-(E,E)	90	2028	2031	*n.d.*	0.04 ± 0.01^a	0.04 ± 0.00^a	0.06 ± 0.01^b	7.086	**0.029**	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.00	4.597	0.100
SFAs					16.54 ± 0.02^b	14.15 ± 0.02^a	14.38 ± 0.02^a	7.322	**0.026**	11.20 ± 0.08^a	10.95 ± 0.39^a	14.98 ± 0.04^b	6.489	**0.039**
MUFAs					83.42 ± 0.23^a	85.82 ± 0.13^b	85.56 ± 0.15^b	7.322	**0.026**	88.77 ± 0.08^b	89.04 ± 0.40^b	84.99 ± 0.06^a	6.489	**0.039**
PUFAs					0.04 ± 0.01^a	0.04 ± 0.00^a	0.06 ± 0.00^b	7.086	**0.029**	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.00	4.597	0.100

In bold Asymptotic Significance indicates significantly different results at p < 0.05.

Different superscript letters within the same row denote significantly different values at different times (by Kruskall–Wallis test), calculated for the two fermentation temperatures (24 °C and 30 °C).

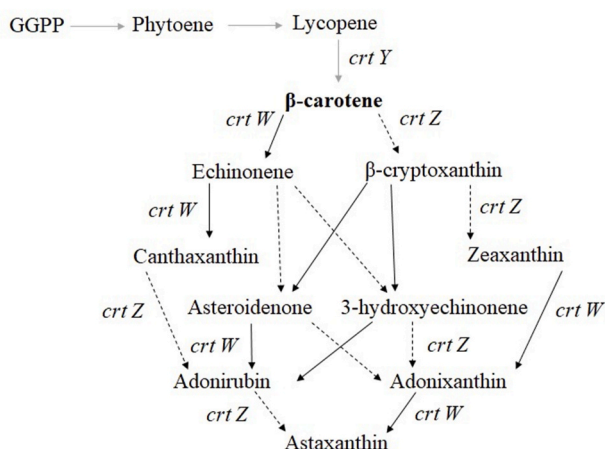[a] **, tentative identification;** *tr*: trace level.

Fig. 3. Carotenoids metabolic pathway (Hayashi et al., 2021).
GGPP was used for geranylgeranyl pyrophosphate; CrtY gene encoding for Lycopene beta-cyclase; CrtW gene encoding for Beta-carotene ketolase; CrtZ gene encoding for Beta-carotene hydroxylase; solid black lines were used for CrtW pathway; dotted lines were used for CrtZ pathway.

characterized (*P. marcusii, P. haeundaensis, P. carotinifaciens, P. seriniphilus, P. homiensis, P. bogoriensis* and *P. zeaxanthinifaciens*), with slight difference in the percentages detected (Osanjo et al., 2009; Lee et al., 2011; McGinnis, 2015), probably due to the differences in experimental conditions (Roh et al., 2009).

With regards to the main representative fatty acids of the species, cis-vaccenic acid and stearic acid, LL1 shown a percentage around the 80% and 13% respectively, in agreement with the range reported for other species of *Paracoccus,* such as *bogoriensis* and *marcusii* (Osanjo et al., 2009; Roh et al., 2009).

3.3. Evaluation of carotenoids profile detected during fermentation processes

Contrary to previous results reported in the literature (Khomlaem et al., 2021; Khomlaem et al., 2023), no extracellular carotenoids have been detected in all the supernatants samples investigated, confirming the intracellular nature of the vesicles containing carotenoids (Mussagy et al., 2019; Papapostolou et al., 2023; Sharma et al., 2024; Rodríguez-Sifuentes et al., 2021).

Intracellular carotenoids produced by *Paracoccus* sp. LL1 have been characterized by HPLC-PDA-APCI/MS analyses.

It was possible to distingue 12 different carotenoids, represented by adonirubin, cis-adonixanthin, adonixanthin, zeaxanthin, 2-hydroxyechinonene, 3-hydroxyechinonene, β-cryptoxanthin, echinonene, 15-cis-β-carotene, 13-cis-β-carotene, β-carotene and 9-cis -β-carotene (reported, as example, in Table 1S and Fig. 5S from the Supplementary Material).

The results of the relative quantification, expressed as carotenoids percentage, at 24 °C and 30 °C are reported in Table 2.

The main carotenoids detected after 24 h of fermentation carried out at 24 °C, were β-Carotene, 13-cis-β-carotene and 9-cis-β-carotene, reaching a percentage of 65.84% ± 1.48%, 11.93% ± 1.00% and 8.73% ± 0.58% respectively. After further 24 h of fermentation the carotenoid profile was not influenced by the process (p > 0.05), excepting for 3-hydroxyechinonene, whose percentage decreased from 1.65% ± 0.12%–0.96% ± 0.08 (p < 0.05). At the end of the process, after 72 h, all the carotenoids produced were affected by the fermentation (p < 0.05) expecting for 2-hydroxyechinonene. The main carotenoids detected after 72 h at 24 °C were β-carotene (37.48% ± 2.27%), adonixanthin (18.06% ± 1.21%), adonirubin (10.30% ± 0.60%) and 13-cis-β-carotene (8.35% ± 0.13%), thus showing a significant increase of carotenoids bearing keto and hydroxy groups in their moieties.

According to the known carotenoids metabolic pathway (Hayashi et al., 2021), all the carotenoids detected in this study were directly produced from β-carotene (Fig. 3). In fact, it was possible to observe a
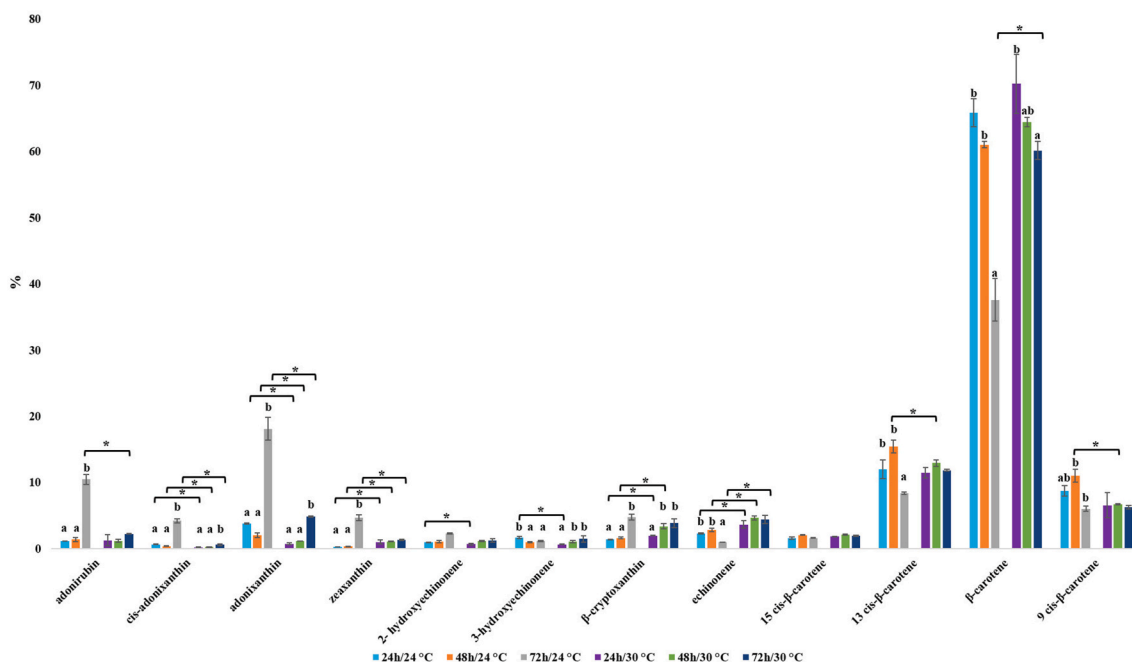

Fig. 4. Carotenoids percentage detected at different fermentation times carried out at 24 and 30 °C.
Columns light blue, orange and grey show the carotenoids percentage detected at 24 °C, after 24, 48 and 72 h respectively.
Columns purple, green and dark blue show the carotenoids percentage detected at 30 °C, after 24, 48 and 72 h respectively.
Different letters above the columns indicate significant differences in the same group (P < 0.05 by Kruskall–Wallis). Significant differences between groups are indicated with asterisk (P < 0.05 by Mann–Whitney *U* test).

Table 2
Carotenoids composition (%) detected by HPLC-PDA-MS analysis in *Paracoccus* sp. LL1 during fermentation at 24 °C and 30 °C after 24h, 48h and 72h, along with H Statistic and Asymptotic Significance values.

Carotenoids	24 h_24 °C	48 h_24 °C	72 h_24 °C	H Statistic	Asymptotic Significance	24 h_30 °C	48 h_30 °C	72 h_30 °C	H Statistic	Asymptotic Significance
Adonirubin	1.10 ± 0.01[a]	1.40 ± 0.23[a]	10.30 ± 0.60[b]	7.200	**0.027**	1.25 ± 0.59	1.15 ± 0.15	2.22 ± 0.03	5.422	0.066
Cis-adonixanthin	0.62 ± 0.02[a]	0.36 ± 0.02[a]	4.14 ± 0.23[b]	7.200	**0.027**	0.16 ± 0.05[a]	0.24 ± 0.03[a]	0.56 ± 0.11[b]	7.200	**0.027**
Adonixanthin	3.69 ± 0.10[a]	2.28 ± 0.47[a]	18.06 ± 1.21[b]	7.200	**0.027**	0.73 ± 0.10[a]	1.13 ± 0.03[a]	4.69 ± 0.23[b]	7.200	**0.027**
Zeaxanthin	0.25 ± 0.01[a]	0.31 ± 0.04[a]	4.72 ± 0.36[b]	7.200	**0.027**	0.99 ± 0.23	1.06 ± 0.05	1.26 ± 0.06	5.600	0.061
2- hydroxyechinonene	0.92 ± 0.03	1.02 ± 0.13	2.23 ± 0.07	5.956	0.051	0.67 ± 0.08	1.10 ± 0.12	1.16 ± 0.24	5.422	0.066
3- hydroxyechinenone	1.65 ± 0.12[b]	0.96 ± 0.08[a]	1.09 ± 0.10[a]	6.489	**0.039**	0.59 ± 0.05[a]	1.07 ± 0.10[b]	1.48 ± 0.36[b]	5.956	**0.049**
β-Cryptoxanthin	1.37 ± 0.05[a]	1.56 ± 0.13[a]	4.82 ± 0.34[b]	7.200	**0.027**	1.94 ± 0.03[a]	3.24 ± 0.33[b]	3.85 ± 0.45[b]	6.489	**0.039**
Echinonene	2.28 ± 0.06[a]	2.84 ± 0.22[b]	0.98 ± 0.01[a]	7.261	**0.027**	3.74 ± 0.46	4.47 ± 0.32	4.46 ± 0.45	3.467	0.177
15-cis-β-carotene	1.60 ± 0.14	2.04 ± 0.03	1.62 ± 0.05	5.600	0.061	1.78 ± 0.05	2.09 ± 0.07	1.87 ± 0.12	5.600	0.061
13-cis-β-carotene	11.93 ± 1.00[b]	15.46 ± 0.69[b]	8.35 ± 0.13[a]	7.200	**0.027**	11.31 ± 0.60	12.79 ± 0.38	11.81 ± 0.12	5.600	0.061
β Carotene	65.84 ± 1.48[b]	61.03 ± 0.34[b]	37.48 ± 2.27[a]	7.200	**0.027**	69.90 ± 3.20[b]	64.24 ± 0.59[ab]	60.11 ± 0.97[a]	7.200	**0.027**
9-cis β-carotene	8.73 ± 0.58[ab]	10.87 ± 0.70[b]	5.62 ± 0.70[b]	7.200	**0.027**	6.54 ± 1.32	6.68 ± 0.07	6.26 ± 0.21	3.200	0.202

In bold Asymptotic Significance indicates significantly different results at p < 0.05.
Different superscript letters within the same row denote significantly different values at different times (by Kruskall–Wallis test), calculated for the two fermentation temperatures (24 °C and 30 °C).

significant β-carotene percentage decreasing during the fermentation process from 65.84% ± 1.48%–37.48% ± 2.27% at 24 °C. This decrease was followed by a simultaneous significant increasing, mainly of adonixanthin (18.06% ± 1.21%) and adonirubin (10.30% ± 0.60%), both representing the direct precursors of astaxanthin. These rare carotenoids are currently gaining more interest by the scientific community due to their antioxidant properties very similar to the astaxanthin ones (Mussagy, Farias, et al., 2024). The identification of these ketocarotenoids in *Paracoccus* sp. LL1 represents an interesting output from scientific and biotechnological points of view.

A different trend was observed when the fermentation process was carried out at 30 °C.

While after 24 h the main carotenoids detected were β-carotene, 13-cis-β-carotene and 9-cis-β-carotene, as already observed at 24 °C, their concentration was not affected by the fermentation process, expecting for the β-carotene that was characterized by a slight decrease, from 69.90% ± 3.20% at 24 h to 60.11% ± 0.97% at 72 h. At the end of the fermentation, after 72 h, the only carotenoids significantly affected by the process were β-carotene, as already stated, followed by β-cryptoxanthin (3.85% ± 0.45%), adonixanthin (4.69% ± 0.23%), 3- 3-hydroxyechinenone (1.48% ± 0.36%) and *cis*-adonixanthin (0.56% ± 0.11%).

Comparing the results obtained from the two different processes carried out at 24 °C and 30 °C (Fig. 4), it was possible to point out that, according to the literature (Chougle & Singhal, 2012; Raita et al., 2023) the temperature can affect the metabolism related to the carotenoids production in *Paracoccus* sp. LL1. Carotenoids biosynthesis is in fact described as a microbial strategy to protect the cellular membrane under low temperature growth conditions having a similar function in regulating the membrane fluidity, as already described for fatty acids (Seel et al., 2020; Mapelli-Brahm et al., 2023; Saubenova et al., 2024). Analysing the results obtained in this study for the strain investigated, it was possible to observe that, in particular, *cis*-adonixanthin, adonixanthin, zeaxanthin and echinenone showed a significant difference between 24 °C and 30 °C for all the fermentation times investigated (p < 0.05). Whereas, the β-cryptoxanthin content changed significantly for the samples collected after 24 and 48 h (p < 0.05), while no significant difference was observed after 72 h between the two different temperatures (p > 0.05). Interestingly, the most abundant carotenoid, β-carotene, at the end of the process showed a significant difference (p < 0.05) due to the temperature, showing its highest content as relative percentage at 30 °C. This trend was in accordance with the results reported by Mostofian et al. (2020) regarding the maintenance of the correct membrane fluidity. In their work authors stated that high percentage of β-carotene caused an increasing in membrane rigidity, while low percentage determined a less rigid membrane structure. Hence, the relative percentage of this carotenoid is strictly correlated to the cultivation temperature.

Whereas, it was noteworthy, that the highest absolute production amount was observed at 24 °C, where the β-carotene concentration increased from 0.14 ± 0.01 mg g^{-1} up to 0.35 ± 0.01 mg g^{-1} at the end of the process, while when the temperature was set at 30 °C the final concentration was lower (0.26 ± 0.01 mg g^{-1}). According to Allahkarami et al. (2021), the carotenoids production was considerably detectable when the steady state was reached. During this phase the pigment production by the bacterium was detectable until the late steady state, held from 36 to 72 h. This behavior was in accordance with previous literature reporting that for the most of bacteria the optimum incubation period for pigmentation was ranging from 24 to 72 h (Allahkarami et al., 2021). In fact, relating the β-carotene production with the growth curves (Fig. 5) it was possible to notice that in the late log phase, at 24 h, the β-carotene was 0.14 ± 0.01 mg g^{-1} and 0.14 ± 0.1 mg g^{-1} at 24 °C and 30 °C respectively. These concentrations were increasing during the stationary phase up to 0.33 ± 0.03 mg g^{-1} and 0.18 ± 0.01 mg g^{-1}, after 48 h to reach then the highest concentration at the end of the processes (72 h), 0.35 ± 0.01 mg g^{-1} and 0.26 ± 0.01 mg g^{-1} at 24 °C and 30 °C respectively.
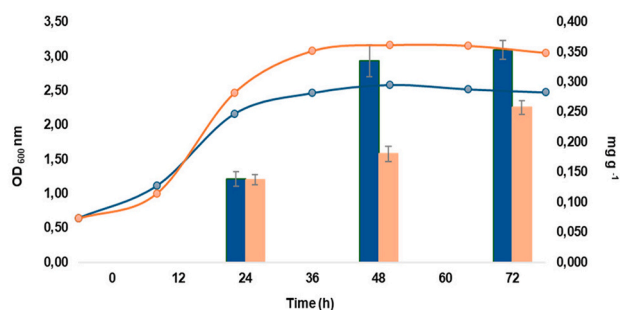
Fig. 5. β-Carotene production and *Paracoccus* sp. LL1 growth curves detected for fermentation processes carried out at 24 °C and 30 °C.
In blue line and columns are reported the optical density (OD_{600}) detected for *Paracoccus* sp. LL1 and β-carotene production, respectively, during the fermentation process carried out at 24 °C.
In orange line and columns are reported the optical density (OD_{600}) detected for *Paracoccus* sp. LL1 and β-carotene production, respectively, during the fermentation process carried out at 30 °C.

A comparison with previous literature results regarding β-carotene production, by the main microorganisms involved, is reported in Table 5. The main studies referred to fungi, yeasts and microalgae, recognized as the main producers, while wild type bacteria are generally reported as the main producers of other carotenoids, or referred for their total carotenoids content. *Blakeslea trispora* and *Dunaliella salina* are the most productive among fungi and microalgae respectively. As a result of deep strains selection and optimization of the cultivation conditions, β-carotene from *Dunaliella salina* is commercialized by BASF as Beta-tene®, NBT in Israel and the Indian E.I.D Parry reaching a yield of 40–50 tons per year, 2–3 tons per year and 1–3 tons per year, respectively (Singh & Sambyal, 2022). These yields are really attractive from an industrial point of view and several researches are currently oriented on the implementation of engineering microorganisms in order to reach similar results. The main microorganisms employed in this sense are represented by *Escherichia coli* and *Saccharomyces cerevisiae*, however this strategy is not believed to be suitable for food industry (Lyu et al., 2022; Singh & Sambyal, 2022; Wang et al., 2021).

Table 3
Genome statistics of *Paracoccus* sp. LL1.

Raw reads	
Total reads of raw dataset	14,789,620
Total reads of filtered dataset	11,611,224
Genome assembly	
Total length (bp)	4,011,047
Number of total contigs	12
Number of total contigs ($\geq$10,000 bp)	8
Number of total contigs ($\geq$100,000 bp)	5
Largest contig (bp)	3,104,234
Minimum contig (bp)	4143
Mean length contig (bp)	334,257
N50	3,104,234
GC (%)	65.91
Genome assembly assessment (compared to bacteria_odb 10)	
% Complete and single-copy BUSCOs (S)	97.58%
% Complete and duplicated-copy BUSCOs (D)	1.61%
% Missing BUSCOs	0.81%
Genome annotation	
Number of predicted genes	3991
Number of protein-coding genes	3932
tRNA	50
5s rRNA	3
16s rRNA	3
23s rRNA	3

Table 4
Genes in the *Paracoccus* sp. LL1 genome which are involved in carotenoid biosynthesis.

Contig	Size (bp)	Genes	Description	Function
1	627	Crt	Enoyl-CoA hydratase	Beta-oxidation of fatty acid: Trans-2-enoyl-CoA + H_2O → 3-hydroxyacyl-CoA trans-2-enoyl-CoA + H_2O → 3-hydroxyacyl-CoA
	882	CrtE	Geranylgeranyl pyrophosphate synthase	Synthesis of Geranylgeranyl Pyrophosphate (GGPP): IPP + DMAPP → GPP (geranyl pyrophosphate) GPP + IPP → FPP (farnesyl pyrophosphate) FPP + IPP → GGPP (geranylgeranyl pyrophosphate)
	915	CrtB	Phytoene synthase	2GGPP → Phytoene + 2PPi (pyrophosphate)
	1506	CrtI	Phytoene desaturase	Phytoene → Phytofluene → ζ-Carotene → Neurosporene → Lycopene
	1161	CrtY	Lycopene beta-cyclase	Lycopene + 2NADPH + $2H^+$ → Beta-carotene + $2NADP^+$
	489	CrtZ	Beta-carotene hydroxylase	Beta-carotene + NADPH + H^+ + O_2 → Zeaxanthin + $NADP^+$ + H_2O
	729	CrtW	Beta-carotene ketolase	Beta-carotene + $2O_2$ → Canthaxanthin or Astaxanthin + $2H_2O$
2	1245	CrtX	Zeaxanthin glucosyltransferase	Zeaxanthin + 2UDP-glucose → Zeaxanthin diglucoside + 2UDP

Comparing the results, it is possible to point out that *Paracoccus* sp. LL1 can represent an interesting wild type β-carotene cell-factory, reaching up a final concentration of this carotenoid comparable to the ones obtained by *Rhodotorula glutinis* and *Sporidiobolus pararoseus* wild type strains, as well as the final β-carotene yield, when cultivated at 24 °C, was also comparable to the one obtained by using the engineering *Saccharomyces cerevisiae* strain. Although the β-carotene final yield obtained by the *Paracoccus* sp. LL1 in this study was comparable with other ones reported in literature, this value is still far from attracting an industrial scale-up, as above mentioned for *Blakeslea trispora* and *Dunaliella salina*. In this regards it is needed to highlight that the stated finding represents the first step for further evaluations directed to the improvement on β-carotene production by the investigated bacterium. In fact, the identification of the carotenoids profile and the comparable β-carotene production with other microorganisms, allow to identify this bacterium as a promising β-carotene cell-factory addressing new researches on the identification of natural mutant capable in higher production of this important carotenoid, as well as on the improvement of the cultivation condition, as already carried out for the best-explored natural producers for β-carotene *Blakeslea trispora*, *Dunaliella salina* and *Rhodotorula glutinis* (Hayashi et al., 2021; Lyu et al., 2022).

Finally, as reported by previous literature, *Paracoccus* sp., enclosing strain LL1, have been identified as astaxanthin producers (Chougle & Singhal, 2012; Khomlaem et al., 2020; Khomlaem et al., 2021; Kumar & Kim, 2019; Ye et al., 2006). In the present study no astaxanthin was detected even if, according with the genome sequencing, the two main genes involved in its production have been identified in *Paracoccus* LL1 genome. The astaxanthin production is related to the combination and the specific copy number ratio of the two enzymes specified by the genes *CrtW* and *CrtZ* (Wang et al., 2023), since the hydroxylase *CrtZ* and ketolase *CrtW* compete for their corresponding substrates, making the

Table 5

Main β-carotene-producing microorganisms.

Wild type microorganisms		β-carotene (mg g^{-1} dry biomass)	Reference
Fungi	*Mucor circinelloides*	0.69	Naz et al. (2020)
	Blakeslea trispora	13–19	Singh and Sambyal (2022)
	Mucor azygosporus	0.38	Singh and Sambyal (2022)
	Blakeslea trispora	45.11	Jing et al. (2016)
Yeast	*Rhodotorula glutinis*	0.35	Yen et al. (2019)
	Rhodotorula mucillaginosa	0.72	Sharma and Ghoshal (2020)
	Rhodotorula glutinis NCIM 3353	0.18–0.25	Singh and Sambyal (2022)
	Sporidiobolus pararoseus	0.34	Petrik et al. (2014)
	Sporobolomyces roseus	0.42	Petrik et al. (2014)
Microalgae	*Coelastrella striolata Var. Multistriata*	7	Abe et al. (2007)
	Dunaliella salina	262.90	Hu et al. (2007)
	Dunaliella salina	138.3	Wang et al. (2021)
	Vischeria stellata	77	Li et al. (2012)
Bacteria	*Paracoccus marcusii LL1*	0.35	This study
	Sphingomonas sp	3.5–5.7	Silva & van Keulen, 2004
	Flavobacterium multivorum	0.2	Dias Ribeiro et al. (2011)
Engineering microorganisms			
Yeast	*Saccharomyces cerevisiae*	0.39	Li et al. (2013)
Bacteria	*Escherichia coli*	44.8	Wu et al. (2019)
	Halomonas elongata	0.55	Dias Ribeiro et al. (2011)

balanced expression of these enzymes a critical step for astaxanthin synthesis starting from the β-carotene with no intermediates accumulation (Li et al., 2020). Moreover, in particular, *CrtW* ketolases, recognized as more crucial to astaxanthin accumulation than *CrtZ*, is reported to show an amino acid sequence, close to other oxygen-dependent and iron-containing integral membrane enzymes, making this enzyme particularly oxygen dependent for its expression (Jin et al., 2018; Ye et al., 2006). Consequently, the impossibility to produce astaxanthin by *Paracoccus* sp. LL1 observed in this study could be ascribable to a low aeration or an insufficient *CrtW* transcription rate, being these identified as crucial points for astaxanthin production (Chougle et al., 2012; Hayashi et al., 2021; Raita et al., 2023). In this regard, it is necessary to highlight that previous study, identifying *Paracoccus* sp. LL1 as astaxanthin producer, reported a percentage of dissolved oxygen >20%, but it was not reported a reproducible/exact value. Considering the large range of values that this parameter could indicate, in this study the DO was maintained at 20%, in order to give an experimental reproducibility. More experiments will be carried out in the future with 40%–60%–80% DO values, according to previous studies suggesting that aerobic microorganisms can enhance astaxanthin synthesis during fermentation when dissolved oxygen concentrations range from 40% to 80% (Park et al., 2018; Stoklosa et al., 2019; Wang & Yu, 2009). Moreover, the nature of the main end product may be determined more by the compatibility of the genes and enzymes from different sources, and the ability of these enzymes to associate into a functional complex than simply by the order of introduction of the respective oxygen

functions. In fact, *in vivo*, even if the necessary complement of genes is present to direct synthesis of the required enzymes, this does not guarantee that the expected carotenoid will be formed as the main biosynthetic end product.

Strengthening the knowledge on the *Paracoccus* sp. LL1 active biosynthetic pathways requires multifaceted investigation. This entails investigating into the genetic makeup, carotenogenic clusters, genes expression levels, and applying approaches like isotope-guided metabolomics. This comprehensive approach will unravel the intricate mechanisms underpinning astaxanthin production (Mussagy, Farias, et al., 2024).

Thus, the optimized controlled conditions for the β-carotene production from the wild type *Paracoccus* sp. LL1reported in this study become relevant considering all the properties attributed to this molecule which is applied as a natural dye in food industry, as a healthy nutraceutical in formulations for its bioactivity, and its well-known fundamental role as precursor of the vitamin A.

3.4. Whole genome sequencing of Paracoccus sp. LL1

The total genome length of *Paracoccus* sp. LL1 was 4,011,047 bp, made of 12 contigs, showing a GC content of 65.91%, which is similar to the total genome size of *Paracoccus* genus of approximately 3.64–4.77 Mbp, with an average GC content of 61.5%–67.8% (Hollensteiner et al., 2023). The total genes of *Paracoccus* sp. LL1 contained 3991 genes, 3932 genes were protein-coding genes, whereas 59 genes were associated with tRNA and rRNA, including 50 genes for tRNA, 3 genes for 5S rRNA, 3 genes for 16S rRNA, and 3 genes for 23S rRNA. The completeness of single-copy, duplicated copy orthologous genes and contamination were 97.6%, 1.61% and 0.81%, respectively as shown in Table 3. The whole genome sequence of *Paracoccus* sp. LL1 has been submitted to the National Center for Biotechnology Information (NCBI) database under accession number PRJNA1178628. According to the genome analysis of *Paracoccus* sp. LL1, which was found in contigs 1 and 2, there was a carotenoid biosynthesis pathway that generates various compounds including lycopene, beta-carotene, zeaxanthin and astaxanthin. The key genes directly involved in the biosynthesis of carotenoid in *Paracoccus* sp. LL1 have been identified in the carotenoid gene cluster, which included *Crt* (encoding enoyl-CoA hydratase, an enzyme involved in the beta-oxidation of fatty acid), *CrtE* (encoding geranylgeranyl pyrophosphate synthase, an enzyme synthesizing geranylgeranyl pyrophosphate (GGPP) as precursor for carotenoid biosynthesis), *CrtB* (encoding phytoene synthase, an enzyme that catalyzes the conversion of GGPP to phytoene), *CrtI* (encoding phytoene desaturase, an enzyme that adds a double bond into phytoene to produce lycopene), *CrtY* (encoding lycopene beta-cyclase, an enzyme that can convert lycopene to beta-carotene), *CrtZ* (encoding beta-carotene hydroxylase, which hydroxylates beta-carotene to produce zeaxanthin), *CrtW* (encoding beta-carotene ketolase, which adds keto groups to beta-carotene to produce canthaxanthin or convert zeaxanthin to astaxanthin), and *CrtX* (encoding zeaxanthin glucosyltransferase, which catalyzes the glucosylation of zeaxanthin to zeaxanthin diglucoside). These carotenoid biosynthesis genes are listed in Table 4.

The 16s rRNA gene sequence of whole genome *Paracoccus* sp. LL1 was found to share 100% similarity with the 16s rRNA gene of *Paracoccus marcusii* (Y12703) and *Paracoccus marcusii* strain MH1 (NR044922) as shown in Fig. 6. These findings confirmed that the 16s rRNA gene of *Paracoccus* sp. LL1 was the closest relative with *Paracoccus marcusii* species. *Paracoccus* sp. LL1 has been renamed *Paracoccus marcusii* strain LL1. Results regarding blast analysis and the whole genome are reported in supplementary material (Fig. 5S).

3.5. Antimicrobial activity of Paracoccus sp. LL1 carotenoids enrich extract

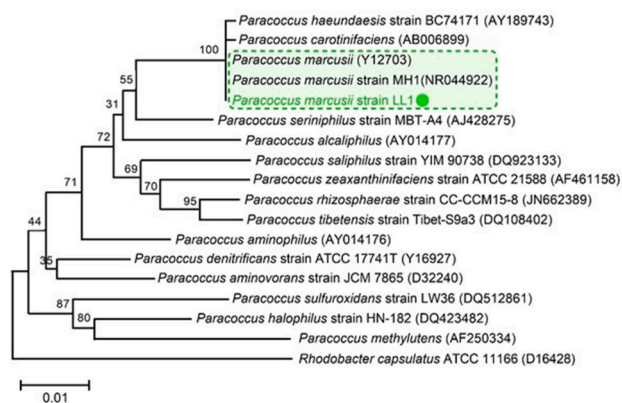*Paracoccus* LL1 biomass extract presented no antimicrobial activity

Fig. 6. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *Paracoccus marcusii* strain LL1 and their closest related taxa of the genus *Paracoccus*. *Rhodobacter capsulatus* ATCC 11166T was added as an outgroup. Bar, 0.01 substitutions per nucleotide position.

against the pathogens investigated. Lee et al. (2012) reported the antimicrobial activity of *Paracoccus* sp. against *Peudomonas aeruginosa* ATCC 10145T, while no activity was detected against *Candida albicans* ATCC 10231T, *Staphylococcus aureus* ATCC 51650T, *S. aureus* (MRSA) ATCC BAA-44T.

Previous studies carried out by Leinberger et al. (2021), reported the potential of *Paracoccus* sp. For the biosynthesis of antimicrobial compounds, detectable in the culture extract, active against strictly related strains combined with self-resistance, providing a competitive advantage over other microorganisms living in the same ecological niche (Egan et al., 2014; Mullis et al., 2019). However, in our study, the agar spot test did not show significant activity as no inhibition halos were observed at the concentrations tested, therefore the potential of the strain to cause antimicrobial resistances or to interfere with the use of antibiotics can be considered remote, representing this an important requisite for its applications in food and feed sectors (EFSA Opinion of the Scientific Panel on additives and products or substances used in animal feed, 2007). The decision to test the extracts on a different range of pathogenic and spoliative microorganisms was driven by the objective of obtaining a comprehensive and representative assessment of its potential antimicrobial capabilities.

In an industrial context, studies have shown that *Paracoccus* spp. Can be used in controlled fermentations to produce carotenoids in a sustainable manner, including using waste biomass as a substrate (Sen et al., 2019). These processes offer the advantage of providing safe and natural pigments that may also be used in food for human consumption, although such use will require additional regulatory approvals beyond those currently required for direct food use.

These results indicate the need for further studies to clarify the conditions under which *Paracoccus* sp. LL1 could produce antimicrobial compounds and identify the factors influencing this production.

4. Conclusion

In this work the wild type *Paracoccus* sp. LL1 has been characterized for the first time for its carotenoids and fatty acids profiles, as well as for whole genome sequencing.

These results allowed us to identify *Paracoccus marcusii* strain LL1 as a possible carotenoids cell factory, in particular for β-carotene production (up to 0.35 ± 0.01 mg g^{-1} of dry cell weight), giving an interesting output for the current research focused on the identification of new microbial sources. In particular, as stated above, the main β-carotene microbial sources are represented by yeasts and microalgae, while just few bacteria are used for this purpose. This study allowed to identify a carotenogenic wild type bacterium as in interesting β-carotene producer,

avoiding the application of engineering techniques.

Moreover, strains belonging to *Paracoccus* genera have been already authorized and employed for feed supplementation and tested for industrial scalability. This makes *Paracoccus marcusii* strain LL1 a possible candidate for further investigations in this regards, considering both the interesting β-carotene production and the absence of antimicrobial activity against the tested pathogenic strains, with remote potential for the induction of antimicrobial resistance. The employment of this bacterium at industrial scale is still challenging, since several factors need to be further investigated. Further study are required for the evaluation of factors affecting the growth of microbial strain for accumulation of β-carotene, the fermentation kinetics, for identifying the effect of the fermentation parameters and the media composition on the final carotenoids production, as well as the designing, execution and manufacturing costs of culture systems/bioreactors.

The identification of the carotenoids patterns produced by *Paracoccus marcusii* LL1 carried out in this study would represent an improvement of the state of the art for bacterial carotenoids production, and an important starting point for addressing researches based also on the identification of natural mutant.

CRediT authorship contribution statement

Roberta La Tella: Writing – original draft, Investigation, Data curation. **Alessia Tropea:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Francesca Rigano:** Writing – review & editing, Investigation, Formal analysis. **Daniele Giuffrida:** Writing – review & editing, Formal analysis, Conceptualization. **Giuseppe Micalizzi:** Writing – original draft, Investigation. **Tania Maria Grazia Salerno:** Writing – original draft, Investigation. **Cassamo U. Mussagy:** Writing – review & editing, Writing – original draft. **Beom Soo Kim:** Writing – original draft, Resources. **Krittayapong Jantharadej:** Writing – original draft, Investigation. **Paola Zinno:** Writing – original draft, Investigation. **Mireille Fouillaud:** Writing – original draft. **Laurent Dufossé:** Writing – review & editing, Supervision, Resources. **Luigi Mondello:** Supervision, Resources, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.105616.

Data availability

Data will be made available on request.

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