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Correlation Between Polysaccharide Biosynthesis and Glycometabolism Related Enzymes of *Chlamydomonas* sp. YB-204 in Different Conditions

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In order to study the enzymatic mechanisms responsible for polysaccharide synthesis in *Chlamydomonas* sp. YB-204, the changes of polysaccharide and glycometabolism related enzymes were analyzed. Under the condition of salt stress, the activities of phosphoglucose isomerase (PGI) and malate dehydrogenase (MDH) were inhibited, but hexokinase (HK) was increased. The higher concentration of nitrogen source would inhibit the activities of glucose-6-phosphate dehydrogenase (G6PDH), PGI and MDH, and not conducive to the synthesis of polysaccharide. $NaHCO_3$ could promote the synthesis of polysaccharide. The activities of G6PDH, PGI and HK decreased, but the activity of MDH increased when $NaHCO_3$ was added. The variation trend of polysaccharide content was very different with the activities of four enzymes during the growth of YB-204. Pearson correlation analysis showed that HK, G6PDH and MDH were significantly negative correlation with polysaccharide synthesis. The correlation between PGI and polysaccharide synthesis was not significant ($p>0.05$). We presumed that HK, G6PDH and MDH were the key enzymes to regulate the biosynthesis of polysaccharide, and there might be more than one branch to synthetize polysaccharide in YB-204.

Key words: Polysaccharide; Glycometabolism related enzymes; Phosphoglucose isomerase; Malate dehydrogenase; Hexokinase; Glucose-6-phosphate dehydrogenase.

Microalgae are autotrophic microorganisms, which utilize light energy and inorganic nutrients (carbon dioxide, nitrogen, phosphorus etc.) and synthesize valuable biomass compounds, such as carotene[1], lutein[2], unsaturated fatty acid[3], astaxanthin[4], lectin[5], phycobiliproteins[6], dinoflagellate toxins, active polysaccharides[7] and other biological active substances.

Polysaccharides have potential uses in several industrial and health applications. Potential application of polysaccharides includes their use as agent for the emulsion stabilization, as bioflocculants or as thickening agent for alteration of water rheological characteristics and as heavy metal removal agents for treatment of polluted water[8]. Also several polysaccharides displayed good activities as antitumor, antiviral and immunostimulant substances[9, 10, 11]. There are several eukaryotic microalgae, like *Chlorella sp.*, *Porphyridium sp.*[12], *Rhodella sp.*[13], *Botryococcus sp.*[14], *Dunaliella sp.*[15] and prokaryotic microalgae[16], known to produce and excrete polysaccharides in relative high amounts.

The polysaccharide production of microalgae has obvious difference in different environment, and the main influencing factors are pH, salt, incubation time etc. For instance, the effect of nitrogen starvation in cyanobacteria gave in

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some species, like *Anacystis nidulans* and several *Cyanothece* strains higher amounts of released polysaccharides, while nitrogen starvation had no effect in *Synechocystis*, *Phormidium* and some *Cyanothece* strains[9]. In the red alga *Rhodella reticulata*, the polysaccharide production per cell was significantly enhanced by nitrogen and sulfate starvation in comparison to the control cultures[13], while in cultures of *Botryococcus brauniithe* rate of polysaccharide production was negatively correlated with the nitrogen concentration. In three nitrogen concentrations studied (0.5, 2 and 8 mM NO_3^-) the alga produced more polysaccharides with 0.5 and 2 mM NO_3^-[14]. Salt stress increased the production of polysaccharide in *D.salina*. By increasing the salt concentration from 0.5 M NaCl to 5 M, the production of polysaccharide increased about 17 times[15].

There are a variety of enzymes involving in the biosynthesis of polysaccharide in the organism. HK, G6PDH and MDH are the important enzymes of glycolysis pathway, pentose phosphate pathway and Krebs cycle in the process of glycometabolism. Starting from the relationship of glycometabolism and glycosynthases of plant, many scholars have found that sucrose synthetase is SPS (sucrose phosphate synthase) [17] and starch synthase are UDP-glucose pyrophosphorylase[18], ADP-glucose pyrophosphorylase[19] and soluble starch synthase[20]. In microbial community, a large number of glycometabolism related enzymes are also found, and they have important influence on the biosynthesis of microbial polysaccharides. The low activity of fructosebisphosphatase was not conducive to the synthesis of exopolysaccharide in *Lactococcus lactis*[21]. The synthesis of polysaccharide in *Streptococcus thermophiles* LY03 was highly relevant to the activity of α-phosphoglucomutase and UDP-glucose pyrophosphorylase[22]. UDP-glucose pyrophosphorylase, dTDP-glucose pyrophosphorylase, phosphoglucose isomerase and α-phosphoglucomutase played an important role in the exopolysaccharide synthesis of *Ganoderma lucidum*[23]. At present, there has been no research on the relationship between polysaccharide accumulation and glycometabolism related enzymes in microalgae.

In this study, the oleaginous microalgae *Chlamydomonas* sp. YB-204 was selected as the experimental strain. The changes of polysaccharide and glycometabolism related enzymes in the process of growth and development were studied under different culture conditions. The aim of this work was to study the relationship between the changes of polysaccharide and glycometabolism related enzymes.

MATERIALS AND METHODS

Microalgae strain and culture conditions

Chlamydomonas sp. YB-204 was isolated by our laboratory. A SE medium was used in this study, and the microalga grew in a temperature controlled incubator at 25±2 °C under a photoperiod of 16:8 h.

The different culture conditions were designed as follows: NaCl concentration from 25 mg/L to 150 mg/L; $NaNO_3$ concentration from 125 mg/L to 750 mg/L; $NaHCO_3$ concentration from 0 to 4.0 g/L; culture time from 5 to 15 days.

The determination of polysaccharide

The microalgae culture was centrifuged at 5,000 rpm for 10min and the harvested biomass was dried in vacuum at 60 °C until it remains at a constant weight. Ultrasonic-associated hot water extraction was used to extract IWSP: extraction medium of NaOH (4%), solid to liquid ratio of 1:25 (g/mL), ultrasonic broken. Then, it was heated in a water bath at 70 °C for 180 min, immediately centrifuged for the supernatant. The supernatant was precipitated by ethanol of 3 times volume, kept overnight, centrifuged for precipitation, then adding 3% TCA, fully stirring, until no more precipitate dissolved, repeating the above operation, while the precipitation was the coarse polysaccharide.

In this experiment, anthrone-sulfuric acid colorimetric method was used to determine the polysaccharide concentration[9].

Enzyme assays

Samples of fresh culture were centrifuged at 12,000g for 10 min at 4 °C and the supernatant fluid was decanted. The sediment was washed with phosphate buffer (pH 6.5, containing 50 mM NaCl, 10mM $MgCl_2$, and 1 mM dithiothreitol), centrifuged, suspended in 3 ml phosphate buffer and kept on ice. The samples were disrupted ultrasonically at 0°C. Cell debris was removed by

centrifugation (12,000g, 10 min, and 4 °C). The supernatant fluid was kept on ice and used as the source of enzymes.

Enzyme assays were performed at 25 °C in a total volume of 1 ml with freshly prepared cell extracts. The formation or consumption of NAD (P) H was determined by measuring the change in the absorbance at 340 nm. Values are the means of results from three independent duplicate measurements. The blank contained the reaction buffer, the cofactors, and the substrate but lacked the cell extract.

The phosphoglucose isomerase (PGI) (EC 5.3.1.9) reverse-reaction mixture contained 50 mM potassium phosphate buffer (pH 6.8), 5 mM $MgCl_2$, 0.4 mM $NADP^+$, 4 U of glucose-6-phosphate dehydrogenase, and cell extract. The reaction was started by adding 5 mM fructose-6-phosphate[21].

The malate dehydrogenase (MDH) (EC 1.1.1.37) reaction mixture contained 100 mM phosphate buffer (pH 7.4), 0.6 mM NADH, and cell extract. The reaction was started by adding 0.6 mM oxaloacetic acid.

The hexokinase (HK) (EC 2.7.1.1) reaction mixture contained 40 mM triethanolamine buffer (pH 7.6), 5 mM $MgCl_2$, 0.8 mM $NADP^+$, 2.5 mM ATP, 3 U of glucose-6- phosphate dehydrogenase, and cell extract. The reaction was started by adding 4% b-D-glucose.

The glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) reaction mixture contained 40 mM Tris buffer (pH 8.5), 10 mM $MgCl_2$, 0.4 mM $NADP^+$, and cell extract. The reaction was started by adding 5 mM glucose-6-phosphate disodium salt.

RESULTS

Effects of NaCl on polysaccharide content and enzyme activities

The content of polysaccharide increased with the rising of NaCl concentration and achieved maximum of 12.17% when NaCl concentration was 100 mg/L. With the continue increase of NaCl concentration, the polysaccharide content decreased (Fig.1).

The activity of G6PDH had no significant change with the rising of NaCl concentration. The activities of PGI and MDH reached maximum values of 18.12 U/g and 14.01 U/g when NaCl concentration was 50 mg/L, and then they sharply decreased and remained at a low level with the increase of NaCl concentration. The maximum activity of HK was observed when NaCl concentration was 100 mg/L, with a slight reduction at 150 mg/L (Fig.1).

Effects of $NaNO_3$ on polysaccharide content and enzyme activities

The polysaccharide content increased 1.6 times when $NaNO_3$ concentration increased from 125 mg/L to 375 mg/L, and then it would decrease with the increase of $NaNO_3$ concentration (Fig.2).

G6PDH and PGI had the highest activities of 18.46 U/g and 6.95 U/g when $NaNO_3$ concentration was 375 mg/L. Then, they sharply decreased with the increase of $NaNO_3$ concentration. The activity of HK had no significant change with the rising of $NaNO_3$ concentration. The maximum activity of MDH was obtained when $NaNO_3$ concentration was 375 mg/L, and it was consistent with the change of polysaccharide content (Fig.2).

Effects of $NaHCO_3$ on polysaccharide content and enzyme activities

The content of polysaccharide reached to the maximum values of 16.24%, when $NaHCO_3$ concentration was 2.0 g/L, and then it would be in rapid decline with the further increase of $NaHCO_3$ concentration (Fig.3).

The activities of G6PDH, PGI and HK were the highest when no $NaHCO_3$ and they would sharply decrease and remain at a low level with the addition of $NaHCO_3$. The highest activity of MDH was obtained when $NaHCO_3$ concentration was 1.5 g/L, and it was 3 times the activity of no $NaHCO_3$ (Fig.3).

The changes of polysaccharide content and enzyme activities in the process of cultivation

The content of polysaccharide increased continuously with culture time and reached to the maximum values of 12.54% at 12 days. Then, it would be in decline with the further increase of culture time (Fig.4).

The activities of G6PDH and MDH were at a very high level at the beginning stage of culture, but they would be in decline with culture time and remain at a low level. The activity of PGI reached highest values of 16.10 U/g at 4 days, and then it decreased and remained at a low level with culture time. The activity of HK began to decrease

after 6 days and remained at a low level (Fig.4).

The variation trend of polysaccharide content was very different with the activities of four enzymes. Pearson correlation analysis showed that the four enzymes were all negatively correlated with polysaccharide synthesis in which HK, G6PDH and MDH were significantly negative correlation. The correlation between PGI and polysaccharide synthesis was not significant (p>0.05) (Table 1).

Table 1. The pearson correlation analysis of glycometabolism related enzymes and polysaccharide synthesis of YB-204

Enzyme	Pearson (2-tailed) correlation	
	Correlation	Significance
HK	-0.768*	0.044
G6PDH	-0.880**	0.009
MDH	-0.966**	0.000
PGI	-0.418	0.350

* Significant correlation at the 0.05 level; ** significant correlation at the 0.01 level

DISCUSSION

Polysaccharides of microalgae have many potential biological activities, such as antitumor, antiviral and immunostimulant substances. So, it's very important to expound the mechanism of polysaccharide synthesis in microalgae.

Under the condition of salt stress, the growth of microalgae and the activities of PGI and MDH were inhibited (Fig.1). However, the activity

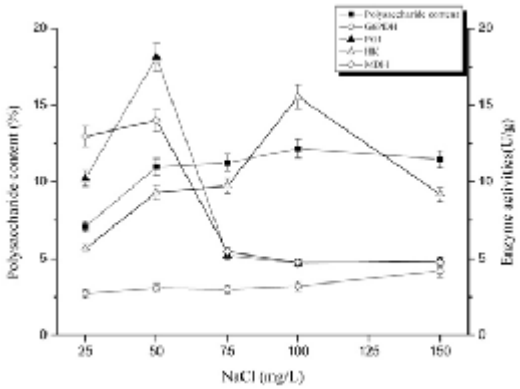

Fig.1. Effects of NaCl concentration on polysaccharide content and enzyme activities. The error bars in the figure indicated the standard deviations from three independent samples.

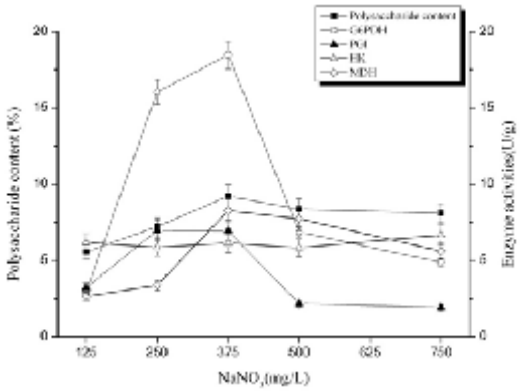

Fig.2. Effects of $NaNO_3$ concentration on polysaccharide content and enzyme activities. The error bars in the figure indicated the standard deviations from three independent samples.

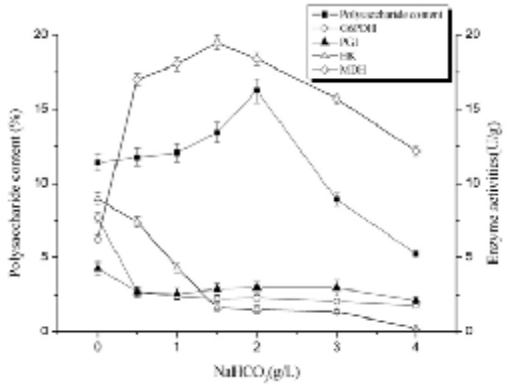

Fig.3. Effects of $NaHCO_3$ concentration on polysaccharide content and enzyme activities. The error bars in the figure indicated the standard deviations from three independent samples.

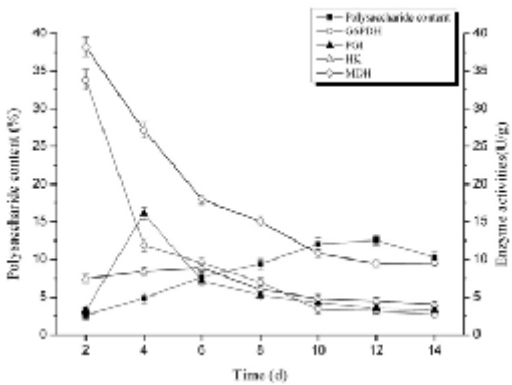

Fig.4. The changes of polysaccharide content and enzyme activities in the process of cultivation. The error bars in the figure indicated the standard deviations from three independent samples.

of HK was increased which might cause the more accumulation of polysaccharide to against the salt stress. It suggested that the synthesis of polysaccharide of YB-204 might have different branches.

The higher concentration of nitrogen source would inhibit the activities of G6PDH, PGI and MDH (Fig.2), and it was not conducive to the synthesis of polysaccharide. Researches had shown that the deficiency of nitrogen source could stimulate the synthesis of polysaccharide in some microalgae[24,25].

$NaHCO_3$ provided carbon source for the photosynthesis of microalgae, and it could promote the growth of microalgae and synthesis of polysaccharide. The activities of G6PDH, PGI and HK decreased, but the activity of MDH increased when $NaHCO_3$ was added (Fig.3), which indicated different metabolic mechanism. It was noteworthy that the polysaccharide content and MDH activity would reduce when the amount of $NaHCO_3$ was excessive, which could induce the initial pH of medium to be alkaline and it was not conducive to the synthesis of polysaccharide.

HK, G6PDH and MDH are the important enzymes of glycolysis pathway, pentose phosphate pathway and Krebs cycle in the process of glycometabolism. They were significantly negative correlation with polysaccharide synthesis of YB-204 (Table 1). The high activities of them at the beginning stage of culture made glycolysis pathway, pentose phosphate pathway and Krebs cycle high efficiency, and they could provide sufficient energy and synthetic precursors, such as nucleotides, amino acids. PGI involved in the conversion between glucose-6-phosphate and fructose-6-phosphate, but it was not significant correlation (p>0.05) with polysaccharide synthesis of YB-204.

We presumed that the synthesis of polysaccharide was regulated by a variety of glycometabolism related enzymes during the development of YB-204, and HK, G6PDH and MDH were the key enzymes to regulate the biosynthesis of polysaccharide. In addition, there might be more than one branch to synthetize polysaccharide in YB-204.

There must be other important enzymes to regulate the synthesis of polysaccharide in the late stage of growth in microalgae, such as UDP-glucose pyrophosphorylase, ADP-glucose pyrophosphorylase, fructosebisphosphatase, ±-phosphoglucomutase and dTDP-glucose pyrophosphorylase which had been proved to have important roles in the biosynthesis of polysaccharide in other species. We needed to do more work to study the correlation between polysaccharide synthesis and other glycometabolism related enzymes in YB-204.

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