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ORIGINAL RESEARCH

Sustainable Diesel Feedstock: a Comparison of Oleaginous Bacterial and Microalgal Model Systems

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Abstract

The key to sustainable and commercially viable biodiesel production relies primarily on species selection. Oleaginous species with high biomass productivity, lipid content, and lipid productivity are desirable. High growth rate of the species results in high biomass productivity, which leads to high lipid productivity. It is known that algal oil technology lacks commercial feasibility predominantly due to low biomass productivity and other factors. The use of a faster-growing organism, such as oleaginous bacteria, could offset this major disadvantage. Thus, the current study analyzes two model oleaginous systems: *Rhodococcus opacus* PD630 (a bacterium) and *Chlorella vulgaris* NIOT5 (a microalga) for their growth rate and lipid productivity. It was found that the bacterial growth rate was 25-fold the microalgal growth rate. The bacterium also showed 57-fold higher biomass productivity and 75-fold higher biodiesel productivity. Further, the analysis of a large number of literature data from relevant studies under different cultivation conditions showed that *R. opacus* PD630 has productivities far higher than various autotrophic microalgae. Similarly, a frequency distribution of data collected from the literature showed that *Rhodococcus* sp. has productivities in the higher range as compared to heterotrophic microalgae. Thus, bacteria could serve as a better alternative to microalgae toward developing a commercially viable biofuel technology. Further, the biodiesel characterization study showed that the quality of diesel from the bacterium was better than that from the microalga.

Keywords Lipid productivity · *R. opacus* · Biodiesel · Sustainability · Growth rate · Biomass productivity

Introduction

Biodiesel, the mono alkyl esters of oil or triacylglycerol (TAG), is a sustainable alternative for petrodiesel [1–3] in the context of an uncertain crude oil supply [4–6]. A suitable feedstock for biodiesel is a crucial need for the development of an economically successful and environmentally sustainable biodiesel production process [7, 8]. Important criteria for rational screening of biodiesel sources include growth rate and oil content [9]. Fast growth ensures high biomass productivity and can reduce the culture area required; high oil content increases the product yield coefficient, and species having both the abovementioned characteristics promise high oil productivity [10]. Oils from seed crops such as grape, soybean, sunflower, and palm were initially considered conventional [11–13]. However, seeds as an oil source lost considerable interest with time as a substitute for crude oil, due to sustainability issues such as seasonal availability, low growth rate, and productivity [14–16].

Microalgae have higher growth rate and oil content amounting to 20–60% of cell dry weight (CDW); hence, they are considered as promising substitutes for oil seed crops [2, 17, 18]. Algae have been claimed to be up to 20 times more productive than oil seed crops [2, 19]. However, algal oil technology lacks commercialization due to its poor process economy, and therefore, algal oils seem to be more expensive than petroleum fuels [20]. The majorly known economic bottlenecks are low biomass and oil productivity [9, 20]. Though the oil content of many microalgal strains is reported to have

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improved, algal productivity is yet to reach the needs of sustainable industrial process [21].

Many oleaginous yeast species are also known to accumulate oil [12, 22, 23] and can reach up to 80% CDW [24, 25]. Yeast, with growth rate higher than algae [26–28], is considered as a better candidate for biodiesel production [29]. Nevertheless, industrial-scale yeast cultivation is often associated with bacterial contamination [30, 31], which results in yeast growth inhibition, and decreased yield and productivity [32, 33]. To overcome this, the process needs specific treatment procedures, which are believed to increase the process expenditure and pose a threat to feasible and sustainable commercialization [30, 34].

Since TAG accumulation has been known as a characteristic of eukaryotes [35], microalgae and yeast are often considered as promising biodiesel feedstock. However, bacteria—known mainly for storing carbon in the form of specialized lipids such as polyhydroxybutyrates (PHB) and polyhydroxyalkanoates (PHA) [36]—are also capable of accumulating TAGs. The oleaginous nature of bacteria gained attention with identification of the bacterial strain *Rhodococcus opacus* PD630, which is capable of accumulating oil up to 80% CDW [37–39]. The advantages of oleaginous bacteria over algae include higher growth rate [26–28], subsequently giving rise to high biomass productivity. Oleaginous bacteria also offer high oil productivity, since the reported oil content of bacteria is also good [38, 39]. Although growth rates of oleaginous yeast and bacteria are not so different [40, 41], metabolic and genetic engineering to improve oil accumulation would be relatively easier in bacteria [42], since expressions of many genes involved in fatty acid synthesis are already understood in bacteria [35, 43]. Further, comprehensive omics study of lipid droplet organelle is available for strains such as *R. opacus* PD630 that aids in easy strain engineering [44].

Although it is known that oleaginous bacteria are faster growing than microalgae, an explicit comparison of their growth, lipid accumulation, and fatty acid characteristics has not been reported yet. Hence, the aim of the current study was to perform a quantitative comparison in terms of growth, biomass, and biodiesel productivity and a qualitative comparison of fatty acid profile, between the strains of oleaginous bacteria and microalgae. The model systems chosen were the bacterium *R. opacus* PD630 and the microalga *C. vulgaris*—a common model system in algal fuel technology. Since the aim was to compare inherent characteristics of the oleaginous system from two different domains, the factors that maximize lipid accumulation in the particular strain such as the effect of substrate, nitrogen limitation, C/N ratio, TAG synthesis pathway manipulation, etc. were not considered in the study. Further, analysis of a large amount of data from relevant literature has been carried out, to compare the productivities of the organism and their related species under optimized and different cultivation conditions. Overall, the paper seeks to highlight the importance of employing bacteria as biodiesel feedstock.

Materials and Methods

Organism and Culture

Rhodococcus opacus strain PD630 (DSMZ 44193) [38] was obtained from DSMZ culture collections, Germany, and *C. vulgaris* NIOT5 was a gift from the National Institute of Ocean Technology (Chennai, India). The strains were maintained and grown in their respective standards and commonly used media under suitable conditions. The bacteria *R. opacus* PD630 were grown aerobically in nutrient broth (NB) medium (M002, HiMedia, Mumbai, India) at 28 °C and 200 rpm [44] in a shaker incubator (OrbitekR LEBT, Scigenics Biotech, Mumbai, India). The seed culture was prepared by inoculating the glycerol stock in 5 ml of NB and was incubated for 32 h. This was further inoculated into 100 ml of NB and was incubated overnight. The overnight-grown culture was used as inoculum for the experimental flask. Cultures of *C. vulgaris* NIOT5 were grown in Guillard and Ryther's f/2 medium (Online Resource 1 Table S1), a widely used seawater-enriched medium [45], and incubated in a shaker incubator (OrbitekR LEBT, Scigenics Biotech, Mumbai, India) at 25 °C, 150 rpm with an illumination regime of 12 h light (1200 lx) and 12 h dark.

Growth Measurement

The growth of *R. opacus* PD630 was assessed with OD measurements at 600 nm. The biomass density (g/l) was obtained from OD_{600} by calibrating against the standard plot (OD_{600} vs. known cell density). The growth rate was obtained from the slope of log (cell density) vs. time. Growth of *C. vulgaris* NIOT5 was measured by taking cell count using Neubauer's improved bright line hemocytometer. The growth rate was obtained from the slope of log (cell count) vs. time. Biomass productivity was calculated during the maximum lipid accumulation period by obtaining dry weight after harvesting.

Intracellular TAG Measurement

Lipid or TAG accumulation in both bacteria and microalgae was monitored real time using Nile red (N3013, Sigma-Aldrich, MO, USA). For measurement in bacteria, sample (1 ml) withdrawn was pelleted at 12,000*g*, washed, and resuspended in 0.85% NaCl (RM 853, HiMedia, Mumbai, India). Sample OD was normalized to 0.2 to which 5 μl of Nile red (0.1 mg ml^{-1}) was added and incubated under the dark for 20 min at room temperature [46]. Lipid measurement in algae was performed with a similar procedure as that of bacteria

using a normalized cell concentration of 10^6 (cells ml^{-1}) [47]. Fluorescence measurements were made using a fluorescence spectrometer (LS55, Perkin Elmer, Llantrisant, UK), and the values calibrated against a standard triglyceride—tri-olein (TO) (37958, SRL, Mumbai, India). Intracellular neutral lipid or TAG accumulation was expressed as specific intracellular lipid accumulation (SILA).

Biodiesel: Fatty Acid Content and Composition

The accumulated TAG was converted to fatty acid methyl esters (FAMEs) or biodiesel by in situ transesterification of biomass, carried out as per previously established methods [48]. In case of the bacterium *R. opacus* PD630, biomass harvested at the maximum lipid accumulation period (12th hour) was used for analysis. For *C. vulgaris* NIOT5, biomass harvested on day 15 was used for FAME analysis. The transesterification was performed in a sealed glass vial with 50 mg of dried biomass. The biomass was incubated with 4 ml of methanol:H_2SO_4 (10:1, *v*/*v*) for 40 min at 100 °C. After cooling, the FAMEs were extracted using hexane:chloroform (4:1, *v*/*v*). The solvent was evaporated by nitrogen purging and the dry weight of the resulting FAMEs was quantified by gravimetry. Separation and identification of the FAMEs were carried out with GC–MS (Clarus 600/Clarus 600 S, Perkin Elmer, Shelton, USA) using a capillary GC column (Omegawax® Capillary GC column, 24136, Supelco, Bellefonte, USA).

Biodiesel Characterization

Based on the fatty acid content and composition obtained through GCMS, the quality of the biodiesel was obtained through certain physiochemical properties like cetane number (CN), iodine value (IV), viscosity, density, and heat of combustion (HC). The properties were calculated using established empirical correlations which are given in detail in Online Resource 1. The calculated properties were then compared with the American (ASTM 6751) [49], European (EN 14214) [50], and Indian (IS 15607) [51] standards and with that of conventional petroleum diesel [52].

Calculation and Units

$$\text{Biomass productivity (BP, g } l^{-1} h^{-1}) = \frac{\text{Biomass weight (g)}}{\text{harvest time (h)} \times \text{culture volume (l)}}$$

$$\text{SILA of bacteria } (\mu g\ g^{-1}) = \frac{\text{Lipid concentration } (\mu g\ \text{TO}/l)}{\text{Biomass density (g}/l)}$$

$$\text{SILA of algae } (\mu g\ \text{cell}^{-1}) = \frac{\text{Lipid concentration } (\mu g\ \text{TO}/l)}{\text{cell count (cells}/l)}$$

$$\text{Lipid or biodiesel yield (LY, g g}^{-1}) = \frac{\text{Biodiesel amount (g)}}{\text{Biomass amount (g)}}$$

$$\text{Lipid or biodiesel content (LC, \% CDW)} = \text{LY} \times 100$$

$$\text{Lipid or Biodiesel productivity (LP, mg } l^{-1} h^{-1}) = \text{BP} \times \text{LY} \times 1000$$

All experiments were carried out in triplicates. The statistical significance was determined with one-way ANOVA (level of significance = 0.05) using MegaStat version 10.4.

Data Collection and Analysis

The articles involving individual studies on oleaginous bacteria and microalgae were selected based on the criteria that they either had the productivities data or the appropriate data to calculate the productivities. In most of the studies, the productivities were not reported directly, and hence, they were calculated from the available data such as biomass density, lipid content, and duration of the study, using the appropriate formula mentioned in the previous section. The data collected was sorted into the following:

Culture method: autotrophic (in case of algae), heterotrophic
Culture strategy: batch, fed-batch

While the literature data for autotrophic algae spanned a variety of species, the heterotrophic studies have been done majorly on *Chlorella* species. In the case of bacteria, the literature data available were comparatively less than the algae and the available studies majorly focused on *R. opacus* PD630 and its strain variants. This is primarily because, in the other *Rhodococcus* species and other prokaryotic TAG producers, the carbon is divided into diverse storage compounds such as glycogen and PHA, in addition to TAG. However, *R. opacus* PD630 stores excess carbon majorly as neutral lipids (TAG) [39, 53, 54] which makes them suitable as a biofuel feedstock. The collected data and the associated details are provided in Online Resource 2 and calculated productivities are summarized as tables in Online Resource 1 (Tables S3–S6).

Based on the data collected from the literature, the following comparisons were made:

1. The productivity of *R. opacus* PD630 from this study was compared with the productivities of various autotrophic algae reported in the literature.
2. The productivity of *R. opacus* PD630 from this study was compared with algal theoretical maximum productivity reported in the literature.
3. The productivity of *R. opacus* PD630 from various studies was compared with the heterotrophic productivities of microalgae reported in various studies through frequency distribution analysis.

Economic Analysis

The biomass productivities of *R. opacus* PD630 and *C. vulgaris* NIOT5 obtained from this study were used to

calculate their respective unit biomass production cost. The production system chosen for the analysis is a tubular reactor system as it is a feasible and practicable configuration for large-scale algal cultivation [2, 55]. The tubular reactors are also considered promising for large-scale industrial bioprocessing of microbial sources due to their uniform plug flow characteristics [56]. The cost estimates from a recent technoeconomic analysis of microalgal biomass production by Slade and Bauen [57] in tubular photobioreactors (TBPR) [57] were taken as the basis for calculating unit biomass production cost of *R. opacus* PD630 and *C. vulgaris* NIOT5. The reactor had a working volume of 7000 m^3 that occupied a land area of 10 ha and was available for 300 days of operation. The cost estimate split-up of Slade and Baune [57] based on a biomass productivity of 20 g m^2 day^{-1} is given in Online Resource 1 (Table S2).

For this analysis, the volumetric productivity or the aerial productivity of biomass was calculated using the following equation:

$$\text{Volumetric productivity}\left(\text{g l}^{-1}\text{ h}^{-1}\right) = \frac{\left(\text{aerial productivity}\left(\text{g m}^2\text{day}^{-1}\right) \times \text{operation days (day)} \times \text{area (m}^2\text{)}\right)}{\text{reactor volume (L)} \times 24\text{ (h)}}$$

The assumptions used in the cost analysis are as follows:

1. The productivities from the shake flask study were assumed as tubular reactor productivities.
2. The effect of raw material cost on the total biomass production cost for *C. vulgaris* NIOT5 and *R. opacus* PD630 was assumed insignificant. This is because, as per the cost estimate of Slade and Baune [57], only 4% of total biomass production cost is comprised of raw material cost.

Results and Discussion

Faster Growth and Higher Biomass Productivity in *R. opacus* PD630

R. opacus PD630 was cultivated in nutrient broth with no additional carbon source and *C. vulgaris* NIOT5 in f/2 media with atmospheric CO_2 as the carbon source. Following inoculation, both the strains displayed no significant lag phase. *R. opacus* PD630 had an exponential phase from 3 to 22 h and *C. vulgaris* NIOT5 had an exponential phase from 24 to 300 h (Fig. 1). Not surprisingly, the bacterial strain *R. opacus* PD630 had higher values of specific growth rate (μ). The strain *R. opacus* PD630 exhibited a specific growth rate of 0.241 ± 0.007 h^{-1}, and for *C. vulgaris* NIOT5, it was 0.009 ± 0.002 h^{-1}. Growth rate or doubling time of an organism is one of the important parameters to be considered while screening a source for biodiesel production. Selection of a fast-growing species is a vital step for developing a mass culture of the species [58]. High growth rate of algae when compared to oil seed crops is often known as one of the major reasons to invest capital in algal fuel technology [59, 60]. Oleaginous bacteria, on the other hand, are expected to perform better than algae, as they are fast growing. For example, as per our study, the specific growth rate of oleaginous bacteria *R. opacus* PD630 was 25-fold higher than that of the microalga *C. vulgaris* NIOT5 (Table 1).

Faster growth also ensured high biomass density and, consequently, high productivity. The maximum biomass density obtained was 2.546 ± 0.15 and 0.763 ± 0.01 g l^{-1} with *R. opacus* PD630 and *C. vulgaris* NIOT5, respectively. The BP at the time of harvest for *R. opacus* PD630 and *C. vulgaris* NIOT5 was 0.121 ± 0.003 and 0.002 ± 0.000 g l^{-1} h^{-1}, respectively. The BP was 65-fold higher than that of the microalga *C. vulgaris* NIOT5 (Table 1). Such a significant increase in BP is vital for low-value products like biodiesel since biomass productivity is the major factor influencing the process production cost [8, 60]. A 100-fold increase in biomass can bring down the unit production cost by 6-fold [2]. In addition to improved BP, faster growth can also assist in rapid screening and manipulation of bacteria.

Higher Lipid Accumulation Rate in *R. opacus* PD630

The accumulated or stored TAG was monitored using the fluorescent dye Nile red. In *R. opacus* PD630, the lipid accumulation phase started much earlier than in *C. vulgaris* NIOT5. For *R. opacus* PD630, lipid accumulation was started from the third hour after inoculation and lipid accumulation happened in parallel with the exponential phase. The maximum lipid accumulation was observed at the 12th hour of growth, after which it showed a decrease. In *C. vulgaris* NIOT5, lipid accumulation started only around 250 h and continued to increase thereafter for the entire period of cultivation (Fig. 2). The bacterial strain *R. opacus* PD630 showed higher lipid accumulation rate of 0.139 ± 0.014 h^{-1} which was 14-fold higher than the microalga *C. vulgaris* NIOT5 (Table 1). Higher lipid accumulation rate contributes to improved productivity, while poor lipid accumulation rate causes delay in harvesting, ultimately affecting productivity and economy of the process [61].

Q5

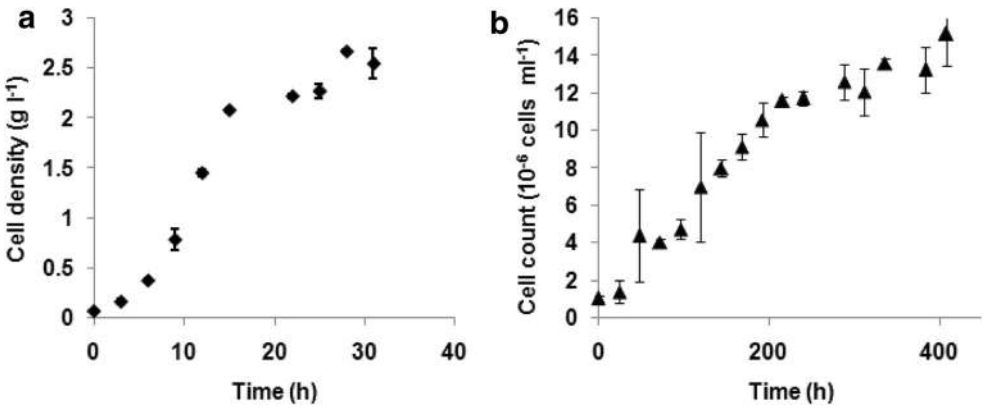

Fig. 1 Growth profile of the oleaginous model organisms. **a** Bacterium *R. opacus* PD630. **b** Microalga *C. vulgaris* NIOT5. Data points are represented as mean ± SD, $n = 3$

Higher Biodiesel Productivity in *R. opacus* PD630

Apart from growth rate and BP, lipid or biodiesel productivity (LP), which is the product of BP and LY, is also an equally important criterion for rational species selection for biodiesel production. The FAME or biodiesel yield (LY) was determined by gravimetric analysis. The LY from *R. opacus* PD630 and *C. vulgaris* NIOT5 were almost the same (Table 2). For *R. opacus* PD630, LY was 0.33 ± 0.003 g g^{-1} (33% CDW), and for *C. vulgaris* NIOT5, the LY was 0.283 ± 0.003 g g^{-1} (28.3% CDW). Though the LY of *R. opacus* PD630 and *C. vulgaris* NIOT5 were in a similar range, the LP of *R. opacus* PD630 was far higher when compared to *C. vulgaris* NIOT5. The LP for *R. opacus* PD630 was 40 ± 2.33 mg l^{-1} h^{-1}, while for *C. vulgaris* NIOT5, it was 0.53 ± 0.01 mg l^{-1} h^{-1}. Thus, the productivity of *R. opacus* PD630 was 75-fold higher (Table 2).

In this section, we argue that the possibility for the microalgae to surpass bacterial performance is low, although many attempts have been reported to improve the LP in microalgae either by screening for a new high producer or by enhancing the BP and the LC of a known strain. For example, the green microalgal strain *Scenedesmus dimorphus* was identified as the highest producer among 43 screened algal strains, that had a LP of 8.79 mg l^{-1} h^{-1} and the corresponding BD and LC were 5.87 g $l^{-1}$and 43.13% CDW, respectively [62]. Despite this high BD and high LC, the LP is still very low compared to that of *R. opacus* PD630, which was 40 ± 2.33 mg l^{-1} h^{-1} (Table 2). Further, both the BP and LP of autotrophic algae reported by various studies (Online Resource 1 Table S2) under physiological stress or optimized conditions were also significantly less when compared to the productivity of *R. opacus* PD630 obtained from this study. The LP of microalgae *S. dimorphus* (8.79 mg l^{-1} h^{-1}), the highest producer among the 43 screened algal strains [62], can meet the productivity of *R. opacus* PD630, only if both the biomass density and lipid content are doubled simultaneously. However, attempts to improve lipid content through physiological stress are often associated with decreased biomass [63, 64]. For example, in the diatom *Phaeodactylum tricornutum* by overexpressing an enzyme glycerol-3-phosphate dehydrogenase (G3PDH), a 60% increase in lipid accumulation was achieved. But, it also resulted in 20% decrease in cell growth [65], thus making the simultaneous improvement in biomass and lipid difficult. However, few reports claim increased lipid accumulation through gene manipulation without affecting the biomass productivity. But the techniques to genetically transform wild microalgae into a superior strain with high biomass and lipid production are unreliable due to lack of efficiency and reproducibility [66]. Moreover, some process improvement techniques might incur significant cost, ultimately affecting the process sustainability. Even if the improvement does increase the economy of production, it was not sufficient enough for a large-scale sustainable process [19]. Further, the technoeconomic analysis of algal biomass production with different systems such as open

t1.1 **Table 1** Growth and lipid accumulation characteristics of the bacterium *R. opacus* PD630 and the microalga *C. vulgaris* NIOT5

t1.2 Oleaginous organism	μ (h^{-1})*	D (h)*	BD (g l^{-1})*	BP (g l^{-1} h^{-1})*	(h^{-1})*
t1.3 *R. opacus*	0.241 (± 0.007) 25[a]	2.878 (± 0.09)	2.546 (± 0.15)	0.121 (± 0.003) 65[a]	0.139 (± 0.014) 14[a]
t1.4 *C. vulgaris*	0.009 (± 0.002)	72.204 (± 11.79)	0.763 (± 0.01)	0.002 (± 0.000)	0.010 (± 0.000)

Data represented as mean (±SD), $n = 3$

μ, specific growth rate; D, doubling time; *BD*, biomass density; *BP*, biomass productivity; , lipid accumulation rate

*One-way ANOVA was carried out for all parameters. All differences were significant, $p < 0.001$

[a] Fold increase in *R. opacus*

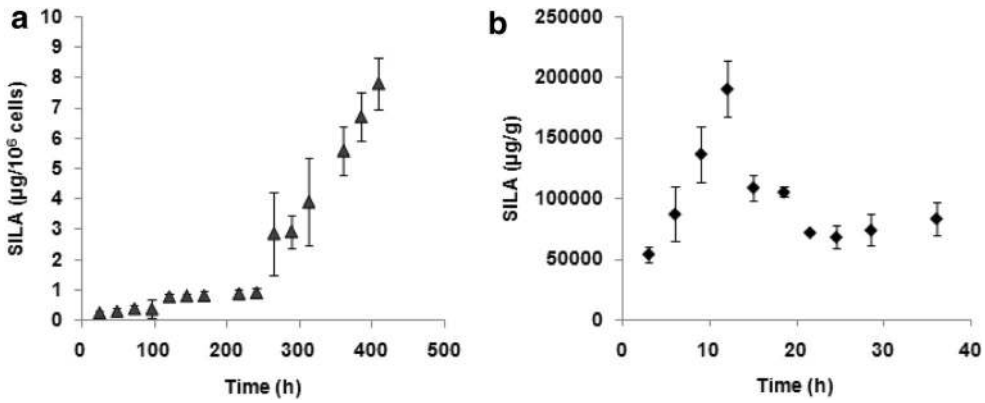

Fig. 2 Intracellular lipid profile of the oleaginous model organisms. **a** Bacterium *R. opacus* PD630. **b** Microalga *C. vulgaris* NIOT5. Data points are represented as mean ± SD, $n = 3$

pond systems, and closed systems that included vertical or horizontal tubular reactors, and flat panel reactors showed economic losses. It is also known that achieving an economically viable algal production technology is associated with high risk and uncertainties [67–69]. The cultivation process by both the open pond and closed system requires a dramatic increase in their biomass productivity and energy efficiency to make the production process economically viable [57]. These difficulties associated with algal fuel technology seem persuasive enough to consider bacteria as an alternative biodiesel feedstock.

Superior Diesel Quality from *R. opacus* PD630

An additional feature for biofuel application of a strain is suitability of its fatty acids for biodiesel. The fatty acid chain length, degree of saturation, and fatty acid proportion influence the quality of biodiesel produced [70]. The fatty acid profile of biodiesel was obtained through GC-MS. The fatty acid composition of the biodiesel obtained from *R. opacus* PD630 (Table 3) and *C. vulgaris* NIOT5 (Table 4) was found to be different. In *R. opacus* PD630, the fatty acid chain length ranged from C14 to C23 with a high degree of saturation in their chains. The relative content of SFA was 60% and that of MUFA was 40% (Fig. 3). Polyunsaturation was not observed in fatty acid chains of *R. opacus* PD630. Palmitic acid (C16:0) and margaric acid (C17:0) were the predominant SFAs and major MUFAs included oleic acid (C18:1) and heptadecenoic acid (C17:1). Unlike *R. opacus* PD630, *C. vulgaris* NIOT5 had a high degree of unsaturation in their fatty acid chains with chain length ranging from C14 to C20. The total USFA content was 74%, of which 38% was MUFA and 35% was PUFA (polyunsaturated fatty acids). The relative content of SFA was 27% (Fig. 3). Stearic acid (C18:0), hexadecenoic acid (C16:1, *cis*-11), and linolenic acid (C18:3, *cis*-9, 12, 15) were the major SFA, MUFA, and PUFA, respectively. Based on the fatty acid composition and structural characteristics such as chain length and degree of unsaturation, few properties that dictate the quality of the diesel were calculated from available empirical correlations [71, 72]. The calculated properties such as CN, IV, viscosity, density, and HC were then compared with previously developed standards. The biodiesel obtained from both *R. opacus* PD630 and *C. vulgaris* is of suitable quality, as the biodiesel's characteristic properties were in accordance with the established standards (Table 5). While comparing across the strains, *R. opacus* PD630 had higher CN of 69 ± 0.512 and lower IV of 39 ± 1.243 gI_2/100 g oil, than alga *C. vulgaris* NIOT5.

The absence of PUFA and a high amount of SFA content (60%) in *R. opacus* PD630 when compared to those of *C. vulgaris* NIOT5 (27%) confer superior oxidative stability [73]. The measure of unsaturation, i.e., the IV of biodiesel from *R. opacus* PD630 being lower than that of *C. vulgaris* NIOT5, also confirms its higher oxidative resistance. However, the absence of PUFA in *R. opacus* PD630's biodiesel may result in its poor flow properties at low temperatures [74]. Nevertheless, this issue could be taken care by the presence of palmitoleic acid (C16:1), which is known to improve

Table 2 Biodiesel yield and productivity of the bacterium *R. opacus* PD630 and the microalga *C. vulgaris* NIOT5

Oleaginous organism	LY (g g^{-1})*	LC (% CDW)*	LP (mg l^{-1} h^{-1})*
R. opacus	0.33 (± 0.003)	33 (± 2.89)	40 (± 2.33), 75[a]
C. vulgaris	0.283 (± 0.003)	28.3 (± 0.29)	0.53 (± 0.01)

Data represented as mean (±SD), $n = 3$

LY, lipid or biodiesel yield; *LC*, lipid or biodiesel content; *LP*, lipid or biodiesel productivity; *CDW*, cell dry weight

*One-way ANOVA was carried out for all parameters. All differences were significant, $p < 0.001$

[a] Fold increase in *R. opacus*

Q6 **Table 3** Fatty acid profile of biodiesel obtained from the bacterium *R. opacus* PD630

Fatty acid chain	Compound	Relative content (%)
C12:0	Methyl laurate	0.21 (± 0.02)
C13:0	Methyl tridecanoate	0.31 (± 0.04)
C14:0	Methyl myristate	2.98 (± 0.624)
C15:0	Methyl pentadecanoate	9.59 (± 0.53)
C16:0	Methyl palmitate	25.92 (± 2.34)
C16:1	Methyl palmitoleate (*cis*-9)	7.64 (± 0.10)
C17:0	Methyl margarate	15.35 (± 0.04)
C17:1	Methyl heptadecenoate (*cis*-8)	15.27 (± 1.67)
C18:0	Methyl stearate	4.25 (± 0.11)
C18:1	Methyl oleate (*cis*-9)	12.98 (± 1.75)
C19:0	Methyl nonadecanoate	1.14 (± 0.05)
C19:1	Methyl nonadecanoate (*trans*-10)	1.59 (± 0.20)
C20:0	Methyl arachidate	0.32 (± 0.06)
C22:0	Methyl behenate	1.01 (± 0.12)
C23:0	Methyl tricosanoate	1.44 (± 0.19)

Data represented as mean (±SD), $n = 3$

cold flow properties [75]. Also, poor flow properties are of concern in countries with relatively low temperature.

Further, the higher degree of saturation in *R. opacus* PD630's biodiesel resulted in the high CN of 69. The CN, which describes the diesel's ignition efficiency, is a prime indicator of biodiesel quality [76, 77]. Diesel with CN of 60 or more is considered as premium quality fuel. The high CN of *R. opacus* PD630's biodiesel helps in quieter combustion, reduces the risk of residue formation in engines, and facilitates smoother engine performance [78]. Further, higher CN promotes reduced mono-nitrogen oxide (NO_x) emissions during combustion and, thus, reduces pollution caused by engine exhausts [79]. Thus, biodiesel from the oleaginous bacteria *R. opacus* PD630 had better quality when compared to that from the microalga *C. vulgaris* NIOT5.

Biomass Productivity and Production Cost

Based on the productivities obtained in the current study, a calculation was made to understand the influence of biomass productivity on unit production cost. The estimates can be considered to be conservative because other optimized bioreactor systems are expected to yield higher productivities. However, the comparison between the strains is expected to be valid because they have been done on the same cultivation basis. Since *R. opacus* PD630 and *C. vulgaris* NIOT5 had comparable FAME content (Table 2), the cost of in situ transesterification of biomass to biodiesel is not expected to significantly differ with respect to the strain. Hence, biomass production cost was considered as a suitable feature for comparative assessment of economics. As mentioned in the

Table 4 Fatty acid profile of biodiesel obtained from the microalga *C. vulgaris* NIOT5

Fatty acid chain	Compound	Relative content (%)
C14:0	Methyl myristate	1.93 (± 0.32)
C16:0	Methyl palmitate	5.71 (± 0.05)
C16:1	Methyl hexadecenoate (*cis*-7)	7.04 (± 0.17)
C16:1	Methyl hexadecenoate (*cis*-11)	13.23 (± 0.64)
C16:1	Methyl palmitoleate (*cis*-9)	3.39 (± 0.03)
C16:2	Methyl hexadecadienoate (*cis*-7, 10)	3.26 (± 0.21)
C16:3	Methyl hexadecatrienoate (*cis*-7, 10, 13)	6.14 (± 0.15)
C18:0	Methyl stearate	14.88 (± 0.42)
C18:1	Methyl elaidate (*trans*-9)	6.69 (± 0.53)
C18:1	Methyl oleate (*cis*-9)	7.15 (± 0.33)
C18:2	Methyl linoleate (*cis*-9, 12)	4.75 (± 0.09)
C18:3	Methyl linolinate (*cis*-9, 12, 15)	19.86 (± 0.40)
C20:0	Methyl eicosanoate	4.13 (± 0.15)

Data represented as mean (±SD), $n = 3$

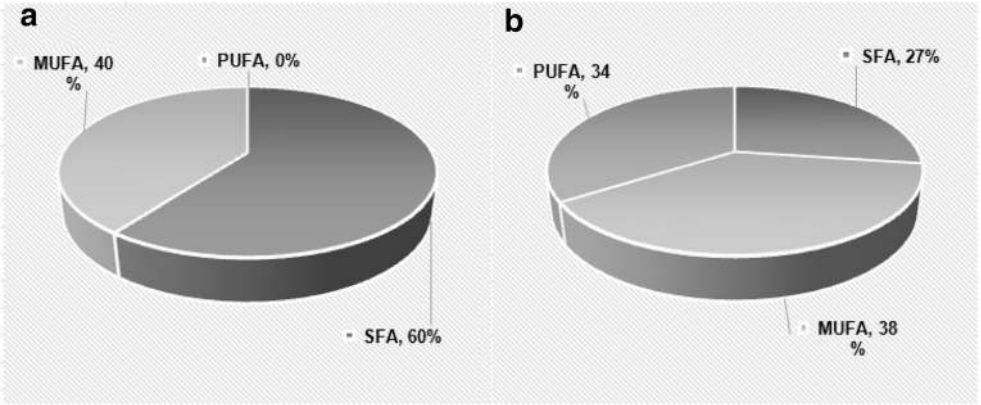

Fig. 3 Fatty acid distribution of biodiesel from the oleaginous model organisms. **a** Bacterium *R. opacus* PD630. **b** Microalga *C. vulgaris* NIOT5

"Materials and Methods" section, data from recent cost estimates of microalgae biomass production in tubular photobiorector (TPBR) through the meta-modeling approach was taken as the basis [57] for the current calculation. The reported model [57] had assumed an aerial biomass productivity of 20 g m^{-2} day^{-1} (Table 6). For our convenience, the corresponding volumetric productivity was calculated, and it was around 0.012 g l^{-1} h^{-1}. Since the calculated volumetric productivity (0.012 g l^{-1} h^{-1}) was in the range (0.001–0.016 g l^{-1} h^{-1}) that can be obtained with algal shake flask study [62] and as the productivity of *R. opacus* PD630 (Table 1) was already higher than 0.012 g l^{-1} h^{-1}, the cost analysis for tubular reactor production system was done based on shake flask productivities. For cost assessment, the same TPBR system that has been mentioned earlier [57] was considered as pilot-scale plant for both *C. vulgaris* NIOT5 and for *R. opacus* PD630 (TPBR without light). By retaining the same pilot-scale TPBR system for this cost analysis, it is reasonable to expect a similar cost estimate for biomass production (Online Resource 1 Table S2). However, as bacteria and algae differ in their nutrient requirements [80], a difference in their raw material costs is expected. But as stated earlier, the difference was considered insignificant, since raw material costs contributed to only 4% of the total biomass production costs (Online Resource 1 Table S2). Further, this assumption can be considered valid as industrial flue gas can source CO_2 for algae and *Rhodococcus* has high potential to grow on waste materials [47, 81]. With the productivity of *R. opacus* PD630 and *C. vulgaris* NIOT5 obtained in this study, the unit production cost per kilogram of biomass was calculated (Table 6). As stated already, enhancing biomass productivity is one important criterion for reducing the production cost [82]. It was observed that with the increase in productivity, a proportionate decrease in unit production cost resulted. The unit cost of production of *C. vulgaris* NIOT5 and *R. opacus* PD630 was found to be 61 and 1 € kg^{-1}, respectively (Table 6). Higher productivity of *Rhodococcus* resulted in a significant decrease in unit production cost and, thus, can improve the economy and process sustainability.

Choosing a suitable species that can feed on versatile waste resources and its resulting conversion to valuable oil would add to environmental sustainability [8]. For instance, strains of the genus *Rhodococcus*, in addition to being oleaginous in nature, have the ability to catabolize the most recalcitrant and toxic organic compounds [83]. The compounds like short and long chain alkanes, aromatics (halogenated, heterocyclic, and polycyclic), organic solvents [84], halogenated organic compounds, recalcitrant herbicides, and textile dyes [85, 86]

Table 5 Biodiesel properties of the bacterium *R. opacus* PD630 and the microalga *C. vulgaris* NIOT5 and their comparison with established standards

Fuel properties	*R. opacus*	*C. vulgaris*	Petroleum diesel[a]	ASTM 6751-02	EN 14214	IS 15607
Cetane number	69.12 (± 0.51)	50.32 (± 0.84)	49–55	≥ 47	≥ 51	≥ 51
Density (g/cm^3)	0.90 (± 0.00)	1.00 (± 0.01)	0.85	NM	0.86–0.9	0.86–0.9
Iodine value (gI_2/100 g)	39.02 (± 1.24)	87.70 (± 0.96)	NR	NM	< 120	NM
Kinematic viscosity (mm^2/s)	4.47 (± 0.01)	3.95 (± 0.05)	2.6	1.9–6	3.5–5	2.5–6
Calorific value (MJ/kg)	39.66 (± 0.12)	38.84 (± 0.35)	42.2	> 35	NM	NM

Data represented as mean (±SD)

NM, not mentioned; *NR*, not reported

[a] Mallick et al. [52]

t6.1 **Table 6** Biomass production cost analysis and comparison

Pilot plant details	Modeled plant[a]	*C. vulgaris* NIOT5	*R. opacus* PD630
Production volume (m^3)	7000	7000	7000
Land area (ha)	10	10	10
Biomass volumetric productivity (g l^{-1} h^{-1})	0.012[b]	0.002[c]	0.12[c]
Biomass aerial productivity (g m^{-2} day^{-1})	20[b]	0.34[d]	202[d]
No. of days operated	300	300	300
Annual biomass production (t)	600	101	6048
Total biomass production cost (€)	6,149,682	6,149,682	6,149,682
Unit production cost (€ kg^{-1})	10	61	1

[a] Parameters according to Slade and Bauen [57]

[b] Values assumed by Slade and Bauen [57]

[c] Data from the current study

[d] Calculated with data from the current study based on specifications of the modeled plant

are examples to cite a few. Strains of *Rhodococcus*, *R. opacus* DSM 1069 and *R. opacus* PD630, have been proven to be oleaginous with oil content amounting over 20% CDW, when grown on aromatic phenyl acetic acid and recalcitrant lignin-related compounds [38, 87], and 22–26% CDW with light oil from pyrolysis of lignocellulosic resources as sole carbon source [88]. Strain *R. opacus* PD630 was capable of accumulating 51% CDW of oil by using dairy wastewater as substrate [81]. Thus, choosing oleaginous species like *Rhodococcus* or similar species, which can degrade a wide range of chemicals, helps in integrating bioremediation and bio-oil production. Such process integration aids in developing an environmentally sustainable and economically viable process [8].

Productivities of Microalgae Vs. *Rhodococcus*: Data Analysis from the Literature

As discussed in previous sections, based on this study and various other studies (Table S3), the productivity of

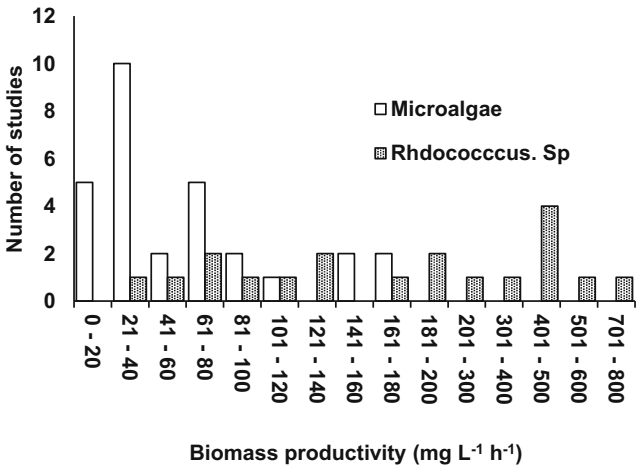

Fig. 4 Frequency distribution of biomass productivities in microalgae and *Rhodococcus* sp., reported across various studies

R. opacus PD630 is far higher than autotrophic microalgae. Further, bacterial productivity was compared with the theoretical (unattainable) maximum productivity of autotrophic algal production system. The theoretical maximum productivity for any algal production system using solar energy, irrespective of their location site, is 196 g m^2 day^{-1} [89]. However, the practically possible productivity of *R. opacus* PD630 was already in the range of 202 g m^2 day^{-1} (Table 6). Though the aerial productivities are comparable, outdoing the theoretical maximum is impossible for photosynthetic algae [89], whereas in the case of bacteria, further improvement is possible.

Nevertheless, the theoretical maximum productivity is not applicable for heterotrophic algal cultivation, where energy supplies such as sugars are added [89] with which it is possible to achieve higher algal productivity [90, 91]. Thus, heterotrophic algal and *Rhodococcus* productivities reported by various studies in the literature, some of which used optimized media for culturing the organisms, were compared. Tables S4 and S6 (Online Resource 1) show the BP and LP of microalgae and *Rhodococcus* sp. The highest BP reported in *Rhodococcus* was 747 mg l^{-1} h^{-1} and the mean BP of the represented dataset was 255.2 mg l^{-1} h^{-1}, whereas the maximum BP reported in microalgae is 170. 8 mg l^{-1} h^{-1} and the mean BP of the represented dataset is 58.7 mg l^{-1} h^{-1}, which are significantly less when compared to *Rhodococcus*. The fact that *Rhodococcus* productivity is better than algae was further demonstrated with the frequency distribution plot (Fig. 4). The most frequently reported BP of microalgae was in the range of 21–40 mg l^{-1} h^{-1} and for *Rhodococcus* a shift in productivity to the right is clearly seen, with studies reporting productivities in higher range of 401–500 mg l^{-1} h^{-1}.

Similarly, the highest LP reported in *R. opacus* was 257 mg l^{-1} h^{-1} and the mean LP of the represented dataset was 103 mg l^{-1} h^{-1}. However, the maximum LP reported in microalgae was only 101 mg l^{-1} h^{-1}. Although the mean LP calculated for *Chlorella* sp. was 28.35 mg l^{-1} h^{-1}, the frequency

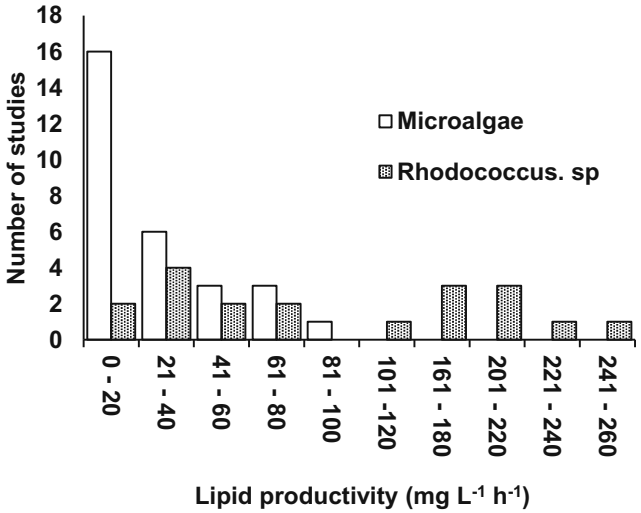

Fig. 5 Frequency distribution of lipid productivities in microalgae and *Rhodococcus* sp., reported across various studies

distribution plot (Fig. 5) shows that most studies report LP in the even lower range of 11–20 mg l^{-1} h^{-1}. As with BP, the LP of *Rhodococcus* also showed a right shift, with studies reporting in a higher range of 161–220 mg l^{-1} h^{-1}. Therefore, despite the use of optimized media in some of these studies, the maximum BP and LP for microalgae from the represented dataset are still significantly lower than those of *Rhodococcus*. While heterotrophic fed-batch cultures of algae can reach higher productivities than batch mode [90], the range of productivity that is obtainable with algal fed-batch is already attainable with *Rhodococcus* batch cultivation (Online Resource 1 Tables S5 and S6). This is due to the latter's high substrate tolerance that enables rapid high-density fermentation when compared to the photosynthetic organism [92].

Conclusion

The oleaginous bacteria *R. opacus* PD630 exhibited higher biomass productivity and high lipid productivity than the microalga *C. vulgaris* NIOT5, although their oil contents were similar. Also, the characteristics of biodiesel from *R. opacus* PD630 were better than those from *C. vulgaris* NIOT5. It is evident from the current study and as well as from the analysis of a large set of literature data that oleaginous bacteria such as *Rhodococcus* sp. due to their higher productivities could serve as a better biodiesel feedstock than microalgae. The current uncertainties associated with developing a sustainable and commercially suitable biofuel technology could be addressed by taking advantage of oleaginous bacteria.

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Compliance with Ethical Standards

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

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

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