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ORIGINAL ARTICLE

Scenedesmus vacuolatus cultures for possible combined laccase-like phenoloxidase activity and biodiesel production

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Abstract A key aspect of the industrial development of microalgal production processes is the excessive cost of biomass production. A solution is a combination of biodiesel production and wastewater treatment. The microalga *Scenedesmus* has a high lipid content and a potential extracellular phenoloxidase activity, which could improve the phycoremediation of phenolic pollutants. In this work, the most suitable growth conditions to obtain this twofold aim were analyzed. First, different strains of *Scenedesmus vacuolatus* microalga were tested at different pH, salinity and CO_2 concentration in the gas phase. The two most promising strains were then cultivated in autotrophic and heterotrophic conditions, and were investigated in terms of efficient nitrogen removal, fatty acid profile and maximized extracellular phenoloxidase activity in the medium. The results showed two extreme conditions: (1) biomass productivity doubled when photobioreactors were sparged with 5% CO_2 supplemented air with respect to cultures sparged with air (the steady state values of strain 53 were 0.138 g L^{-1} day^{-1} in the presence of air, and 0.243 in the presence of CO_2 addition), and N-starvation under 5% CO_2enhanced the transesterified fraction of lipids (strain 53 FAME fraction in the presence of N-starvation was 33%, in the presence of nitrogen FAME fraction was 22%); (2) phenoloxidase activity was completely suppressed by presence of 5% CO_2 in the gas phase (strain 53 0.21 U mL^{-1}), indicating clear catabolite repression for the induction of this enzyme in the algal metabolism.

Keywords *Scenedesmus vacuolatus* · Laccase-like phenoloxidase activity · Biodiesel · Lipids · Photobioreactor

Introduction

In recent years, the coupling of wastewater algal treatment (phycoremediation) with bioenergy production has been suggested to resolve the problems of the high cultivation costs of biomass production (Rawat et al. 2011; Hena et al. 2015); indeed, autotrophic cultivation is more costly then heterotrophic and mixotrophic cultivation, provided that the organic carbon source is not expensive as in the case of carbon-rich wastewaters.

The presence of phenols in the environment is related mainly to the production and degradation of numerous pesticides, plastics, dyes, drugs, resin paint (Michalowicz and Duda 2007; Gad and Saad 2008) and the generation of industrial and municipal sewage (Fleeger et al. 2003). Phenols are harmful exotoxins that can cause different diseases and induce mutagenesis and carcinogenesis (Busca et al. 2008; Gad and Saad 2008; Wasi et al. 2013).

Biological techniques to remove phenols in contaminated water can be based on the use of laccases—multi-copper oxidases that catalyze the oxidation of phenolic substances (Si et al. 2013; Riva 2006). Laccase-like enzymes occur in a wide

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range of microorganisms, including microalgae (Otto et al. 2010; Strong and Claus 2011).

Several microalgae species have been tested to evaluate their capacity to remove pollutants in wastewater, and their potential in phycoremediation processes (Pacheco et al. 2015; Paskuliakova et al. 2016; Kong et al. 2010), in particular in degrading phenols. Most frequently reported species probably belong to the genus *Scenedesmus* (Ajayan et al. 2015; Escapa et al. 2016; Jais et al. 2015; Jàcome-Pilco et al. 2009; Zhou et al. 2012). Within this genus, several *Scenedesmus* strains have demonstrated very great potential ability to degrade phenols and their derivatives (Pollio et al. 1993; Pinto et al. 2002, 2003) as well as produce high concentrations of polysaccharides and triacylglycerides (Mata et al. 2010; He et al. 2014; Yen et al. 2013). However, to date, it has proved more difficult to find results about *Scenedesmus* strains that can simultaneous accumulate a high content of energy-rich molecules and metabolize almost completely phenols and their derivatives. Thus, the twofold simultaneous aim of abating pollutant loading in contaminated water (both nitrogen and phosphorus content, and phenolic compounds), and accumulating the relevant amount of triacylglycerides requires searching for more suitable candidates and optimizing their growth conditions.

In this work, these dual aims were addressed using a sequential laboratory-scale approach based on: (1) selecting strains showing high growth rates under several cultivation conditions characterized by a wide range of values of selective pressure factors as pH, salinity, and CO_2 content in the gas phase; and (2) identifying the effect of CO_2 on the selected strains in terms of both lipid content and the production of extracellular phenoloxidase activity.

Scenedesmus vacuolatus was selected as a model organism because, in previous experiments, it achieved high biomass concentration and showed the ability to grow in different type of cultivation (Carbone et al. 2017a, b; Gargano et al. 2015, 2016).

Materials and methods

Screening tests—microorganism and medium

S. vacuolatus (Chlorophyta), strains 53, 54, 61, 235, 315, 316, and 317 were taken from the algal collection at the Department of Biology of the University "Federico II" of Naples (ACUF) (http://www.acuf.net). Bold Basal Medium (BBM), supplemented with $NaNO_3$ (40 mg/L) as nitrogen source, was used. BBM was autoclaved for 20 min at 120 °C. The pH of the autoclaved medium was about 7. Glycylglycine and NH_4Cl were supplemented to BBM as buffer for tests carried out at pH 3.0 and pH 8.5, respectively. HCl and NaOH were used to adjust the pH. NaCl and glucose were added to BBM cultures to have final concentrations of 0,25, 0,50, 1, 2, and 3% for NaCl, and 0.25, 0,50 and 1% for glucose. The specific growth rate μ (day^{-1}) was determined as follows: $\mu = (\mathrm{Ln}X_2 - \mathrm{Ln}X_1)/(t_2 - t_1)$ where X_1 and X_2 were the biomass concentrations on days t_1 and t_2, respectively (Guillard 1973). Biomass concentration was determined by optical density. The optical density of the cultures was measured with a Specord 50–Analytic Jena spectrophotometer at a wavelength of 680 nm with a 1 cm light path, and, when the biomass exceeded OD > 1, a dilution was applied. Under these conditions, the optical density can be considered proportional to the biomass dry weight concentration (Silva and Pirt 1984).

Growth in photobioreactors—operating conditions and procedure

Vertical bubble columns (VBC) were used (Olivieri et al. 2013) for microalgal growth. The VBC was a 1 L cylinder with an operating volume of 600 mL. Gas stream was sparged at the bottom of the photobioreactor by means of a porous ceramic diffuser; the irradiance was continuous, and was set at 140 μE/m^2 s. The head of photobioreactors was equipped with three ports for gas inlet, gas outlet and sampling operations. The photobioreactors were housed in climate chambers (M2M Engineering, Naples, Italy) equipped with lamps at the wall. Temperature in climate chambers was set at 23 ± 1 °C. The volumetric flow rate of the gas stream—sterilized through a 0.22 μm filter—was set at 20 nL/h. A gas mixing device (M2M Engineering) provided a mix of pure CO_2 in the gas stream with a CO_2 concentration equal to 5%.

Photobioreactors were inoculated with 1/10 of the final working volume. Tests were carried out in three different phases with respect to the liquid phase: batch, fed-batch and semi-continuous. Under fed-batch conditions, a concentrated BBM ([ten times the normal concentration of medium was supplemented to the photobioreactors when the total nitrogen (TN) < 10 mg L^{-1}]. The volume of concentrated BBM was 1/10 of initial culture volume, and it almost balanced the liquid losses by sampling and water evaporation. Under semi-continuous operations, 35% of microalgal suspension was replaced weekly with fresh medium. The average dilution rate (D), defined as the ratio between the weekly replaced suspension volume (F) and the photobioreactor working volume (V), was 0.05 day^{-1}. Steady state conditions were typically approached in about 1 month. The sampling operation took place two or three times a week. Data at steady state conditions were calculated as the average values: biomass concentration (X), volumetric productivity (P_X), areal productivity (A_X), photon yield ($Y_{X/E}$), lipid and fatty acid methyl esters (FAME) content within the biomass.

Liquid phase characterization

Liquid phase was characterized in terms of pH (pH meter Consort R305, Consort, Turnhout, Belgium), TN, and total inorganic carbon (IC) concentrations (TOC-V CSH analyzer, Shimadzu, Tokyo, Japan).

The activity of laccase-like phenoloxidase (POX) in the culture medium was estimated by biochemical assay with 2,2′-azino-bis (3-etilbenzotiazolin-6-sulfonic acid) (ABTS) (Li et al. 1999; Johannes and Majchercsyk 2000). The phenoloxidase catalyzes the oxidation of the ABTS to $ABTS^+$ in McIlvaine buffer solution, and the increase of $ABTS^+$ concentration is proportional to this oxidizing activity. The rate of oxidation was assayed at 25 °C and was monitored in a spectrophotometer at 420 nm; the results were expressed in the enzymatic units per milliliter of supernatant (U/mL) (Piscitelli et al. 2005).

Biomass characterization

The procedure for the analysis of the microalgae lipid content was the following: (1) biomass harvesting by centrifugation for 20 min, 6000 *g* at 5 °C (Eppendorf-5804 R, Eppendorf, Germany); (2) biomass freeze-drying at −50 °C (Labconco Freezon, Labconco, Kansas City, MO); (3) lipid extraction with a 2:1 chloroform-methanol solvent mixture in a Soxhlet apparatus for 8 h; (4) lipid transesterification with methanol and 1.5%v/v NaOH at 65 °C for 3 min; (5) methyl esters analysis through HPLC (Agilent 1100) (mobile phase: water and acetonitrile; column: Synergy 4u; detector: UV/Vis).

Statistical analysis

The main data obtained at different operating conditions were worked out by means of principal component analysis (PCA). In particular, the matrix representing the analyzed set of real data were reduced and transformed by calculating the eigenvectors and the eigenvalues. Then, the most relevant eigenvectors were considered based on the absolute value of their related eigenvalues. Based on the final composition of the reduced eigenvectors by the real data, the performance indicators, which depend mainly on the selected operating conditions, can be identified.

Results and discussion

Screening of *S. vacuolatus* strains

The ecophysiological features of *S. vacuolatus* strains are presented in Fig. 1a–c. All the strains were able to grow in the investigated pH range (3.5–8.0), but some were less tolerant to low pH, particularly strain 235. Strain 53 showed the highest growth rate at low pH, and can well grow in a wide range of pH, while strain 315 exhibited the best growth rate at pH 6.0 and 8.0. A pH tolerance in the range between 4.0 and 11.0 has been reported for a *Scenedesmus* strain ®-16; Ren et al. 2013), but generally a pH neutral or slightly alkaline has been found to be the optimum for growth in other *Scendesmus* species, such as *S. almeriensis* and *S. obtusiusculus* (Sánchez et al. 2008).

The salt tolerance of *Scenedesmus* seems to be species-specific: *S. obliquus* is sensitive to NaCl concentrations ≥0.05 M (Zhang et al. 1997); *S. quadricauda* growth was inhibited by NaCl >0.02 mM (Kirroliaa et al. 2011); *S. almeriensis* showed higher biomass productivities at 0.1 M NaCl (Benavente-Valdés et al. 2016). In our case of *S. vacuolatus*, NaCl concentrations from 0.25% to 1% strongly stimulated the growth rate of the majority of strains. Some strains, particularly 61, 315, and 317, were sensitive to 2% and 3% NaCl; strain 316 showed the highest tolerance to the selected range of salt concentration.

The addition of glucose did not stimulate the growth rate of most *S. vacuolatus* strains. Only at 1% of glucose in the medium did strains 53 and 54 exhibit growth rate values slightly higher than those observed without the addition of organic substrates in the culture medium.

Analysis of the screening results indicates that the majority of *S. vacuolatus* strains presents non-homogeneous features, suggesting selection of strains 053 and 316, which showed the best performances of growth in the range of pH and salinity tested, for subsequent experiments.

Effects of CO_2 concentration in the gas phase on growth and laccase-like activity of *S. vacuolatus* strain 53 and 316

Figure 2a, b reports the data obtained with *S. vacuolatus* strains 53 and 316 grown in VBC, and fed with only an air gas stream. *S. vacuolatus* strains were cultured under batch conditions with respect to the liquid phase for 14 days: the algal biomass concentration increased from 0.1 to 0.5 g L^{-1} for both strains. Fed-batch conditions started on the 14th day. The microalgae grew constantly and the biomass concentration was, at day $t = 56$, about 3.16 g L^{-1} and 2.84 g L^{-1} for *S. vacuolatus* 53 and 316, respectively. The semi-continuous operation was then started. Replacements with fresh fluid were repeated for about 45 days. At day $t = 100$, microalgae cultivation was continued by supplementing BBM without nitrogen source for 10 days. The culture was then stopped and the biomass collected for lipid analysis.

Cell-free supernatants derived from *S. vacuolatus* cultures during the different phases of growth were assessed for their ABTS oxidation activity. As observed, both strains showed an extracellular laccase activity only during the semi-continuous phase of growth, with strain 53 exhibiting better performances

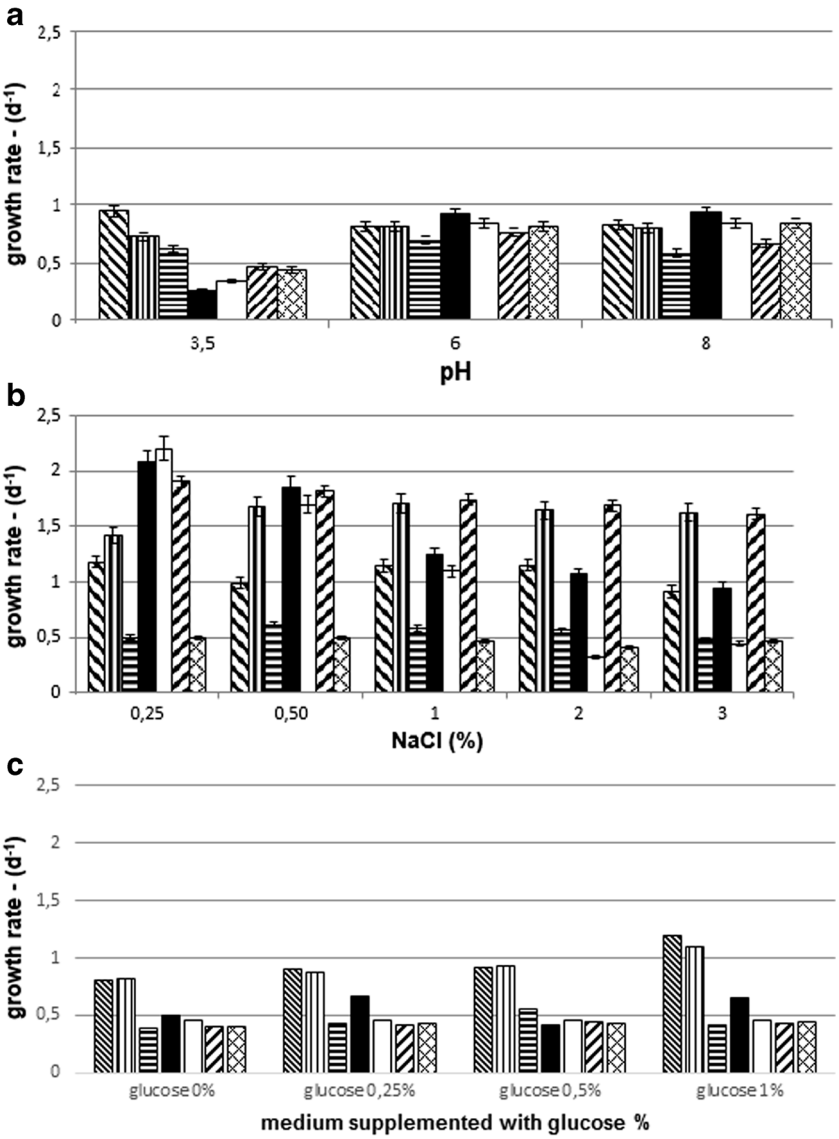

Fig. 1a–c Growth rates of *Scenedesmus vacuolatus* strains grown under different conditions. **a** pH 3,5, 6.0 and 8.0 in the presence of 0% glucose and 0.25% NaCl. **b** NaCl 0.25%, 0.50%, 1%, 2% and 3% in the presence of pH 6.6 and 0% glucose. **c** Glucose 0%,0.25%, 0.50% and 1% in the presence of 0.25% NaCl and pH 6.6. Strains: \\\ 53, ||| 54, ≡ 61, ▉ 235, ∏ 315, /// 316, ♯ 317

(> 4 U/mL^{-1}) than strain 316. Supernatants from cultures grown under nitrogen depletion did not shown POX activity.

The results of the microalgae characterization under steady state conditions are reported in Table 1 for the tests carried out in VBC photobioreactors with only air to gas stream and with 5% CO_2 supplemented air.

Even if both strains of *S. vacuolatus*, 53 and 316, were able to grow with or without CO_2 supplemented to air, the results appear quite contradictory in terms of biomass growth and POX activity:

- cultures aerated with air achieved only lower biomass concentration, specific biomass productivity, areal productivity photon yield as also indicated in Table 2;
- relevant POX activity was observed only in cultures grown under an air flow not enriched with CO_2 (20 times lower values were observed when 5% CO_2 was used as gas phase);
- POX activity was always detected when biomass stopped growing, indicating a complete decoupling of the two phases of the process.

Extracellular POX activity occurs particularly in *Chlamydomonas* and related genera of *Volvocales*, and in *Scenedesmus* clade. Algae belonging to *Chlamydomonas*, *Tetracystis* and allied genera show an oxidizing activity related to the presence of laccase enzymes, whereas a thermostable non-enzymatic compound released by *Scenedesmus* cells is responsible for the observed oxidation reactions (Otto et al. 2010). The phenol-oxidizing algal products are probably released by our *S. vacuolatus* strains under peculiar stress

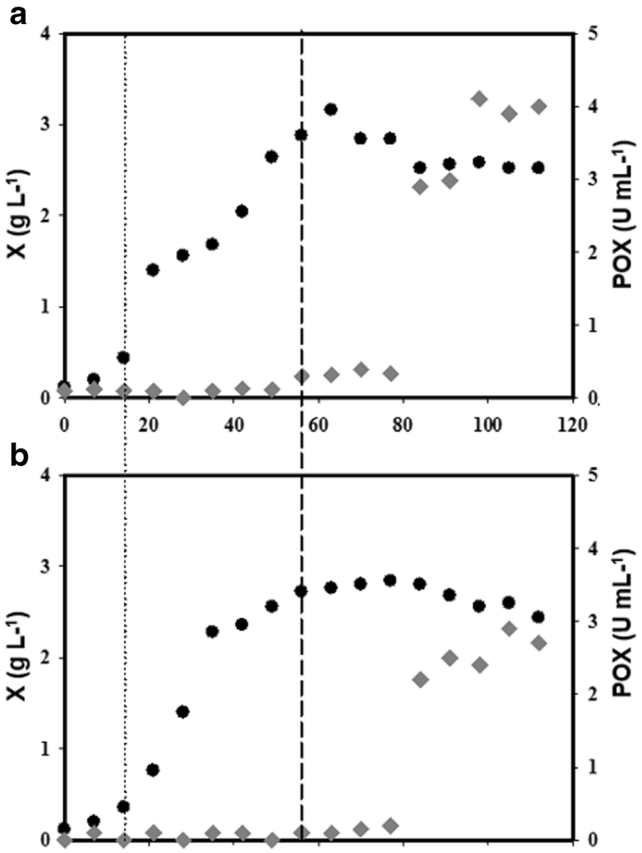

Fig. 2a,b Cultures with only air to gas stream in vertical bubble columns (VBC). **a** *S. vacuolatus* 53; **b** *S. vacuolatus* 316. *Circles* Biomass productivity, *diamonds* POX activity, *dotted line* start of fed-batch mode, *dashed line* start of semi-continuous mode

conditions: the trigger could be the high values of pH, which, in cultures with air sparged, rose to 10.0 during the steady state of semi-continuous growth (data not shown).

The data in Table 1 were subjected to PCA. In particular, from the coefficients of the most relevant eigenvectors components, it was possible to understand which performances are more affected by the CO_2 concentration and by selected strain (see Supplemental Material 1). The analysis indicated that the areal productivity and the level of POX expression are the most sensitive key parameters for determining the efficiency of the process. It is also evident that their respective optimization is achieved under extreme operating conditions. Moreover it seems that when a combination of both—given proper weights—is adopted, optimum operating conditions between the two extremes can be found.

Table 2 Lipid content and fatty acid methyl esters (FAME) composition of *S. vacuolatus* 53 and 316 cultivated in VBC with only air sparged and with 5% CO_2 added to the gas phase. *N+* Culture not in nitrogen starvation, *N−* culture in nitrogen starvation. Lipid content is reported with respect to the biomass dry weight; FAME content is reported with respect to the overall lipid content

	Air				5% CO_2			
	53		316		53		316	
	N+	N−	N+	N−	N+	N−	N+	N−
%Lipid	13.4	15.4	12.3	14.6	29.8	24.8	21.1	23.9
%FAME	22.4	33.3	17.5	16.6	24.4	31.3	28.1	24.0
%Linolenate	1.1	2.7	2.5	2.2	4.4	4.7	5.0	3.9
%Linoleate	/	/	/	/	1.3	0.7	0.5	1.0
%Oleate	21.4	30.6	14.9	12.5	18.6	25.9	22.7	19.1

Effects of CO_2 concentration in the gas phase on lipid content and FAME composition

The lipid content and FAME composition were also investigated for *S. vacuolatus* 53 and 316 grown in VBC with different CO_2 concentrations in the gas phase (with only air sparged and with 5% CO_2 added) and under different nitrogen supplies (initially sufficient supply N+; late nitrogen starvation N−). Table 2 reports the data obtained in terms of the percentage of successfully extracted lipids from algal cells with respect to biomass dry weight (%Lipid) and of percentage of successfully esterified lipids with respect to total extracted lipids from algal cells (%FAME). We recall that FAME are considered as the lipids that can be used as biodiesel, because the other lipids can be assumed to be pigments, and

Table 1 Steady state data of semi-continuous tests of *Scenedesmus vacuolatus* strains grown in vertical bubble column (VBC) photobioreactors with air and 2%CO_2 sparged. X_{max} Maximum biomass concentration, P_X specific biomass productivity, A_X areal productivity, $Y_{X/E}$ photon yield, $POX_{steady\ state}$ POX activity at steady state

	Air		5% CO_2	
	S.vacuolatus 53	*S. vacuolatus 316*	*S. vacuolatus 53*	*S. vacuolatus 316*
X_{max}(g L^{-1})	3.16	2.84	6.4	5.98
P_X (g L^{-1} d^{-1})	0.138	0.136	0.243	0.233
A_X (g m^{-2} d^{-1})	1.385	1.365	2.426	2.335
$Y_{X/E}$ (g E^{-1})	0.115	0.113	0.201	0.194
$POX_{steady\ state}$ (U mL^{-1})	4.09	2.89	0.21	0.19

long chain hydrocarbons such as waxes and sterols. We also report differences in lipid composition under nitrogen sufficient (N+) and nitrogen starvation (N−) conditions.

The total lipid fraction achieved maximum at 5% additional CO_2 with *S. vacuolatus* 53 in N+ conditions. Values in N−, in all conditions and for both strains, were similar when the gas phase composition was constant, but definitely lower values were always obtained in tests with air sparged for both strains, with respect to tests with 5% CO_2 in the gas phase.

The highest amount of FAME was achieved with *S. vacuolatus* 53 grown in VBC with only air sparged during nitrogen starvation, but similar result was obtained for *S. vacuolatus* 53 grown in VBC with 5% CO_2 added to air.

The amount of linolenate was the highest in cultures grown at 5% CO_2 with *S. vacuolatus* 316 in N+ conditions. Linoleate showed similar results for both strains in different conditions: it never appeared in tests carried out with only air sparged; in cultures with CO_2 sparged, it was ~1%. Oleate was the dominating fatty acid in all cultures, with a maximum with only air sparged under nitrogen starvation. The culture of *S. vacuolatus* 316 grown with only air sparged showed the lowest oleate content.

S. vacuolatus 53 appears to be a good source for lipid productivity and biomass yield. Its results were certainly competitive with data obtained from other *Scenedesmus* species: *S. obliquus*, in the presence of 5% CO_2, achieved a maximum biomass values of 2.4 g L^{-1} and a lipid content of ~44.5% (Sforza et al. 2014); in the presence of 2.5% CO_2, a biomass yield of 2.1 g L^{-1} and lipid content of 10% were obtained (Shih et al. 2012); a strain of *S. quadricauda* achieved a biomass value of ~1.65 g L^{-1} and 33% lipid contents (Dev Goswami et al. 2011).

Also in the case of FAME production, the yield was higher in strain 53. However, the production of FAME behaved differently in the two microorganisms: in strain 53, FAME yield was influenced by N starvation but not by CO_2 concentration; strain 316 showed the opposite behavior.

Conclusions

Among seven *S. vacuolatus* strains, two were selected as potentially suitable candidates for coupling phycoremediation of phenol-polluted wastewater and lipid accumulation for biodiesel production under a wide range of pH, salinity and organic C supplemented conditions.

The results of optimization of cultivation conditions for these two strains in terms of N and CO_2 supply gave quite a complicated framework: N and CO_2 supplies stimulate biomass production, late N starvation and continuous CO_2 starvation positively affect the phenol-oxidase activity expressed by both the strains in the liquid phase, sufficient CO_2 supply favors the accumulation of lipids, and no effect of N supply was found.

So, a strategy to couple phycoremediation of phenol-polluted wastewater and biodiesel production could consist of designing a process with three operational stages: N and CO_2 supplies to enhance biomass growth → N starvation and CO_2 supply to accumulate lipids → N and CO_2 starvations to express POX activity.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

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