

Evaluation of common wastewaters on the growth of alga *Spirulina*

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ABSTRACT

To evaluate the suitability of wastewater on the growth of alga *Spirulina*, reclaimed wastewaters, fishpond wastewater, industrial wastewater and mariculture water were used to culture *Spirulina subsalsa* and *Spirulina platensis*. *S. subsalsa* showed better adaptability to fishpond wastewater and higher specific growth rate than that of *S. platensis*. Thus, effects of factors such as the amount of baking soda, inoculation, and sodium nitrate on the growth, biomass and protein content of *S. subsalsa* were evaluated through the single factors design. Results showed that *S. subsalsa* had good growth under conditions of 4–12 g/L baking soda, 1–2 g/L NaNO₃, 25–40°C, and 6000–12000 lux illumination. The maximum dry biomass and protein content were 3.48 g/L and 33.08%, respectively, suggesting that it was feasible to culture *S. subsalsa* in freshwater aquaculture wastewater.

1. INTRODUCTION

Spirulina is one of the oldest photosynthetic autotrophic microalgae, named for its spiral shape. *Spirulina* contains 50–70% protein and 10–14% carbohydrate [1]. In addition, it has high photosynthetic efficiency, strong environmental adaptability, short growth period and high yield, making *Spirulina* a promising commercial application [2].

The growth of *Spirulina* is not strict with the cultivation environment. In general, the pH value ranges from 7 to 11, the water depth is between 0.2 and 0.3 m, and the water temperature is 18–38°C [1-3]. However, suitable cultivation environment might significantly improve the profit of commercial cultivation of *Spirulina*.

There are some common commercial *Spirulina* cultivation methods based on the cultivation scales and mode, like family cultivation, natural lake cultivation, outdoor industrialized cultivation, and indoor intensive cultivation [1,4,5]. The cultured algae species mainly include *Spirulina platensis*, *S. maxima*, and *Spirulina subsalsa* [4]. Due to *Spirulina* naturally lives in salty water, gathering of the alga from natural lakes is still using a primitive but economical productive method. However, *Spirulina* requires special condition of light intensity, temperature and pH range; therefore, not all natural lakes are suitable for *Spirulina* growth [6]. Thus, outdoor artificial cultivation become an economical and high efficiency way.

To further reduce the cost of management, application of wastewater to culture microalgae becomes a promising way recently [7,8].

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Wastewater sources mainly include domestic sewage, aquaculture waste, various factory and mariculture wastewater, etc. The wastewater contains certain nutrients such as nitrogen, phosphorus, and potassium which are the basic demands for the growth of microalgae. Besides the necessary nutrition, carbon source is also the most important fertilizer for microalgae cultivation. The common carbon source includes NaHCO₃, HCO₃⁻ and CO₂. CO₂ is an efficient carbon resource which is not only able to directly supply the photosynthesis process, but also able to adjust the solution pH providing a relatively stable pH to favor the alga growth [9]. However, commercial CO₂ is a relatively expensive product and easily dispersed, leading to increment of cultivation cost. Whereas NaHCO₃ is rather stable and relatively cheaper carbon resource [10], Wu *et al.* [11] reported the addition of baking soda in outdoor cultivation of *Dunaliella salina* and found the baking soda could not only maintain high yield and relatively stable pH during the logarithmic growth phase, but also decreased the cultivation cost.

Southern part of Thailand is an area of sufficient light, heat and water resources, which is suitable for microalgae cultivation. With the growth of population and economy, various kinds of sewage discharge are also increased. To reduce the damage of sewage to the local environment and make useful of these sewage resources, seven kinds of local common wastewater were selected to culture microalgae. Considering the difference between actual production and laboratory cultivation, this study did not sterilize various local wastewaters. After simple treatment, *Spirulina* strains were directly inoculated to these wastewaters mixed with cheap edible baking soda as carbon source and pH regulator. The best alga species and suitable wastewater resource were evaluated through the alga growth, biomass yield and protein content under different cultivation environment, discussing the

feasibility of actually cultivating *Spirulina* using wastewater to guide the further outdoor large scale microalgae cultivation.

2. MATERIAL AND METHODS

2.1. Wastewater Collection

Seven kinds of local common wastewaters in Sikao District, Trang Province, Thailand were collected for the test. Details were shown in Table 1. The reclaimed water was taken from the local sewage treatment plant. The domestic sewage was anaerobic fermented and then flowed into the three-stage series purification tank. The purified water of each stage was used as the water source for alga cultivation, recorded as reclaimed water 1, 2 and 3. Freshwater aquaculture wastewater was taken from the local fishpond in the fishing off season. Industrial wastewater was also taken from the outlet of the local sewage treatment plant. The sewage from the local industrial plant was treated by purification tank and then used for alga cultivation. The mariculture water was taken from the local mariculture plant. The seawater was mixed with fresh water with a ratio of 3:1, and then processed by primary and secondary sedimentation removing some heavy metal ions.

2.2. Microalgae culture

S. subsalsa and *S. platensis* were provided by Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya. Two algae were cultured in Zarrouk medium to logarithmic growth stage as inoculation mother solution [12]. Different baking soda (2, 4, 8 and 12 g/L) were added into seven collected local wastewaters respectively, then using 1 mol/L NaOH to adjust pH value of all solution to 9. All solution were stood overnight and then filtered by 300 mesh cloth sieves; 200 mL filtered water was taken into a 500 mL flask, and then inoculated by mother algal solution. The initial OD₅₅₀ values of all treatments were around 0.06; the temperature was 30°C; the light illumination was 10000 lux; the light-dark cycle was 12 h: 12 h. All cultures were shaken regularly 3 times per day for 5 min each time, and recorded the color variance and OD₅₅₀ value by ultraviolet spectrophotometer meanwhile.

2.3. Single Factor Test

The environmental adaptability of alga was evaluated by single factor test under the conditions of edible baking soda, inoculation amount, sodium nitrate, illumination and temperature, respectively [Table 2].

Table 1: Seven kinds of wastewater resource.

Name	Resource
Reclaimed water 1	Domestic sewage purified by the first purification tank after anaerobic fermented
Reclaimed water 2	Domestic sewage purified by the second purification tank after anaerobic fermented
Reclaimed water 3	Domestic sewage purified by the third purification tank after anaerobic fermented
Freshwater aquaculture wastewater	Taken from the local fishpond in the fishing off season
Industrial plant wastewater	The local plant sewage treated by the local sewage treatment plant
Mariculture water	Sea water mixed with fresh water treated by primary and secondary sedimentation, that was ready to culture sea fish
Mariculture wastewater	Wastewater after mariculture

S. subsalsa was cultured with freshwater aquaculture wastewater based on the result of each single factor test orderly, so as to find the best cultivation conditions for biomass and protein yield. The specific growth rate and the average growth rate were calculated by the equation of $\ln(A_x/A_0)$ and $\ln(A_x/A_0)/T$, respectively, where A_x was the OD₅₅₀ value on the day x, A_0 was the initial OD₅₅₀ value, T was the culture time to the harvest day.

2.4. Detection method

2.4.1. Wastewater quality analysis

The detection methods for the contents of total nitrogen, ammonia-nitrogen, total phosphorus, and phosphate phosphorus in wastewater were applied according to standard methods as published by the American Public Health Association [13].

2.4.2. Dry weight determination

Algae solution colors were daily observed and algae were harvested when the color changed from dark green to yellow green or the OD₅₅₀ value did not increase again. The algal solution was filtered by 300 mesh cloth sieve, and the alga was washed by fresh water to neutral. The alga was dried at 60°C until total weight stable.

2.4.3. Protein content determination

About 0.15 g dry algae powder was taken and mixed with 30 ml distilled water, and put into a centrifuge tube. After repeated freezing at -20°C and thawing for 5 times, the solution was centrifuged at 4000 rpm for 15 min. The supernatant was taken to determine the protein content following the Coomassie brilliant blue method [10].

2.5. Data Analysis

All data statistical analysis was performed using the SPSS 19.0 software. All data displayed a normal distribution checked by Kolmogorov-Smirnov test. The results were given as a mean with standard deviation (\pm SD). Figures were created with Microsoft Excel 2010 software.

3. RESULTS AND DISCUSSION

3.1. Wastewater Quality Analysis

Contents of various nutrients in wastewater were shown in Table 3. Phosphorus was little in industrial plant wastewater, and all nutrients in seawater wastewater were lower than that in freshwater wastewater, which may limit the alga growth [2,14].

3.2. Growth of *Spirulina* Strains in Freshwater Wastewater

Colors of *S. subsalsa* in freshwater wastewater were shown in Table 4. *S. subsalsa* in the mother solution showed dark green. After inoculation to fishpond wastewater with different contents of baking soda, the algal solution color gradually recovered from fresh green to dark green with the extension of culture time. The maximum OD₅₅₀ value was 0.422 when adding baking soda 8 g/L. Colors and the maximum OD₅₅₀ values from reclaimed waters were all lighter than that in fish pond wastewater. In addition, a few dead yellow-brown algae clusters floating in reclaimed waters were found on the 4th day. Colors in industrial plant wastewaters always showed brownish green, and their OD₅₅₀ values did not increased since the cultivation, which might be caused by the low content of ammonium nitrogen and phosphorus.

Colors of *S. platensis* in freshwater wastewater were shown in Table 5. *S. platensis* in the mother solution showed blue-green. While colors of algal solution were mostly yellow-green since inoculation to various

Table 2: Single factor test conditions.

Factor	Gradient						Other conditions	
							Illumination (lux)	Temperature(°C)
Edible baking soda (g/L)	2	4	6	9	12	-	10000	32
Inoculation (%)	2	5	10	15	-	-	10000	32
NaNO ₃ (g/L)	0.5	1	1.5	2	2.5	3	10000	32
Illumination (lux)	3000	6000	9000	12000	-	-	NaNO ₃ (1 g/L)	32
Temperature (°C)	20	25	30	35	40	-	9000	NaNO ₃ (1 g/L)

Note - Meant not set

Table 3: Nutrients content in wastewater (mg/L).

Index	Reclaimed water 1	Reclaimed water 2	Reclaimed water 3	Freshwater aquaculture wastewater	Industrial plant wastewater	Mariculture water	Mariculture wastewater
Total nitrogen	224.9±10.6	223.6±9.7	232.6±8.1	180.3±9.1	233.2±13.1	3.2±0.12	3.51±0.21
Ammonia-nitrogen	22.7±1.41	14.2±0.89	8.48±0.33	2.45±0.11	0.35±0.01	2.01±0.11	2.18±0.12
Total phosphorus	10.1±0.53	1.5±0.08	8.69±0.24	3.65±0.12	<0.01	0.55±0.02	0.28±0.01
Phosphate phosphorus	0.41±0.03	0.24±0.02	0.38±0.02	0.04±0.01	<0.01	0.03±0.01	0.09±0.01

Table 4: The maximum OD₅₅₀ value and color of *Spirulina subsalsa* in freshwater wastewater.

Baking soda (g/L)	Freshwater aquaculture wastewater		Reclaimed water 1		Reclaimed water 2		Reclaimed water 3	
	OD ₅₅₀	Color	OD ₅₅₀	Color	OD ₅₅₀	Color	OD ₅₅₀	Color
2	0.308±0.02	Green	0.157±0.01	Green	0.164±0.01	Green	0.142±0.02	Green
4	0.401±0.03	Dark green	0.293±0.02	Green	0.251±0.01	Green	0.225±0.01	Green
8	0.422±0.02	Dark green	0.333±0.03	Green	0.226±0.02	Green	0.313±0.02	Green
12	0.413±0.03	Dark green	0.335±0.03	Green	0.267±0.02	Green	0.316±0.02	Green

Table 5: The maximum OD₅₅₀ value and color of *Spirulina platensis* in freshwater wastewater.

Baking soda (g/L)	Freshwater aquaculture wastewater		Reclaimed water 1		Reclaimed water 2		Reclaimed water 3	
	OD ₅₅₀	Color	OD ₅₅₀	Color	OD ₅₅₀	Color	OD ₅₅₀	Color
2	0.201±0.02	Died algal clusters	0.065±0.01	Yellow-green	Not check	More died algal clusters	Not check	Died all
4	0.283±0.01	Yellow-green	0.187±0.01	Yellow-green	0.192±0.01	Yellow-green	0.272±0.02	Yellow-green
8	0.312±0.02	Yellow-green	0.273±0.02	Yellow-green	0.228±0.01	Yellow-green	0.321±0.02	Yellow-green
12	0.282±0.03	Light blue-green	0.265±0.01	Yellow-green	0.371±0.02	Died algal clusters	0.341±0.02	Yellow-green

kinds of freshwater wastewater and also dead algae clusters appeared in all wastewaters. The maximum OD₅₅₀ value was 0.371 when adding baking soda 8 g/L. Relatively, colors in claimed water 2 and 3 performed better than that in other wastewaters. Colors in industrial wastewater showed more yellow than that in other wastewaters and more dead algae clusters were found.

3.3. Growth of *Spirulina* Strains in Seawater Wastewater

A few of precipitations appeared when adjusting pH value to 8 by NaOH. Seawaters after filtering precipitations to culture two algae *Spirulina*, results found that two species of *Spirulina* both grew slow and their solutions showed light yellow, which might be the absence of carbon source [15]. However, adding more baking soda to adjust pH value to 9 or above, large amounts of precipitation occurred, and parts of floating milk-white particles adsorbed the algal filaments forming granular algal

clusters, which led to the daily growth cannot be measured due to too many impurities. Thus, before inoculation, these precipitations should be filtered and/or the pH value of seawater was adjusted by few baking sodas to maintain pH around 8. However, some precipitations still occurred during the process of culturing algae. These precipitations could absorb *Spirulina* cells to form algal clusters which could not be re-suspended again leading to the low efficiency of absorption and utilization of light energy by *Spirulina*. Therefore, it was the key to adjust pH value or avoid the occurrence of precipitation when culturing *Spirulina* using seawater.

3.4. Single Factor Test Results and Analysis

3.4.1. Effects of baking soda on the growth of *S. subsalsa*

The main component of baking soda is NaHCO₃ which can adjust pH value providing a suitable growth environment and carbon sources [10]. The growth of *S. subsalsa* was shown in Figure 1.

The specific growth rate of *S. subsalsa* decreased sharply on the 4th day; the algal solution color was yellow, and this color intensity decreased with increase of the amount of baking soda. It may be caused by a large consumption of nutrients like nitrogen or phosphorus. Then, the content of nitrogen and phosphorus in fishpond wastewater was checked and found their content decreased significantly with the growth of algae. Test also found that addition 1 g/L of baking soda which was able to recover the algal growth within 12 h and color of algal solution turned to bright green. Based on the result, if 1 g/L of baking soda was added to each treatment, algal growth could be continued. Results found that color of algal solution which turned yellow could adhere to bottle wall after 13 days for treatments of 3 g/L, 4.5 g/L and 6 g/L of baking soda. Whereas the remaining treatments, 9 g/L and 13.5 g/L of baking soda, algal patches started to adhere to bottle wall on the 16th day. According to these results, it could be suggested that the addition of baking soda over 9 g/L might provide sufficient carbon source and retain strong buffer capacity to keep relatively stable pH value.

Baking soda mainly composed of NaHCO_3 , low price and no toxicity, was more suitable for commercial algae cultivation. During algal cultivation, pH value of culture solution was increased with the consumption of HCO_3^- by algae, caused the precipitation declining the purity of algae powder [3,16]. If the pH value was controlled by HCl, algae filaments were easily turned yellow and dead. Therefore, it was advisable to add baking soda before culturing algae and remove the precipitation, decreasing the concentration of fine particles in the culture solution, so as to decrease the precipitation caused by increased pH value, algal filaments entanglement and agglomeration.

3.4.2. Effect of inoculation amount on the growth of *S. subsalsa*

The inoculation amount was the main factor affecting algal growth. Different amounts of inoculation were tested and results were shown in Figure 2.

Results found that colors in all algal solutions showed bright green in the first 4 days, and the specific growth rate of 2.5% inoculation treatment was higher than that of other treatments, and that of 5% inoculation treatment was higher than that of 7.5% and lower than that of 15% inoculation treatment. This result was in agreement with previous report that the growth was higher at low inoculation amount than that at high inoculation amount, but the yield was low [17]. When cultured to the 4th day, colors of all treatments started to turn yellow and became completely yellow on the 5th day, and broken algal filaments were found. The OD_{550} value of 2.5% inoculation treatment sharply decreased, which may relate with the large consumption of

nitrogen resource. Thus, 1 g/L NaNO_3 was added to all treatments on the 6th day, and result found that colors of all algal solutions returned to bright green and algal growth increased again. Among of them, the growth of 2.5% inoculation treatment recovered rapidly, and the specific growth rate was higher than that of other treatments from the 8th day. The specific growth rate of 5% inoculation treatment was higher than that of 15% treatment after adding nitrogen source, while there was little difference between 7.5% and 15% treatments after the 9th day. It could be inferred that the ability of algae to resist nitrogen deficiency increased with the increase of inoculation amount. The reason may be that the more the inoculation amount, the greater the algae density and the stronger the ability to resist adverse environment [17]. It may also be that when the inoculation amount exceeded 7.5%, the mother solution added to the culture system supplementing more nutrients for the growth of *Spirulina*, and delaying the decline of OD_{550} value.

3.4.3. Effect of sodium nitrate on the growth of *S. subsalsa*

The nitrogen source in freshwater aquaculture wastewater was not enough to maintain the growth of *S. subsalsa* [18]. After adding nitrogen source, the growth time was prolonged, seen from Figure 3.

The specific growth rate of the treatment of 2 g/L NaNO_3 was higher than that of other treatments, but not significantly higher than that in 1 g/L. Therefore, the addition of 1 g/L NaNO_3 was recommended for cost saving.

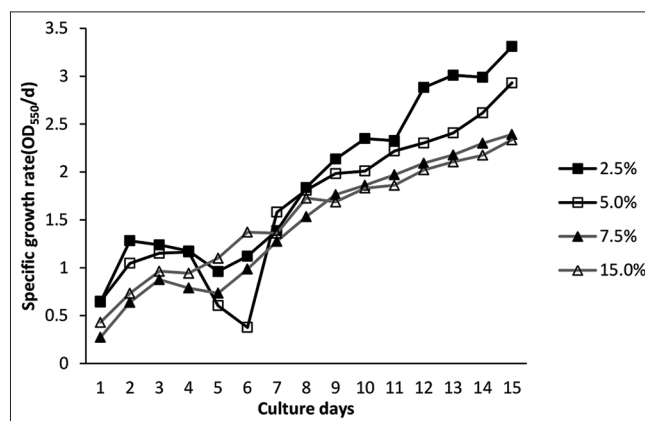


Figure 2: Growth rate of *Spirulina subsalsa* under different inoculation amounts.

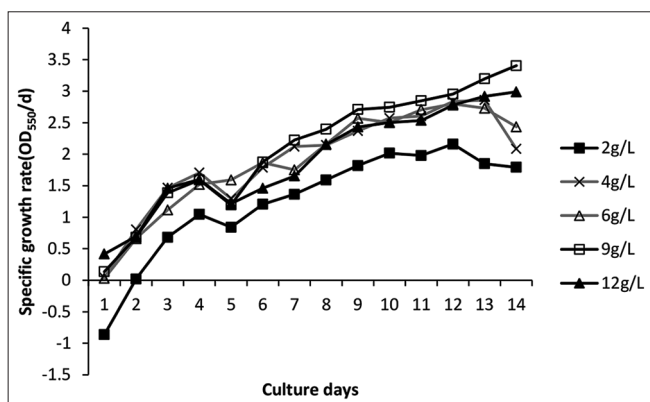


Figure 1: Growth rate of *Spirulina subsalsa* under different amounts of baking soda.

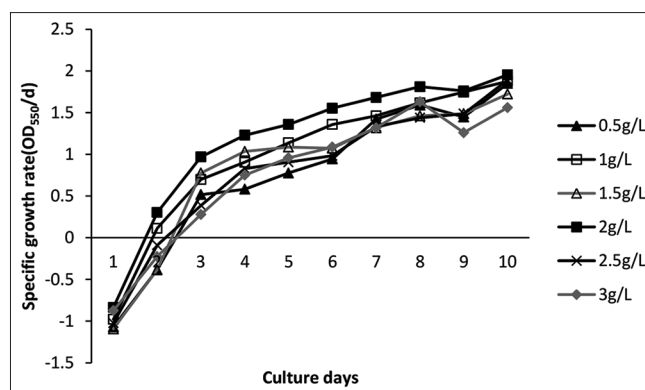


Figure 3: Growth rate of *Spirulina subsalsa* under different NaNO_3 concentrations.

3.4.4. Effect of light illumination on the growth of *S. subsalsa*

Growth conditions of *S. subsalsa* under different illuminations at 32°C and 1 g/L NaNO₃ were shown in Figure 4.

Illumination of 3000 lux was unfavorable to the growth of *Spirulina*, the specific growth rate increased slowly, and the maximum specific growth rate was lower than that of other treatments. The specific growth rate of 6000 lux and 10000 lux treatments had little difference in the first 4 days, and the specific growth rate of 6000 lux treatment was higher in the last 2 days. The specific growth rate of 12,000 lux

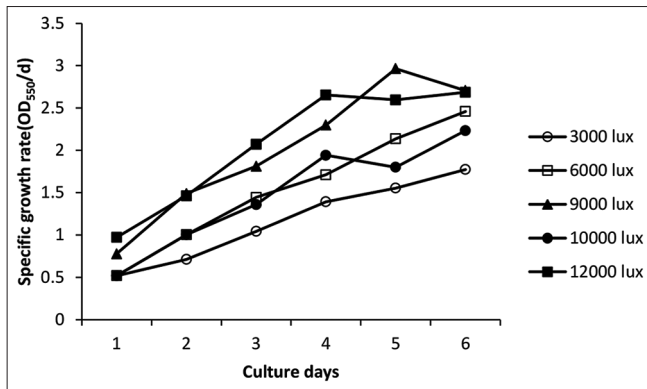


Figure 4: Growth rate of *Spirulina subsalsa* under different light illuminations.

treatment achieved to the maximum 1 day earlier than that of 9000 lux treatments, and then tended to be stable. However, the final specific growth rate in treatment of 9000 lux was the highest than all other treatments. It could be speculated that high illumination was conducive to the growth of *Spirulina*, so that *Spirulina* could complete the logarithmic growth period in a relatively short time [14].

3.4.5. Effect of temperature on the growth of *S. subsalsa*

Growth of *S. subsalsa* under different temperatures was shown in Figure 5 the specific growth rate was negative in the first 2 days at 20°C, indicating that *Spirulina* had an adaptation period at low-temperature. After that, alga started to grow, the specific growth rate turned positive, and the OD₅₅₀ value increased. At 25-35°C, the specific growth rate increased with the increase of temperature, and the specific growth rate at 40°C was lower than 35°C. Therefore, the optimal culture temperature of 35°C was recommended considering cost saving and shorten culture time. This result was in agreement with report that the optimum growth temperature of *S. subsalsa* was between 31 and 40°C [3].

3.4.6. Biomass, protein content and average growth rate under various culture conditions

High algae powder yield and quality were the key targets to cultivate *Spirulina*. Biomass and protein yield of *Spirulina* were shown in Table 6.

The maximum yield of dry algae powder could reach 3.48 g/L, and the maximum protein content was 33.08%. Treatment groups with baking soda and different inoculation amounts produced low protein content may be caused by the lack of nitrogen in the cultivation period

Table 6: Algal biomass, protein content and growth rate under different cultivation conditions.

	Conditions	Dry weight (g/L)	Protein content (%)	Average growth rate
Baking soda (g/L)	2	1.85±0.13	4.50±0.08	0.038±0.002
	4	1.94±0.12	7.34±0.15	0.068±0.002
	6	1.97±0.11	8.93±0.39	0.066±0.004
	9	3.06±0.14	5.18±0.38	0.075±0.003
	12	2.44±0.08	9.88±0.21	0.068±0.003
Inoculation amount (%)	2.5	2.10±0.13	3.29±0.42	0.086±0.004
	5	2.43±0.17	4.83±0.14	0.079±0.003
	7.5	3.36±0.08	1.04±0.16	0.069±0.002
NaNO ₃ (g/L)	15	3.48±0.11	1.99±0.06	0.072±0.004
	0.5	1.15±0.03	20.45±0.08	0.055±0.002
	1	1.10±0.05	21.29±0.79	0.099±0.003
	1.5	1.05±0.07	20.14±0.87	0.085±0.003
	2	0.77±0.02	10.12±0.88	0.101±0.003
	2.5	0.93±0.02	15.65±0.34	0.081±0.003
Illumination (lux)	3	1.03±0.05	17.32±0.84	0.062±0.005
	3000	1.16±0.07	3.85±0.73	0.129±0.007
	6000	2.00±0.11	9.84±0.15	0.172±0.009
	9000	3.11±0.13	7.65±0.42	0.223±0.007
	10000	3.44±0.15	2.51±0.33	0.164±0.008
Temperature (°C)	12000	2.59±0.16	2.17±0.08	0.231±0.006
	20	0.81±0.03	24.84±0.12	0.115±0.008
	25	0.64±0.04	33.08±1.21	0.194±0.008
	30	0.86±0.04	32.14±2.12	0.212±0.009
	35	1.19±0.06	19.51±1.31	0.214±0.009
	40	0.75±0.02	17.11±0.85	0.201±0.007

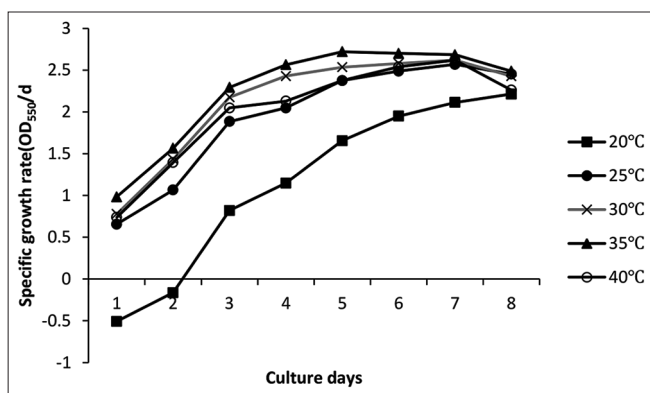


Figure 5: Growth rate of *Spirulina subsalsa* under different temperatures.

and long cultivation time after supplementing nitrogen source [15,19]. In addition, during the culture process, with the consumption of carbon source, the pH value of culture solution increased caused the occurrence of white precipitates in the culture solution, which adhered to the algal filaments and were not easy to remove, that decreased the purity of algal powder and was also a reason for the low protein content [16]. The average growth rates in treatment groups of illumination and temperature were higher than that of other treatments, which may be caused by the relatively short time required to reach the maximum biomass and the higher specific growth rate of *Spirulina*. With a summary, the current research suggested that the algae products with high yield or high protein content could be obtained by culturing *S. subsalsa* with freshwater aquaculture wastewater by adding an appropriate amount of baking soda and NaNO_3 and controlling the culture time [15,18].

4. CONCLUSION

S. subsalsa had stronger adaptability to wastewaters than *S. platensis*, and *S. subsalsa* cultured in freshwater fish pond wastewater showed the best growth. High algae powder yield and protein content could be obtained by adjusting the amount of baking soda, inoculation, sodium nitrate, illumination, and temperature. The maximum dry algal biomass weight was 3.48 g/L under conditions of 8 g/L baking soda, 1 g/L NaNO_3 , 10000 lux illumination and 32°C; and the maximum protein content was 33.08% under conditions of 8 g/L baking soda, 1 g/L NaNO_3 , 9000 lux illumination and 25°C. Hence, it was feasible to use freshwater wastewaters to culture *S. subsalsa*.

5. ACKNOWLEDGMENTS

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6. CONFLICT OF INTEREST

Authors declare that they do not have any conflicts of interest.

7. ETHICAL APPROVALS

Not Applicable.

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