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Linear alkylbenzene sulfonate (LAS) promotes sedimentation and lipid accumulation in *Scenedesmus obliquus*

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The lipids found in microalgae are promising raw materials for bio-diesel production. This study tests the effects of linear alkylbenzene sulfonate (LAS) on the growth, colony formation, sedimentation and lipid content of the green alga *Scenedesmus obliquus*. Growth of *S. obliquus* was unaffected by LAS below concentrations of 100 mg L^{-1}. LAS treatment promoted colony formation and the number of cells per aggregate significantly increased with rising LAS concentration. Both optic and scanning electron microphotoes showed that the induced colony of *S. obliquus* was regular and there were clear attachment points between two neighboring cells. The sedimentation efficiency at 10 min significantly increased with the increasing LAS concentration. Under treatment of 100 mg L^{-1} LAS, the sedimentation efficiency was 69.0% after 40 minutes' settling, which was 30% higher than that in the control group. The sedimentation efficiency was positively related to cells per colony at all sedimentation time except for 60 min. Moreover, the lipid content was 21.2% when treated with 100 mg L^{-1} LAS, which was 1.18 times higher than in the control group. The main fatty acid composition of *S. obliquus* was α-linolenic acid (C18:3) and palmitic acid (C16:0). The degree of fatty acid unsaturation was not obviously affected by LAS addition but the proportion of fatty acid with the chain length ranging from C16 to C18 was promoted from 70.9% to 84.2% by LAS addition. This result indicated that LAS addition was beneficial to improve the quality of algal biodiesel. Our results suggested that moderate doses of LAS can effectively enhance the sedimentation efficiency of *S. obliquus* by inducing colony formation and improving the lipid content of the cells without inhibiting their growth.

1. Introduction

Microalgae is considered a promising raw material for bio-diesel production because of their fast growth rate and high lipid contents.[1] *Scenedesmus obliquus* is among the most widely used lipid-producing microalgae. Although its lipid content is not very high,[2] its lipid composition is suitable for biodiesel synthesis.[3] Moreover, this algal species can efficiently remove nitrogen and phosphorus from wastewater, so it can be cultivated for nutrient removal from wastewater.[1] Nevertheless, *S. obliquus* cells are too small (3–20 μm) to be harvested without energy-intensive filtration and centrifugation methods, which greatly increases the cost of microalgae diesel production.[4]

Consequently, it is still necessary to develop economical methods for biomass harvesting of *S. obliquus*.

Algal biomasses are frequently harvested by adding synthetic polymer flocculants and metal coagulants, which stimulate single cells to form large aggregates.[4] Generally, this method cannot be widely applied because synthetic polymer flocculants are expensive and metal coagulants are toxic to algal cells.[5] Alkaline flocculation is also an alternative method for algae harvest. However, the sedimentation efficiency of alkaline flocculation was always inhibited by the extracellular polymeric substances (EPS) produced by algal cells even though addition of minim alkali is cheap.[4] Therefore, a cheap and effective chemical that promotes algal biomass harvesting is imperative.

In an alternative method, Yang and Li[6] stimulated colony formation of unicellular *S. obliquus* by adding *Daphnia* culture filtrates. The filtrates were considered to significantly increase the colony size of *S. obliquus* and hence improve the harvesting efficiency.[7] Nevertheless, large *Daphnia* culture filtrates themselves consume much energy and demand significant financial resources. Low light intensity was also reported to induce colony formation of *S. obliquus*[8] whereas algal biomass growth would also be inhibited due to low light intensity. Therefore, the gains of this method cannot compensate the losses.

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Li *et al.*[9] demonstrated that 100 and 1000 mg L^{-1} linear alkylbenzene sulfonate (LAS) induced colony formation of *S. obliquus*. This phenomenon revealed that LAS would promote sedimentation of *S. obliquus* by inducing colony formation. Meanwhile, LAS is a widely used representative surfactant.[9] It is cheap, and easily manufactured and degraded. As a consequence, LAS is considered as a promising additive for promoting colony formation and sedimentation of *S. obliquus*. However, Li *et al.*[9] studied the effects of LAS on colony formation of *S. obliquus* and found two defects limiting the development of technology to promote sedimentation of *S. obliquus* by LAS addition. On one hand, the concentration gradient of LAS used in their work was too big to analyze the appropriate concentration of LAS.[9] On the other hand, the authors only investigated colony size of *S. obliquus* rather than the sedimentation efficiency.[9] Hitherto, there was not a clear finding illustrating the relationship between colony size and sedimentation efficiency of *S. obliquus* even in the work of Zhu *et al.*,[7] in which combined effects of nitrogen levels and *Daphnia* culture filtrate on colony size of *S. obliquus* were well studied. Therefore, it is still important to study the effects of LAS on colony size and sedimentation efficiency of *S. obliquus* with proper concentration gradient of LAS.

The algal fatty acids composition was another important factor affecting algal biodiesel production.[10,11] The increase in the degree of fatty acid unsaturation was in favor of high-quality biodiesel production because this production was more suitable for cold weather use due to a typically gel point.[12] Moreover, the chain length of fatty acids are also selective and the most optimum chain length was ranging from C16 to C18.[10,11] Our knowledge about the effects of LAS on algal fatty acid composition was seriously inadequate. Therefore, the effects of LAS addition on algal fatty acid composition should also be evaluated.

This study tests the effects of LAS addition on colony formation and sedimentation efficiency of *S. obliquus*. As lipid productivity and fatty acid composition are two important reference standards in algal diesel production,[13] the growth, lipid content and fatty acid composition of LAS-stimulated *S. obliquus* were also analyzed.

2. Materials and methods

2.1 Organisms and culture conditions

A unicellular strain of *S. obliquus* (FACHB 416) was purchased from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Science, Wuhan, China. This strain was cultured in BG-11 medium for more than 3 months under standard conditions with a 12:12 h light:dark cycle at 50 μmol photons m^{-2} s^{-1}. The temperature was set to 25 °C. In this environment, most of the algal cells were single or paired.

Under the same culture conditions, the algae in logarithmic-phase were inoculated in 150 mL BG-11 medium in 250 mL conical flasks with an initial cell density of 5×10^5 cells per mL. Various amount of LAS were added to the culture medium, and their concentrations were adjusted to 0, 25, 50, 100, 200 and 500 mg L^{-1}. LAS with a chemical formula of $C_{18}H_{29}NaO_3S$ was

obtained from Sigma Chemical Company (USA). Six replicates of each culture were prepared. The flasks were hand-shaken two to three times daily to prevent the cells from clinging to the inner walls of the flasks. Colony formation of algae always occurred in the logarithmic growth phase[14] but lipid content in the stable phase was much higher than that in the logarithmic growth phase.[15] Gan *et al.*[16] showed that the growth of *S. obliquus* entered the stable phase for day 16 and thus the experiment ran for 14 days in the current study as a compromise.

2.2 Cell counting

Cell density was analyzed daily. The cells were directly counted three times in a hemocytometer placed under an Olympus CX31 microscope (Olympus Corporation, Japan) at 400× magnification. If the three calculated results differed by less than 10%, they were averaged to obtain the final cell density. Otherwise, additional counting was conducted. At the end of the experiment, every two replicates were combined into a mixed sample. The number of cells per particle (where a particle denotes a single cell, a small aggregate or a colony) and the percentages of various particles were calculated by individually recording the numbers of particles and the numbers of cells per particle. At least 400 particles were counted in each sample.

2.3 Optic and scanning electron microphotoes

Microphotographs of *S. obliquus* were taken using the microscope equipped with an Olympus C-5050 digital camera. The scanning electron microphotoes were taken by a scanning electron microscope (S-4800, Hitachi, Japan). A drop of algal sample was placed on a cover slip and air dried. Then, it was chemically fixed using 4% (w/v) glutaraldehyde for 24 h. Afterwards, the samples were rinsed in distilled water thrice and dehydrated in graded series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) for 10 min each and air dried. Finally, these prepared samples were kept in desiccators until analysis of scanning electron microscope.

2.4 Assessment of sedimentation rate

The OD_{665} of the mixed sample was measured by a spectrophotometer (UV-1780, Shimadzu, Japan) and recorded as OD_i. Afterwards, six replicates of 15 mL culture of each mixed sample were injected into 15 mL centrifugal tubes respectively. All tubes were stored undisturbed at room temperature in darkness. The OD_{665} of the supernatant liquid in one of the replicates of each mixed sample was measured at 10 min intervals for 1 h, and recorded as OD_t, where t denotes the stand-by time. The sedimentation efficiency was then calculated by eqn (1) described by Gan *et al.*:[16]

$$\text{Sedimentation efficiency (\%)} = (1 - OD_t/OD_i) \times 100 \quad (1)$$

2.5 Lipid content analysis

The remaining culture medium of the mixed sample was centrifuged at $10\,000 \times g$ rpm for 6 min. The pellet was rinsed twice with deionized water and then dried at 60 °C for lipid

extraction. The photos of dried algae were taken by a camera (D60, Cannon, Japan). Algal lipid was extracted using the methanol–chloroform method proposed by Bligh and Dyer[17] and 100 mg dried biomass was used to be extracted for each replicate.

2.6 Fatty acid composition analysis

Fatty acid methyl esters were obtained following the method suggested by Lepage and Roy.[18] The dried lipid obtained in above section was completely dissolved by 4 mL 14% w/v solutions of BF_3–methanol and then, was refluxed by boiling water bath for 15 min. After cooling, 2 mL *n*-heptane and 4 mL NaCl saturated solution was added and the mixture was fully shocked. After stratification, the supernatant was filtrated using 0.2 μm polyvinylidene fluoride syringe microfilters. The fatty acid methyl esters (FAMEs) was analyzed using Shimadzu GC-2014C gas chromatography equipped with FID. A 60-m DB-5ms column was used. The injection temperature was 250 °C. The initial column temperature was 120 °C, held for 3 min. Then, the temperature increased to 220 °C at a increasing rate of 4 °C min^{-1}, held for 5 min. The final temperature increased to 280 °C at a increasing rate of 3 °C min^{-1}, held for 20 min.

2.7 Data statistics

All data in this study are presented as their means ± standard deviations. The specific growth rate (μ) was calculated by eqn (2):

$$\mu = \ln(D_t/D_0)/t \tag{2}$$

where D_t is the cell density at time t, D_0 is the initial cell density, and t is the culture time of this experiment. The differences among treatments were tested by ANOVA implemented in SPSS 10.0. A Pearson correlation analysis was also performed to determine the relationship between sedimentation efficiency at varying sedimentation time and cells per particle of *S. obliquus*.

3. Results

3.1 Growth of *S. obliquus*

The growth of *S. obliquus* was not obviously influenced by LAS concentrations below 100 mg L^{-1}. Merely, it was inhibited at higher concentrations (200 and 500 mg L^{-1}; Fig. 1). The maximum cell density reached 1.6×10^7 cells mL^{-1} at LAS concentrations below 100 mg L^{-1}, reducing to 1.4×10^7 and 1.2×10^7 cells mL^{-1} in the 200 and 500 mg L^{-1} LAS treatments, respectively. The specific growth rate was 0.25 per day when LAS concentration was below 100 mg L^{-1}. This value slightly decreased to 0.24 and 0.23 per day while LAS concentration increased to 200 and 500 mg L^{-1}, respectively.

3.2 Colony formation of *S. obliquus*

The LAS concentration affected the percentages of various particles in the cultures (Fig. 2a). In the control culture, most of the cells were single or double, with a considerable percentage (38.5%) of 4-cell aggregates. Under treatment with 25, 50 and 100 mg L^{-1} LAS, the proportion of 4-cell particles increased to
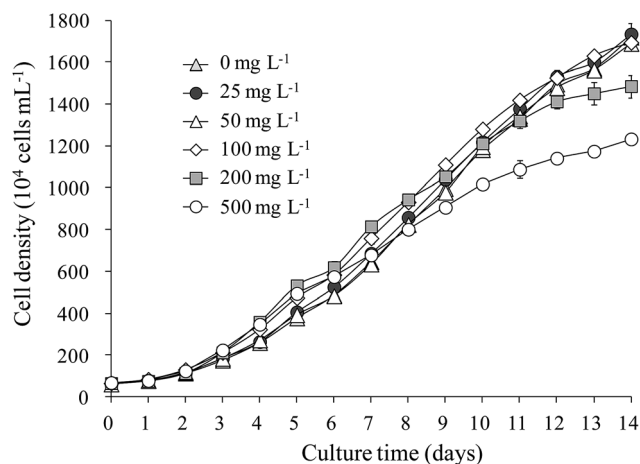

Fig. 1 Growth curves of *Scenedesmus obliquus* treated with various concentrations of LAS.

approximately 60%, while the percentages of single and double cells decreased to half of the control values. When treated with 200 and 500 mg L^{-1} LAS, the percentage of ≥8-cell particles was 38.5% and 51.7%, respectively, *versus* 1.2% in the control. The
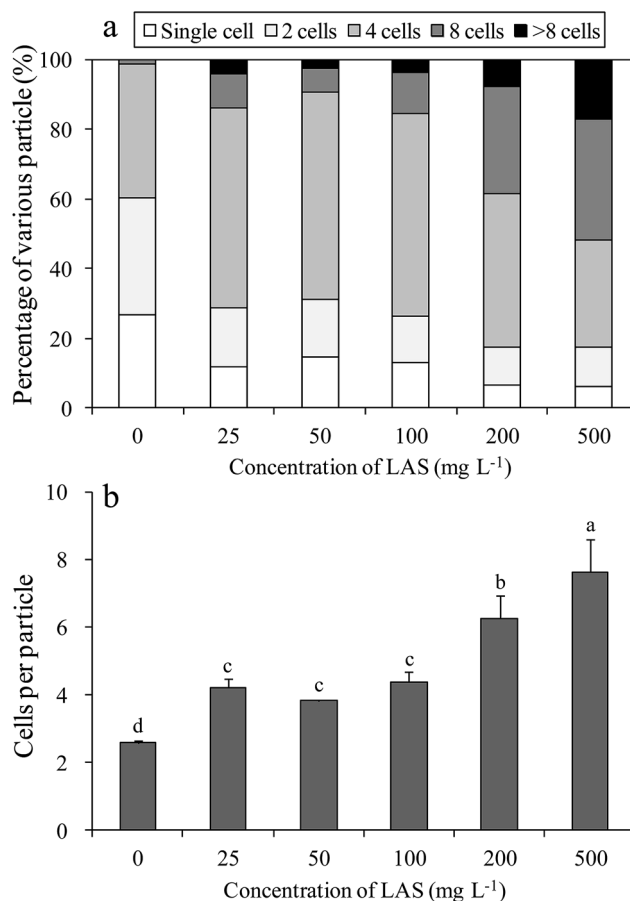

Fig. 2 Proportions of variously sized *S. obliquus* particles (a) and numbers of cells per particle (b) at various concentrations of LAS. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments.

number of cells per particle also increased significantly under LAS treatment (Fig. 2b). In the presence of 0 (control), 25, 50, 100, 200 and 500 mg L^{-1} LAS, the cells-per-particle counts were 2.6, 4.2, 3.8, 4.4, 6.3 and 7.6 respectively. The values in all the treatment were significantly higher than the control ($P < 0.05$).

3.3 Optic and scanning electron microphotoes

Fig. 3 showed the photos of harvested biomass as well as optical and scanning electron microphotoes (SEM) of *S. obliquus*. There was not obvious difference in the appearance of harvested biomass among various treatments. Both optic and scanning electron microphotoes showed that the induced colony of *S. obliquus* was regular and there were clear attachment points between two neighboring cells. The length and width of a single cell of *S. obliquus* was respectively 8 and 4 μm but the value of a colony comprised of four cells was 9 and 8 μm, respectively.

3.4 Sedimentation efficiency of *S. obliquus*

The sedimentation efficiency of *S. obliquus* increased with increasing LAS concentration. After 10 minutes' settling, the sedimentation efficiency was maximized at 67.2% under treatment with 500 mg L^{-1} LAS (Fig. 4), which was significantly higher than other treatments ($P < 0.05$). The values in all treatments increased at later times. At the end of the sedimentation experiment (60 minutes), the sedimentation efficiencies were 69.5%, 76.1% and 79.3% in the 0 (control), 100 and 500 mg L^{-1} LAS treatments, respectively. Notably, the sedimentation efficiency after 40 minutes' settling was 69.0% in the presence of 100 mg L^{-1} LAS, 30% higher than in the control group at that time, and approximately equal to the control value (69.5%) after 60 minutes' settling.

The relationship between sedimentation efficiency at varying sedimentation time and cells per particle of *S. obliquus* was shown in Fig. 5. The sedimentation efficiency was positively
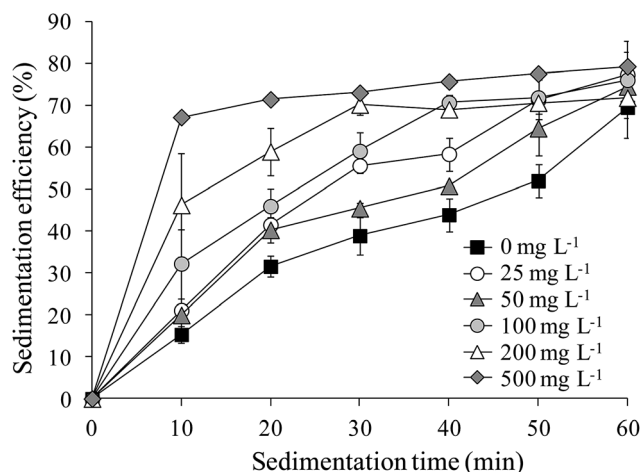
Fig. 4 Sedimentation efficiencies of *S. obliquus* treated with various concentrations of LAS.

related to cells per colony at all the sedimentation time except for 60 min. If cells per particle reached 8, sedimentation efficiency reached 60% at 10 min and the value was much higher than 70% at 20 min.

3.5 Lipid content and composition

All treatments significantly enhanced the lipid content over the control value (18.0%; Fig. 6). The highest lipid content (24.0%) was found in the 25 mg L^{-1} LAS treatment. The increase in this treatment was significantly greater than in other treatments. In the 100 mg L^{-1} treatment, the lipid content was 21.2% (1.18 times the control value).

The main fatty acid composition of *S. obliquus* was α-linolenic acid (C18:3) and palmitic acid (C16:0) showing in Table 1. The proportion of α-linolenic acid (C18:3) was not affected by
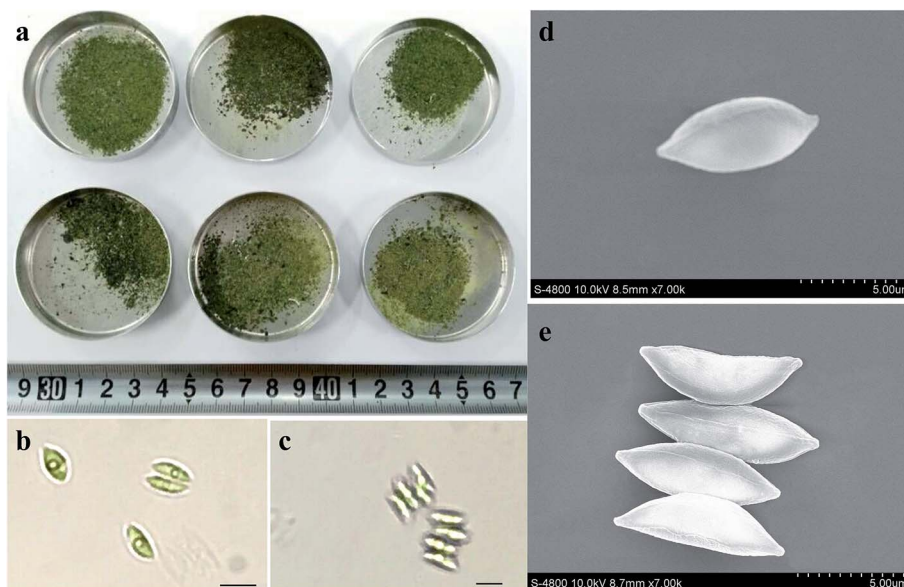
Fig. 3 Photos of harvested biomass as well as optical and scanning electron microphotoes (SEM) of *S. obliquus*.
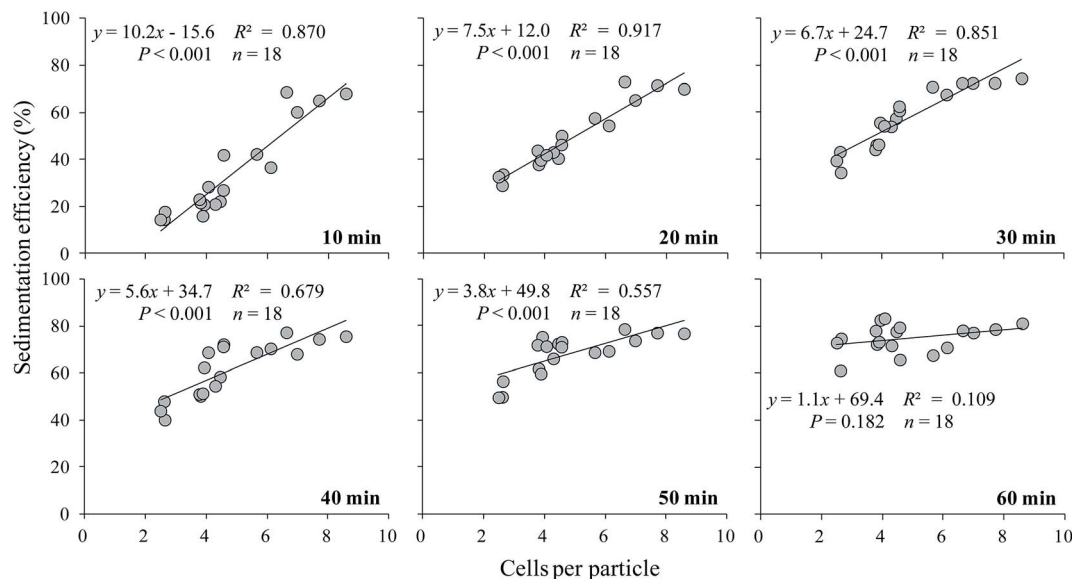
Fig. 5 The relationship between sedimentation efficiency and cells per particle of *S. obliquus* at varying sedimentation time.
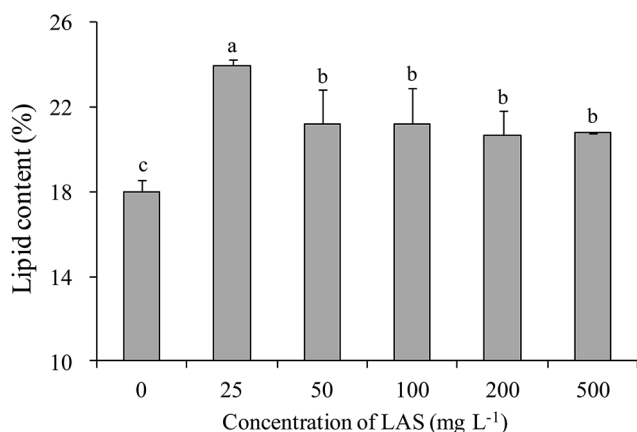

Fig. 6 Lipid contents of *S. obliquus* treated with various concentrations of LAS. Different lowercase letters indicate significant differences ($P < 0.05$) among treatments.

Table 1 Fatty acid composition (%) of *S. obliquus* treated with various concentrations of LAS

Fatty acid	Concentration of LAS in the treatment (mg L^{-1})					
	0	20	50	100	200	500
C15:0	0	3.3	3.8	0	0	0
C16:0	16.3	22.6	18.9	20.0	22.0	23.7
C18:0	0	1.8	1.6	0	0	0
C18:1	0	8.6	9.1	8.6	7.7	22.5
C18:2	9.1	3.5	4.6	0	4.7	4.9
C18:3	45.5	47.7	46.7	47.4	46.3	32.2
C20:5	16.2	10.4	13.7	16.2	13.5	11.7
C22:0	12.9	2.1	1.6	7.8	5.8	5.0
Unsaturated	70.8	70.2	74.1	72.2	72.2	71.3
C16–C18	70.9	84.2	80.9	76.0	80.7	83.3

LAS addition except for the treatment with highest level, in which the value decreased from 45.5% to 32.2% when treated with 500 mg L^{-1} LAS. On the contrary, the proportion of palmitic acid (C16:0) increased from 16.3% to 23.7% with increasing LAS concentration. The degree of fatty acid unsaturation was not obviously affected by LAS addition. However, the proportion of fatty acid with the chain length ranging from C16 to C18 was promoted from 70.9% to more than 76% (highest value reached 84.2%) by LAS addition.

4. Discussion

The current study clearly showed that sedimentation efficiency of *S. obliquus* was promoted by addition of LAS. Even though, some other factors such as culture pH, addition of FeCl$_3$, chitosan and Al$_2$(SO$_4$)$_3$ could also promote sedimentation of *S. obliquus*,[19] the mechanism in the current study was different from previous studies. The SEM photos showed that floc was found in the previous study[20] but was not found in the current study. In the present work, the sedimentation process was accompanied by colony formation of *S. obliquus* induced by LAS. Therefore, flocculation was the main pathway promoting sedimentation in previous studies[19,20] but colony formation was the main pathway in the current study.

It was also found that sedimentation efficiency at 10, 20, 30, 40 and 50 min was significantly related to cells per particle of *S. obliquus*. These regression analysis results could be used to predict the sedimentation performance of *S. obliquus* based on a large number research results about the effects of environmental factors on colony size of *S. obliquus*.[7,8] These predictions would provide some references to choose proper agent to promote sedimentation of *S. obliquus* for harvesting.

Our results also illustrated that colony formation occurred in all the treatment with varying concentrations of LAS from 25 mg L^{-1} to 500 mg L^{-1}. Li *et al.*[9] reported that 100 mg L^{-1} LAS

successfully induced colony formation of *S. obliquus* but 10 mg L^{-1} LAS did not succeed in inducing colony formation. Here, we obtained the missing information that 25 and 50 mg L^{-1} LAS can also induce colony formation. Colony formation in *S. obliquus* is thought to be triggered by increased EPS production.[8,21] LAS can inhibit protein and DNA synthesis in *S. obliquus*, and reduce its dark respiration activity.[22] Thus, the reduced polysaccharide consumption could increase the EPS content and hence induce colony formation. This mechanism has been proved previously by Li *et al.*[9] There were clear attachment points between two neighboring cells of induced colony of *S. obliquus*. Frame and Sawa[23] showed that single cells of *S. obliquus* were wrapped by a structureless slimy layer. Therefore, the pathway of colony formation in the current study was that cells of *S. obliquus* remain attached after binary fission due to the wrapping of slimy layer.

In the current study, the proportion of fatty acid with the chain length ranging from C16 to C18 was promoted by LAS addition. That is to say LAS addition benefitted to improve the quality of algal biodiesel.[10,11] The algal fatty acid composition, related to algal physiological state, was usually affected by salinity.[12,24] LAS addition also increased salinity and thus affected fatty acid composition in the current study. However, the effects of increasing alkyl benzene sulfonic acid on algal fatty acid composition was still not clear and need further studies.

Compared with control, 25 mg L^{-1} LAS significantly stimulated lipid accumulation in *S. obliquus*. Merely, there was no significant change in the lipid content after addition of 50–500 mg L^{-1} LAS. Similar phenomenon that lipid content increased with increasing NaCl when NaCl concentration was low but decreased slightly when NaCl concentration was high was also reported by Salama *et al.*[12] Wang *et al.*[25] reported that low concentrations of LAS enhanced the maximal photochemical efficiency of *Microcystis aeruginosa*. It could also been inferred that low concentration of LAS might also enhance the maximal photochemical efficiency of *S. obliquus* improving its light harvesting for lipid synthesis and thus promoted the accumulation of lipid. When LAS concentration was high, photosynthesis of *S. obliquus* was inhibited[22] and thus no significant change in the lipid content was found in the treatment of 50–500 mg L^{-1} LAS. As is well documented, inorganic substances such as nutrients,[26] Fe,[27] CO_2 (ref. 2) and salt[12] can promote lipid accumulation in *S. obliquus*. Glucose addition also reportedly increases the lipid content,[26] because some *S. obliquus* strains are facultative heterotrophs.[28] However, the lipid content of *S. obliquus* did not monotonically increase with LAS concentration, indicating that LAS is not an organic carbon source for *S. obliquus* in the current study. The effects of Na^+ can also be excluded, because the maximum Na^+ concentration in the present treatments was 39.9 mg L^{-1}, too low to exert any significant effect. Additionally, the levels of triacylglycerol (TAG) and other small molecules (such as glycerol) increase under changes in osmotic pressure,[29] which could be induced by surfactant addition. These molecules promote lipid accumulation in the algal cells.[12]

Although the 25 mg L^{-1} LAS treatment significantly stimulated lipid accumulation in *S. obliquus* (Fig. 6), its effect on sedimentation efficiency was not superior. In contrast, 100 mg L^{-1} LAS maximized the sedimentation efficiency at 40 minutes (to >30% above the control group), but yielded moderate lipid content (1.18 times the control lipid content). The filtrates can be recycled to reduce costs. Lürling[30] reported that surfactant (FFD-6) induces colony formation of *S. obliquus* within 24 h. Therefore, the LAS concentration in the culture medium could be adjusted to 25 mg L^{-1} using recycled filtrate, then adjusted to 100 mg L^{-1} by LAS addition one day before harvesting. The first step would boost the cell yield and the second would enhance the lipid content.

5. Conclusions

Our results indicated that LAS concentrations below 100 mg L^{-1} did not affect the growth of a potential biofuel producer, *S. obliquus*. The number of cells per particle significantly increased with increasing LAS concentration from 2.6 to 7.6. The sedimentation efficiency at 10 min significantly increased along with increasing LAS concentration. In the 100 mg L^{-1} LAS treatment, the sedimentation efficiency was 69.0% after 40 minutes' settling (30% higher than in the control treatment). Meanwhile, the lipid content was 1.18 times higher than in the control. The proportion of fatty acid with the chain length ranging from C16 to C18 was promoted from 70.9% to 84.2% by LAS addition. Synthesis consideration of sedimentation efficiency and lipid production, the LAS concentration in the culture medium could be adjusted to 25 mg L^{-1} using recycled filtrate, then adjusted to 100 mg L^{-1} by LAS addition one day before harvesting. The first step would boost the cell yield and the second would enhance the lipid content.

Acknowledgements

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