Supplementary Materials

Development of a microalgae-based continuous starch-to-hydrogen conversion approach

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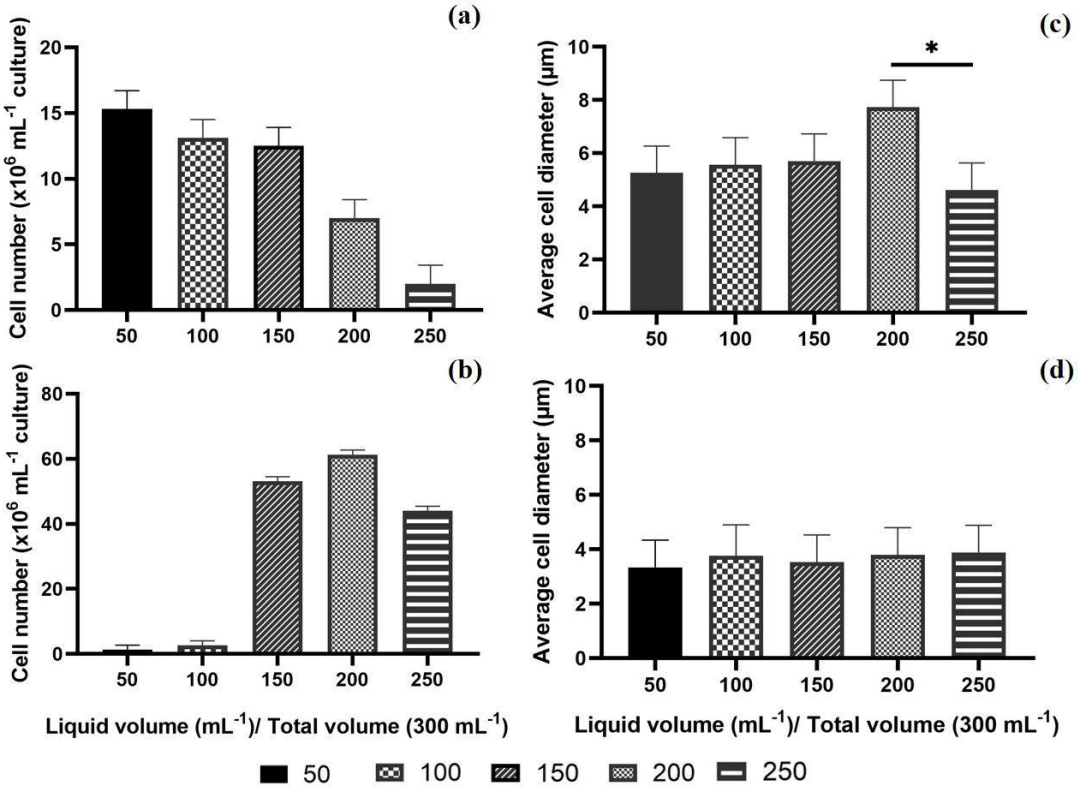
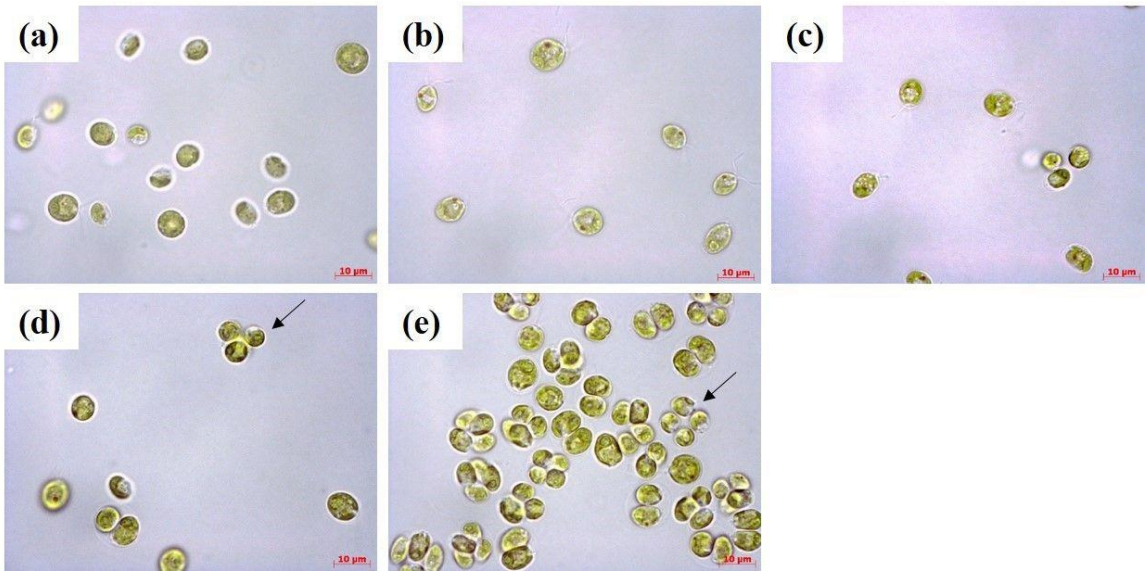
Supplementary Text S1. Optimization of algae growth conditions

Microalgae were cultured for a period of 7 days in closed 300 mL Erlenmeyer flasks. The growth of the selected two green algae was investigated under different liquid culture-to-gas volume ratios (1:5, 1:2, 1:1, 2:1 and 5:1). Thus, the volume of the gas phase (headspace in the closed batch bioreactors) gradually decreased (in 50 mL steps) from 250 mL to 50 mL. Algae cell size and morphology as well as chlorophyll content were analysed on the 7th day of growth. The increase of liquid-to-gas volume ratio was inversely proportional to the cell number of *C. reinhardtii* cc124 (Fig. S1 a). The algae cell sizes of microalgae cultures slightly increased when the liquid-to-gas volume ratio increased from 1:5 to 2:1 (average cell diameter increased from 5,35 μm to 7,84 μm) (Fig. S1 c). However, when the liquid-to-gas volume ratio further increased to 5:1, algae cell size values (average cell diameter of 4,73 μm) showed significant decrease, which was confirmed by one-way analysis of variance (ANOVA) ($P<0.05$) (Fig. S1 c). The light microscopy analysis revealed morphological variations of *C. reinhardtii* cc124 cells in 200 mL and 250 mL cultures (2:1 and 5:1 liquid-to gas ratios), the presence of high number of palmelloid structures (multi-cell aggregates in a joint sheath) indicated that the algae might be stressed under these low-oxygen conditions (Fig. S2). Palmelloids in *C. reinhardtii* cc124 have been observed under various stress conditions including osmotic and nutrient depletion stresses. *Chlorella* sp. MACC-360 algae was investigated under the same growth conditions. The results showed that by increasing the liquid-to-gas volume ratio from 1:5 to 2:1 the algal cell numbers increased (Fig. S1 b). The average algae cell diameter of the *Chlorella* sp. MACC-360 cultures increased from 3,36 μm to 3,87 μm. In case of the average algae cell diameter values of *Chlorella* sp. MACC-360 the one-way analysis of variance (ANOVA) did not reveal any significant differences (Fig. S1 d). The light microscopy analysis showed exclusively unicellular cell morphology in all *Chlorella* sp. MACC-360 cultures and did not reveal any cell aggregates as it was observed for *C. reinhardtii* cc124 in 200 mL (2:1 gas-to-liquid ratio) and 250 mL (5:1 gas-to-liquid ratio) cultures (Fig. S3). No significant differences were observed in the chlorophyll concentrations of the algae cultures with

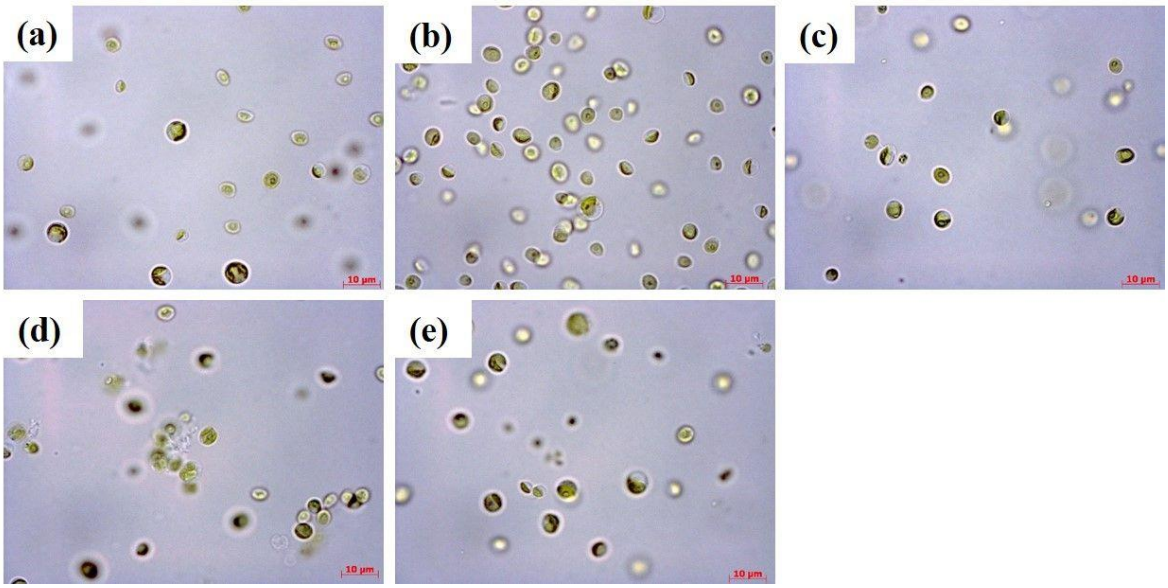
various liquid-to-gas volume ratios (Fig. S4). Overall, liquid-to-gas volume ratio influenced the cell numbers of the two algae in a different way. In case of *C. reinhardtii* cc124 increasing the liquid-to-gas volume ratio from 1:5 to 2:1 resulted in a decrease in the algae cell number, while the cell number of *Chlorella* sp. MACC-360 showed a clear increase. Moreover, liquid-to-gas volume ratio had a moderate impact on the average cell diameter of *C. reinhardtii* cc124, while that of *Chlorella* sp. MACC-360 was not influenced.



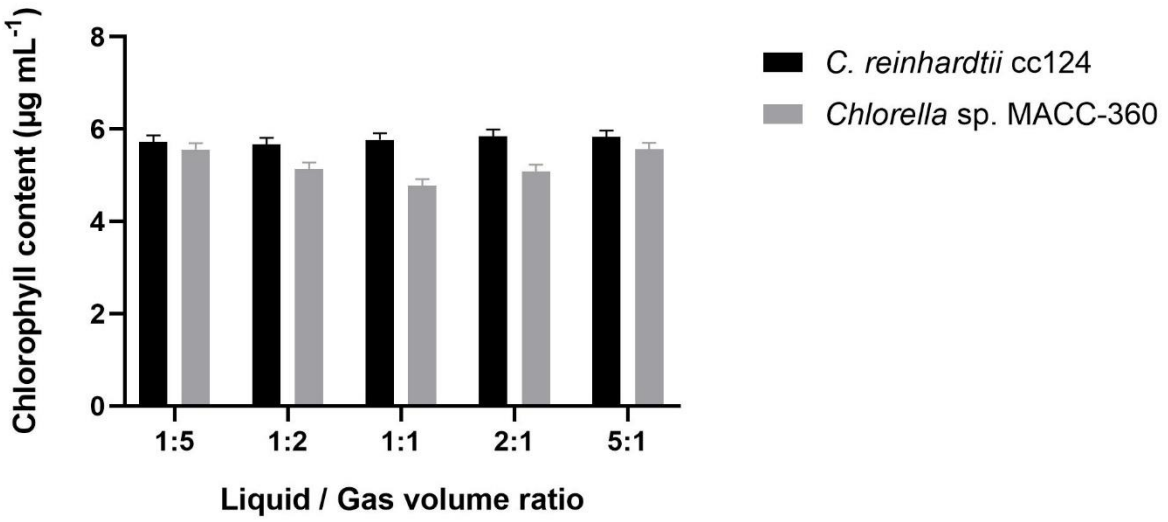
Supplementary Figure S1. Algae cell numbers of *C. reinhardtii* cc124 (a) and of *Chlorella* sp. MACC-360 (b) and average cell diameter of *C. reinhardtii* cc124 (c) and of *Chlorella* sp. MACC-360 (d). Erlenmeyer flasks with a total volume of 300 mL were used for cultivation. The one-way analysis of variance (ANOVA) showed significant difference between the cell size values of 2:1 and 5:1 liquid-to gas ratio cultures of *C. reinhardtii* cc124 ($P<0.05$). Error bars are standard deviations based on three replicates.



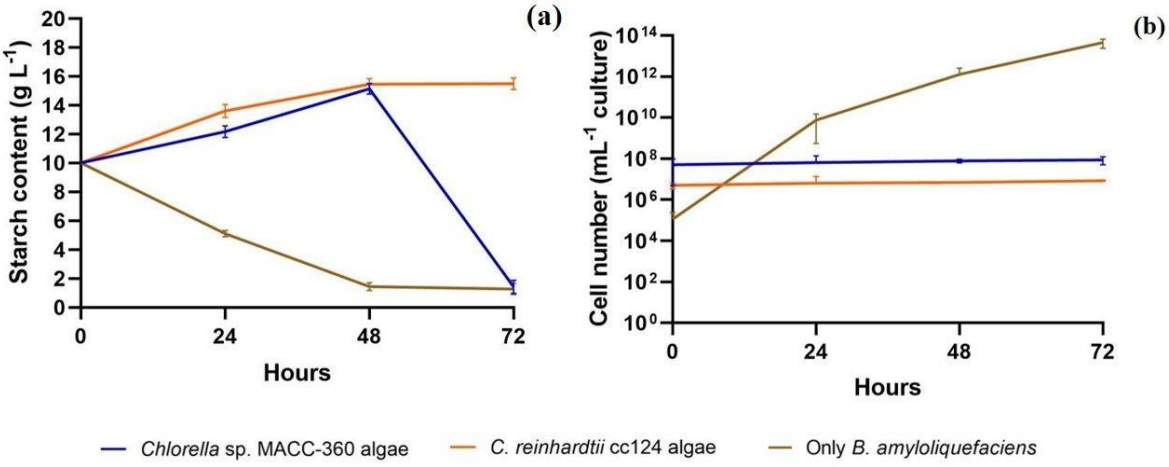
Supplementary Figure S2. Optical microscopy images of *C. reinhardtii* cc124. Morphology of algae cells grown in 50 mL (a), in 100 mL (b), in 150 mL (c), in 200 mL (d) and in 250 mL TAP medium (e) in Erlenmeyer flasks of 300 mL total volume. Panel a-c show the presence of unicellular algae.



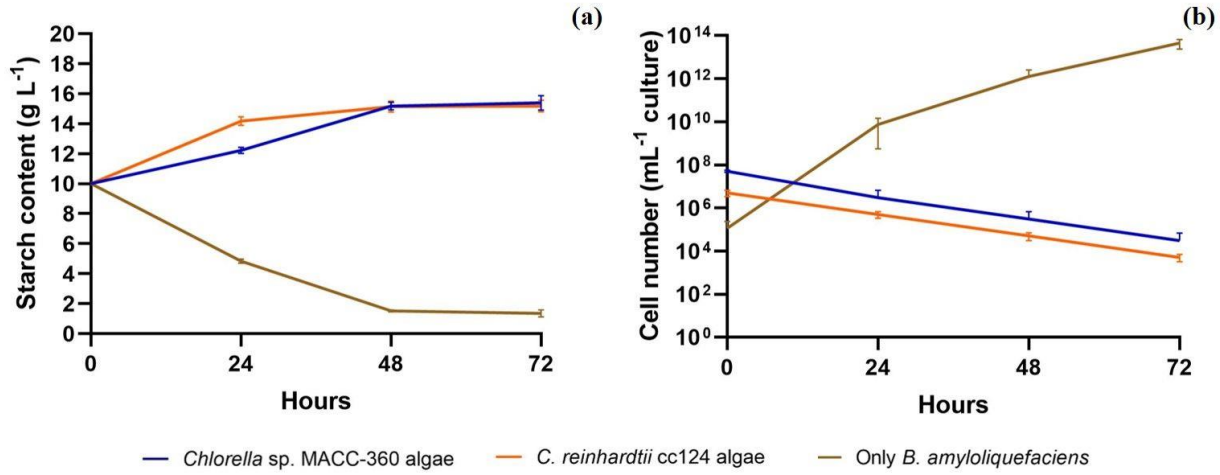
Supplementary Figure S3. Optical microscopy images of *Chlorella* sp. MACC-360. Morphology of algae cells grown in 50 mL (a), in 100 mL (b), in 150 mL (c), in 200 mL (d) and in 250 mL TAP medium (e) in Erlenmeyer flasks of 300 mL total volume.



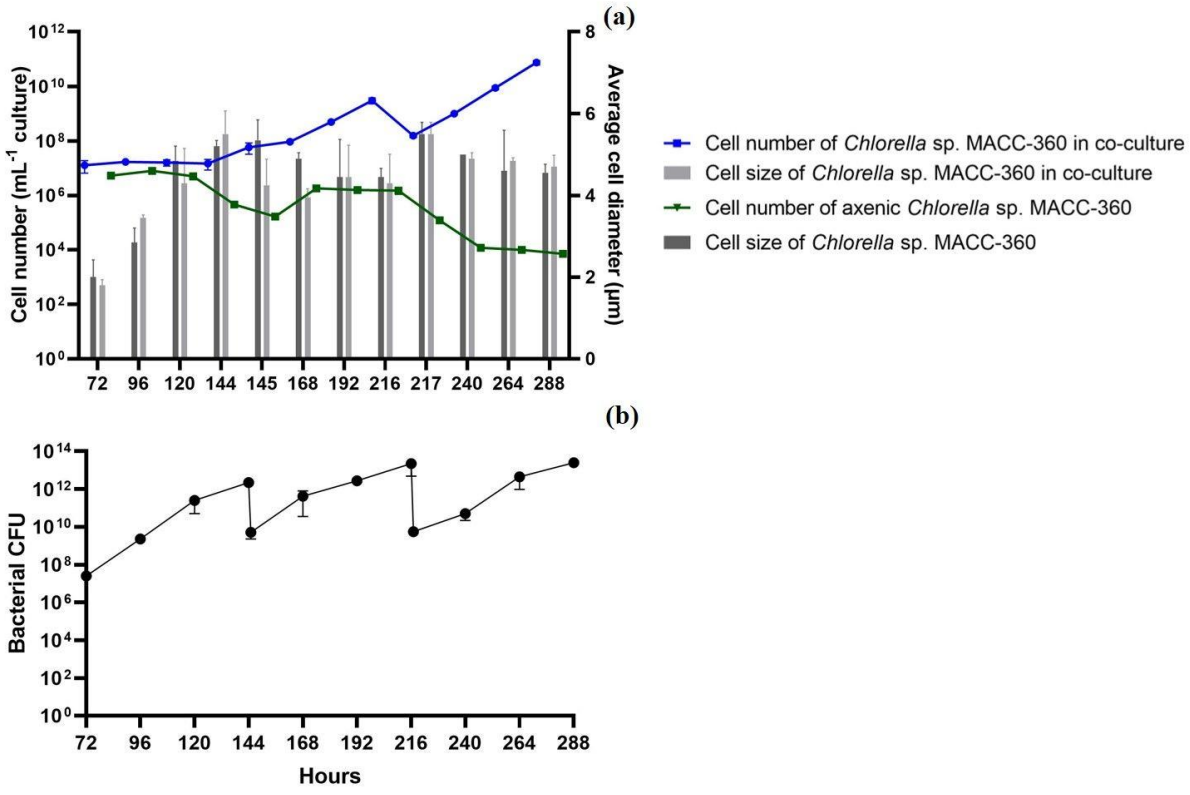
Supplementary Figure S4. Chlorophyll concentrations in green algae grown under various liquid-to-gas volume ratio conditions.



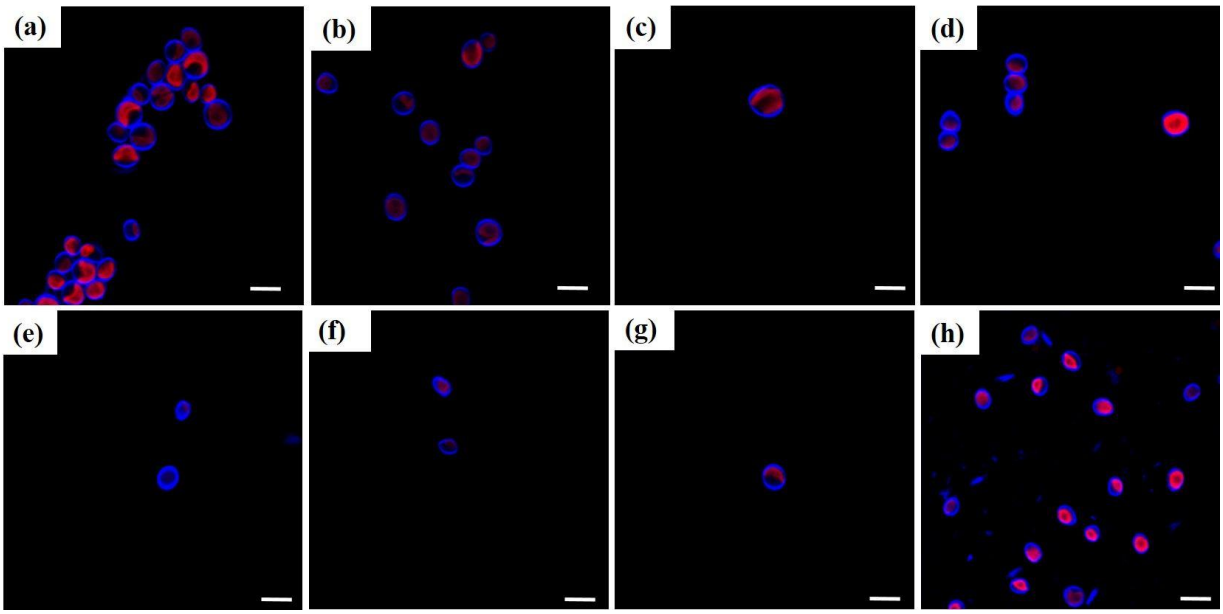
Supplementary Figure S5. Starch degrading capacity (a) and cell numbers (b) of green algae and the partner bacterium in TAP containing 10 g L^{-1} starch (the initial bacterial OD_{600} of 0.07). Error bars are standard deviations based on three replicates.



Supplementary Figure S6. Starch degrading capacity (a) and cell numbers (b) of green algae and the partner bacterium in TP containing 10 g L^{-1} starch (the initial bacterial OD_{600} of 0.07). Error bars are standard deviations based on three replicates.



Supplementary Figure S7. Algal cell number and average cell size (diameter) of *Chlorella* sp. MACC-360 in acetate-free TP medium containing 8 g L^{-1} starch (a), in which algae cell number is OD_{680} of 0.7 and the bacterial cell number is OD_{600} of 0.07. Colony forming units (CFU) of the partner bacterium *B. amyloliquefaciens* (b). Error bars are standard deviations based on three replicates.



Supplementary Figure S8. CLSM analysis of *Chlorella* sp. MACC-360 cultures in both medium and supplemented with 8 g L^{-1} starch. Images were taken on the 7th day of cultivation. Panel a-b show algae cultures in TAP medium. Axenic algae cells (a) and algae co-cultured with *B. amyloliquefaciens* (b). Panel c-d show algae cultures in TAP medium supplemented with 8 g L^{-1} starch. Axenic algae cells (c) and algae co-cultured with *B. amyloliquef*aciens (d) Panel e-f show algae cultures in TP medium. Axenic algae cells (e) and algae co-cultured with *B. amyloliquefaciens* (f) Panel g-h show algae cultures in TP medium supplemented with 8 g L^{-1} starch. Axenic algae cells (g) and algae co-cultured with *B. amyloliquefaciens* (h). Algae cell walls were stained with CFW (blue fluorescence), while red depicts live chloroplast autofluorescence. Scale bar is 10 μm.