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Growth and lipid accumulation properties of a freshwater microalga *Scenedesmus* sp. under different cultivation temperature

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ABSTRACT

Microalgal lipid is a promising feedstock for biodiesel production. Effect of cultivation temperature on the growth and lipid accumulation properties of a freshwater microalga *Scenedesmus* sp. LX1 was studied. *Scenedesmus* sp. LX1 could grow in a wide range of temperature (10 ~ 30 °C), and the growth activation energy was 49.3 kJ·mol^{-1}. The optimal temperature to produce microalgal biomass and lipid was 20 °C, and after 15 days of batch cultivation the productivities of 313.3 g biomass·(g P)$^{-1}$, 112 g lipid (g P)$^{-1}$ and 14.7 g TAGs·(g P)$^{-1}$ were obtained. The content of polyunsaturated fatty acids decreased with the increase of cultivation temperature. The reactive oxygen species (ROS) levels at 10 °C and 20 °C were higher than that under higher temperature. For the first time the cultivation temperature, ROS level, specific growth rate and lipid content per microalgal biomass were correlated together.

1. Introduction

Due to the quick development of human activities and over consumption of fossil fuels, the energy crisis is representing the largest challenge of the 21st century, and the oil and natural gas storage on earth has been estimated to be depleted in 40 and 64 years, respectively (Vasudevan and Briggs, 2008). Meanwhile, the over consumption of fossil fuels also releases amounts of greenhouse gases and aggravates the global warming (Bruce, 2008). Therefore, sustainable and green energy needs to be exploited to handle the depletion of fossil fuels.

Biodiesel is a renewable, non-toxic, biodegradable and CO_2-neutral energy source (Miao and Wu, 2006). So in recent years it has become a hot topic for the exploitation of renewable and environment-friendly energy forms (Groom et al., 2008). Comparing with the conventional oil crops, microalgae are more attractive as feedstock for biodiesel production, due to their high photosynthesis efficiency and lipid content. The microalgal lipid productivity/biomass (dry weight) is about 15–300 times that of conventional crops (Chisti, 2008). Besides that, microalgae have the function of removing nitrogen and phosphorus (Khan and Yoshida, 2008; Li et al., 2010b) and fixing CO_2 (Fernández et al., 2008), making the coupling of bioenergy production and wastewater treatment based on microalgae as a promising technology in the future (Li et al., 2010c).

Therefore, microalgae-based biodiesel has attracted more and more attention worldwide. However, the high cost of biodiesel production is the main bottleneck of its commercial application (Behzadi and Farid, 2007). In order to reduce the total cost of biodiesel production, increase of the lipid content per microalgal biomass is one of the efficient methods to realize this purpose.

Recently much work has been done in this research field. Nutrient limitation is one of the most efficient triggers to enhance lipid accumulation in single microalgal cell when energy source (light) and carbon source (CO_2) are available (Courchesne et al., 2009), but high lipid content and high lipid productivity are in contradiction with each other, due to the low microalgal biomass productivity at low nutrient concentration (Li et al., 2010a). Adding organic carbon into heterotrophic microalgal cultures can increase both lipid content and microalgal biomass productivity (Han et al., 2006), but this increases the extra feedstock cost and the energy transformation efficiency is low (Li et al., 2010c). Other factors affecting lipid accumulation in microalgal cells have also been studied, such as light conditions (Solovchenko et al., 2008), ferric ions (Liu et al., 2008), temperature (Converti et al., 2009), ethyl-2-methyl acetoacetate (EMA, antialgal allelochemical) (Li et al., 2010d), and so on.

Among all these factors mentioned above, temperature is a very sensitive factor for microalgal growth and metabolic activities in microalgal cells. Meanwhile, it is also an easy-control factor in the practical operation of microalgae cultivation. In Converti et al. (2009) study an increase of the cultivation temperature from 20 to 25 °C doubled the lipid content of *Nannochloropsis oculata* (from 7.9% to 14.9%), showing that temperature control may be a possible approach to enhance the lipid productivity.

Previously a freshwater microalga *Scenedesmus* sp. LX1 which is suitable for the coupling system of biodiesel production and

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wastewater treatment was isolated by the authors. In this paper the effect of temperature on the growth and lipid accumulation properties of *Scenedesmus* sp. LX1 was studied, and for the first time the reactive oxygen species (ROS) level was investigated to explain the lipid-content change under different cultivation temperature.

2. Methods

2.1. Microorganism

The *Scenedesmus* sp. LX1 (Collection No. CGMCC 3036 in China General Microbiological Culture Collection Center) was isolated previously (Li et al., 2010a).

2.2. Experimental set up

The microalga was cultured in 200 mL modified 50% BG11 medium (Li et al., 2010a) in 500-mL Erlenmeyer flasks. TN and TP in the culture medium was 15 and 1.5 mg·L^{-1}, with NO_3-N and PO_4-P as nitrogen and phosphorus sources, respectively. Other elements in the culture medium were the same as 50% BG11 medium.

The initial microalgal density was about 6.5×10^5 cells·mL^{-1}. The cultivation conditions were: light intensity 55 ~ 60 μmol photon·m^{-2}·s^{-1}, light/dark periods of 14/10 h, relative humidity 75%. The cultivation temperature was controlled at 10, 20, 25 and 30 °C, respectively.All tests were carried out in triplicate (n = 3).

2.3. Analytical methods

2.3.1. Microalgal growth property

The microalgal density was determined by measuring the OD_{650} (the optical density of algal culture at 650 nm) every 24 h. The relationship between microalgal density (D, cells·mL^{-1}) and OD_{650} is as shown in Eq. (1) (Li et al., 2010a):

$$D = 9.52 \times 10^6\ OD_{650} + 70957, R = 0.997 \quad (1)$$

The dry weight of algal biomass was determined using the method of suspended solid (SS) measurement according to Li et al. (2010c).

The Logistic model was used to describe the microalgal growth (Li et al., 2010a):

$$N = \frac{K}{1 + e^{a - rt}} \quad (2)$$

$$R_{\max} = \frac{rK}{4} \quad (3)$$

where N (cells·mL^{-1}) is the algal density at time t (h), K (cells·mL^{-1}) is the carrying capacity (the maximum algal density reached in the culture), a is a constant in the logistic model which indicates the relative position from the origin, r (d^{-1}) is the intrinsic growth rate, and $R_{\max}$ (cells·(mL·d)$^{-1}$) is the maximum population growth rate.

The Arrhenius Model was applied to study the relationship between the microalgal specific growth rate and temperature (Eq. (4)).

$$\mu = A \cdot \exp\left(-\frac{E_a}{RT}\right) \quad (4)$$

Where μ (d^{-1}) is the specific growth rate in logarithmic growth phase, which can be calculated by Eq. (5), A (d^{-1}) is the Arrhenius constant, E_a (kJ·mol^{-1}) is the growth activation energy, R [J·(mol·K)$^{-1}$] is the universal gas constant, and T (K) is thermodynamic temperature.

$$\mu = \frac{\ln N_{t_2} - \ln N_{t_1}}{t_2 - t_1} \quad (5)$$

where t_1 and t_2 is the beginning and end of the logarithmic growth phase, and N_{t_1} and N_{t_2} is the microalgal density at the time of t_1 and t_2, respectively.

Eq. (4) could be transformed into a linear form as in Eq. (6). In specific temperature range, $\ln\mu$ has a linear relationship with $1/T$, and a regression line with a slope equal to $-\frac{E_a}{R}$ could be obtained.

$$\ln \mu = \ln A - \frac{E_a}{R \cdot T} \quad (6)$$

2.3.2. Lipid accumulation property

The total lipid content (dry weight) was measured as described by Li et al. (2010a).

After the measurement of total lipid, the dried lipid was dissolved in 0.4 mL of isopropyl alcohol. Then the triacylglycerols (TAGs) were estimated as Li et al. (2010d).

For the fatty acid analysis, about 20 mg freeze-dried microalgal powdered sample was added with 2 mL 6% KOH of methanol–water solution ($V_{methanol}$:V_{water} = 4:1) and 0.2 mL chloroform, sonicated for 20 min and then saponified for 2 h in 80 °C water bath. After the saponification the microalgal sample was added with 1 mL saturated NaCl solution and the pH was adjusted to below 1 by HCl, then the sample was added with 2 mL extraction solution ($V_{chloroform}$:V_{hexane} = 1:4) and the extracted liquid was blew to dryness by nitrogen flow. Then 0.5 mL HCl of methanol solution (2 mol·L^{-1}) were added and the sample was esterified for 20 min in 70 °C water bath under nitrogen flow. After that 1 mL saturated NaCl solution was added and the sample was extracted with 2 mL petroleum ether. Then the fatty acid methyl esters (FAMEs) in extracted liquid were analyzed by GC–MS (QP2010, SHIMADZU) equipped with FID using HP-5MS capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness) with a temperature programming 80 to 280 °C at a rate of 6 °C·min^{-1}. The FAMEs were identified by comparing their fragmentation pattern with NIST library.

2.3.3. Flow cytometric analysis of reactive oxygen species (ROS) level

The effect of temperature on ROS level was investigated by flow cytometric analysis using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Fluka Company). DCFH-DA is a popular indicator for monitoring the intracellular generation of ROS. ROS in the microalgal cells promotes the oxidation of DCFH to yield the fluorescent product 2′, 7′-dichlorofluorescein (DCF). The treatment of microalgal cells and the ROS level determination were according to Hong et al. (2008). The flow cytometric measurements were performed on a FACS Calibur (Becton Dickinson, USA) at the FL-1 parameter. The DCF fluorescence (emission wavelength: 530 nm) is excited with Argon-ion laser (excitation wavelength: 488 nm).

2.3.4. Significant difference analysis

Paired-samples t-test and Independent-samples t-test were used for significant difference analysis, respectively, by using SPSS (version 13.0) statistical software.

3. Results and discussion

3.1. Effect of temperature on Scenedesmus sp. LX1's growth

The growth curves of *Scenedesmus* sp. LX1 in the growth medium for different cultivation temperature are shown in Fig. 1. After 15 days of cultivation *Scenedesmus* sp. LX1 grew into the stable phase and the maximum microalgal densities under

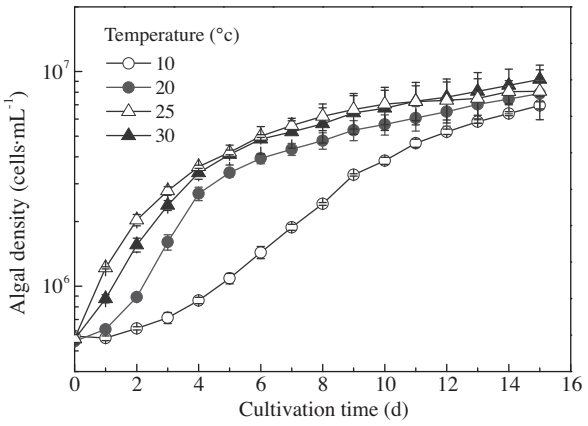

Fig. 1. Growth curves of *Scenedesmus* sp. LX1 in growth medium for different cultivation temperature.

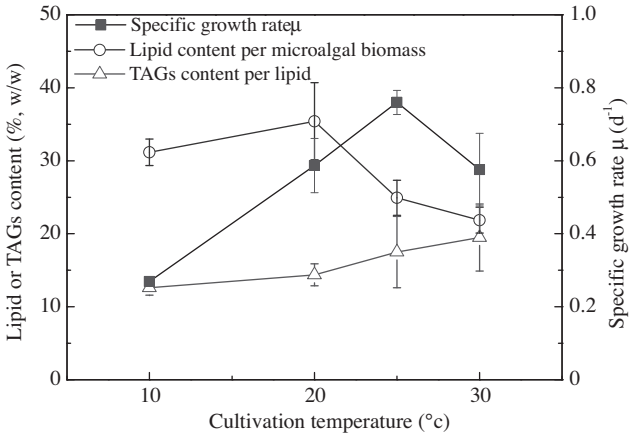

Fig. 2. Specific growth rate μ, lipid content per microalgal biomass (%, g/g, dry weight) and TAGs content per lipid (%, g/g, dry weight) of *Scenedesmus* sp. LX1 for different cultivation temperature.

different temperature had no significant difference ($p > 0.12$). Through Paired-samples *t*-test, microalgal densities in the cultivation process at the temperature of 20, 25 and 30 °C were significantly higher than the one at 10 °C ($p < 0.001$). At 10 °C *Scenedesmus* sp. LX1 grew very slowly at the beginning of cultivation. However, after 3 days of adaptation the microalgal growth rate began to increase and finally the microalga had almost the same maximum microalgal density with the ones at higher temperature, which indicates that *Scenedesmus* sp. LX1 has the ability of growing in cold area.

Through linear regression analysis (Logistic Model) (Li et al., 2010a) of the growth data in Fig. 1, the *K*, R_{max} and *r* of *Scenedesmus* sp. LX1 for different cultivation temperature were obtained and are shown in Table 1. There was positive correlation between *K* (R_{max}) and cultivation temperature from 10 to 30 °C, but the differences under different temperature were not significant ($p > 0.12$ for *K* and $p > 0.09$ for R_{max}). The *r* indicates the intrinsic multiplication rate of a population under the ideal growth conditions without environmental limitations (e.g. enough food and space, no predators or disease). The *r* of *Scenedesmus* sp. LX1 under different temperature had no significant difference ($p > 0.05$), indicating the growth potential of this microalga under different cultivation temperature was the same.

Through Eq. (5) the specific growth rate μ of *Scenedesmus* sp. LX1 in logarithmic growth phase under different cultivation temperature could be obtained and is shown in Fig. 2. From 10 to 25 °C, the specific growth rate μ had a positive correlation with the cultivation temperature. When the temperature increased to 30 °C, the μ began to drop. The maximum μ (0.76 d^{-1}) was obtained at the cultivation temperature of 25 °C.

By linear regression analysis (Eq. (6)) it is found that the specific growth rate μ of *Scenedesmus* sp. LX1 in the temperature range of 10 ~ 25 °C fit the Arrhenius Model. The following Arrhenius parameters were obtained: Arrhenius constant $A = \exp(19.7)$ d^{-1}, the growth activation energy $E_a = 49.3$ kJ·mol^{-1} ($p < 0.01$). Therefore, the Arrhenius Model in the temperature range of 10 ~ 25 °C can be calculated as Eq. (7).

$$\mu = \exp\left(19.7 - \frac{49.3}{R}\cdot\frac{1}{T}\right) \quad (7)$$

The influence of cultivation temperature on microorganism's growth may be species-dependent. Converti et al. (2009) and Chen et al. (2008) studied the optimal cultivation temperature for the specific growth rate of *Nannochloropsis oculata* and *Nitzschia laevis*, and the result was 20 and 23 °C, respectively. In Westerhoff et al.'s study of *Scenedesmus* and *Chlorella*, the exponential growth rate constant ($\mu = 0.03$ h^{-1}) did not vary between 27 and 39 °C, but at 42 °C the microalgae could not grow (Westerhoff et al., 2010). In our study, at 25 °C *Scenedesmus* sp. LX1 got the maximum specific growth rate μ (Fig. 2); and at 30 °C it had the maximum carrying capacity *K* and population growth rate R_{max} (Table 1). Meaningfully, at the cultivation temperature between 10 and 30 °C *Scenedesmus* sp. LX1 could all grow well after it adapts to the ambient environment. This indicates *Scenedesmus* sp. LX1 is a promising microalgal species which could adapt to a wide temperature range (10 ~ 30 °C) for its growth.

3.2. Effect of temperature on lipid accumulation property of *Scenedesmus sp. LX1*

The lipid content per microalgal biomass (%, g/g, dry weight) and TAGs content per lipid (%, g/g, dry weight) of *Scenedesmus* sp. LX1 after 15 days of cultivation for different cultivation temperature are shown in Fig. 2. At 25 °C which is the normal temperature for microalgal cultivation, the lipid content per microalgal biomass was about 25% (w/w). At lower temperature of 10 and 20 °C, the lipid content per microalgal biomass was about 31% (w/w) and 35% (w/w), significantly (by Independent-samples *t*-test) higher than the one at 25 °C by 25% ($p < 0.02$) and 42% ($p < 0.05$), respectively. At higher temperature of 30 °C, the lipid content per microalgal biomass was 22% (w/w), but the difference from the one at 25 °C was not significant ($p > 0.15$). The TAGs content per lipid had a positive correlation with the cultivation temperature, especially above 20 °C, but the increasing trend was not significant ($p > 0.06$).

The microalgal biomass, lipid and TAGs productivities (dry weight) of *Scenedesmus* sp. LX1 for different cultivation temperature are shown in Table 2. At low temperature of 10 °C the microalgal biomass productivity was the lowest ($p < 0.001$) among all the experimental results. And due to the low microalgal biomass,

Table 1
Carrying capacity (*K*), maximum population growth rate (R_{max}) and intrinsic growth rate (*r*) of *Scenedesmus* sp. LX1 for different cultivation temperature ($p < 0.01$).

Temperature (°C)	10	20	25	30
K ± S.D. (10^6 cells·mL^{-1})	7.0 ± 0.3	7.9 ± 1.2	8.5 ± 1.7	9.0 ± 1.5
R_{max} ± S.D. [10^6 cells·(mL·d)$^{-1}$]	0.60 ± 0.02	0.71 ± 0.13	0.80 ± 0.15	0.85 ± 0.14
r ± S.D. (d^{-1})	0.35 ± 0.01	0.36 ± 0.01	0.38 ± 0.02	0.37 ± 0.01

Table 2
Microalgal biomass, lipid and TAGs productivities (dry weight) of *Scenedesmus* sp. LX1 for different cultivation temperature.

Temperature (°C)	10	20	25	30
Biomass ± S.D.(g biomass·(g P)$^{-1}$)	200.0 ± 6.7	313.3 ± 6.7	340.0 ± 13.3	340.0 ± 6.7
Lipid productivity ± S.D.(g lipid·(g P)$^{-1}$)	62.0 ± 3.3	112.0 ± 18.7	85.3 ± 4.7	74.7 ± 6.0
TAGs productivity ± S.D.(g TAGs·(g P)$^{-1}$)	8.0 ± 0.7	14.7 ± 1.3	14.7 ± 4.0	14.7 ± 3.3

the lipid and TAGs productivities were also very low. The microalgal biomass and TAGs productivities were almost the same at the temperature of 20, 25 and 30 °C. At 20 °C the lipid productivity was the highest, and comparing with the results at 25 and 30 °C, it was significantly ($p < 0.05$) increased by 31% and 50%, respectively. Therefore, for the optimization of microalgal cultivation to produce microalgal biomass and lipid, the optimal cultivation temperature for *Scenedesmus* sp. LX1 should be 20 °C.

Converti et al. (2009) found that higher temperature (from 20 to 25 °C) was benefit for the lipid accumulation in *N. oculata*'s cells. In Chen et al. (2008) study, they reported that the cultivation temperature had little effect on the lipid content of microalgal biomass, but the TAGs content decreased with the decrease of temperature. The effect of temperature on the lipid content per microalgal biomass of *Scenedesmus* sp. LX1 was different from either of the above. In our study, low temperature would induce the lipid accumulation in *Scenedesmus* sp. LX1's cells and result in a high lipid content. However, the TAGs content per lipid was decrease with the decrease of temperature, the same as reported by Chen et al. (2008).

Many other approaches have also been studied to enhance the microalgal lipid productivity besides temperature adjustment. Li et al. obtained high lipid content per microalgal biomass of *Scenedesmus* sp. LX1 under nitrogen or phosphorus limitation, but the lipid productivity was not high due to the low microalgal biomass productivity (Li et al., 2010a). In another study of Li et al., trace amount of ethyl-2-methyl acetoacetate (EMA) could increase TAGs accumulation in microalgal cells of *Scenedesmus* sp. LX1 without negative effect on microalgal biomass productivity, which resulted in both high TAGs content per microalgal biomass and high TAGs productivity (Li et al., 2010d). Temperature is a sensitive parameter to microalgal growth and their metabolic activities. In the coupled system of wastewater treatment and microalgal biodiesel production, enhancing lipid productivity via temperature adjustment has an advantage that the original composition of wastewater does not need to be changed or added by other chemicals, making it convenient for operation and ecologically safe.

The fatty acid composition of *Scenedesmus* sp. LX1 for different cultivation temperature is shown in Fig. 3. While the cultivation temperature increasing, the ratio of the sum of saturated and monounsaturated fatty acids increased, and the polyunsaturated fatty acids decreased. At relatively low (10 °C) or high (30 °C) temperature, the fatty acids in *Scenedesmus* sp. LX1's cells were almost 100% composed of long-chain species (C16 ~ C18). In the middle temperature range (20 and 25 °C) the fatty acids were mainly very-long-chain species (C22).

The saturation and fatty acid profile would affect the properties of biodiesel. Hu et al. (2008) reported that saturated fats produce a biodiesel with superior oxidative stability and a higher cetane number, but rather poor low-temperature properties (to gel at ambient temperature). And feedstock rich in polyunsaturated fatty acids produce a biodiesel with good cold-flow properties, but susceptible to oxidation. In our study, at relatively low cultivation temperature (⩽25 °C) the fatty acids of *Scenedesmus* sp. LX1 were mainly composed of polyunsaturated fatty acids, at high cultivation temperature (30 °C) the fatty acids are mainly composed of saturated and monounsaturated fatty acids. The fatty acid profile of *Scenedesmus* sp. LX1 under different temperature makes the microalgal cells (cell structures containing lipid) adaptable to the ambient temperature, and also makes the microalgae-based biodiesel suitable to be utilized in cold or warm area in accordance with the cultivation temperature under which the feedstock are obtained.

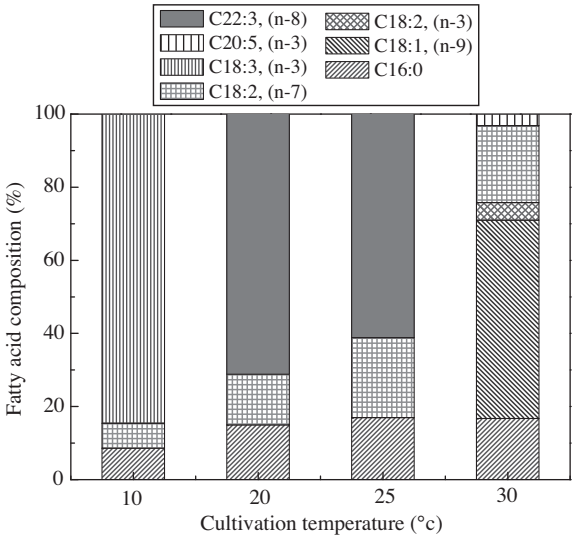

Fig. 3. Fatty acid composition (%) of *Scenedesmus* sp. LX1 for different cultivation temperature.

3.3. Effect of temperature on ROS level of Scenedesmus sp. LX1

The Fluorescence intensity of microalgal cells of *Scenedesmus* sp. LX1 for different cultivation temperature is shown in Fig. 4. At 25 or 30 °C, the fluorescence intensity for most of the microalgal cells (F_{main}, which is relevant to the ROS level) was the same (1.5×10^2 AU). When the cultivation temperature decreased to 20 °C, F_{main} (4×10^2 AU) was increased a little. At the lowest cultivation temperature of 10 °C, F_{main} (30×10^2 AU) was increased a lot very obviously. This indicates that low cultivation temperature could

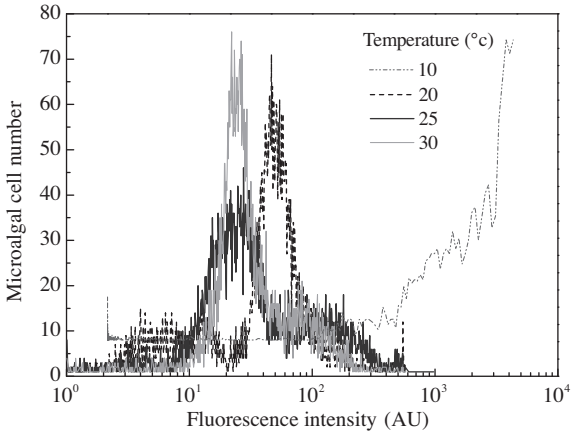

Fig. 4. Fluorescence intensity of microalgal cells of *Scenedesmus* sp. LX1 for different cultivation temperature.

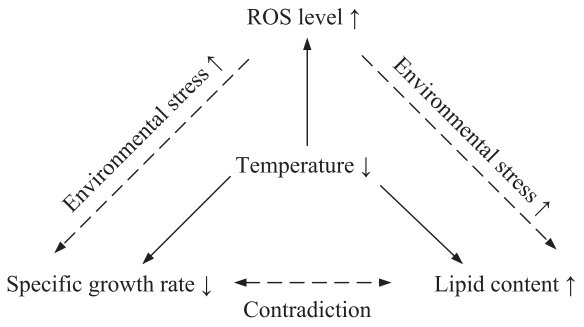

Fig. 5. Relationship among cultivation temperature, ROS level, specific growth rate and lipid content per microalgal biomass of *Scenedesmus* sp. LX1 (↑, increase; ↓, decrease).

induce the accumulation of ROS in the microalgal cells of *Scenedesmus* sp. LX1.

To our knowledge, the lipid accumulation of microalgae has never been correlated with the ROS level of microalgal cells before. The relationship among cultivation temperature, ROS level, specific growth rate and lipid content per microalgal biomass of *Scenedesmus* sp. LX1 is shown in Fig. 5. The ROS is well known for its role in the stress-response signal transduction pathway, and it would accumulate in microalgal cells when microalgae are exposed to the various environmental stresses, but over-accumulation of ROS can result in cell death (Hong et al., 2008). At low cultivation temperature the ROS level in microalgal cells of *Scenedesmus* sp. LX1 was arising, indicating that low temperature was a certain environmental stress for *Scenedesmus* sp. LX1. Therefore, under this environmental stress the specific growth rate of *Scenedesmus* sp. LX1 decreased, and the lipid content per microalgal biomass increased (Fig. 2). The increase of lipid content was in accordance with the common idea that environmental stress is an effective trigger of lipid accumulation in microalgal cells (Hu et al., 2008). However, the high growth rate and high lipid content of microalgae are in contradiction, they can hardly be both high simultaneously. This contradiction is also a difficult problem in the research field of microalgal lipid production (Sheehan et al., 1998).

4. Conclusions

Scenedesmus sp. LX1 could grow in a wide range of temperature ($10 \sim 30$ °C), and the growth activation energy E_a was 49.3 kJ·mol^{-1}.

The optimal temperature for *Scenedesmus* sp. LX1 to produce microalgal biomass and lipid was 20 °C. The productivities of 313.3 g biomass·(g P)$^{-1}$, 112 g lipid·(g P)$^{-1}$ and 14.7 g TAGs·(g P)$^{-1}$ were obtained.

Low temperature (10 and 20 °C) would increase the ROS level of microalgal cells. For the first time the high lipid content of microalga was correlated with high ROS level at low cultivation temperature.

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