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Optimum temperature and salinity conditions for growth of green algae *Chlorella ellipsoidea* and *Nannochloris oculata*

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ABSTRACT: The effects of temperature and salinity on growth of green algae *Chlorella ellipsoidea* and *Nannochloris oculata* were determined to compare the optimum culture conditions. A four-temperature (15, 20, 25, and 30°C) × three-salinity (10, 20, and 30) factorial design with triplicates was applied. Specific growth rate (SGR), maximum density, and duration to reach maximum density of *C. ellipsoidea* were significantly affected by both temperature and salinity. The highest SGR was observed in *C. ellipsoidea* at 25°C and salinity 10, but the maximum density was very low. The highest maximum density was achieved in *C. ellipsoidea* at 15°C and 10. The slope constant of the linear relationship between semilogarithmic growth of *C. ellipsoidea* and day of culture was highest at 15°C and 10. The SGR and duration to reach maximum density of *N. oculata* were significantly affected by both temperature and salinity. However, maximum density of *N. oculata* was significantly affected by temperature, but not salinity. The highest maximum density was achieved in *N. oculata* at 25°C and 30, but SGR was significantly lower than that of *N. oculata* at 25°C and 10. The slope constant of the linear relationship between semilogarithmic growth of *N. oculata* and day of culture was highest at 25°C and 30. Based on these results, the condition of 15°C and salinity 10 seemed to be optimal for maximum density of *C. ellipsoidea*, and the condition of 25°C and 10 and 30 for SGR and maximum density for *N. oculata*, respectively.

KEY WORDS: ***Chlorella ellipsoidea*, *Nannochloris oculata*, salinity, temperature.**

INTRODUCTION

Application of green algae has been tried for marine fish hatcheries because the algae are commonly used as food for the first live foods (such as rotifers and *Artemia* nauplii) for early larval fish, or directly added to the rearing tanks for larval fish production to achieve the 'green water effect'.[1] Supplementation of algae into the larval rearing tanks improved survival and/or growth of larval fish.[2–5]

In addition, the use of microalgae lead to a decrease in numbers of bacteria associated with the rotifer culture when compared with the rotifer culture fed yeast-based diets in the rotifer culture tanks.[6] The high nutrient content in microalgae improved the lipid and fatty acid compositions of rotifers when either fed to rotifers, or supplied to the larval fish tanks, and eventually resulted in an improvement of fish production.[7–11] Therefore, the constant production of high-quality microalgae is needed in marine fish hatcheries, and application of microalgae is highly recommended for larval fish production.

Growth of microalgae is likely to be affected by culture conditions such as temperature, salinity, illumination, and/or nutrients.[12–22] Green algae commonly used for marine fish hatcheries are *Chlorella ellipsoidea* and *Nannochloris oculata*, class Chlorophyceae,[23] because of the ease of mass culture and management, and high nutrient content leading to improvement in larval fish production.[5,9,24–28] Hur[19] demonstrated that *N. oculata* was superior to *C. ellipsoidea* as food for rotifers in terms of rotifer growth rate and fatty acid

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composition during summer. However, no study on the combined effect of temperature and salinity on growth of *C. ellipsoidea* and *N. oculata* have been reported.

In this study, therefore, the effects of temperature and salinity on growth of *C. ellipsoidea* and *N. oculata* were determined to compare the optimum culture conditions.

MATERIALS AND METHODS

Preparation of green algae and culture conditions

Chlorella ellipsoidea (KMCC C-20 clone) and *Nannochloris oculata* (KMCC C-31 clone) were purchased from the Korea Marine Microalgae Culture Center (KMCC, Busan, Korea). Algal cultures were performed in f/2 media[29] and intensity of illumination was maintained at 31 μmol photons/m^2 per second by a digital lux meter (INS DX-100, Japan) with a 24:0 h (light–dark) cycle at various temperature zones in a low temperature incubator (Dongwon Scientific System, Busan, Korea). Salinity was adjusted by mixing the filtered sea water and distilled water while monitoring with a salinometer (SM-2000, TS-EL, Japan).

Before inoculation with green algae, all flasks with 100-mL working volumes were sterilized by autoclave at 121°C for 20 min. *Chlorella ellipsoidea* and *N. oculata* were initially inoculated into thirty-six 250-mL flasks at a concentration of 100×10^4 cells/mL each. All flasks were hand-agitated twice a day (09:00 and 17:00 hours) and alternated within the same temperature zone every day to minimize the effects of differences in intensity of illumination on growth of algae.

Design of experiment

A four-temperature (15, 20, 25, 30°C) × three-salinity (10, 20, 30) factorial design with triplicates was prepared for this study.

Criteria measured for growth of green algae

Growth of green algae was measured by using a hematocytometer (0.0025 mm^2, Superior, Marienfeld, Germany). Samples of green algae from each flask were taken then dropped on the hematocytometer by means of a straw. Ten minutes were allowed for focusing before counting the green algae. Density of green algae at each condition was the mean of three counts observed under a microscope (CH20, Olympus, Tokyo, Japan) and specific growth rate (SGR/day) was calculated by the formula of Guillard:[30] $\text{SGR/day} = 3.322 \times \log_{10}(N_1/N_2)/t_1 - t_2$, where N_1 and N_2 are the cell concentrations at t_1 and t_2, and t_1 and t_2 are final and initial days of the experiment, respectively. The optimum conditions for SGR, maximum density ($\times 10^4$ cells/mL) and duration to reach maximum density (d) were determined for each alga. Data on daily growth of algae were semilogarithmically transferred, and relationships between semilogarithmic growth of algae and days of culture were calculated. The cell sizes of *C. ellipsoidea* and *N. oculata* were measured as approximately 3 and 1 μm in diameter, respectively, using a microscope (Zeiss, Jena, Germany).

Statistical analysis

One-way and two-way analysis of variance (ANOVA) and Duncan's multiple range test[31] were used to analyze the significance of the difference among the means of treatments. In addition, regression analysis for SGR and maximum density of each alga were conducted by using regression analysis with SAS v9.12 (SAS Institute, Cary, NC, USA).

RESULTS

Growth of *Chlorella ellipsoidea*

SGR, maximum density, and duration to reach maximum density of *C. ellipsoidea* at the various temperature and salinity conditions are given in Table 1. SGR, maximum density, and duration to reach maximum density of *C. ellipsoidea* were significantly ($P < 0.05$) affected by both temperature and salinity. The highest SGR (0.60) was observed in *C. ellipsoidea* at 25°C and salinity 10, but the maximum density was very low (Fig. 1). A similar trend was observed in *C. ellipsoidea* at 30°C at all salinity conditions tested. Maximum density and duration to reach maximum density of *C. ellipsoidea* at 15°C and 10 were 131.7×10^6 cells/mL and 18 days after inoculation, respectively. Relatively high SGR and maximum density was achieved in *C. ellipsoidea* at 25°C and 30. Significant ($P < 0.0001$) effects of temperature and salinity on SGR, maximum density, and duration to reach maximum density of *C. ellipsoidea* were observed.

Results of regression analysis for specific growth rate (SGR) and maximum density of *C. ellipsoidea* on salinity levels at constant temperature are given in Table 2. A linear regression was obtained

Table 1 Specific growth rate, maximum density and duration to reach maximum density of *Chlorella ellipsoidea* at various temperature and salinity conditions

Temperature (°C)	Salinity	SGR	Maximum density (10^6 cells/mL)	Duration to reach maximum density (d)
15	10	$0.37 \pm 0.013^{e,f}$	131.7 ± 12.36^{a}	18 ± 0.0^{b}
	20	$0.36 \pm 0.010^{e,f,g}$	$115.2 \pm 7.60^{a,b}$	18 ± 0.0^{b}
	30	0.31 ± 0.004^{g}	$097.3 \pm 1.78^{b,c}$	20 ± 0.0^{a}
20	10	$0.40 \pm 0.002^{d,e,f}$	$112.5 \pm 6.91^{a,b}$	16 ± 0.0^{b}
	20	$0.37 \pm 0.007^{e,f}$	$113.3 \pm 11.94^{a,b}$	$17 \pm 0.0^{b,c}$
	30	$0.35 \pm 0.009^{f,g}$	087.3 ± 5.49^{c}	$17 \pm 0.0^{b,c}$
25	10	0.60 ± 0.042^{a}	010.2 ± 0.99^{e}	05 ± 0.0^{g}
	20	$0.41 \pm 0.017^{d,e}$	041.2 ± 15.80^{d}	12 ± 2.1^{e}
	30	$0.45 \pm 0.006^{c,d}$	$103.1 \pm 27.11^{b,c}$	14 ± 1.5^{d}
30	10	$0.48 \pm 0.078^{b,c}$	2.6 ± 0.44^{e}	3 ± 0.6^{h}
	20	0.52 ± 0.044^{b}	012.9 ± 1.80^{e}	07 ± 0.0^{f}
	30	$0.44 \pm 0.030^{c,d}$	050.8 ± 10.69^{d}	12 ± 0.0^{e}
Two-way ANOVA				
Temperature		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Salinity		$P < 0.0001$	$P < 0.0006$	$P < 0.0001$
Interaction		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

Values (mean ± standard deviation) with different superscript letters in the same column are significantly different ($P < 0.05$). SGR, specific growth rate.

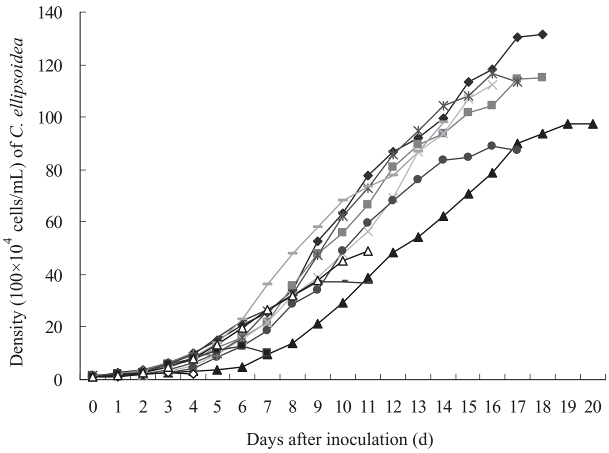

Fig. 1 Density of *Chlorella ellipsoidea* after inoculation at temperature and salinity conditions: 15°C and salinity 10 (◆), 20 (■), and 30 (▲); 20°C and salinity 10 (×), 20 (★), and 30 (●); 25°C and salinity 10 (+), 20 (-), and 30 (–); 30°C and salinity 10 (◇), 20 (■), and 30 (△).

between SGR of *C. ellipsoidea* and salinity at 15°C and 20°C ($P < 0.0003$ and $P < 0.0001$, respectively), but quadratic regression was obtained at 25°C ($P < 0.0009$). Linear regression was also obtained between maximum density of *C. ellipsoidea* and salinity at 15, 20, 25, and 30°C ($P < 0.003$, $P < 0.02$, $P < 0.0009$, and $P < 0.0001$, respectively).

Relationships between semilogarithmic growth of *C. ellipsoidea* and day of culture at various temperature and salinity conditions are presented in Table 3. The slope of the linear relationship was highest in *C. ellipsoidea* at 15°C and salinity 10, and lowest for this alga at 30°C and 10. However, a relatively lower constant was observed in *C. ellipsoidea* at 25°C and 10, at which the highest SGR was achieved.

Growth of *Nannochloris oculata*

SGR, maximum density, and duration to reach maximum density of *N. oculata* at various temperatures and salinities are presented in Table 4. SGR and duration to reach maximum density of *N. oculata* were significantly ($P < 0.05$) affected by both temperature and salinity. However, maximum density of *N. oculata* was significantly ($P < 0.05$) affected by temperature, but not salinity. The highest SGR (0.46) was observed in *N. oculata* at 25°C and salinity 10, and its maximum density was 227.0×10^6 cells/mL (Fig. 2). A relatively poor SGR was achieved in *N. oculata* at 15°C at all salinities tested. However, the highest maximum density, 308.0×10^6 cells/mL was achieved in *N. oculata* at 25°C and 30, but SGR was significantly ($P < 0.05$) lower than that of *N. oculata* at 25°C and 10. Further, duration to reach maximum density of *N. oculata* at 25°C and 30 was longest, 21 days after inoculation. Significant effect of temperature and salinity on maximum density and duration to reach maximum density of *N. oculata* were observed.

Table 2 Regression analysis for specific growth rate and maximum density of *Chlorella ellipsoidea* and *Nannochloris oculata* on salinity levels at constant temperature

Item	Temperature (°C)	*Chlorella ellipsoidea*	*Nannochloris oculata*
SGR	15	L ($P < 0.0003$)	Q ($P < 0.005$)
	20	L ($P < 0.0001$)	ns
	25	Q ($P < 0.0009$)	L ($P < 0.03$)
	30	ns	Q ($P < 0.0004$)
Maximum density	15	L ($P < 0.003$)	L ($P < 0.02$)
	20	L ($P < 0.02$)	ns
	25	L ($P < 0.0009$)	L ($P < 0.03$)
	30	L ($P < 0.0001$)	L ($P < 0.02$)

L, linear regression; ns, no significant difference; Q, quadratic regression; SGR, specific growth rate.

Table 3 Relationships between semilogarithmic growth of *Chlorella ellipsoidea* and *Nannochloris oculata* and day of culture at various temperature and salinity conditions

Temperature (°C)	Salinity	*Chlorella ellipsoidea*	*Nannochloris oculata*
15	10	Y = 8.30X – 17.63, R^2 = 0.97	Y = 8.34X – 21.60, R^2 = 0.95
	20	Y = 7.47X – 15.91, R^2 = 0.96	Y = 8.08X – 18.57, R^2 = 0.97
	30	Y = 5.73X – 18.01, R^2 = 0.94	Y = 7.04X – 24.94, R^2 = 0.92
20	10	Y = 7.54X – 18.91, R^2 = 0.93	Y = 10.10X – 18.54, R^2 = 0.97
	20	Y = 8.09X – 18.49, R^2 = 0.95	Y = 10.25X – 20.35, R^2 = 0.95
	30	Y = 6.32X – 14.12, R^2 = 0.94	Y = 9.50X – 18.97, R^2 = 0.97
25	10	Y = 1.88X + 0.67, R^2 = 0.90	Y = 15.31X – 41.64, R^2 = 0.95
	20	Y = 3.96X – 2.60, R^2 = 0.96	Y = 14.59X – 33.29, R^2 = 0.97
	30	Y = 7.59X – 12.94, R^2 = 0.96	Y = 17.19X – 49.98, R^2 = 0.96
30	10	Y = 0.34X + 1.08, R^2 = 0.66	Y = 12.10X – 29.04, R^2 = 0.96
	20	Y = 1.73X + 0.57, R^2 = 0.88	Y = 14.05X – 35.73, R^2 = 0.95
	30	Y = 4.74X – 5.89, R^2 = 0.96	Y = 15.15X – 32.93, R^2 = 0.97

R^2, residual.

Results of regression analysis for SGR and maximum density of *N. oculata* on salinity levels at constant temperature are given in Table 2. Linear regression was obtained between SGR of *N. oculata* and salinity at 25°C ($P < 0.03$), but quadratic regression was obtained at 15°C and 30°C ($P < 0.005$ and $P < 0.0004$, respectively). Linear regression was obtained between the maximum density of *N. oculata* and salinity at 15, 25, and 30°C ($P < 0.02$, $P < 0.03$, and $P < 0.02$, respectively).

Relationships between semilogarithmic growth of *N. oculata* and day of culture at the various temperature and salinity conditions are presented in Table 3. The slope of the linear relationship was highest in *N. oculata* at 25°C and salinity 30, and lowest for this alga at 15°C and 30, respectively.

DISCUSSION

Green algae such as *C. ellipsoidea* and *N. oculata* have been widely used not only as a food for live foods such as rotifers and *Artemia* nauplii for early larval fish, but also as a source for the green water effect in larval production of marine fish. Therefore, preparation of these algae in high quantity and quality is needed for successful seedling production of marine larval fish.

Many algae grew well over the broad ranges in both temperature and salinity, showing high tolerance to changes in these factors.[12] SGR, maximum density, and duration to reach maximum density of *C. ellipsoidea* and *N. oculata* were significantly affected by both temperature and salinity, except for maximum density of *N. oculata* in this study. Regardless of salinity, the maximum density was very low in *C. ellipsoidea* at 30°C in this study. This result probably indicates that high temperature is not optimum for growth of *C. ellipsoidea*, and agrees with Hur's[19] study. SGR and maximum density of *C. ellipsoidea* linearly decreased with an increase in salinity at 15°C and 20°C, but maximum density linearly increased with increase in salinity at 25°C and 30°C (Table 2). However, since

Table 4 Specific growth rate, maximum density, and duration to reach maximum density of *Nannochloris oculata* at various temperature and salinity conditions

Temperature (°C)	Salinity	SGR	Maximum density (10^6 cells/mL)	Duration to reach maximum density (d)
15	10	0.36 ± 0.004^{e,f}	154.0 ± 19.32^{d}	19 ± 0.6^{b,c}
	20	0.36 ± 0.014^{e,f}	139.4 ± 4.30^{d}	20 ± 0.6^{a,b,c}
	30	0.35 ± 0.005^{f}	121.0 ± 4.67^{d}	20 ± 0.0^{a,b}
20	10	0.44 ± 0.014^{a,b}	154.2 ± 5.72^{d}	16 ± 0.0^{d,e}
	20	0.44 ± 0.012^{a,b,c}	150.7 ± 7.15^{d}	16 ± 0.0^{f}
	30	0.41 ± 0.009^{b,c,d}	139.8 ± 2.67^{d}	17 ± 0.0^{e,f}
25	10	0.46 ± 0.017^{a}	227.0 ± 45.03^{b,c}	18 ± 1.7^{f}
	20	0.43 ± 0.003^{b,c}	238.1 ± 38.11^{b}	19 ± 0.6^{b,c}
	30	0.42 ± 0.023^{b,c}	308.0 ± 11.51^{a}	21 ± 0.6^{a}
30	10	0.41 ± 0.009^{c,d}	192.7 ± 11.31^{c}	19 ± 0.6^{c,d}
	20	0.37 ± 0.012^{e,f}	208.0 ± 16.17^{b,c}	19 ± 0.6^{b,c}
	30	0.38 ± 0.036^{d,e}	230.7 ± 1947^{b}	18 ± 0.0^{d,e}
Two-way ANOVA				
Temperature		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Salinity		$P < 0.0020$	$P < 0.0800$	$P < 0.0070$
Interaction		$P < 0.2000$	$P < 0.0010$	$P < 0.0040$

Values (mean ± standard deviation) with different superscript letters in the same column are significantly different ($P < 0.05$).
SGR, specific growth rate.

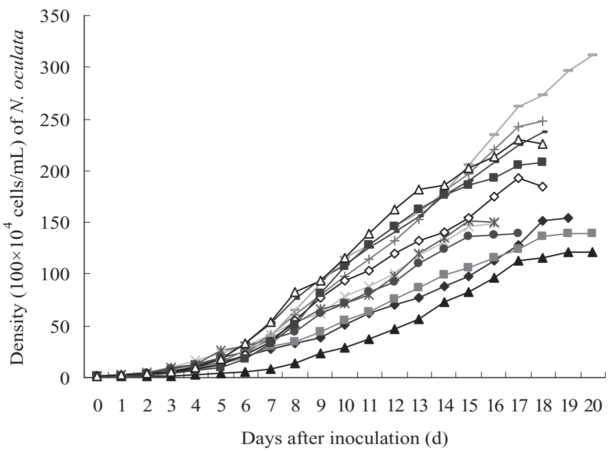

Fig. 2 Density of *Nannochloris oculata* after inoculation at temperature and salinity conditions: 15°C and salinity 10 (◆), 20 (■), and 30 (▲); 20°C and salinity 10 (×), 20 (★), and 30 (●); 25°C and salinity 10 (+), 20 (-), and 30 (–); 30°C and salinity 10 (◇), 20 (■), and 30 (△).

maximum density was achieved in *C. ellipsoidea* at 15°C and salinity 10, which was the lowest temperature in this study, it is possible that the optimum temperature for this alga is lower than 15°C.

In addition, a relatively high maximum density of *C. ellipsoidea* at low temperature (15 and 20°C) rather than high temperature (25 and 30°C) at all salinities tested in this study, except for 25°C and salinity 30, indicated that lower temperature is better to achieve the maximum density of *C. ellipsoidea*. Although the highest SGR was achieved in *C. ellipsoidea* at 25°C and 10, the relatively low slope between growth of this alga and days of culture, and maximum density probably indicate that the condition of 25°C and 10 is not optimum for growth of *C. ellipsoidea*.

SGR and duration to reach maximum density of *C. ellipsoidea* at 25°C and 30 were relatively high and short, respectively, compared to those of *C. ellipsoidea* at 15°C and 20°C at all salinities tested in this study. Hur[32] also showed that *C. ellipsoidea* achieved satisfactory growth at 28°C, but there was exponential growth retardation above 30°C. However, the condition of 15°C and salinity 10, which achieved the highest slope between growth of *C. ellipsoidea* and days of culture, seemed to be the best for growth of *C. ellipsoidea* at all conditions tested in this study.

Unlike growth of *C. ellipsoidea*, *N. oculata* seemed to grow relatively well at all conditions tested in this study. SGR of *N. oculata* linearly decreased with the increase in salinity at 25°C (Table 2). The maximum density linearly decreased with increase in salinity at 15°C, but linearly increased at 25°C and 30°C. The highest SGR and maximum density were achieved for *N. oculata* at 25°C and salinities 10 and 30, respectively, indicating the optimal temperature was 25°C for growth of *N. oculata*. This result is supported by the relatively high slope of the linear relationship between growth of *N. oculata* and day of culture at 25°C for all salinities tested in this study. Similarly, Terlizzi and Karlander[33] reported a positive correlation between temperature and growth of *N. oculata* from 20–25°C. Thus, the optimal salinity condition for growth of *N. oculata* in this study could vary depending on its purpose. For the purpose of achieving the maximum density of *N. oculata*, the optimal salinity condition was 30, and it was 10 for the purpose of fast growth (high SGR), at 25°C.

The relatively high maximum density of *N. oculata* at high temperature (25 and 30°C) rather than low temperature (15 and 20°C) at all salinities tested in this study indicate that high temperature is better for achieving the maximum density of *N. oculata*. Similarly, Hur[19] mentions that *N. oculata* is more stable than marine *Chlorella* as food for rotifers above 30°C.

The results of two-way ANOVA of growth of *C. ellipsoidea* and *N. oculata* on temperature and salinity showed significant effects of temperature, salinity, and a temperature–salinity interaction on the SGR, maximum density, and duration to reach maximum density (Tables 1 and 4). For both *C. ellipsoidea* and *N. oculata*, the main effect of temperature was significant at $P < 0.01\%$, which was equal to or greater than that of salinity and two–factor interaction, and it probably indicates that temperature is more important than salinity in determining growth of both algae. A similar result was observed in red tide flagellates *Heterocapsa circularisquama* and *Chattonella verruculosa*.[34]

The maximum density of *C. ellipsoidea* was lower than that of *N. oculata* at the same culture conditions in this study, probably resulting from the differences in cell size of the two algae. The cell size of *C. ellipsoidea* (3 μm diameter) was three times larger than that of *N. oculata* (1 μm diameter). Optimal culture conditions (temperature and salinity) for growth of two green algae were determined in 250-mL flasks (i.e. lab scale) in this study. Since large quantities of green algae are needed for marine fish hatcheries, further study is needed to determine optimal culture conditions for outdoor tanks or at a commercial scale.

Direct application of these algae as food for the first live foods, or to the rearing tanks for marine larval fish should be avoided due to high gap in salinity, although relatively high maximum density was achieved in *C. ellipsoidea* and *N. oculata* at low salinities (10 and 20) in this study. The chemical composition (fatty acid composition) of microalgae is also affected by environmental culture conditions such as temperature, salinity, light, and nutrients.[16,18,35] Therefore, further studies on nutrient composition of green algae at the various culture conditions are needed.

CONCLUSION

Low temperature (15 and 20°C) with low salinity (10 and 20) conditions were better for growth of *C. ellipsoidea*. On the contrary, high temperature (25 and 30°C) with high salinity (30) were better conditions for growth of *N. oculata*. Optimal temperature and salinity to achieve maximum density of *C. ellipsoidea* were 15°C and 10, respectively. However, for *N. oculata*, optimal temperature and salinity conditions were, respectively, 25°C and 10 for SGR, and 25°C and 30 for maximum density.

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