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Purple bacteria screening for photoautohydrogenotrophic food production: Are new H_2-fed isolates faster and nutritionally better than photoheterotrophically obtained reference species?

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ABSTRACT

Photoautohydrogenotrophic enrichments of wastewater treatment microbiomes were performed to obtain hypothetically high-potential specialist species for biotechnological applications. From these enrichment cultures, ten photoautohydrogenotrophic species were isolated: six *Rhodopseudomonas* species, three *Rubrivivax* members and *Rhodobacter blasticus*. The performance of these isolates was compared to three commonly studied, and originally photoheterotrophically enriched species *(Rhodopseudomonas palustris, Rhodobacter capsulatus* and *Rhodobacter sphaeroides),* designated as reference species. Repeated subcultivations were applied to improve the initial poor performance of the isolates (acclimation effect), which resulted in increases in both maximum growth rate and protein productivity. However, the maximum growth rate of the reference species remained 3–7 times higher compared to the isolates (0.42–0.84 d^{-1} at 28 °C), while protein productivities remained 1.5–1.7 times higher. This indicated that H_2-based enrichment did not result in photoautohydrogenotrophic specialists, suggesting that the reference species are more suitable for intensified biomass and protein production. On the other hand, the isolates were able to provide equally high protein quality profiles as the references species, providing full dietary essential amino acid matches for human food. Lastly, the effect of metabolic carbon/electron switching (back and forth between auto- to heterotrophic conditions) initially boosted μ_{max} when returning to photoautohydrogenotrophic conditions. However, the switch negatively impacted lag phase, protein productivities and pigment contents. In the case of protein productivity, the acquired acclimation was partially lost with decreases of up to 44 % and 40 % respectively for isolates and reference species. Finally, the three reference species, and specifically *Rh. capsulatus*, remained the most suitable candidate(s) for further biotechnological development.

Introduction

Conventional animal-based protein production is severely altering biogeochemical cycles of nitrogen and phosphorus, biodiversity and land-use [1]. An alternative and more sustainable route for protein production is based on microorganisms, otherwise known as 'microbial protein' (MP), 'microbial food', or 'single-cell protein' [2]. Nowadays, MP production is still predominantly based on the use of agricultural products or fossil fuels [3]. However, a shift towards land-and-fossil free MP production would improve their sustainability [4,5]. Purple bacteria

Abbreviations: MP, Microbial protein; PB, Purple bacteria; PH, Photoheterotrophically; PA_{H2}, Photoautohydrogenotrophy; K_s, Substrate affinity constant; VFA, Volatile fatty acids; COD, Chemical oxygen demand; PH_{VFA}, Photoheterotrophic with VFA as carbon source; μ_{max}, Maximum growth rate; t_{lag}, lag time; TSS, Total suspended solids; VSS, Volatile suspended solids; TAA, Total amino acids; EAA, Essential amino acids; EAAI, Essential amino acids index; Bchl a, Bacteriochlorophyll a; HOB, Hydrogen oxidizing bacteria.

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(PB), known for their metabolic versatility, have the ability to switch between all combinations of photo-, chemo-, hetero-, and autotrophic conditions enabling them to tap into a broad range of feedstocks and resources, thus rendering them perfect candidates for MP products [6].

PB are a highly diverse group represented by more than 160 known species which are classified into 57 genera [7]. Purple non-sulphur bacteria belong to the alpha- and beta-division of the proteobacteria, while purple sulphur bacteria are represented by the gamma division. Photoheterotrophically (PH) cultivated PB have been extensively studied and have already been proposed as a protein source within biological life support systems for space missions [8], as well as for animal and plant production (e.g. [9,10]). Only recently, advances were made for photoautotrophic growth with H_2 as electron donor, i.e. photoautohydrogenotrophy (PA_{H2}), rendering them as a high-potential sustainable source of MP, which can be produced on land-and-fossil free light, CO_2 and H_2. Kinetic and nutritional characterization of PA_{H2} growth has exclusively been carried out with 'conventional' photoheterotrophically enriched PB and was limited to *Rhodobacter capsulatus* [11–13], *Rhodobacter sphaeroides* [13], *Rhodopseudomonas palustris* [13,14] and *Rhodospirillum rubrum* [15]. In a comparative study among the first three species, *Rhodobacter capsulatus* had the best kinetic and nutritional performance, expressing protein productivities comparable to (phototrophic) microalgae and higher protein yields than H_2 oxidizing bacteria (HOB), while providing a full dietary protein match for humans [13].

With the future prospect of producing PA_{H2} purple bacteria on a larger scale, the selection of specialist species is essential to guarantee optimized biotechnological applications [16]. The ecological principle of specialist (r-strategy) and generalist (K-strategy) species describes the growth and reproduction strategies of organisms. While generalists have the competitive advantage of ensuring their survival in a broad range of environments due to their low substrate affinity constants (K_S), specialists are known for their higher growth rates in uncrowded conditions. Hypothetically, PA_{H2} specialists are expected in habitats in which a combination of inorganic carbon, light and H_2 are present. While inorganic carbon has a wide distribution range, light availability is limited to surface conditions and H_2 presence is mostly linked to anaerobic/anoxic digestion processes or geothermal manifestations [17]. One previous PA_{H2} enrichment from typical natural systems (ponds and stagnant ditches) resulted in the isolation of *Rhodospirillum rubrum, Rhodopseudomonas palustris*, *Rhodobacter capsulatus* and *Rubrivivax gelatinosus* [18].

However, it appears that no PA_{H2} enrichment of engineered systems (i.e., non-natural, man-made processes) has been reported thus far. For example, wastewater treatment processes often provide high nutrient levels (i.e. eutrophic), harbour rich microbial communities and combine aerobic and anaerobic/anoxic processes, which could provide high-potential inocula for the isolation of specialists for biotechnological PA_{H2} applications. Moreover, it has been shown that PB were able to acclimate to certain substrates over the course of subcultivations, resulting in improved kinetic performance. This has been previously seen for the optimization of biohydrogen production by photofermentation [19,20] and the cultivation under acetate conditions [21].

Finally, PB are mostly known for their metabolic versatility, yet the effect of switching between metabolic conditions on the performance remains a gap in literature. Thus far, metabolic switching has been focused on heterotrophic conditions in which switches between the energy source were made (photo- and chemotrophic) to study the gene regulation of the photosystem [22,23] or the possible reduction in production cost [24]. The effect of switching between autotrophic and heterotrophic conditions (and thereby also switching electron source) on the performance of PB, while remaining phototrophic, has not been studied previously. As the reducing equivalents needed for inorganic carbon are higher compared to organic carbon sources, autotrophic growth is more energy-demanding and therefore typically slower than heterotrophic. Thus, hypothetically, under the energetically favourable heterotrophic conditions, some excess energy could be stored as biopolymers (e.g., polyhydroxyalkanoates, polyphosphates), which could result in a performance boost when returning to PA_{H2} conditions. On the other hand, the switch from heterotrophic conditions could negatively impact the achieved acclimation of the subcultivations, resulting in a decreased performance when switching back to PA_{H2} conditions.

In this study, the first PA_{H2} enrichment and isolation of PB species from wastewater treatment microbiomes of engineered systems was performed in the search for PA_{H2} specialists. The hypothesis that specialist species would emerge was evaluated by comparing the performance against three PH enriched species (*Rhodopseudomonas palustris, Rhodobacter capsulatus* and *Rhodobacter sphaeroides*). These latter species were considered baseline or reference species as they are both (i) highly represented in studies on anoxygenic photosynthesis [6,25] and (ii) were previously kinetically and nutritionally characterized under the same PA_{H2} conditions as this study [13]. Moreover, the PB isolates obtained were characterized over the course of subcultivations in PA_{H2} conditions to study the effect of acclimation. Lastly, the effect of metabolic switches on the performance of both isolates and reference species was studied.

Materials and methods

Purple bacteria growth media for enrichment and cultivation

The autotrophic medium used for enrichment, isolation and subcultivations was based on [12], adapted in the dosage of nitrogen and phosphorus as well as the dosage and source of carbon. Instead of adding CO_2 (0.1 g C L^{-1}), $NaHCO_3$ (0.33 g-C L^{-1} or 27 mmol C L^{-1}) was used as inorganic carbon source. Nitrogen (originally 0.21 g N L^{-1}) was dosed as $(NH_4)_2SO_4$ at a concentration of 0.31 g-N L^{-1} (22 mmol N L^{-1}), while the phosphate buffer (originally 0.59 g P L^{-1}) was adapted to a strength of 30 mmol P L^{-1} (0.93 g-P L^{-1}). The pH was adjusted to 6.9 with 0.5 M NaOH. The heterotrophic growth medium used for the metabolic switch cultivations was based on the AT ("Athiorhodaceae") medium [26,27], but modified in carbon/electron source with the use of volatile fatty acids (VFA). Acetic acid, butyric acid and propionic acid were added in a chemical oxygen demand (COD) ratio of 1:1:1 (1 g COD L^{-1} or 0.33 g C L^{-1}) instead of sodium acetate (0.29 g C L^{-1}) [28]. Nitrogen was dosed as NH_4Cl at a concentration of 0.26 g-N L^{-1} (19 mmol N L^{-1}), while the phosphate buffer was added to a strength of 7 mmol P L^{-1} (0.23 g-P L^{-1}). Inorganic carbon $NaHCO_3$ was added in a concentration of 0.43 g-C L^{-1} or 36 mmol C L^{-1}. The pH was adjusted to 7.3 with 0.5 M NaOH. All chemicals were provided by Sigma-Aldrich (Saint Louis, Missouri, USA).

Batch cultivations of PA_{H2} and PH_{VFA} (photoheterotrophic with VFA as carbon source) conditions were performed in Erlenmeyer flasks without baffles (DURAN, Mainz, Germany) of 500 mL, with a working volume of 300 mL. First, all autoclavable components of the growth medium were added, after which the bottles were autoclaved at 121 °C for 15 min. The remaining medium components were then added axenically added after filter sterilization (Chromafil Xtra PVDF-20/25, Macherey-Nagel, Dueren, Germany), while the inoculum was added last. The initial biomass concentration was set to an optical density (OD) of 0.05 at 660 nm. The Erlenmeyer flasks were subsequently closed with a gas-tight septum and the headspace was completely flushed with either a commercial mixture of 80 % H_2/20% N_2 (autotrophy) or 100 % N_2 (heterotrophy) and set at a gauge pressure of + 0.4 bar (absolute pressure of 1.4 bar). The flasks were continuously mixed at 28 °C on a magnetic stirring plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 400 rpm in an incubation chamber (Snijders Scientific, Tilburg, The Netherlands). The light in the incubation chamber was provided by 2 × 7 fluorescent tubes at an intensity of 17 W m^{-2} (in the range of 175–1000 nm). All flasks were randomized on a daily basis to provide an even distribution of light. When the gauge pressure dropped below + 0.2 bar, the headspace was spiked with the corresponding gas (80% H_2/20% N_2 or 100% N_2) to a pressure of + 0.4 bar.

Origin of inocula, photoautohydrogenotrophic enrichment and isolation

Nine microbiome samples of engineered systems (2 anaerobic, 2 aerobic and 5 aerobic/anoxic conditions) from the wastewater treatment of industrial (brewery) and municipal streams in Belgium and the Netherlands were used as inocula in a search for PA_{H2} specialists. The enrichment was performed with autotrophic medium with H_2 as electron donor in the headspace. Each inoculum was individually added in a concentration of 30 mg volatile suspended solids L^{-1} and a cultivation of 8–10 days was performed (Fig. 1 A). In contrast to the unfiltered light conditions in cultivation batches, an infra-red passing filter black perspex 962 (Bay Plastics Ltd., North Shields, UK) was added for the specific enrichment of PB. Subsequently, a dilution to extinction method was applied on these enriched culture batches. Serial dilutions (10^{-4}, 10^{-6} and 10^{-8}) were made in phosphate buffered saline and spread plated on Petri plates prepared with autotrophic medium to which agar (15 g L^{-1}) was added before autoclaving. The plates were incubated in a gas bag filled with 80% H_2/20% N_2 using halogen light of 39.8 W m^{-2} for 10–14 days at 28 °C. Twenty colonies were picked and streak plated on new plates to isolate them. Once isolates were achieved, they were re-cultivated in liquid autotrophic medium in small 50 mL Erlenmeyer flaks without baffles (DURAN) under the same conditions as the cultivation batches. The identification of these isolates was performed via Sanger sequencing at VIB Genetic Service Facility UAntwerp, Belgium (methodology can be found in Supplementary materials).

Three commonly used reference species

Three PB species, all originally stemming from PH enrichments, were used as reference species in this study. *Rhodopseudomonas palustris* LMG 18881 *(Rps. Palustris)* and *Rhodobacter sphaeroides* LMG 2827 *(Rh. Sphaeroides)* were ordered through BCCM (Belgian Coordinated Collections of Microorganisms, Ghent). *Rhodobacter capsulatus (Rh. Capsulatus)* was obtained from [28] who isolated it from a mixture of (i) activated sludge from a sewage treatment plant, (ii) activated sludge from a dairy wastewater treatment plant and (iii) sediment from a local pond.

Subcultivations and metabolic switch experiments

Fig. 1B depicts the experimental procedure of the subcultivations and metabolic carbon switch experiments, in which the experiments were coded to simplify the discussion of the results. Light (phototrophy) was used as an energy source in all experiments, while as carbon source both autotrophic (A) and heterotrophic (H) conditions were applied. The electron source for the autotrophic conditions was H_2, while in heterotrophic conditions the carbon source also served the purpose of electron donor. The superscript numbers depict the ongoing subcultivation in a certain condition.

The subcultivation experiments (inocula taken at stationary phase from previous cultivation) were performed with the 10 isolates and samples were analysed in the first (A^1), second (A^2) and sixth batch cultivation (A^6). Data from [13] was used for the performance of the reference species, which were not themselves included during the subcultivation experiments. As the 3 reference species, cultivated in the

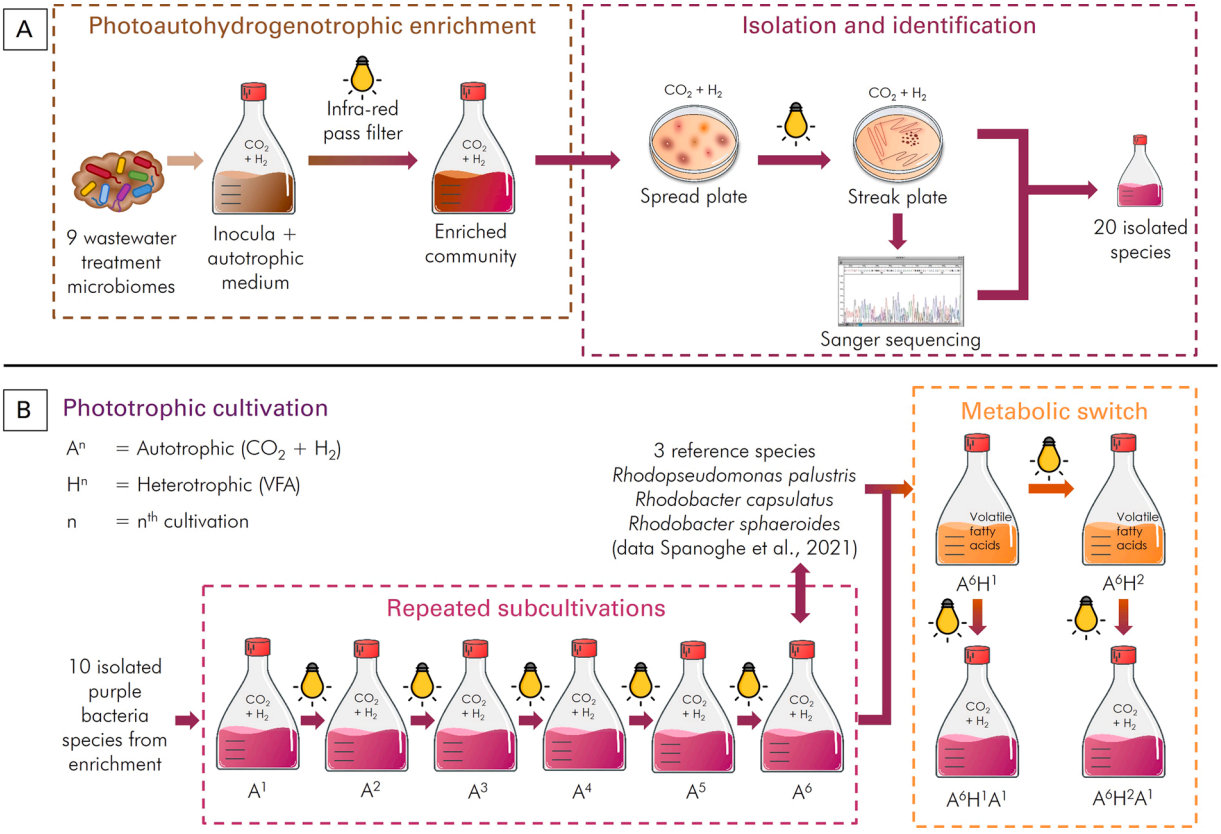

Fig. 1. Experimental procedure for enrichment, isolation and identification of the isolates under photoautohydrogenotrophic conditions (panel A) and the subcultivations and metabolic carbon/electron switch experiments (panel B).Various inocula of engineered water treatment systems (industrial and municipal) were used to enrich and isolate photoautohydrogenotrophic purple bacteria species. The 10 isolates from this enrichment were subsequently cultivated in repeated subcultivations (autotrophic). To compare the performance of the isolates, the 3 reference species (originally photoheterotrophically enriched) were incorporated in the last stage of the subcultivations (data from Spanoghe et al. (2021) [13]). Next, both the isolates and reference species were studied in metabolic carbon/electron switch (back -and-forth between autotrophic and heterotrophic) experiments.

exact same conditions, had been actively used in PA_{H2} subcultivations, they were considered acclimated and their performance correlated with the sixth cultivation of the isolates. In a last experimental procedure, a metabolic switch to heterotrophic conditions was performed with either one (A^6H^1) or two cultivations (A^6H^2). From both cultivations, the switch back to autotrophic conditions was studied ($A^6H^1A^1$ and $A^6H^2A^1$ respectively). The metabolic switch experiments were performed both with the 10 isolates and the 3 reference species. Due to the large extent of samples, experiments were performed with one biological replicate.

Growth analyses and calculations: temperature, pH, biomass and H_2

Temperature and pH were measured with a digital metre (Hanna Instruments, Woonsocket, Rhode Island, USA). OD at 660 nm was measured with an UV–VIS spectrophotometer (Shimadzu, Kyoto, Japan). Details for the growth rate determinations can be found in [13]. In short, the OD data were fitted to the Gompertz model modified by [29] which provided the maximum specific growth rate (μ_{max}) and the lag time (t_{lag}). The total suspended solids (TSS) and volatile suspended solids (VSS) during batch experiments were determined via calibration curves based on the ODs by diluting a batch culture at stationary phase. TSS and VSS were respectively determined via the APHA methods 2540B/2540D and 2540E [30]. Overall TSS productivities (g TSS L^{-1} d^{-1}) were calculated dividing the difference in TSS concentration ($[X]_0$ to $[X]_i$) by the total cultivation time (t_0 to t_i).

The percentage of H_2 gas in the headspace was monitored by gas chromatography (GC-2014, Shimadzu, Kyoto, Japan, TCD detector, Shincarbon-ST 50/80 Stainless steel 2.0 m × 3.0 mm, Argon as carrier gas) during cultivations A^2, $A^6H^1A^1$ and $A^6H^2A^1$. This analysis could not be performed for all experimental runs due to the inability to store the gas samples without losses of H_2 to the atmosphere. The pressure in the Erlenmeyer flasks was monitored with a manual manometer (Bourdon MEX5-D30. B74, Baumer, Huskvarna, Sweden) and temperature of the headspace was assumed to be equal to the liquid. H_2 gas consumption was then calculated with the ideal gas law. The biomass yield (g TSS g^{-1} H_2) was calculated at the end of the batch experiment by dividing the net TSS production by the cumulative H_2 consumption.

Nutritional analyses: Protein, essential amino acids and pigments

Protein analysis was performed colorimetrically with a modified Lowry analysis [31]. The biomass protein content was expressed as a fraction of the biomass (g protein g^{-1} TSS). The protein productivity (g protein L^{-1} d^{-1}) was calculated by dividing the difference in protein concentration ($[Protein]_0$ to $[Protein]_i$) by the total time of cultivation (t_0 to t_i).

Total amino acids (TAA), dietary match, essential amino acids (EAA) and EAA index (EAAI) were determined for 3 isolates (*Rhodobacter blasticus*, *Rubrivivax* sp 3 and *Rhodopseudomonas*. Sp 3) at their maximum protein productivity in cultivation A^6 as described in [13]. In short, the protein quality (g EAA 100 g^{-1} protein) was expressed both on adapted Lowry or TAA protein content and a range of the quality was given. As target organisms, humans, pigs and penaeid shrimp were chosen and respectively meat (beef) [32,33], soybean meal and fishmeal [34,35] were used as conventional and comparative protein sources. The digestibility of the bacterial product was assumed to be 87.0 % [36]. The quality of the protein is classified according to the EAAI as superior (>1), high (0.95–1), good (0.86–0.95), useful (0.75–0.86) or inadequate (<0.75) [37].

Lastly, bacteriochlorophyll a (bchl a) and total carotenoid content was determined by an acetone/methanol solvent (7:2 v/v) extraction, followed by spectrophotometric analysis and conversion with the Lambert-Beer law [38,39] as described in [13].

Statistical analyses

A standard deviation (SD) is given for the maximum growth rates and the lag phases, which is the SD on the fit of the Gompertz model (95% confidence). Averages and SDs are given if comparisons are made between the isolates and the reference species as groups. The number of observations is reported between brackets (n = …) in the legends of figures and tables.

Statistical analyses were conducted in the programme "IBM SPSS statistics 26″. The results of isolated and reference species were grouped, based on careful consideration of their individual results, as these groups did not contain significant outliers. This enabled performance of one-way ANOVA analyses. The normality of data residuals was tested using the Shapiro-Wilk normality test. The assumption of homoscedasticity was verified through a Levene's test. The Bonferroni post-hoc test was then used to determine significant differences. The non-parametric Kruskal-Wallis rank sum test was executed when normality was rejected, followed by the Dunn post-hoc method. The Welch's t-test was used in case of heteroscedasticity. Statistical differences ($p < 0.05$) on the figures are denoted by letters and compare means between experiments (grouped per type: either for reference species or isolates). Finally, statistical differences between isolates and reference species during a cultivation were computed, but were not shown in the figures to preserve readability.

Results and discussion

Photoautohydrogenotrophic enrichment and isolates identification

After enrichment and isolation, 20 colonies were obtained which were identified using Sanger sequencing. Five out of the 20 isolates could not be identified due to a low sequence quality (Supplementary Table S3). For most of the remaining 15 isolates, more than one possible identification was obtained, as closely related species are expected to have a similar percentage ID. A complete genome sequencing or dedicated PCR with several signature genes would be necessary to identify these isolates to the species level. However, in this study the kinetic and nutritional performance was the key parameter and thus only strains that showed an exceptional performance will be sequenced for their whole genomes in follow-up research.

Five isolates (colonies 1,4,7,9 and 15) were identified as either *Shewanella* sp., *Pannonibacter* sp. or *Hydrogenophaga electricum,* which do not represent purple bacteria, and thus were omitted from further experiments. *Shewanella* species are known for their ability to bioremediate hydrocarbons and metallic pollutants, which would explain their presence in wastewater treatment plants [40]. The same applied to *Pannonibacter* species, which were known as facultative anaerobic chemoorganoheterotrophs that can degrade undesirable compounds (e.g., tert-butyl alcohol or 4-aminobenzene sulphonate) [27]. Finally, others discovered *Hydrogenophaga electricum* as a novel species of the *Hydrogenophaga* genus (i.e. HOB) that could explain their presence in the enrichment if small O_2 levels were present [41].

The 10 achieved PB isolates (*Rhodopseudomonas, Rubrivivax* and *Rhodobacter* species) could be classified as alpha- and beta-proteobacteria [7]. As an exception, *Rhodobacter blasticus* (*Rh. blasticus*) (colony 3) could be identified to the species level, the species having been used earlier for the treatment of anaerobically digested swine wastewater [42]. Due to the circular lamellar structure of its photosynthetic membranes and budding replication mechanism, it differs from other *Rhodobacter* species that divide by binary fission and contain vesicular membranes (cfr. *Rh. capsulatus* and *Rh. sphaeroides)* [43]. As far as we are aware, *Rh. blasticus* has not been PA_{H2} enriched or cultivated.

Interestingly, 3 colonies were identified to the genus level as *Rubrivivax* species (denoted as *"Rvi.* species 1–3″ in the results), with equal likelihood of being identified as *Rvi. benzoatilyticus or Rvi. gelatinosus*,

which belong to the beta division of the proteobacteria. Only a limited number of studies have focused on photosynthetic betaproteobacteria [44]. *Rvi. benzoatilyticus* was previously described as an aromatic hydrocarbon-degrading purple bacterium [45], while *Rvi. gelatinosus* was able to use hydrolysed starch and gelatine as carbon source, due to hydrolysing enzymes it produces [44]. These species divide by binary fission in the same manner as *Rh. capsulatus* and *Rh. sphaeroides,* but their photosynthetic membranes are less developed than other purple bacteria, appearing as small finger-like intrusions [43].

Six colonies were linked to various species of the *Rhodopseudomonas* genus (denoted as *"Rps.* species 1–6″ in the results). These were either closely related or identical to the reference species *Rps. palustris.* Both *Rps. thermotolerans* and *Rps. pentothenatexigens* were previously isolated before from paddy soils [46], while *Rps. faecalis* was once isolated from chicken faeces [47]. Characteristic of these species from the Rhizobiales order is the budding mode of growth (no binary fission), which is, as mentioned earlier, associated with lamellar internal photosynthetic membranes [43].

Growth characterization

Subcultivations

The 10 PB isolates from the enrichment experiment were re-cultivated in liquid autotrophic conditions and used as inocula for a first PA_{H2} cultivation, after which subcultivations were performed to study the effect of acclimation. As mentioned above, the reference species were not included in these subcultivations as data from [13] provided their performance. The cultivation conditions in this study were identical, and the reference species had actively been used in PA_{H2} subcultivations (i.e. considered acclimated). The performance data therefore correlated with the sixth cultivation of the isolates.

As seen in Fig. 2A, the μ_{max} of the reference species (2.30–2.70 d^{-1} at 28 °C) remained 3–7 times higher after the full acclimation period (A^6, ± 16–31 generations) compared to the isolates (0.42–0.84 d^{-1} at 28 °C). Initial improvement in maximum growth rate performance was seen for the isolates (Fig. 2C), with 9 out of 10 isolates increasing in μ_{max} after one repetition (ratio>1), while only 4 isolates further improved after 5 repetitions (both ratios >1). The lower μ_{max} for isolates versus reference species likely indicated that the enrichment did not result in photoautohydrogenotrophic PB specialist species. The reference species, originally photoheterotrophically enriched, perform as specialist species in this case, suggesting that specialist species are likely not linked to a specific metabolism, but rather interchangeable due to the fact that most PB species can switch between all metabolisms.

Another important kinetic performance indicator, the lag phase (t_{lag}) was shown in Fig. 2B in absolute terms. The large standard deviations were due to inaccuracy of the Gompertz model. If there were insufficient samples taken in the expected lag phase period, the model will express a larger error on the best-fit value. However, the relative lag phase in Fig. 2D, calculated as the Δ between two subcultivations, displayed a clearer trend. After one repetition, 6 out of 10 isolates showed a decrease in t_{lag} ($\Delta < 0$), while only 4 showed further improvement after 5 repetitions (both $\Delta < 0$).

Furthermore, in Fig. 3 A, the maximum TSS productivities for isolates and reference species (based on calibration curves in Supplementary Table S4) were grouped per subcultivation experiment in a boxplot. An increasing trend in average maximum TSS productivity was seen for the isolates over the course of the experiments (with a significant increase in A^6). Additionally, the required time to reach maximum TSS productivity significantly decreased in batch A^2 (shown as the connected scatterplot). Moreover, the maximum TSS productivity in A^6 of the reference species was 2.6 times higher than that of the isolates ($p < 0.05$, not shown on the graph), while this productivity was reached 1.9 times faster (not significant).

The last studied indicator for kinetic performance was the biomass yield (Supplementary Fig. S1). This parameter was only measured at A^2 for the isolates and A^6 for the reference species, which implied that comparison was not possible over the course of the subcultivation experiments. However, it was observed that the biomass yield, for both isolates and reference species, exceeded the maximum biomass yield reached by HOB [48].

Metabolic switch

A metabolic carbon/electron switch from PA_{H2} to PH_{VFA} conditions was performed to analyze the effect on the kinetic and nutritional performance for the 3 reference species and the 10 isolates. The absolute and relative maximum growth rates in PA_{H2} conditions after metabolic switching showed varying results (Fig. 4A and C). A boost in μ_{max}

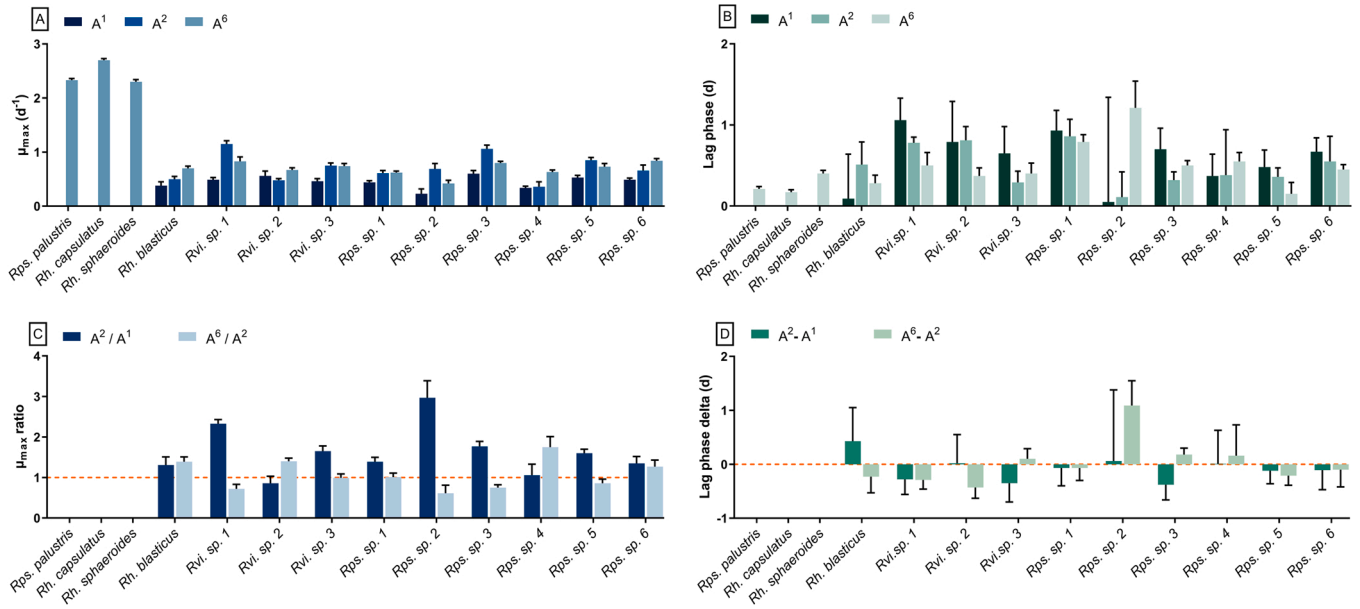

Fig. 2. The absolute maximum growth rates (panel A), relative maximum growth rates as ratio (panel C), absolute lag phase (panel B) and relative lag phase as delta (panel D) during the subcultivation experiments of the reference species (n = 3) and isolates (n = 1). The standard deviation shown is based on the fit of the Gompertz model (95% confidence).

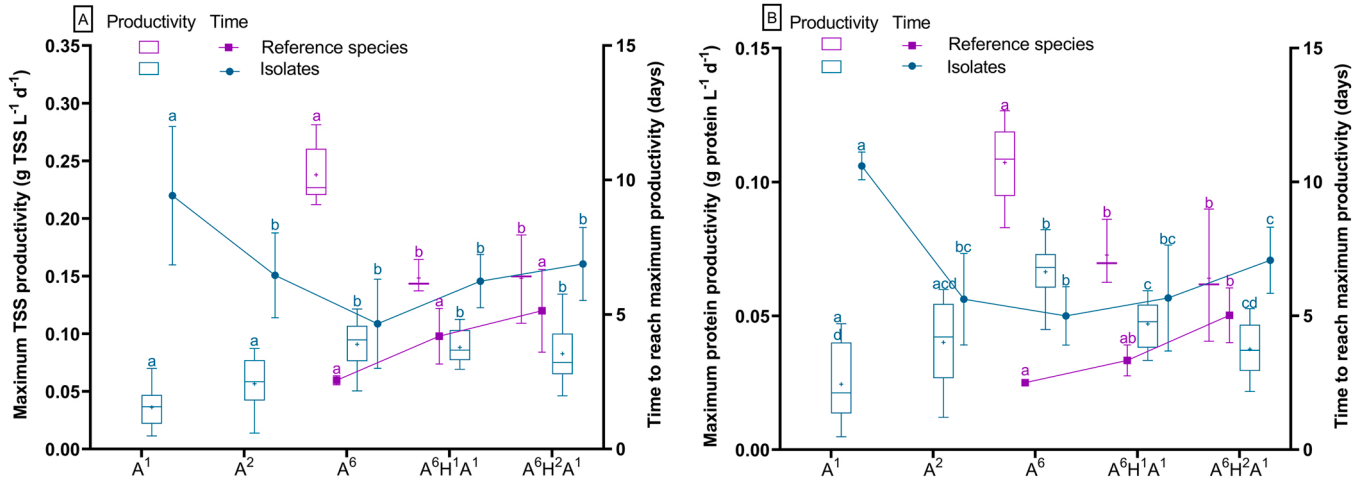

Fig. 3. Overall maximum productivities and the time to reach these maxima of total suspended solids (panel A) and protein (panel B). Productivities are shown as boxplots for each experiment with the average given as "+ ", while the time is shown as averages with their standard deviation for reference species (n = 9 for A^6, n = 3 $A^6H^1A^1$ and $A^6H^2A^1$) and isolates (n = 10). Statistical differences ($p < 0.05$) on the figure are denoted by letters and compare means between experiments (grouped per type: either for reference species or isolates).

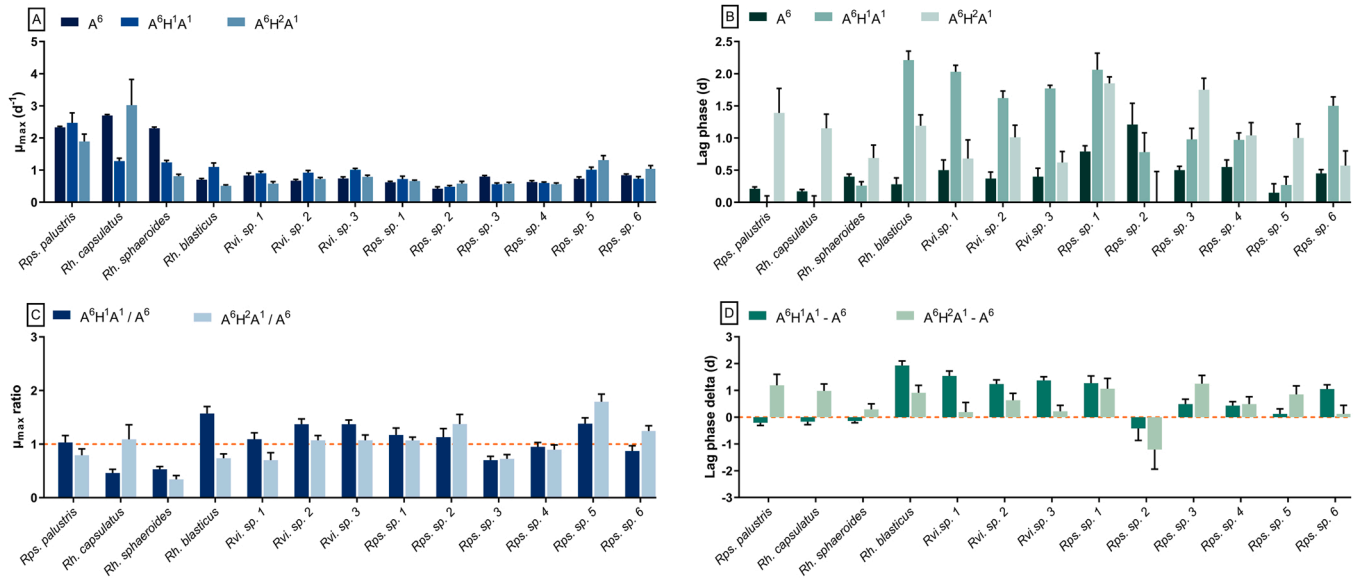

Fig. 4. The absolute maximum growth rates (panel A), relative maximum growth rates as ratio (panel C), absolute lag phase (panel B) and relative lag phase as delta (panel D) during the metabolic switch experiments of the reference species (n = 3 for A^6, n = 1 for $A^6H^1A^1$ and $A^6H^2A^1$) and isolates (n = 1). The standard deviation shown is based on the fit of the Gompertz model (95 % confidence).

occurred for 8 out of 13 species (7 isolates and 1 reference species) after one intermediary cultivation in PH_{VFA} conditions ($A^6H^1A^1$), while this was no longer the case after a period of two PH_{VFA} subcultivations ($A^6H^2A^1$). As expected, even though the focus of this study was not on photoheterotrophy, this boost also occurred for 11 out of 13 species (10 isolates and 1 reference species) when switching to PH_{VFA} conditions (A^6H^1) due to the energetically favourable heterotrophic conditions (Supplementary Fig. S2).

While μ_{max} rate did not necessarily deteriorate after the metabolic switching (Fig. 4C), t_{lag} was negatively impacted (Fig. 4D). One intermediary cultivation under PH_{VFA} conditions resulted in an increase in t_{lag} ($A^6H^1A^1$) for 9 out of 13 species ($\Delta > 0$), interestingly all isolates. However, the increase in t_{lag} only continued for 3 isolates with a second intermediary cultivation in PH_{VFA} conditions ($A^6H^2A^1$). The three reference species only increased in t_{lag} after switching back after two cultivations in PH_{VFA} conditions ($A^6H^2A^1$). The average maximum TSS productivity slightly decreased (not significant) in $A^6H^1A^1$ for the isolates (Fig. 3A). The reference species, however, decreased significantly ($p < 0.05$), which could be explained by the fact that these species were only involved at the start of the metabolic switch experiments and could have been less active (i.e., acclimated) than the isolates. Despite the more pronounced decrease, the average maximum productivity of the reference species remained significantly higher than the isolates ($p < 0.05$) in both $A^6H^1A^1$ and $A^6H^2A^1$. The required time to reach these maximum TSS productivities showed an increasing trend for both reference species and isolates (not significant), which can be linked to the effects seen for t_{lag}. In $A^6H^2A^1$, the time difference between the reference species and isolates was no longer significant.

Finally, the biomass yield of the subcultivations could not be interpreted due to the lack of data points. However, the metabolic switch did not impose a negative impact on the biomass yield of the reference species (Supplementary Fig. S1). The effect on the isolates was more difficult to interpret, since data of A^6 (hypothetically at their most acclimated) was not recorded.

Protein

Content

For the isolates, the average protein content varied significantly over the course of the subcultivation experiments (Supplementary Fig. S3). The average protein content improved after one repetition (A^2), but showed no further improvement after 5 repetitions (A^6). The metabolic switch decreased the protein content significantly ($A^6H^1A^1$), which did not decline further after two cultivations in PH_{VFA} conditions ($A^6H^2A^1$). The average protein content for the reference species over the course of the metabolic switch experiments compared to the baseline value (A^6) remained equal, which indicated a better performance resilience. Finally, the difference in protein content between the reference species and isolates was only significant ($p < 0.05$) in experiment $A^6H^1A^1$.

Quality

In terms of protein quality for feed or food formulation, a favourable EAA (histidine, isoleucine, phenylalanine/tyrosine, leucine, lysine, methionine/cysteine, tryptophan, threonine and valine) profile is required. The isolates were able to provide protein quality profiles which were as high as the reference species (Supplementary Fig. S4). The dietary match and EAAI for humans (Fig. 5A), pigs and penaeid shrimp (Supplementary Fig. S4) were calculated as a min-max range, based on the protein content determined using the adapted Lowry or TAA method. For human consumption, both the reference species and isolates were able to provide full dietary matches, which is also reflected in the EAAI (Fig. 5B). All reference species were classified as superior protein sources (EAAI>1), while the isolates ranged from good (0.95 >EAAI>0.86) to superior (EAAI>1) quality. The EAA profile of microbial protein originating from PB would therefore be able to provide an alternative for the conventional EAA derived from meat (beef).

The purple bacteria could be foreseen as a feed component in a full dietary match for 5 out of 10 EAA for both pigs and penaeid shrimp (Supplementary Fig. S4). Interestingly, the PB were even able to outcompete the conventional protein sources (soybean meal and fish meal) on a few occasions. Based on the EAAI (Fig. 5B), PB could reach high (1 >EAAI>0.95) to superior (EAAI>1) quality respectively for pigs and penaeid shrimp. Although, differences in the EAA quality among the species was difficult to determine, a blend of PB species per target organism could lead to tailored profiles that maximize the dietary match.

Productivity

The protein productivity of the isolates increased significantly ($p < 0.05$) during the subcultivation experiments, suggesting an acclimation potential to PA_{H2} conditions, while decreasing significantly ($p < 0.05$) after the metabolic switching, indicating a partial loss of acclimation and thus poor resilience of their nutritional performance (Fig. 3B). As expected, the time to reach maximum protein productivities followed the opposite trend. Compared to the average protein productivity of A^6, the isolates lost 29 % and 44 % of their productivities in $A^6H^1A^1$ and $A^6H^2A^1$ respectively. The reference species significantly ($p < 0.05$) decreased their protein productivities after the metabolic switch experiments as well, with decreases of 32 % and 40 % respectively. The maximum average protein productivity of the reference species remained significantly higher ($p < 0.05$, not shown on graph) compared to the isolates by factors of 1.6, 1.5 and 1.7 for A^6, $A^6H^1A^1$ and $A^6H^2A^1$ respectively. Furthermore, the required time to reach these maxima remained significantly ($p < 0.05$) lower for the reference species for experiment A^6 and $A^6H^2A^1$ with a factor of 2.0 and 1.4 respectively.

Yield

The protein yield of the subcultivations could not be interpreted due to the lack of data points. On the other hand, the metabolic switch had no negative impact on the protein yield of the reference species (Supplementary Fig. S1), as seen before for the biomass yield. Once again, data for the isolates at their, hypothetically, most acclimated were lacking. The trend can therefore only indicate that the same order of magnitude for protein yield was seen in all experimental runs performed of the isolates. Finally, all PB protein yields were higher than the maximum yield reached by HOB [48], showing their competitive advantage.

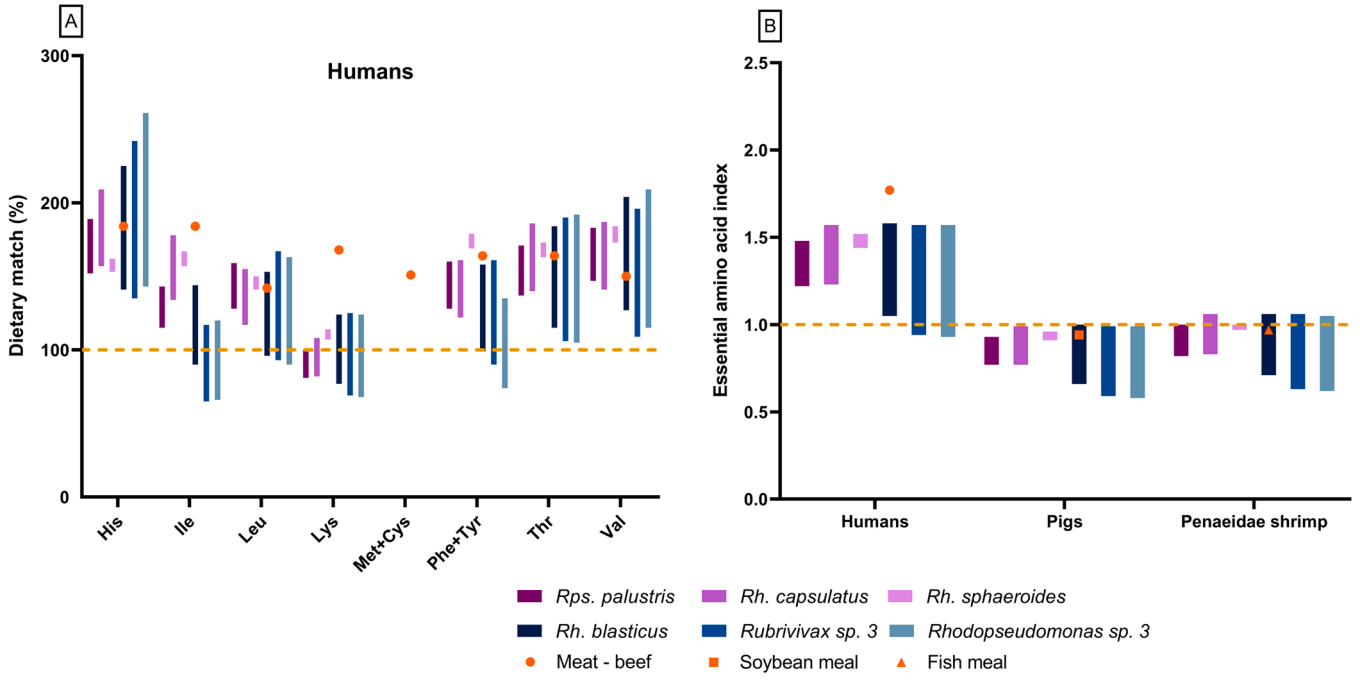

Fig. 5. Protein quality of the reference species and isolates expressed as the dietary match for humans (panel A) and the essential amino acid index (EAAI) for humans, pigs and penaeid shrimp (panel B). The results are shown as a min-max range, with the minima correlating with Markwell protein content (n = 1) and the maxima with total amino acid (TAA) protein content (n = 1). As comparison, conventional protein sources meat-beef (for humans), soybean meal (for pigs) and fish meal (for penaeid shrimp) are given. The 100% dietary match or EAAI score of 1 is shown as the dashed yellow line.

Pigment content

Nutritional added value in PB could be found in their Bacteriochlorophyll (BChl) a and carotenoids content, which both possess antioxidant properties [49]. Exceptionally, the PH_{VFA} cultivations were included in the results, as they showed interesting effects. BChl a remained stable for the isolates during the subcultivation experiments, but significantly decreased throughout the metabolic switching experiments (Fig. 6 A). In contrast, the BChl a content in the reference species did not decrease by the metabolic switch in the first PH_{VFA} experiment and its corresponding switch back ($A^6H^1A^1$). However, the content also decreased after two cultivations in PH_{VFA} conditions and its switch back ($A^6H^2A^1$). The BChl a content only differed significantly ($p < 0.05$) between reference species and isolates during the PH_{VFA} experiments. Similar trends were found for the carotenoid content for both reference species and isolates (Fig. 6B). The reference species remained at the same level of carotenoids in their first PH_{VFA} cultivation, but all levels dropped significantly in the second PH_{VFA} cultivation and the switches back to PA_{H2}. The isolates showed a declining trend towards the metabolic switching experiments, but this could not be shown to be statistically significant, due to the large variation in experiments A^2 and A^6. In all experiments, the difference between reference species and isolates was significant ($p < 0.05$).

Previous metabolic switches for heterotrophic PB showed, as expected, reduced BChl a when changing from phototrophic to chemotrophic conditions [50]. However, as the energy source (i.e. light) in this study remained constant, the photosystem should have remained intact and active to maintain the ATP production. Furthermore, the BChl a and carotenoid content found in the repeated subcultivations were of the same order of magnitude as the pigment content found in [13], indicating that the pigment content was not exceptionally high after acclimation. The switch in carbon source could have initiated a stress response in which carbon allocation towards the photosystems was spared [51], while investing in other macromolecules or even energy-reserves (e.g. polyphosphate) [52,53].

Conclusion

The goal of this study was to obtain specialist species by performing photoautohydrogenotrophic enrichments of wastewater treatment microbiomes. The main findings were:

(i) The enrichments resulted in 10 isolates (6 *Rhodopseudomonas* species, 3 *Rubrivivax* species and 1 *Rhodobacter blasticus*) of which *Rh. blasticus* and the *Rubrivivax* species were not previously cultivated in photoautohydrogenotrophic conditions.

(ii) The repeated subcultivation experiments indicated that kinetic properties (μ_{max}, lag phase and TSS productivities) and protein productivities could be improved due to the acclimation potential. However, μ_{max} and protein productivities remained respectively 3–7 and 1.5–1.7 times higher for reference species compared to the isolates. Interestingly, isolates reached the same protein qualities as the reference species.

(iii) The photoautohydrogenotrophic enrichments did not result in the isolation of specialist species, as the originally photoheterotrophically enriched reference species reached the best kinetic and nutritional performances, suggesting that they could act as photoautohydrogenotrophic specialists.

(iv) Metabolic carbon/electron switching (back and forth between auto- to heterotrophic conditions) initially boosted μ_{max}, but negatively impacted t_{lag}, protein productivities and pigment contents, resulting in partial loss of the acclimation. Protein productivities decreased by up to 44 % and 40 % respectively for isolates and reference species.

(v) Finally, the reference species, and specifically *Rh. capsulatus*, remained the most suitable candidates for further biotechnological development.

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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.

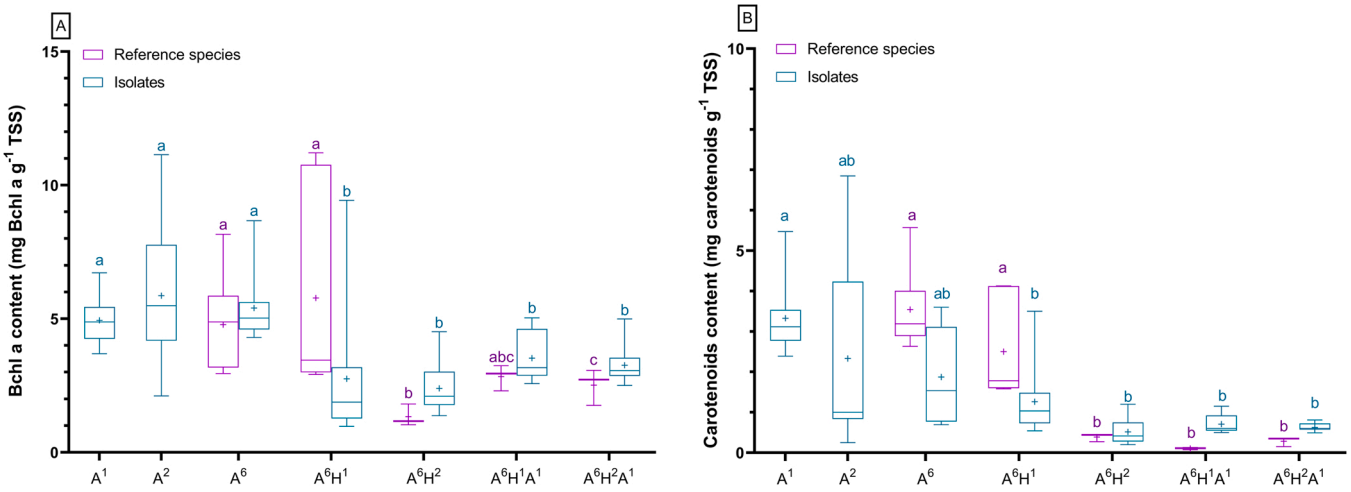

Fig. 6. Bacteriochlorophyll a (panel A) and total carotenoids (panel B) content in the total suspended solids (TSS) of the reference species and isolates. Results are shown as boxplots for each experiment with the average given as "+" for reference species (n = 9 for A^6, n = 6 for A^6H^1, n = 3 for remaining experiments) and isolates (n = 14 for A^1, n = 20 A^6H^1, n = 10 for remaining experiments). Statistical differences ($p < 0.05$) on the figure are denoted by letters and compare means between experiments (grouped per type: either for reference species or isolates).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2022.08.005.

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