

# Demonstration of the Antioxidant Potential of Three Newly Isolated Carotenoid-Producing Microscopic Algae from KwaZulu-Natal (South Africa)

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## Abstract

Natural carotenoids from green microalgae exhibit beneficial effects in treating health-related diseases, primarily due to their antioxidant capacity. Therefore, carotenoid-producing microalgae were isolated and characterized from aqueous samples in KwaZulu-Natal (South Africa) under photoautotrophic conditions. Three isolates were characterized comprehensively using light and electron microscopy. In addition, the growth of the three selected microalgae was evaluated under photoautotrophic and photoheterotrophic conditions. Based on the cytological characteristics, the three strains matched the genera *Haematococcus*, *Parachlorella*, and *Scenedesmus*, the latter two additionally confirmed via analysis of the 18S rRNA gene sequence at the genus level. Light and electron microscopy and UV-Vis spectroscopy confirmed pigment production by all three microalgal strains. Both isolate *Parachlorella* sp. AA1 and *Scenedesmus* sp. AA2 showed the highest growth rate when cultured with acetate (25 mM) photoheterotrophically, while the isolate *Haematococcus* sp. AA3 grew best with glucose (50 mM). In addition, all three isolates utilized salicylate as a carbon source under photoheterotrophic conditions, evidently resulting in increased carotenoid production in strains AA1 and AA3. The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) antioxidant assay confirmed that methanol extracts of all three isolates contained carotenoids that can scavenge radicals, and thin layer chromatography (TLC) analysis showed that  $\beta$ -carotene and astaxanthin were formed by strain AA3 as main carotenoids.

**Keywords:** Microalgae, Cytology, Carotenoid Antioxidants, 18S rRNA, Photoautotrophic and Photoheterotrophic Growth, Radical Scavenging

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## INTRODUCTION

Algae are widely distributed and commonly located in various aqueous habitats and soil.<sup>1</sup> Most algae perform oxygenic photosynthesis to produce biomass. Still, numerous microalgae can use simple sugars or organic acids as an additional source of energy and carbon with or without illumination. Green microscopic algae have been studied for the production of valuable chemicals such as pigments.<sup>2</sup> Such pigments, which have attracted commercial interest, are astaxanthin,  $\beta$ -carotene, and lutein.<sup>3</sup> Among the known microalgal producers of the prominent antioxidant carotenoid astaxanthin, *Ankistrodesmus braunii* (now *Chlorolobion braunii*), *Chlorella zofingiensis* (now *Chromochloris zofingiensis*), *Dunaliella salina*, *Coelastrella rubescens*, and *Scenedesmus vacuolatus* are well-known for producing large quantities of this pigment under mixotrophic growth conditions, albeit *Haematococcus pluvialis* is considered the most potent producer.<sup>3,4</sup> However, cytological changes during the formation of astaxanthin in this microalga vary from other producers, such as *Dunaliella* spp., *Parachlorella* spp., *Chlorolobion* spp., and *Scenedesmus* spp.<sup>5,6</sup> Under favorable cultivation conditions, highly motile biflagellated green cells are characteristic in *Haematococcus* spp. Unfavorable conditions such as radiation or starvation induce the loss of motility and enlargement of cells, along with the formation of thick cell walls and the characteristic immotile red-colored haematocysts, indicating carotenoid accumulation. The aplanospores of *Haematococcus* spp. synthesize considerable amounts of astaxanthin, mainly as mono and diesters.<sup>7</sup> However, in *Scenedesmus* spp. varying illumination, nutrient deprivation, and redox stress induce cell colorization from green to brick red, indicating an accumulation of carotenoids protecting the photosynthetic apparatus from photo-oxidative damage under stress conditions such as high light intensity.<sup>8</sup> While *Chlorella* spp. may contain up to 0.7% and *Scenedesmus* spp. up to 0.3% of its dry weight of the carotenoid astaxanthin, in *Haematococcus* spp. astaxanthin can reach up to 4% of the dry weight.<sup>5,8</sup> Generally, microscopic green algae can be cultured autotrophically in open or closed photobioreactors or heterotrophically in conventional bioreactors

when supplemented with additional sources of carbon. The latter cultivation approach enables increased biomass yields and carotenoid production by implementing appropriate growth conditions.<sup>9</sup>

Thus far, few studies on the isolation and characterization of green microalgae intended for the biotechnological production of beneficial carotenoids with antioxidant potential, like the carotenoid astaxanthin, are available from South Africa. Therefore, this study assessed the antioxidant potential of newly isolated carotenoid-producing microalgae from KwaZulu-Natal, South Africa.

## MATERIALS AND METHODS

### Microalgae isolation and cultivation

Msunduzi river water samples collected in sterile Schott glass bottles (Pietermaritzburg, KwaZulu-Natal, South Africa, S 29°37'27.40", E 30°22'22.49") yielded two freshwater green algal isolates. An additional third isolate was obtained from a local bird bath displaying the typical reddish coloration demonstrating a possible occurrence of *Haematococcus* spp. Defined media, according to Bourrelly,<sup>10</sup> Chu,<sup>11</sup> and Bristol,<sup>12</sup> were used to enrich and isolate microalgae. Decimal dilutions of samples in sterile medium were spread plated onto solidified medium followed by incubation at 20±1°C with constant illumination for 10 days (40  $\mu\text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$ ). Axenic cultures of three distinct green microalgal isolates were established and maintained via sequential purification, subculturing, and regular monitoring.

### Microbiological characterization

The cells of the three axenic green microalgal isolates were analyzed microscopically using an Axioscope A1 (Zeiss, Germany) or a Motic BA 310 (China). Aqueous potassium iodide (0.2 % w/v, 30 minutes) was used to microscopically establish the number of pyrenoids in individual cells of the three isolates.<sup>13</sup>

### Electron Microscopy

Cells were collected from individual microalgal colonies, soaked in millipore water, fixed in glutaraldehyde (3% v/v, Sigma-Aldrich, USA), and washed in a sodium cacodylate buffer (pH 7.4,

50 mM, Sigma-Aldrich, USA), post-fixed in osmium tetroxide (2% w/v, Sigma-Aldrich, USA), and serially dehydrated using ethanol (10/30/50/70/100%) and propylene oxide (Sigma-Aldrich, USA), with final embedding in Spurr (50/50) resin (Sigma-Aldrich, USA).<sup>14</sup> Embedded microalgal cells were sectioned (EM UC7 ultramicrotome, Leica, Germany), followed by staining with uranyl acetate (2% w/v), and analyzed (JEOL 1400 TEM, Japan). For SEM analysis, cells of the three isolates were fixed with glutaraldehyde (3% v/v), dehydrated using ethanol as above and carbon dioxide critical point drying (Quorum K850), and analyzed (Zeiss EVOLS 15, Germany) after sputter coating with gold.

#### Phylogenetic analysis of selected isolates

As isolate AA3 was clearly identified as a member of the genus *Haematococcus* due to its distinct cellular features and its unique lifecycle, the sequence of the partial 18S rRNA gene of isolate AA1 and AA2 was analyzed after amplification employing two different primer pairs (S35DB, S35DC; S3F24, S3F25) to confirm assignment at the genus level.<sup>15,16</sup> The microalgal DNA was extracted from cells using a micro prep kit (D6007, Zymo, USA). The final reaction mix contained 10  $\mu$ M of each primer, 12.5  $\mu$ l Taq master mix (Thermo, USA), 10  $\mu$ l nuclease-free water, and 1.5  $\mu$ l DNA template. Amplification was done using 2 minutes of initial denaturation at 94°C and 35 cycles of 30 seconds denaturation at 94°C, annealing for 60 seconds at 60°C, extension for 60 seconds at 72°C, and finalized using 7 minutes extension at 72°C, with 4°C holding temperature. The size of the amplicons (~850bp and ~1400bp) was determined using agarose gel electrophoresis and ethidium bromide staining, as reported previously.<sup>17</sup> The sequences obtained by bidirectional sequencing of amplicons (CAF, Stellenbosch University, South Africa) were assembled into sequence contigs using Geneious prime.<sup>17</sup> The resulting sequences for the two isolates AA1 and AA2 were compared with 18S rRNA gene sequences for closely related *Parachlorella* and *Scenedesmus* strains available in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using the basic local alignment search tool (BLAST). Clustal W (used to establish sequence alignments) and the neighbor-joining algorithm implemented under Mega X (V10)<sup>18</sup> were used to generate phylogenetic

trees for strains AA1 and AA2. In addition, the maximum likelihood algorithm implemented under Mega X (V10)<sup>18</sup> was used to verify tree topologies. The sequences of *Ochromonas danica* SAG 933.7 (GU 935657.1) and *Chlamydomonas reinhardtii* UTEX 90 (AB 511834.1) served as out-group for isolate AA1 and AA2, respectively. The consensus sequences obtained for strains AA1 and AA2 were deposited with GenBank under accession numbers MT 984304 and MT 984303.

#### Growth of isolates

For photoautotrophic growth, 100 ml Erlenmeyer flasks with 20 ml sterile medium were inoculated to an initial cell density of about  $3 \times 10^6$  cells/ml. For isolate AA1 and AA2, the defined medium described by Chu<sup>11</sup> was used, while isolate AA3 was grown in the defined medium described by Bourrelly.<sup>10</sup> The inoculated cultures were incubated under continuous illumination ( $40 \mu\text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$ ) at  $20 \pm 1^\circ\text{C}$  over a period of 14 days with shaking at 100 rpm. 20  $\mu$ l culture samples were collected at 12-hour intervals for cell counts using a Neubauer counting chamber (Wertheim, Germany) and bright field light microscopy. For photoheterotrophic growth, the sterile defined medium was supplemented with either 25 mM sodium-acetate or 50 mM glucose as a carbon source.

Total carotenoid content was evaluated for the algal cultures by taking 500  $\mu$ l from each flask at 12 hourly intervals. Algal cells were harvested by centrifuging at  $10,000 \times g$  for 5 min, and microalgae cell pellets were washed twice using millipore water. The harvested cells were broken using 0.3 mm glass beads, extracted using 1ml acetone, and centrifuged at  $10,000 \times g$  for 5 min. The total carotenoid content in acetone extracts was determined using a Shimadzu UV-VIS spectrophotometer (UV mini1240, Japan) at 470 nm, as described by Lichtenthaler and Wellburn.<sup>19</sup>

For carotenoid analysis, algal cells at the stationary growth phase were harvested by centrifugation with two times washing (15 mins,  $10,000 \times g$ ; millipore water was used to wash pellets) from photoautotrophically grown cultures of the three strains (200 ml culture flasks). The harvested biomass was broken using 0.3 mm diameter sterile glass beads, extracted using 1 ml acetone, and centrifuged for another

15 mins (10,000 × g). The extraction process was carried out thrice until cell debris lost coloration. The total carotenoid content in acetone extracts was evaluated using a Shimadzu UV-VIS spectrophotometer (UV 1800, Japan) between 400-700 nm.<sup>20</sup>

### Effects of sodium salicylate on growth and carotenoid production

Sodium salicylate is known to boost the growth and carotenoid accumulation of green algae.<sup>21</sup> To determine if salicylate supports the growth and carotenoid production of the three isolates, 100 ml Erlenmeyer flasks containing 20 ml of sterile defined medium supplemented with various concentrations of sodium salicylate (0, 0.5, and 1.5 mM) were inoculated in triplicate to an initial cell density of about  $3 \times 10^6$  cells/ml. The medium described by Chu<sup>11</sup> was used to cultivate AA1 and AA2, while isolate AA3 was grown in the culture medium described by Bourrelly.<sup>10</sup> Growth and total carotenoid content were monitored as described above. To determine if the isolates could use added salicylate, cell-free sample volumes (1 ml) obtained during the growth analysis were used to assess the consumption of salicylate during the growth of isolate AA1, AA2, and AA3, respectively. Flasks containing salicylate and algae cells of all three isolates that were heat inactivated (90°C, 1 hour) and flasks without algal cells but salicylate served as abiotic controls. Each algae culture sample, including controls, was diluted 5-fold and subjected to spectral analysis, and the salicylate concentration was established using the molar extinction coefficient at 295 nm reported for this compound ( $\epsilon = 3840 \text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ ).<sup>22</sup>

### Analytical TLC

For isolate AA3, acetone extracts of cells were spotted on a 5 × 10 cm analytical silica gel thin layer chromatography plate (Merck, Germany). The TLC plates were analyzed in an enclosed chamber in the dark via hexane:acetone (70:30) (v/v) and acetone:petroleum ether (25:75) (v/v) as mobile phase.<sup>23,24</sup> Authentic astaxanthin and  $\beta$ -carotene (Sigma-Aldrich, USA) were used as standard.

### Determination of antioxidant capacity of cell extracts

The radical scavenging ability of cell extracts generated using methanol was estimated using the stable organic radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to Blois.<sup>25</sup> A stock solution (5 mM) of DPPH (Sigma-Aldrich) was dissolved in methanol. Ascorbic acid and  $\beta$ -carotene (Sigma-Aldrich, USA) were used as reference antioxidants. A 1 ml assay cuvette contained DPPH (100  $\mu\text{M}$ ) and various concentrations of ascorbic acid (0-2.5 mM) dissolved in methanol, cell extracts in methanol ranging from 0-900  $\mu\text{M}$  based on the molar extinction coefficient of  $\beta$ -carotene at 450 nm of  $136400 \text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ .<sup>26</sup> Controls containing only methanol and DPPH were used as blank. After 30 min of incubation without illumination, decolorization of the solution containing DPPH from violet to yellow was visually observed, while the absorbance was determined at 517 nm (Shimadzu, UV1800, Japan).<sup>25</sup> The effect concentration ( $\text{EC}_{50}$ ) value was estimated as the concentration of ascorbic acid,  $\beta$ -carotene, and the carotenoids in extracts required to scavenge 50% DPPH compared to controls using only methanol.

## RESULTS AND DISCUSSION

### Phenotypic Characteristics

The morphological characterization of the three purified algal isolates by light microscopy showed that cells of strain AA1 were planktonic, ovoidal-shaped, and non-motile, ranging between 3-10  $\mu\text{m}$ , which is typical of *Parachlorella* spp. (Figure 1A). In addition, the cells contained a pyrenoid (Figure 2A) within the chloroplast, again a regular feature of *Parachlorella* spp. Based on the cell characteristics, strain AA1 was provisionally assigned to the genus *Parachlorella*.<sup>27</sup> Isolate AA2 cells appeared spindle-shaped (8-15  $\mu\text{m}$ ), non-motile, and planktonic (Figure 1B) with a single pyrenoid and cup-shaped chloroplast (Figure 2B). Cells of strain AA2 were fused together with nipple-shaped projections on the obtuse apices of strain AA2 cells. Isolate AA2 was, therefore, provisionally assigned to the genus *Scenedesmus*.<sup>28</sup> These unique cell features differentiate strain AA2

from the cells of microalgae such as *Coelastrum* and *Coelastropsis* that are spherical and compact in shape with intracellular spaces.<sup>29</sup> Cells of strain AA3 in young cultures were typically ellipsoidal-shaped, motile biflagellate cells (Figure 1C). In addition, cells contained a central pyrenoid (Figure 2C). Furthermore, the cells of isolate AA3 were undergoing a developmental growth cycle including different cytological stages as reported for the genus *Haematococcus* such as the formation of a non-motile vegetative cell stage (Figure 1D). As adverse conditions occur, cells of strain AA3 form the characteristic non-motile spherical carotenoid accumulating aplanospores (Figure 1E).<sup>30</sup> The ellipsoidal-shaped flagellate motile cells are surrounded by a mucous envelope and are between 13-15 µm in diameter, and hematochrome (e.g., carotenoid) formation was visible in the protoplast (Figure 1C). In addition, the periplasmic space between the protoplast and the cell wall is connected by delicate radiating cytoplasmic strands, features present in isolate AA3 that are distinctive and typical of *Haematococcus* at the motile stage (Figure 1C).<sup>5,30</sup>

#### Ultrastructural analysis

A smooth, double-layered cell wall was observed in isolate AA1 by TEM analysis (Figure 3A). The chloroplast stroma showed a single pyrenoid. These cytological features match the findings of Bock et al.<sup>31</sup> for the genus *Parachlorella*. Scanning electron microscopy showed that isolate AA1 reproduces asexually via cell division (Figure 3B).

Thin sections of isolate AA2 proved that the cell wall had both an inner polysaccharide and an outer trilaminar layer (Figure 3C). The chloroplast was located near the periphery of isolate AA2 cells, containing the pyrenoid and starch grains scattered in the stroma (Figure 3C). Lipid bodies containing carotenoids were situated near the center of the cells, confirming carotenoid production by strain AA2. The SEM analysis further confirmed asexual reproduction via multiple fission as coenobia of isolate AA2 were formed during the exponential growth phase (Figure 3D), which agreed with an earlier report showing that a single mother cell can reproduce 2-16 coenobia in *Scenedesmus* spp.<sup>28</sup>

TEM analysis of thin sections of isolate AA3 revealed a thick electron-dense cell wall encompassing strain AA3 cells, matching a report by Hagen et al.<sup>32</sup> Cytological analysis (Figure 3E) additionally confirmed the expected presence of a nucleus with partially condensed chromatin visible. As noted by Buchheim et al.<sup>33</sup> *Haematococcus* spp. produces the carotenoid astaxanthin both in vegetative and late life cycle stages, thereby matching isolate AA3. The SEM analysis indicated asexual reproduction, as autospores of strain AA3 were detected (Figure 3F). The outer cell sheath surfaces showed protuberates and were irregularly wavy. Based on the observed distinct cytological features (e.g., the distinct and characteristic biflagellated motile cells) and the specific and unique lifecycle, strain AA3 was provisionally allocated to the genus *Haematococcus*.

#### Phylogenetic analysis of isolates AA1 and AA2

If morphological characteristics are not distinct and reliable enough, 18S rRNA gene sequence analysis is commonly applied for the phylogenetic characterization of green microalgae to confirm an assignment of isolates to genus level.<sup>33</sup> Comparing the resulting sequences (18S rRNA) of the isolated green microalgae (isolate AA1 and AA2) to sequences deposited in GenBank showed the highest resemblance level of more than 99% for isolate AA1 to *Parachlorella kessleri* and for isolate AA2 to members of the family *Scenedesmaceae*, with more than 98% sequence similarity to members of the genera *Scenedesmus*, *Coelastrum*, and *Coelastropsis*, which is adequate to assign algal isolates to genus level.<sup>28,31</sup> Therefore, isolate AA1 can be assigned tentatively to the genus *Parachlorella* with 100% sequence resemblance to *Parachlorella kessleri* strain SAG 211-11c (accession number KM 020114.1). Even though the sequence similarity of strain AA2 to *Scenedesmus ovalternus* with 98.51% (accession number X 81966.1) was similar to that for strains of the genera *Coelastrum* and *Coelastropsis*, isolate AA2 can be assigned tentatively to the genus *Scenedesmus* based on its distinct cell morphology. Toledo-Cervantes et al.<sup>34</sup> highlighted the identification of *Scenedesmus* spp. based exclusively on the 18S rRNA sequence similarity is not always sufficient and recommends

**Table.** Growth kinetic data for the three isolates AA1, AA2 and AA3 under photoautotrophic and photoheterotrophic growth conditions using acetate, glucose, and salicylate supplemented medium

Isolates	Photoautotrophic conditions			Photoheterotrophic conditions						
	No supplement Specific growth rate ( $\text{hr}^{-1}$ )	Generation time (Hrs)	Acetate (25mM) Specific growth rate ( $\text{hr}^{-1}$ )	Generation time (Hrs)	Glucose (50mM) Specific growth rate ( $\text{hr}^{-1}$ )	Generation time (Hrs)	Salicylate (0.5mM) Specific growth rate ( $\text{hr}^{-1}$ )	Generation time (Hrs)	Salicylate (1.5mM) Specific growth rate ( $\text{hr}^{-1}$ )	Generation time (Hrs)
<i>Parachlorella</i> sp. strain AA1	0.010±0.001	69	0.020±0.006	35	0.017±0.003	41	0.011±0.002	63	0.014±0.002	49
<i>Scenedesmus</i> sp. strain AA2	0.011±0.004	63	0.019±0.005	36	0.012±0.025	58	0.012±0.005	58	0.016±0.045	43
<i>Haematococcus</i> sp. strain AA3	0.009±0.001	72	0.015±0.035	46	0.016±0.005	43	0.012±0.045	58	0.010±0.004	69

Data shown are the means ( $\pm$  range) of two independently performed experiments

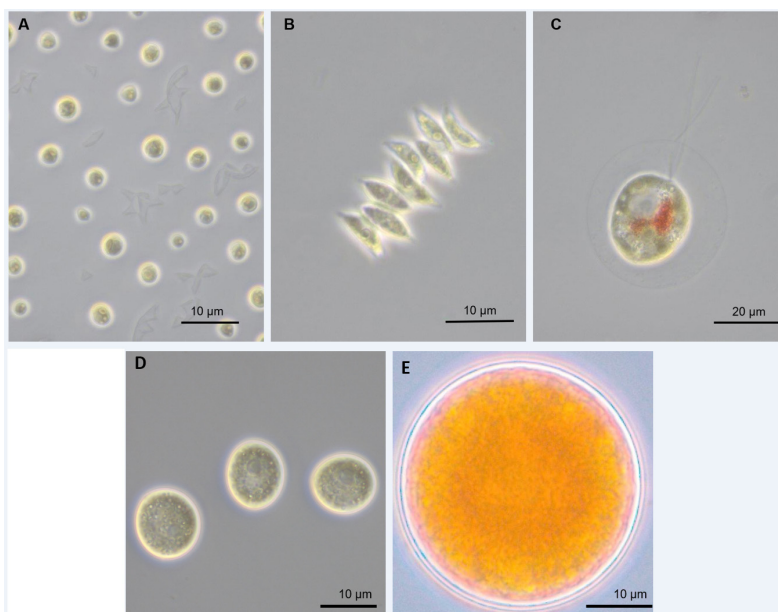
the further use of morphological characteristics. The unique spindle shape of isolate AA2 clearly differentiates it from the genera *Coelastrum* and *Coelastropsis*, the cells of which are spherical and compact in shape. According to Hegewald et al.,<sup>35</sup> *Coelastrum* and *Coelastropsis* possess net-like ridges produced by an inner cellulosic cell wall. This morphological feature was not observed in strain AA2, thereby allowing for the assignment of strain AA2 to the genus *Scenedesmus*. The phylogenetic relationship of isolates AA1 and AA2 with type strains of *Parachlorella kessleri*, *Scenedesmus ovalternus*, and *S. raciborskii* is demonstrated in supplementary Figures S1 and S2.

### Growth of the three isolates

Under photoautotrophic conditions without an organic carbon source, isolate AA1 achieved a growth rate of  $0.010 \text{ hr}^{-1}$  with a generation time of 69 hours (Table, Figure 4A). Praveen et al.<sup>36</sup> reported a two-fold lower growth rate of  $0.005 \text{ hr}^{-1}$  for *Chlorella pyrenoidosa* (now *Auxenochlorella pyrenoidosa*) under similar photoautotrophic conditions. Maroneze et al.<sup>37</sup> recorded a specific growth rate of  $0.023 \text{ hr}^{-1}$  for *Scenedesmus obliquus* (now *Tetradesmus obliquus*), which is higher than the result for isolate AA2 (specific growth rate  $0.011 \text{ hr}^{-1}$ , generation time 63 hours) under photoautotrophic conditions (Table, Figure 4B). Isolate AA3 grew under photoautotrophic conditions at  $0.009 \text{ hr}^{-1}$  (generation time 72 hours) (Table, Figure 4C), which is lower than the specific growth rate of  $0.025 \text{ hr}^{-1}$  reported previously by Giannelli et al.<sup>38</sup> However, these differences are probably due to varying culture conditions, as constant illumination with  $70 \mu\text{mol photons} \times \text{m}^{-2} \times \text{s}^{-1}$  was used by Giannelli et al.<sup>38</sup> Under photoautotrophic conditions, the chlorophyll a concentration increased concomitantly with cell numbers, the highest chlorophyll a concentration of  $14.48 \mu\text{g/ml}$  was observed for isolate AA3, followed by  $12.50 \mu\text{g/ml}$  in isolate AA1, while isolate AA2 recorded the lowest chlorophyll concentration of  $11.15 \mu\text{g/ml}$  (Figures S3.1, S3.2, S3.3). Comparable results were reported for *Haematococcus pluvialis*, with the chlorophyll a concentration increasing alongside astaxanthin accumulation.<sup>39</sup> Similarly, under photoautotrophic conditions, carotenoid yields increased alongside cell numbers in

*Parachlorella* sp. strain AA1, yielding 0.44 mg/L total carotenoids, followed by isolate AA2 with a total carotenoid yield of 0.81 mg/L, while isolate AA3 yielded 0.65 mg/L of total carotenoids (Figure 4). The total carotenoid yields observed for strains AA1 and AA2 were higher than data reported by De Jesus et al.<sup>40</sup> for *Parachlorella kessleri*, while the carotenoid yield of 0.81 mg/L recorded for isolate AA2 was higher than the yields reported for *Scenedesmus almeriensis* (now *Tetradesmus almeriensis*) under photoautotrophic conditions.<sup>41</sup> Leonardi et al.<sup>42</sup> reported a carotenoid yield of 0.63 mg/L for *Haematococcus pluvialis*, matching the result obtained for isolate AA3. Different organic carbon sources, like glucose, glycerol, salicylate, and acetate, are available to boost microalgae growth.<sup>7</sup> The three algal isolates from KwaZulu-Natal showed higher growth rates under photoheterotrophic conditions supplemented with 25 mM of acetate compared to photoautotrophic growth without supplement (Table). Under photoheterotrophic conditions with acetate as a carbon source, isolate AA1 had the highest growth rate of 0.020 hr<sup>-1</sup> (generation time 35 hours), isolate AA2 of 0.019 hr<sup>-1</sup> (36 hours), and isolate AA3 of 0.015 hr<sup>-1</sup> (46 hours) (Table, Figure S3.2). Heredia-Arroyo et al.<sup>43</sup>

reported a higher growth rate of 0.040 hr<sup>-1</sup> in a 25 mM acetate-supplemented medium for *Chlorella protothecoides* (now *Auxenochlorella protothecoides*), while the growth rate recorded for isolate AA2 with acetate resembles the growth rate of 0.022 hr<sup>-1</sup> reported for *Scenedesmus obliquus* (now *Tetradesmus obliquus*).<sup>44</sup> Thus, acetate enhanced cell growth under moderate light intensity compared to photoautotrophic growth conditions. The enhanced growth rate of isolate AA3 in the presence of 25 mM acetate matches a report by Kobayashi et al.,<sup>45</sup> showing similarly that 25 mM acetate enhanced the growth rate in *Haematococcus pluvialis* along with changes in morphological features. Acetate increased the chlorophyll a concentration to 17.80 µg/ml in isolate AA1 and 18.28 µg/ml in isolate AA2 (Figure S3.2). Acetate, however, reduced the chlorophyll a concentration in strain AA3 (Figure S3.2), with only 7.54 µg/ml chlorophyll a observed for isolate AA3. The results for strain AA1 agreed with the study by Papageorgiou<sup>46</sup> for *Chlorella pyrenoidosa* (now *Auxenochlorella pyrenoidosa*), while the result for isolate AA2 matches the reported chlorophyll a yield data for *Scenedesmus dimorphus* (now *Tetradesmus dimorphus*).<sup>47</sup> Growth analysis of photoheterotrophic cultures of different green



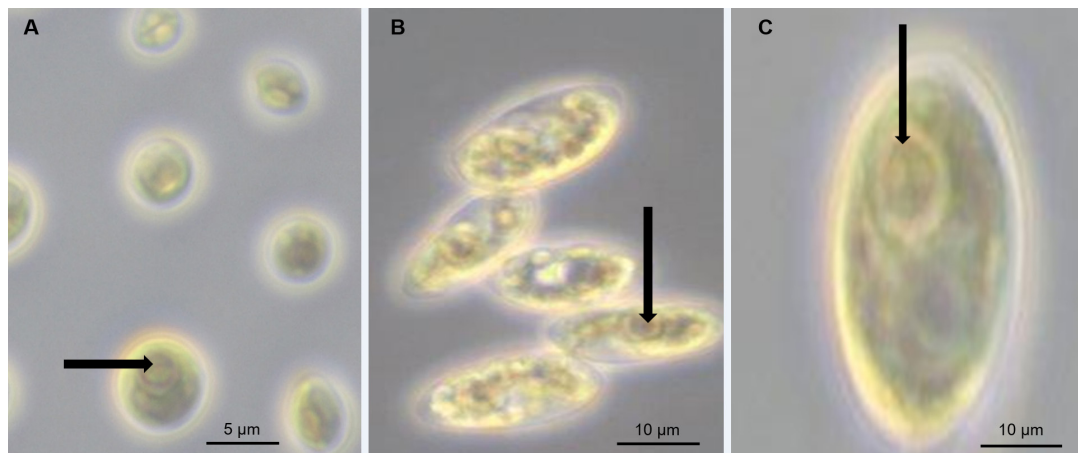
**Figure 1.** Typical cell shape of isolates AA1 (A), AA2 (B), and life cycle stages of AA3 (C, D, E) during the exponential (A, B, C) and the late stationary (D, E) growth phase under photoautotrophic conditions

algae, such as *Parachlorella* spp., *Ankistrodesmus* spp., *Haematococcus* spp., *Dunaliella* spp., and *Scenedesmus* spp., demonstrated that growth kinetics are strain dependent and affected by the light intensity.<sup>48</sup> Isolate AA1, when grown photoheterotrophically with glucose supplement ( $0.017 \text{ hr}^{-1}$ ), achieved a higher growth rate than isolate AA2 ( $0.012 \text{ hr}^{-1}$ ) and was similar to that of isolate AA3 ( $0.016 \text{ hr}^{-1}$ ) (Table, Figure S3.3). A study on the assimilation of glucose and carbon dioxide in *Chlorella vulgaris* by Martinez and Orus<sup>49</sup> showed that glucose enhanced growth rate and biomass yield, similar to the present study, suggesting that acetate stimulated the vegetative growth of strains AA1, AA2, and AA3, while glucose specifically stimulated the growth of strain AA3. Similar results were reported by Kim et al.<sup>50</sup> for *Haematococcus pluvialis*. A total chlorophyll a content of  $22.01 \mu\text{g/ml}$  was achieved by isolate AA1 when grown in the presence of glucose, which is higher than the  $17.15 \mu\text{g/ml}$  reported by Amin et al.<sup>51</sup> for *Chlorella vulgaris* (Figure S3.3). The highest total chlorophyll content of  $22.40 \mu\text{g/ml}$  was recorded for *Haematococcus* sp. strain AA3, agreeing with Kim et al.,<sup>50</sup> reporting a chlorophyll a content of  $19.8 \mu\text{g/ml}$  for *Haematococcus pluvialis*.

#### Effects of salicylate on carotenoid yields

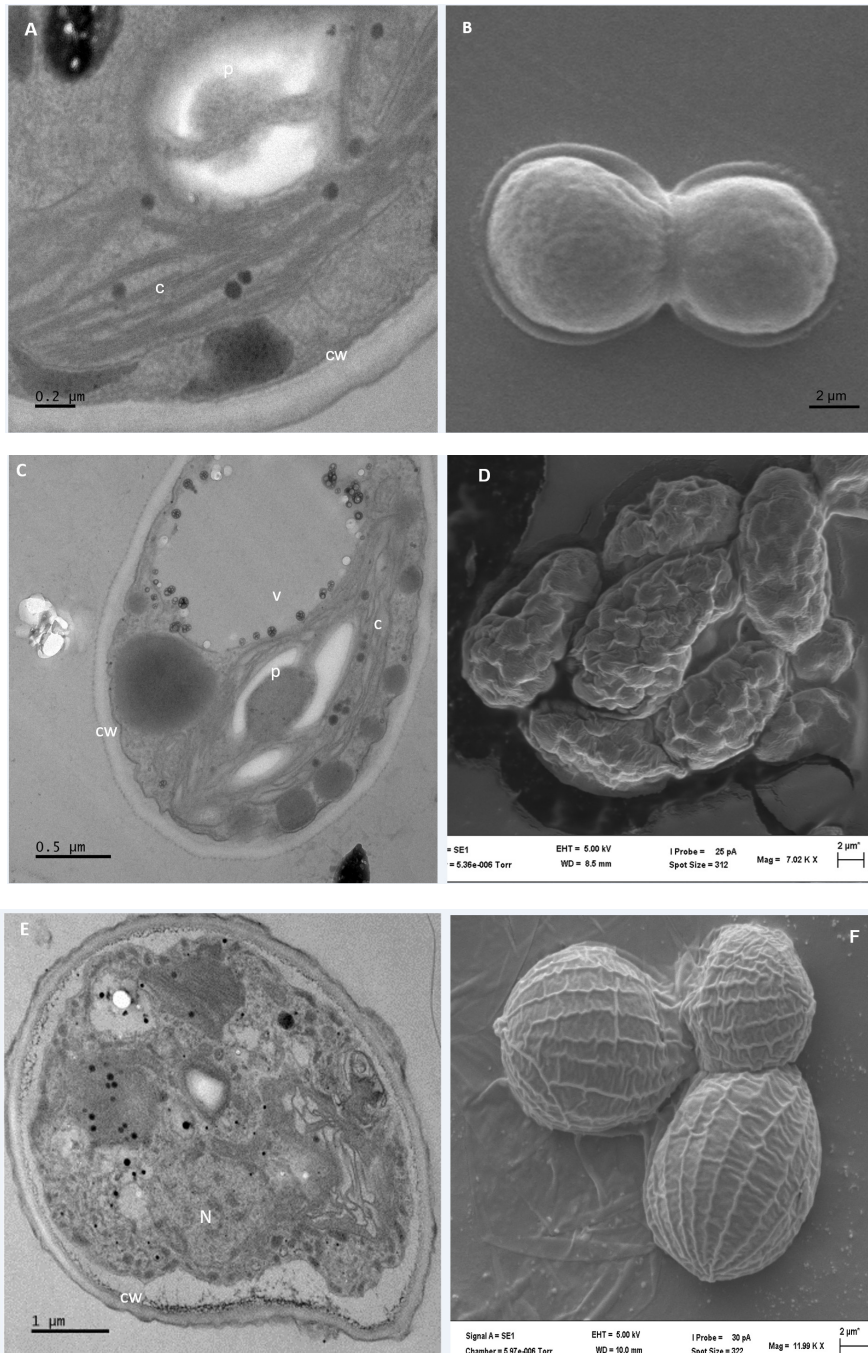
Growth with salicylate (0.5 mM, 1.5 mM) under photoheterotrophic conditions in isolate AA1 led to optimum specific growth rates of  $0.011$

$\text{hr}^{-1}$  and  $0.014 \text{ hr}^{-1}$ , matching generation times of 63 and 49 hours (Table). This is lower than the specific growth rate of  $0.087 \text{ hr}^{-1}$  reported by Martinez et al.<sup>52</sup> for *Chlorella pyrenoidosa* (now *Auxenochlorella pyrenoidosa*) in 1.5 mM salicylate-supplemented medium. However, the difference might be due to varying experimental conditions (e.g., illumination). The total carotenoid yield of  $1.30 \text{ mg/L}$  achieved for isolate AA1 is higher than the total carotenoid concentration of  $0.72 \text{ mg/L}$  reported by Heo et al.<sup>53</sup> for *Parachlorella kessleri* (Figure S4.1C). For strain AA2, the results showed that 1.5 mM salicylate stimulated better growth ( $0.016 \text{ hr}^{-1}$ , generation time 43 hours) than a control without salicylate (figure S4.2, Table), and the highest carotenoid yield of about  $0.99 \text{ mg/L}$  was recorded with cultures supplemented with 1.5 mM salicylate (Figure S4.2C). This observation corresponded with Guedes et al.<sup>54</sup> reporting a carotenoid productivity of  $0.84 \text{ mg/L}$  for *Scenedesmus obliquus* (now *Tetradasmus obliquus*) but is two-fold higher than the  $0.4 \text{ mg/L}$  earlier reported by Bao et al.<sup>9</sup> for *Scenedesmus dimorphus* (now *Tetradasmus dimorphus*). Depending on cultivation conditions, salicylate, similar to other simple aromatic compounds, can serve as a carbon source for microscopic algae, increasing cell growth and inducing carotenoid accumulation.<sup>55</sup> For isolate AA3, a low concentration of salicylate (0.5 mM) enabled a specific growth rate of  $0.012 \text{ hr}^{-1}$ , a generation time of 58 hours, and a



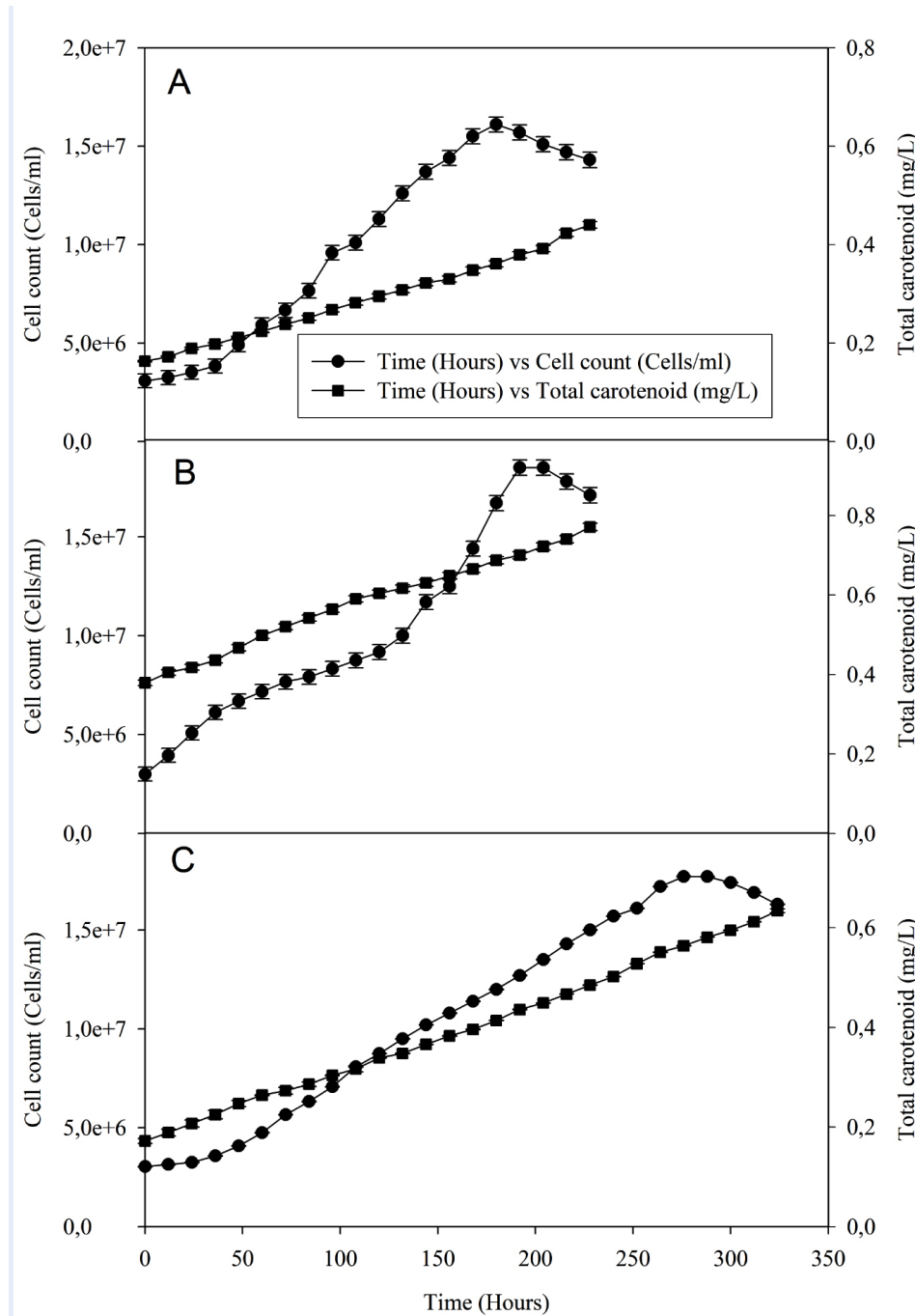
**Figure 2.** Detection of pyrenoids in cells of isolate AA1 (A), isolate AA2 (B), and isolate AA3 (C) after staining with 0.2% (w/v) aqueous potassium iodide by light microscopy. The arrow highlights the presence of a stained pyrenoid





**Figure 3.** Analyses of isolate AA1, AA2, and AA3 cells from the exponential growth phase by transmission and scanning electron microscopy. The symbol CW indicates the cell wall, C indicates chloroplasts, and P indicates a pyrenoid in AA1 (A), while the SEM micrograph indicates asexual reproduction by binary fission in isolate AA1 (B). The symbol CW indicates a double-layered cell wall; V indicates a vacuole, C indicates the chloroplast, and P represents a pyrenoid in AA2 (C), while the SEM micrograph shows asexual reproduction by multiple fission of cells in isolate AA2 (D). The symbol N indicates the nucleus with condensed chromatin, CW represents the cell wall with plasmalemma in AA3 (E), while the SEM micrograph indicates possible autospores (F)

carotenoid yield of about 1.20 mg/L; this is more than the growth rate of 0.009 hr<sup>-1</sup>, the generation time of 72 hours and carotenoid yield of 0.65 mg/L under photoautotrophic conditions (Table, Figure 4C). These results for isolate AA3 agreed with Gao et al.,<sup>56</sup> reporting that salicylate stimulated

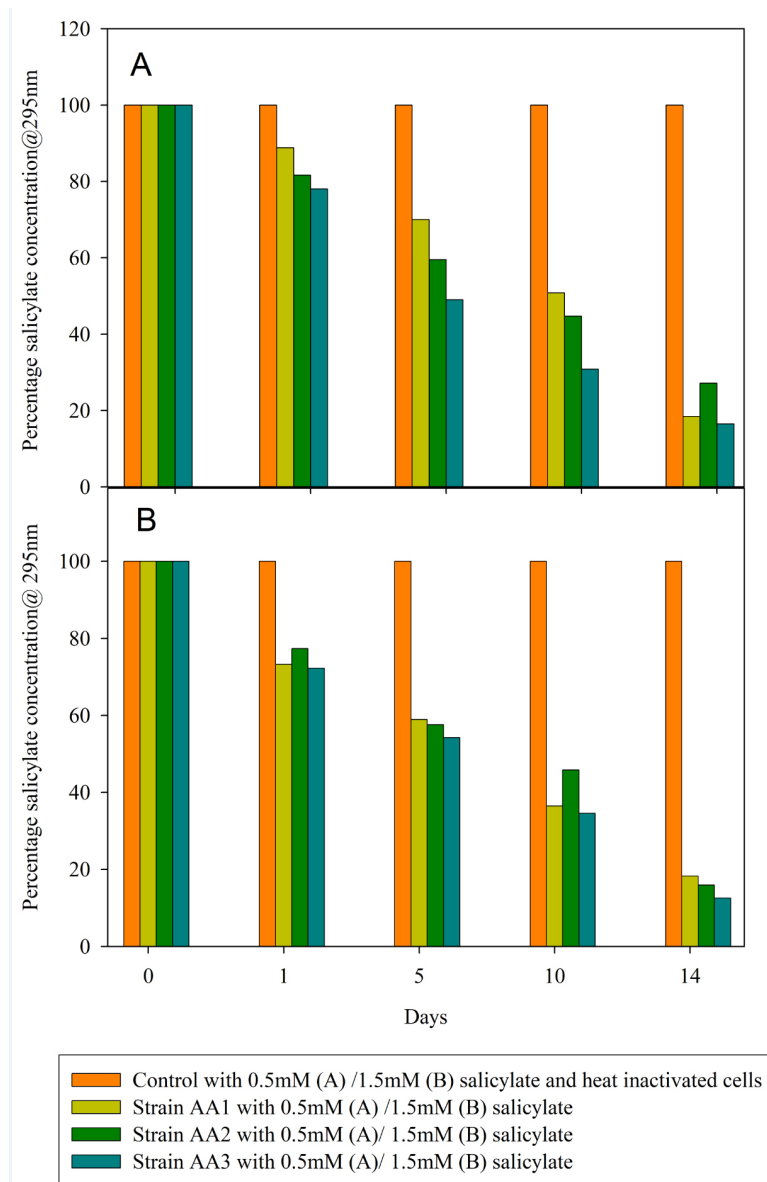


**Figure 4.** The growth and carotenoid production of strain AA1 (A), strain AA2 (B), and strain AA3 (C) under photoautotrophic conditions in Chu (A, B) and Bourelly medium (C). All data points shown are the means of two independently performed experiments. The bars shown indicate the range of the mean.

carotenoid accumulation in *Haematococcus pluvialis* compared to photoautotrophic conditions. Hu et al.<sup>21</sup> showed that sodium salicylate at similar concentrations (e.g., 0.5 to 1mM) was removed from active cultures and enhanced cell growth and production of carotenoid in *Haematococcus pluvialis*, matching results for isolate AA3 as cultures supplemented with 1.5mM salicylate achieved a carotenoid yield of about 1.36 mg/L

(Figure S4.3C), thus similar to earlier carotenoid yield data reported by Cifuentes et al.<sup>57</sup> for *Haematococcus pluvialis*.

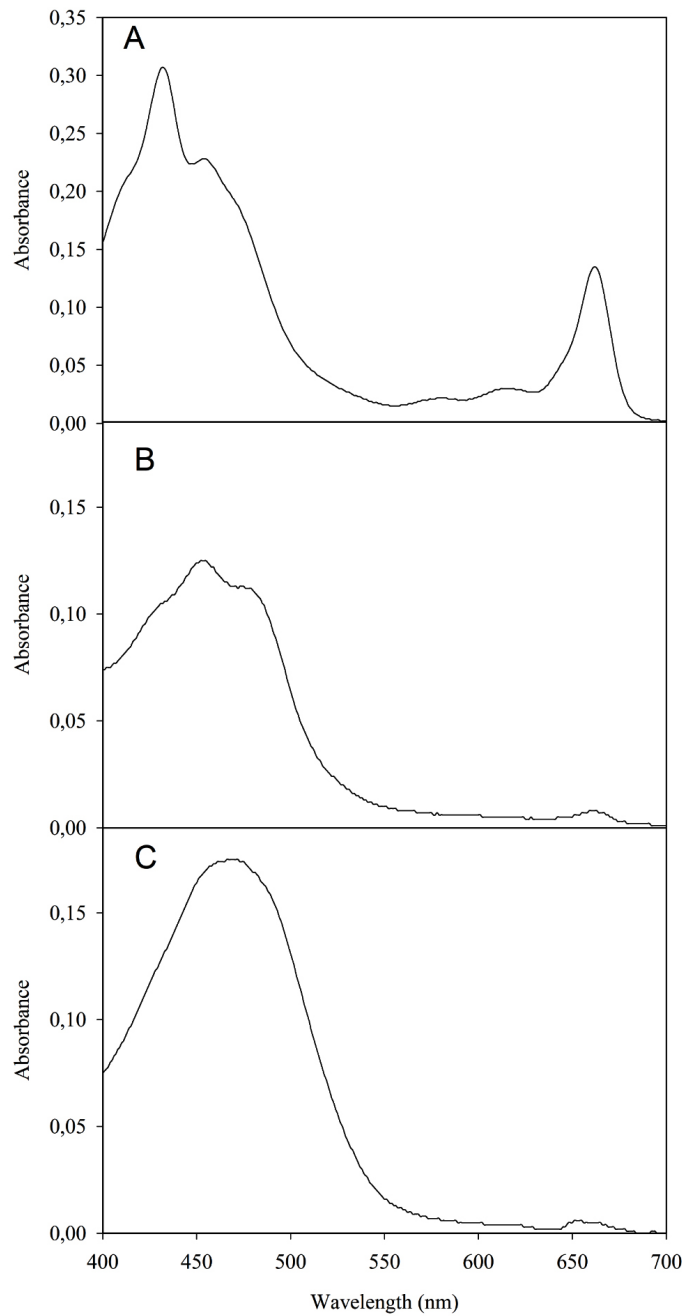
For cultures of isolates AA1, AA2, and AA3 supplemented with 0.5 mM salicylate, the initial concentration of salicylate decreased by >82% (AA1), >73% (AA2), >84% (AA3) after 14 days (Figure 5A). Similarly, the initial concentration of 1.5mM salicylate decreased by >82%, >84%, and



**Figure 5.** The photoheterotrophic growth of strains AA1, AA2, and AA3 with 0.5mM (A) and 1.5mM salicylate (B) as the sole carbon source over 14 days. The controls contain 0.5mM or 1.5mM salicylate and heat-inactivated cells of strains AA1, AA2, and AA3. The values shown are the means of two independently performed experiments

>87% for strains AA1, AA2, and AA3 after 14 days incubation (Figure 5B). The control with heat-inactivated algae cells of strains AA1, AA2, and AA3 and salicylate indicated that the salicylate concentration did not change in the absence of metabolically active cells. Therefore, only in

the presence of active cultures of strains AA1, AA2 and AA3 was salicylate eliminated from the medium. This finding matches a report of Fu et al.<sup>58</sup> wherein salicylate was applied as a carbon source and induced carotenoid production in *Chlorella regularis* (now *Coelastrum microporum*).



**Figure 6.** The visible absorption spectrum of diluted acetone extracts of late stationary cells of strain AA1 (A), strain AA2 (B), and strain AA3 (C) after photoautotrophic growth

Furthermore, Kovacik et al.<sup>59</sup> showed that *Scenedesmus quadricauda* utilized salicylate as carbon source to produce carotenoids.

Similarly, *Scenedesmus* sp. strain AA2 decreased the initial salicylate concentration in both 0.5 mM and 1.5 mM salicylate supplemented medium. Hu et al.<sup>56</sup> showed that salicylate served as source of carbon for *Haematococcus pluvialis*, improving cell growth and boosting astaxanthin accumulation, matching our observation that *Haematococcus* sp. strain AA3 utilized salicylate for cell growth and carotenoid production. *Haematococcus pluvialis* has been reported to use carbon sources like glycerol, glucose, and acetate to boost astaxanthin production under photoheterotrophic conditions.<sup>44</sup> However, while salicylate was categorized by Gao et al.<sup>55</sup> as a phytohormone, inducing increased astaxanthin biosynthesis in *Haematococcus pluvialis*, our results show that active cells of isolate AA3 utilized more than 80% of available salicylate as a carbon source for biomass and carotenoid production (Figure 5, S4.3). Therefore, salicylate can function as a carbon source to enhance carotenoid biosyntheses like acetate and glucose.

### Pigment Production

Acetone extracts of cells of isolate AA1 exhibited a spectrum with absorbance maxima of 450 and 665nm (Figure 6A), matching the spectral properties for  $\beta$ -carotene and chlorophyll a in acetone.<sup>60</sup> The spectral characteristics of acetone cell extracts of isolate AA2 (Figure 6B) revealed a higher  $\beta$ -carotene (A450 nm) to chlorophyll a (665 nm) ratio than isolate AA1, matching spectral characteristics previously reported by Qin et al.<sup>8</sup> for *Scenedesmus* spp. The spectral characteristics of acetone extracts from cells of isolate AA3 (Figure 6C) revealed low chlorophyll a content based on the absorbance at 665 nm and demonstrated astaxanthin production, as astaxanthin shows an absorption maximum at 474 nm in acetone.<sup>20</sup> The absorption spectrum revealed a ratio of about 10 for astaxanthin (474 nm) to 1 for chlorophyll a (665 nm) (Figure 6C). Similar ratios were reported by Kim et al.<sup>49</sup> for astaxanthin and chlorophyll a in a strain of *Haematococcus pluvialis*. The absorption spectrum of TLC-purified astaxanthin from strain AA3 matched the spectrum for authentic

astaxanthin (Figure S5A and B) and spectral data reported by Dragos et al.<sup>20</sup> for astaxanthin.

### TLC analysis of strain AA3 extracts

Two major bands were detected in strain AA3 acetone cell extracts. Based on the Rf values obtained when using two different mobile phases, both  $\beta$ -carotene and astaxanthin were identified when compared to authentic  $\beta$ -carotene and astaxanthin (Table S1), additionally matching results reported previously.<sup>22,23</sup>

### Evaluation of antioxidant activity

As expected, vitamin C scavenged the DPPH radical with an EC<sub>50</sub> value of about 10  $\mu$ M, which is in agreement with the value of 10.2  $\mu$ M reported by Mishra et al.<sup>61</sup> Burton and Gold<sup>62</sup> reported  $\beta$ -carotene as an effective radical quencher with an EC<sub>50</sub> of 50  $\mu$ M for DPPH, matching our experimental results for  $\beta$ -carotene with an EC<sub>50</sub> value of 50  $\mu$ M. Methanolic cell extracts of isolate AA1 could quantitatively decolorize DPPH (100  $\mu$ M) within 30 mins of incubation, demonstrating that cells of isolate AA1 contain carotenoids possessing antioxidant capacity, with the EC<sub>50</sub> value estimated as 330  $\mu$ M. Comparable results were reported recently<sup>63</sup> for *Parachlorella kessleri* methanol extracts with an estimated EC<sub>50</sub> of 215  $\mu$ M.<sup>63</sup> Based on  $\beta$ -carotene content, *Scenedesmus* sp. strain AA2 methanol cell extracts yielded an estimated EC<sub>50</sub> of 280  $\mu$ M, likewise *Haematococcus* sp. strain AA3 extracts neutralized DPPH with an estimated EC<sub>50</sub> of 264  $\mu$ M, matching earlier results showing that *Haematococcus pluvialis* methanol extracts possessed substantial antioxidative capacity.<sup>64</sup> The scavenging of DPPH by methanol extracts of strain AA3 and the TLC-purified astaxanthin with an estimated EC<sub>50</sub> value of 100  $\mu$ M is expected as astaxanthin is considered a more efficient radical scavenger than  $\beta$ -carotene; comparable data were reported by Oh et al.<sup>65</sup> with an estimated EC<sub>50</sub> value of 105  $\mu$ M for astaxanthin using the DPPH assay.

### CONCLUSION

This study demonstrated that potent carotenoid-producing algal strains representing different algal genera, such as *Parachlorella*,

*Scenedesmus*, and *Haematococcus*, could be isolated from local aqueous samples in KwaZulu-Natal, South Africa. All the microalgal isolates obtained in the current study evidently used salicylate under photoheterotrophic conditions as a carbon source and produced carotenoids exhibiting antioxidant properties. Thus, these isolates might have the potential to provide such carotenoids as food supplements to tackle diseases caused by radicals.

#### SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at <https://doi.org/10.22207/JPAM.17.2.46>

**Additional file:** Additional Table S1 and Figures S1-S5.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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#### DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or in the supplementary files.

#### ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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