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Prospecting for Oleaginous and Robust *Chlorella* spp. for Coal-Fired Flue-Gas-Mediated Biodiesel Production

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Abstract: Prospecting for robust and high-productivity strains is a strategically important step in the microalgal biodiesel process. In this study, 30 local strains of *Chlorella* were evaluated in photobioreactors for biodiesel production using coal-fired flue-gas. Three strains (M082, M134, and KR-1) were sequentially selected based on cell growth, lipid content, and fatty acid composition under autotrophic and mixotrophic conditions. Under autotrophic conditions, M082 and M134 showed comparable lipid contents (*ca.* 230 mg FAME [fatty acid methyl esters derived from microalgal lipids]/g cell) and productivities (*ca.* 40 mg FAME/L·d) versus a reference strain (KR-1) outdoors with actual flue-gas (CO_2, 13%). Interestingly, under mixotrophic conditions, M082 demonstrated, along with maximal lipid content (397 mg FAME/g cell), good tolerance to high temperature (40 °C). Furthermore, the fatty acid methyl esters met important international standards under all of the tested culture conditions. Thus, it was concluded that M082 can be a feedstock of choice for coal-fired, flue-gas-mediated biodiesel production.

Keywords: *Chlorella*; coal-fired flue-gas; screening; biodiesel property; mixotrophic cultivation

1. Introduction

Microalgae have attracted much global attention for their potential as biodiesel feedstock [1,2]. This interest reflects not only microalgae's higher photosynthetic efficiency and lipid yield compared with conventional oil crops but also their ability to mitigate atmospheric CO_2 and to grow in non-arable land and a variety of wastewaters [3–7]. Moreover, microalgae are easily isolated: they are ubiquitously present in nature and represent a vast diversity of (>50,000) species existing over a wide range of environment conditions [1,8].

Despite its several advantages, microalgae-based biodiesel production, when considered on a commercial scale, remains challenging. First, for competitive and sustainable biodiesel production, the cost of the culture system needs to be significantly reduced, and lipid productivity needs to be substantially improved [1]. Industrial exhaust flue-gases are an inexpensive and rich source of CO_2 (~14% v/v), the utilization of which for microalgal biomass production would be a fiscally and environmentally better option than compressed CO_2 [9,10]. However, high concentrations of CO_2

and the presence of inhibitory compounds such as NOx, Sox, and CO in flue-gases can significantly suppress the metabolic activities of microalgae [6,10]. Hence, for the successful application of flue-gases to microalgal biomass production, the selection of a flue-gas-tolerant and high-lipid-yield strain is warranted.

On the other hand, several microalgae, if not all, can grow mixotrophically by utilizing organic carbons along with light, thereby yielding high lipid productivity. Liang et al. reported that a mixotrophic cultivation of *Chlorella vulgaris* with 1–2% glucose yielded a 10-to-20-fold increase in the biomass (254 mg/L·d) and lipid (54 mg/L·d) productivities relative to an autotrophic cultivation [11]. Exploiting such a mixotrophic cultivation system with a suitable microalga would open up opportunities to utilize waste organics such as carbon sources and, at the same time, help to bring down cultivation costs.

Amongst the several oleaginous microalgae, *Chlorella* is one of the most widely studied genus for its potential to grow in outdoors at high cell densities and accumulate high levels of intracellular triacylglycerol (TAG), a desirable biodiesel feedstock [11–15]. However, the literature indicates that the growth rates and lipid contents of *Chlorella* are both species-specific and possibly significantly variant according to culture conditions. Recently, Sun et al. reported the differences in biomass productivity (0.41–0.58 g/L·d) and lipid content (36–49 wt. %) among 9 *Chlorella* spp. cultured with artificial CO_2 gas under autotrophic conditions [15].

What also must be noted is the fact that many highly productive microalgae selected under laboratory conditions fail to withstand the actual, fluctuating environmental conditions and contamination outdoors [7,16,17]. While accounting for such differential behavior among the *Chlorella* spp., it is imperative to select a native isolate that is appropriate not only in having a high lipid content but also in its capacity to adapt to environmental fluctuations under a suitable cultivation system.

The aim of the present work was to screen various native *Chlorella* spp. to identify a robust, fast-growing, and high-lipid-accumulating strain for high-quality biodiesel production under autotrophic/mixotrophic systems mediated by coal-fired flue-gas (CO_2, 13%). The employed screening strategies included comparisons of growth rate, lipid content, high-temperature tolerance, and fatty acids quality with respect to key biodiesel properties. The robustness of each of the selected high-productivity stains was finally evaluated under outdoor culture conditions mediated by coal-fired flue-gas.

2. Results and Discussion

2.1. Preliminary Screening Based on Growth Rate

In a preliminary screening, 30 local strains of *Chlorella* were evaluated for their growth rates in bubble-column photobioreactors (b-PBRs) with 10% CO_2 (v/v) under autotrophic and mixotrophic conditions. The mixotrophic cultures were supplemented with 5 g glucose/L. After 50 h incubation, and the growths (OD_{660}) of the strains were compared (Figure 1). Most of the strains showed relatively higher growths under the mixotrophic than under the autotrophic conditions. This phenomenon is consistent with other studies that have reported organic carbon's induction of growth promotion in *Chlorella* [11,18,19]. The 30 stains, as based on their growth characteristics, were divided into the following three groups.

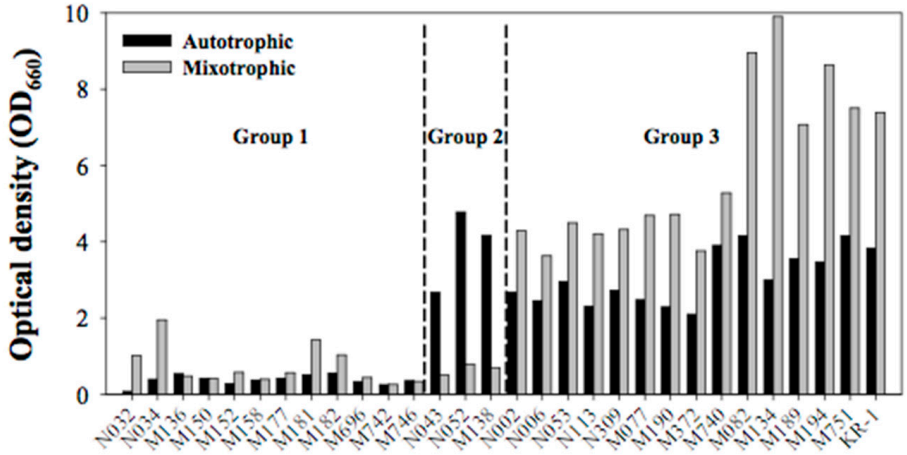

Figure 1. Growth of *Chlorella* spp. (30 strains) under autotrophic and mixotrophic conditions. The optical density was measured at 660 nm at the end of 50 h cultivation in b-PBRs. The mixotrophic cultures were supplemented with 5 g glucose/L.

Group 1: Twelve strains of the tested *Chlorella* showed a very low growth rate under both autotrophic and mixotrophic conditions. This delayed growth was expected, based on their demonstrated intolerance to the high concentrations of CO_2 (10% v/v in air) applied in this study. Many microalgae reportedly tolerate CO_2 concentrations up to 5% (v/v in air), whereas concentrations above this can be harmful, not to mention inhibitive of microalgal growth [9]. Hence, these strains might not be suitable for flue-gas-mediated cultivation, in which CO_2 concentrations often exceed 10%.

Group 2: Interestingly, this group of 3 strains (N043, N052, and M138) exhibited decreased growth under mixotrophic, compared with autotrophic, conditions. Lower growth rate under mixotrophy could possibly be due to the inhibitory effects of glucose on the microalgal cells. Such effects are species-dependent; high concentrations of glucose have been reported to substantially inhibit *Chlorella* and Nannochloropsis growths [11,18]. The three group 2 strains, seemingly sensitive to organic carbon supplementation, were not further studied, considering their poor future prospects for utilization in lipid-productivity-improvement using waste organics.

Group 3: For these 15 strains of *Chlorella*, the mixotrophic cultures resulted in higher growth rates than the autotrophic condition. That is to say, these 15 strains utilized glucose efficiently to achieve impressive growth. Among them, six (M082, M134, M189, M194, M751, and KR-1) exhibited the highest growth under mixotrophic conditions and also appeared to tolerate a high concentration of CO_2 (10% v/v in air). Hence, these six strains, thus identified as fast-growing, were held over for further investigation.

2.2. Secondary Screening Based on Lipid Content and Composition

Growth rate and lipid content are the most important factors in assessing prospective microalga for biodiesel production [1,20]. Additionally, the composition of the fatty acids produced by the microalga determines the quality of the biodiesel produced [21,22]. Hence, the six selected fast-growing strains (M082, M134, M189, M194, M751, and KR-1) were allowed to grow for a longer period of time (until 96 h), and then they were further evaluated for their lipid contents (in this study, expressed as FAME, i.e., amount of fatty acid methyl esters derived from microalgal lipids) and their compositions under autotrophic and mixotrophic conditions (Figure 2). After 96 h of cultivation, the microalgal cells would have already experienced nitrogen depletion [6]. Indeed, despite their fast-growing nature, strains M082 and M134 accumulated high levels of intracellular lipids: 283 and 298 mg FAME/g DCW (dry cell weight), respectively, under autotrophic conditions. Moreover, under mixotrophic conditions, these strains accumulated 397 and 310 mg FAME/g DCW, respectively, which is slightly less than the corresponding content for control strain KR-1 (411.3 mg FAME/g DCW). Notwithstanding, owing to

their higher biomass productions, the small differences in the lipid contents of M082 and M134 could be very well compensated by their higher biomass productivities (Figure S1). As for *Chlorella* sp. KR-1, it has been well studied in the past for its fast-growth and high-lipid-accumulation potential under mixotrophic conditions [6,23]. Thus, M082 and M134 strains, based on their indoor performances, could be suggested to be potentially comparable with KR-1.

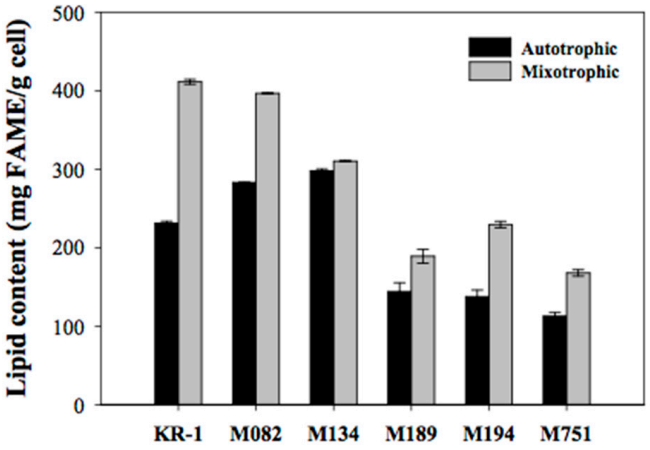

Figure 2. Lipid contents of six fast-growing *Chlorella* strains under autotrophic and mixotrophic conditions. The lipid contents of the cultures were estimated by FAME analysis after 96 h cultivation. The mixotrophic cultures were supplemented with 5 g glucose/L.

Further, the FAME compositions of the biodiesel produced from lipids by the six fast-growing strains also were examined (Table 1). The data were similar under both autotrophic and mixotrophic conditions: the C16 and C18 species, the common fatty acids in vegetable oils, were the main constituents [15,22,24]. Also, at different distribution (%) levels, major fatty acid methyl esters such as palmitate (C16:0), stearate (C18:0), oleate (C18:1n9C), linoleate (C18:2n6C), and linolenate (C18:3n3) were observed (Figure S2). Among these, palmitate, oleate, and linoleate accounted for more than 70% of the total fatty acids identified. The balance between these fatty acids determines the key qualities of biodiesel, such as oxidative stability, cold flow point, lubricity, viscosity, cetane number, iodine number, heat of combustion, and NOx emission [15,22,24]. The details are discussed in a later section below (see Section 2.4.—estimation of biodiesel properties from fatty acids profiles). In consideration of the high lipid contents and FAME compositions of the 3 *Chlorella* strains M082, M134, and KR-1, these were chosen for further investigation in an outdoor culture mediated by coal-fired flue-gas.

2.3. Outdoor Flue-Gas Cultivation and High-Temperature Tolerance Experimentation

The 3 high-productivity strains (M082, M134, and KR-1) were cultivated outdoors in 1 L b-PBRs for 160 h with a supply of actual flue-gas collected from a 2 MW demonstration-scale coal-burning power plant [6]. The changes in the light intensity and temperature were continuously monitored; the maximum sunlight was 1000 μmol/m^2·s with an average daylight of 500 μmol/m^2·s; meanwhile, the day temperature ranged between 30 and 38 °C and came down to 19 °C during the night (Figure 3a). M082 and M134 were tolerant to flue-gas and grew well in the outdoor conditions: M134, like the reference strain KR-1, readily adapted to the conditions, starting to grow in quick time. Irrespective of the sharp lag phase, M082, very similarly to the other two strains, grew rapidly to reach the final OD_{660} (Figure 3b). At the end of 160 h of cultivation, the lipid contents of M082 and M134 were estimated to be 228 and 235 mg FAME/g cell, for FAME productivities of 39 and 42 mg/L·d, respectively (Figure 3c). These values were comparable with the lipid productivity of the reference strain KR-1 (41 mg FAME/L·d). Under the optimized culture conditions, the FAME productivity of KR-1 was shown to have increased as high as 155 mg/L·d [6], which highlights the likelihood of improving the

productivities of the selected strains M082 and M134. Thus, despite growing outdoors, with a supply of actual flue-gas and under fluctuating environmental conditions, the productivities of these two strains did not deteriorate; moreover, the FAME compositions remained less altered when compared with the indoor cultivations (Figure S3).

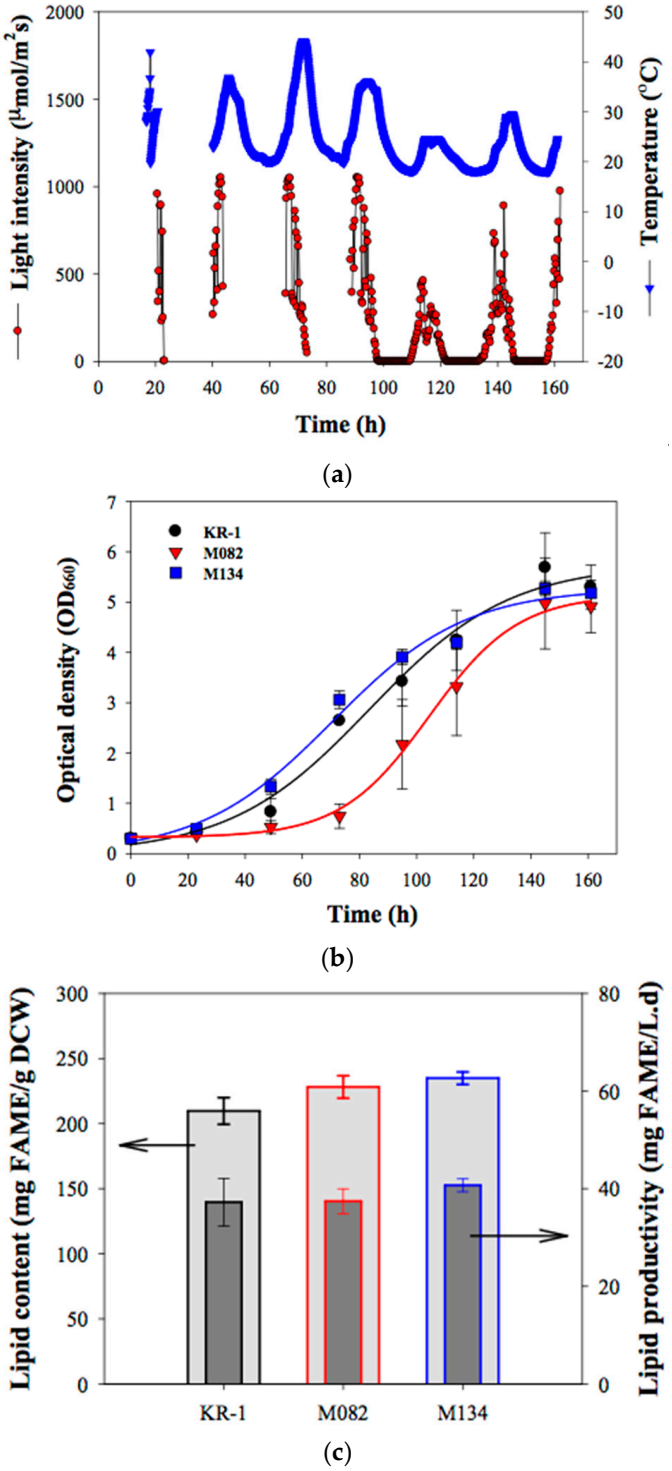

Figure 3. (**a**) Time-course changes in light intensity and temperature during cultivation; (**b**) growth; and (**c**) lipid content of three high-productivity *Chlorella* strains in outdoor b-PBR culture supplied with coal-fired flue-gas.

Table 1. FAME compositions of lipid extracted from six fast-growing *Chlorella* strains under autotrophic and mixotrophic conditions.

Fatty Acid Methyl Ester		Ratio (%) of Each FAME to Total FAME (Mean ± Standard Deviation)											
		M082		M134		M189		M194		M751		KR-1	
		Auto	Mixo	Auto	Mixo	Auto	Mixo	Auto	Mixo	Auto	Mixo	Auto	Mixo
Caprate	C10:0	0.05 ± 0.07	0.08 ± 0.00	nd	0.1 ± 0.01	nd	nd	nd	nd	nd	nd	nd	0.19 ± 0.01
Tridecanoate	C13:0	0.26 ± 0.01	nd	0.26 ± 0.01	nd	0.34 ± 0.03	0.18 ± 0.00	0.37 ± 0.01	0.14 ± 0.01	0.49 ± 0.06	0.32 ± 0.00	0.23 ± 0.03	0.11 ± 0.01
Myristate	C14:0	nd	0.94 ± 0.01	nd	0.57 ± 0.10	0.61 ± 0.03	0.59 ± 0.00	0.64 ± 0.03	0.48 ± 0.00	0.36 ± 0.02	0.51 ± 0.51	0.23 ± 0.00	0.22 ± 0.00
Pentadecanoate	C15:0	0.12 ± 0.00	0.19 ± 0.01	0.1 ± 0.00	0.22 ± 0.00	nd	0.15 ± 0.00	0.21 ± 0.01	0.14 ± 0.00	nd	0.16 ± 0.00	nd	0.13 ± 0.00
Palmitate	C16:0	23 ± 0.08	26.78 ± 0.06	25.99 ± 0.05	26.79 ± 0.06	22.36 ± 0.99	24.55 ± 0.37	22.21 ± 1.34	24.6 ± 0.08	20.96 ± 0.16	23.91 ± 0.52	24.49 ± 0.30	28.55 ± 0.02
Palmitoleate	C16:1	0.49 ± 0.01	0.29 ± 0.00	0.55 ± 0.01	0.37 ± 0.00	0.35 ± 0.01	0.4 ± 0.01	0.31 ± 0.00	0.36 ± 0.00	0.14 ± 0.20	0.3 ± 0.02	0.36 ± 0.01	0.32 ± 0.00
Stearate	C18:0	3.72 ± 0.01	6.26 ± 0.01	4.32 ± 0.01	5.71 ± 0.02	0.78 ± 0.00	1.01 ± 0.00	0.72 ± 0.03	1.52 ± 0.35	0.5 ± 0.04	0.97 ± 0.00	4.57 ± 0.02	4 ± 0.02
Oleate	C18:1n9c	21.45 ± 0.02	21.8 ± 0.05	20.76 ± 0.02	19.25 ± 0.03	11.54 ± 0.03	16.06 ± 0.10	10.07 ± 0.22	22.02 ± 0.03	7.28 ± 0.01	15.26 ± 0.05	19.07 ± 0.10	18.7 ± 0.02
Linoleate	C18:2n6c	24.64 ± 0.04	25.42 ± 0.17	23.66 ± 0.07	27.47 ± 0.14	20.61 ± 0.55	21.43 ± 0.18	20.2 ± 0.69	21.6 ± 0.02	18.3 ± 0.21	19.43 ± 0.25	25.6 ± 0.30	24.08 ± 0.03
γ-Linoleate	C18:3n6	0.34 ± 0.00	0.31 ± 0.00	0.32 ± 0.00	0.35 ± 0.01	0.4 ± 0.03	0.35 ± 0.03	0.43 ± 0.01	0.33 ± 0.01	0.4 ± 0.02	0.38 ± 0.00	0.35 ± 0.01	0.29 ± 0.01
Linolenate	C18:3n3	6.69 ± 0.00	4.22 ± 0.06	6.22 ± 0.01	4.43 ± 0.01	12.87 ± 0.38	12.12 ± 0.16	13.09 ± 0.56	9.89 ± 0.01	14.52 ± 0.19	12.18 ± 0.21	6.5 ± 0.09	7.49 ± 0.02
Arachidate	C20:0	0.14 ± 0.19	0.35 ± 0.00	0.25 ± 0.01	0.15 ± 0.01	nd	nd	nd	nd	nd	nd	0.32 ± 0.03	0.26 ± 0.01
cis-11-iocosenoate	C20:1	0.14 ± 0.20	0.05 ± 0.07	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Behenate	C22:0	0.12 ± 0.01	0.19 ± 0.00	nd	0.05 ± 0.07	nd	nd	nd	nd	nd	nd	nd	0.1 ± 0.00
Lignocerate	C24:0	nd	0.15 ± 0.00	nd	0.15 ± 0.05	nd	nd	nd	nd	nd	nd	nd	nd
Saturated		27.41 ± 0.19	34.95 ± 0.07	30.91 ± 0.06	33.75 ± 0.16	24.1 ± 1.05	26.49 ± 0.36	24.14 ± 1.40	26.62 ± 0.05	22.31 ± 0.09	25.87 ± 0.53	29.85 ± 0.32	33.55 ± 0.04
Monounsaturated		22.08 ± 0.21	22.14 ± 0.02	21.31 ± 0.03	19.63 ± 0.03	11.89 ± 0.03	16.47 ± 0.11	10.38 ± 0.22	22.38 ± 0.04	7.42 ± 0.22	15.55 ± 0.07	19.43 ± 0.09	19.02 ± 0.02
Polyunsaturated		31.66 ± 0.05	29.95 ± 0.22	30.2 ± 0.07	32.25 ± 0.14	33.89 ± 0.90	33.9 ± 0.32	33.73 ± 1.26	31.82 ± 0.01	33.22 ± 0.39	31.99 ± 0.46	32.45 ± 0.40	31.85 ± 0.03
Others		18.85 ± 0.04	12.96 ± 0.13	17.58 ± 0.02	14.37 ± 0.01	30.12 ± 0.19	23.15 ± 0.15	31.75 ± 0.36	19.18 ± 0.09	37.05 ± 0.51	26.59 ± 0.13	18.26 ± 0.01	15.57 ± 0.09
Total		100	100	100	100	100	100	100	100	100	100	100	100

Auto: autotrophic condition; Mixo: mixotrophic condition; nd: not detected.

The three strains were further tested for their tolerances to high temperatures (35–40 °C) under indoor culture condition. Selection of a high-temperature-tolerant strain carries an added advantage for practical application of microalgal culture to higher-temperature flue-gas from emission sites, especially in summer [23]. Although *Chlorella* sp. KR-1 has been extensively studied due to its high biomass/lipid productivity and flue-gas tolerance [2,5,6,23], its lowered metabolic activities, especially at 40 °C, should be noticed. Interestingly, *Chlorella* sp. M082 demonstrated better tolerance to temperatures up to 40 °C than those of M134 and the reference strain KR-1 (Figure S4). The results indicated, thereby, that *Chlorella* sp. M082, because it is endowed with not only a high temperature/flue-gas tolerance but also high lipid productivity, might be a highly robust strain.

2.4. Theoretical Estimation of Biodiesel Properties from Fatty Acid Profiles

Based on fatty acid profiles (Table 1) and literature-based correlations [21,24], the biodiesel properties (especially viscosity, specific gravity, cloud point, cetane number, iodine number, and HHV-high heating value) of the six *Chlorella* strains (M082, M134, M189, M194, M751, and KR-1) grown under indoor and three strains under outdoor cultures were estimated (Table 2). The values of these key properties were then compared with the Europe (EN14214) and USA (ASTM D6751) standards and a variety of vegetable oil values.

Kinematic viscosity (mm/s^2) is one of the important properties of a biodiesel: the value must be high enough to provide sufficient lubrication for the engine parts, but also low enough not to affect the flow properties at the operational temperature. The viscosities of the *Chlorella* strains under the indoor and outdoor culture conditions were determined to be approximately 4.6 and 4.5 mm/s^2, respectively, which meet the specifications established by the international standards. These values, it should also be emphasized, are very similar to the viscosities of the vegetable oils. Sun et al. (2015), similarly, reported that the kinematic viscosities of 9 *Chlorella* strains all measured around 4.4 mm/s^2 [15]. Indeed, in the present study, no significant viscosity value changes were observed between the indoor and outdoor culture conditions, which suggests that these conditions have a negligible effect on such changes, especially in saturated fatty acids. The specific gravity of a biodiesel is the ratio of the density of the FAME to that of water at 15 °C. A denser biodiesel has a higher energy content and thus will deliver better engine performance. Irrespective of the culture conditions, the biodiesels from all of the tested strains tested in the present investigation measured a specific gravity of 0.88, which is similar to the values of the vegetable oils and petroleum diesel, and which, in any cases, falls within the limits of international standards.

The cloud point (°C) is defined as the temperature at which crystals appear in biodiesel as it becomes cooler. The cloud point increases with increased saturated fatty acids. The lower the cloud point, the better the fuel quality. Insignificant cloud point differences were observed among the strains under the different culture conditions; however, there were no common trends. In general, the cloud point values were within the 6.7–8.4 °C range indoors and the 5.4–6.5 °C range outdoors, whose values are slightly higher than those of the Jatropa oil that is commonly used as a biodiesel in many countries. Cloud point values ranging from 3.6 to 5.5 °C have been reported for different *Chlorella* strains tested under autotrophic conditions [15]. Nevertheless, the addition of anti-gel additives is widely recommended in order to lower the cloud point of biodiesel sufficiently to prevent filters from clogging under lower temperatures [24]. The cetane number (CN) represents the combustion quality of a biodiesel during compressor ignition. In a particular diesel engine, higher-CN fuels will have shorter ignition delay periods compared with lower-CN ones. The CN values of the tested strains were within a 56.26–57.10 range indoors and a 55.58–56.16 range in the flue-gas-mediated outdoor cultures, whose values were well above the standard specifications. Moreover, the microalgal biodiesels had higher CN values compared with Jatropa and most of the other vegetable oils.

The iodine value (IV) indicates the total unsaturation degree regardless of the relative proportions of mono to polyunsaturated compounds. A high IV value for a fuel represents a decreased oxidation stability, which causes the formation of various degradation products that can negatively affect

engine operability. The IV values of the tested *Chlorella* strains ranged from 77.05 to 94.50 under the different culture conditions and were under the maximum limit of 120 established in the European biodiesel standard (no limits have been specified for IV in the U.S. standard). The higher heating value (HHV, MJ/kg) shows the energy content, otherwise known as the calorific value, of biodiesel. Hence, a higher HHV generally is recommended for diesel automobiles. The HHV of a given biodiesel increases with increasing fatty acid chain length and decreases with unsaturation [25]. The HHVs of the present tested strains were unaffected by the culture conditions and were around 40 MJ/kg (Table 2). Similar values of HHV (40.5 MJ/kg) had been reported among 9 *Chlorella* strains under indoor cultivation conditions [15]. Moreover, these values were almost the same as those of other vegetable oils. There are no specifications in the international standards. It should be noted that overall, irrespective of the culture conditions, the biodiesel properties of all of the tested *Chlorella* strains were within the ranges specified in the international standards.

Table 2. Biodiesel properties of *Chlorella* strains under autotrophic and mixotrophic conditions indoors and outdoors compared with literature and standards.

	Chlorella Strain	Viscosity [mm/s^2]		*Specific Gravity		Cloud Point [°C]		Cetane Number		Iodine Value		HHV [MJ/kg]	
		Auto	Mixo	Auto	Mixo	Auto	Mixo	Auto	Mixo	Auto	Mixo	Auto	Mixo
Indoor culture	KR-1	4.63 ± 0.00	4.63 ± 0.00	0.88 ± 0.00	0.88 ± 0.00	7.81 ± 0.07	7.91 ± 0.01	56.79 ± 0.04	56.84 ± 0.00	80.53 ± 0.42	80.02 ± 0.05	40.14 ± 0.01	40.13 ± 0.00
	M082	4.62 ± 0.00	4.66 ± 0.00	0.88 ± 0.00	0.88 ± 0.00	7.67 ± 0.01	8.44 ± 0.05	56.72 ± 0.00	57.10 ± 0.03	81.35 ± 0.05	77.05 ± 0.29	40.16 ± 0.00	40.05 ± 0.01
	M134	4.65 ± 0.00	4.64 ± 0.01	0.88 ± 0.00	0.88 ± 0.00	8.21 ± 0.01	8.12 ± 0.03	56.99 ± 0.00	56.94 ± 0.02	78.34 ± 0.06	78.83 ± 0.16	40.09 ± 0.00	40.09 ± 0.00
	M189	4.62 ± 0.01	4.59 ± 0.00	0.88 ± 0.00	0.88 ± 0.00	7.58 ± 0.20	7.07 ± 0.06	56.68 ± 0.10	56.42 ± 0.04	81.83 ± 1.11	84.65 ± 0.35	40.17 ± 0.03	40.23 ± 0.01
	M194	4.63 ± 0.01	4.60 ± 0.00	0.88 ± 0.00	0.88 ± 0.00	7.79 ± 0.27	7.14 ± 0.00	56.78 ± 0.14	56.46 ± 0.00	80.66 ± 1.52	84.28 ± 0.02	40.14 ± 0.04	40.23 ± 0.00
	M751	4.64 ± 0.01	4.62 ± 0.00	0.88 ± 0.00	0.88 ± 0.00	8.14 ± 0.11	7.69 ± 0.11	56.95 ± 0.05	56.74 ± 0.05	78.74 ± 0.61	81.20 ± 0.56	40.09 ± 0.02	40.15 ± 0.02
¶Outdoor culture	KR-1	4.52 ± 0.00	na	0.88 ± 0.00	Na	5.37 ± 0.04	na	55.58 ± 0.02	na	94.12 ± 0.23	na	40.46 ± 0.01	na
	M082	4.55 ± 0.00	na	0.88 ± 0.00	Na	6.02 ± 0.00	na	55.90 ± 0.00	na	90.50 ± 0.01	na	40.37 ± 0.00	na
	M134	4.57 ± 0.00	na	0.88 ± 0.00	Na	6.54 ± 0.02	na	56.16 ± 0.01	na	87.64 ± 0.12	na	40.31 ± 0.00	na
Vegetable oil	†Jatropa	4.48		0.88		4.67		55.23		98.02		40.55	
	‡Palm	4.61		0.87		14.00		61.90		54.00		40.60	
	‡Rapeseed	4.50		0.88		-3.00		53.70		116.10		41.10	
	‡Soy bean	4.26		0.88		0.00		51.30		125.50		39.70	
‡Petroleum diesel		2-3		0.85		Country specific		40-45		na		na	
International standard	EN14214	3.5–5.0		0.86–0.9		na		Minimum 51.0		Maximum 120		na	
	ASTM D6751	1.9–6.0		0.85–0.9		na		Minimum 47.0		na		na	

* Specific gravity = (1/1000) density at 15 °C; ¶ outdoor autotrophic are mediated by coal-fired flue-gas; † values from literature [21]; ‡ values from literature [24]; na: not available.

3. Materials and Methods

3.1. Microalgal Strains and Culture Medium

Twenty-nine strains of *Chlorella* were evaluated for biodiesel production in comparison with a reference strain *Chlorella* sp. KR-1 (Table 3). These strains were obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). Meanwhile, *Chlorella* sp. KR-1, which has been extensively studied for its high lipid productivity and flue-gas tolerance, was maintained at the Korea Institute of Energy Research (KIER; Daejeon, Korea) [2,5,6,23,26,27]. The modified N8 medium used in this study was prepared and filter-sterilized through a 0.2 μm membrane, and its pH was adjusted to 6.5 [16].

Table 3. List of tested *Chlorella* strains and their origins.

No.	Strain	Species	Origin
1	N002	*Chlorella vulgaris*	Paddy field/Ssukgol, Gyeonggi
2	N006	*C. vulgaris*	Paddy field/Suwon, Gyeonggi
3	N032	*C. vulgaris*	Reservoir/Andongho, Gyeongbuk
4	N034	*C. vulgaris*	Reservoir/Andongho, Gyeongbuk
5	N043	*C. vulgaris*	Reservoir/Imhaho, Gyeongbuk
6	N052	*C. vulgaris*	Reservoir/Youngsanho, Chungnam
7	N053	*Chlorella* sp.	Reservoir/ Andong, Gyeongbuk
8	N113	*Chlorella* sp.	Youngpyung, Gangwon
9	N309	*Chlorella* sp.	Local fresh water body
10	M077	*Chlorella* sp.	Local fresh water body
11	M082	*Chlorella* sp.	Local fresh water body
12	M134	*Chlorella* sp.	Local fresh water body
13	M136	*Chlorella* sp.	Local fresh water body
14	M138	*Chlorella* sp.	Local fresh water body
15	M150	*Chlorella* sp.	Local fresh water body
16	M152	*Chlorella* sp.	Local fresh water body
17	M158	*Chlorella* sp.	Local fresh water body
18	M177	*Chlorella* sp.	Local fresh water body
19	M181	*Chlorella* sp.	Local fresh water body
20	M182	*Chlorella* sp.	Local fresh water body
21	M189	*Chlorella* sp.	Local fresh water body
22	M190	*Chlorella* sp.	Local fresh water body
23	M194	*Chlorella* sp.	Local fresh water body
24	M372	*Chlorella* sp.	Local fresh water body
25	M696	*C. vulgaris*	Reservoir/Yongsoho Jeju
26	M740	*C. sorokiniana*	Reservoir/Daedongho, Hampyung
27	M742	*C. vulgaris*	Reservoir/Daedongho, Hampyung
28	M746	*C. vulgaris*	Reservoir/Daedongho, Hampyung
29	M751	*C. vulgaris*	Local fresh water body
30	KR-1	*Chlorella* sp.	Yeongwol, Gangwon

3.2. Seed Flask Culture

All of the strains were initially cultured in 250 mL Erlenmeyer flasks (working vol., 100 mL). The flasks were incubated under continuous illumination (white fluorescent lamps, *ca.* 40 μmol photons/m^2·s) in a shaking incubator (IS-971RF, Lab Companion, Korea) at 150 rpm and 25 °C.

3.3. Indoor Culture Using Photobioreactor with 10% (v/v) CO_2

Pyrex glass bubble-column photobioreactor (b-PBRs; working vol., 500 mL) [23] was utilized to culture *Chlorella* strains. The biomass from the flask cultures was used as the b-PBRs inoculum, a concentration of which was fixed to an initial optical density (OD) of 0.1 at 660 nm. The strains were grown under either autotrophic or mixotrophic conditions for 96 h. For the latter, the modified N8 medium was supplemented with filter-sterilized glucose (5 g/L, Sigma, USA; 0.2 μm Minisart

High-Flow filter, 16532-K, Sartorius Stedium Biotech., Germany). The b-PBR was continuously supplied with 10% CO_2 in air (v/v) from the bottom of the reactor. The supplied gas was conveyed by a 0.2 µm PTFE syringe filter (Minisart SRP15, Satorius Stedium Biotech., Germany) and controlled by mass-flow controllers (MKP, Korea) and flow meters (RM Rate-Master, Dwyer instruments Inc., Michigan, IN, USA). The reactor was maintained under continuous illumination (white fluorescent lamps, ca. 170 µmol/m^2·s) in a temperature-controlled room (28–31 °C). Preliminary screening of 30 *Chlorella* spp. was done using single PBR, whereas the rest of the experiments were carried out with duplicate PBRs.

A high-temperature-tolerance experiment was carried out for the fast-growing three strains under autotrophic conditions including continuous illumination (white fluorescent lamps, ca. 69 µmol/m^2 s) in a temperature-controlled growth chamber (GC-300, Jeio Tech, Korea). The b-PBR was incubated at 35 °C for 45 h, and then the temperature was increased to 40 °C, where it was maintained for a further 25 h.

3.4. Outdoor Culture Using Photobioreactor with Actual Flue-gas

The three strains selected under indoor experimentation were grown outdoors in b-PBRs under autotrophic conditions for 160 h. The biomass from the flask cultures was used as an inoculum with a fixed initial OD of 0.3 at 660 nm. The cultures were mediated by a supply of coal-fired flue-gas (obtained from a 2 MW demonstration-scale coal-burning power plant located at KIER, Daejeon, Korea) at a flow rate of 0.6 vvm [23]. The typical composition of the flue-gas supplied was CO_2, 13.3%; O_2, 7.6%; CO, 39.1ppm; NOx, 6.9 ppm [6]. Details on the storage, pre-processing, and transfer of flue-gas are available elsewhere [6]. Throughout the experiment, the cultures were maintained at ambient temperature, and the average day time light intensity in this period was 500 µmol/m^2·s.

3.5. Analytical Methods

Growth of the microalgal strains was evaluated based on their OD values measured at 660 nm using a UV-Vis spectrophotometer (Optizen 2120UV, Mecasys Co., Daejeon, Korea). The dry-cell weight (DCW) was measured by GF/C filtration, washing with distilled water, and 105 °C drying. The pH and light intensity were determined using a pH meter (DKK-TOA Co., Japan) and a quantum meter (LI-250A, LI-COR Inc., Lincoln, NE, USA), respectively. For outdoor cultures, data on parameters such as temperature and light intensity were continuously recorded. The temperature probe (CIMON-SCADA V 2.10, KDT Systems, Seongnam, Korea) was immersed inside the b-PBR in the culture medium. Changes in the light intensity were monitored through a light sensor (LI-250A, LI-COR Inc., USA) that was fixed on the top of the b-PBR-setting panel, and the data was collected by a data logger (LI-1400, LI-COR Inc., USA). The compositions (CO, NO, NO_2, SO_2, CO_2, and O_2) of the flue gas supplied for the cultures were analyzed using a portable flue-gas analyzer (Vario Plus, MRU, Neckarsulm, Germany). The FAME (fatty acids methyl ester) contents of the dried cells were prepared by following a modified *in-situ* transesterification method [18] and analyzed by gas chromatography (GC; Agilent 6890, Agilent Technologies, Wilmington, DE, USA). The relevant detailed methodologies have previously been reported [3,23]. All the analyses were done in triplicates, and the results were represented as mean ± standard deviation.

3.6. Estimation of Biodiesel Properties

Important biodiesel properties including viscosity, specific gravity, cloud point, cetane number, iodine number, and higher heating value (HHV) were estimated from the fatty acid compositions based on the empirical formulae proposed by Tanimura et al. (2014) [21].

4. Conclusions

In this study, 30 local strains of *Chlorella* were evaluated for application to flue-gas-mediated biodiesel production. Among the 30 strains, 3 strains (M082, M134, and KR-1) could be selected on the

basis of their growth rates, lipid contents, and fatty acid profiles under autotrophic (10% CO_2) and mixotrophic (10% CO_2 plus 5 g glucose/L) nutrition modes. These strains were further tested under outdoor conditions using the actual coal-fired flue-gas from demonstration-scale plant. M082 showed good lipid production performances (228 mg FAME/g cell and 39 mg FAME/L·d), as well as a high, up-to-40 °C temperature tolerance. Thus, *Chlorella* sp. M082 can be considered a potential strain for simultaneous utilization of CO_2 from flue-gas and of waste organics for biodiesel production.

Supplementary Materials: The following are available online at http://www.mdpi.com/1996-1073/11/8/2026/s1, Figure S1: Growth curve of 6 fast-growing *Chlorella* strains under autotrophic and mixotrophic conditions for 96 h of cultivations, Figure S2: FAME distributions of 6 fast-growing *Chlorella* strains under autotrophic and mixotrophic indoor conditions for 96 h of cultivation, Figure S3: FAME distribution of 3 high-productivity *Chlorella* strains in outdoor culture mediated by coal-fired flue-gas, and Figure S4: Growth patterns of 3 high-productivity *Chlorella* strains under high-temperature conditions.

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