EFFECTS OF SODIUM AND MAGNESIUM SUPPLEMENT ON LIPID PRODUCTION AND WASTEWATER TREATMENT BY *RHODOSPORIDIUM TORULOIDES*

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ABSTRACT

Effects of different concentrations (200-600 mg/L) of sodium (Na^+, provided as Na_2SO_4 and NaCl) and magnesium (Mg^{2+}, provided as $MgSO_4$ and $MgCl_2$) supplement on lipid (biodiesel) production simultaneously with wastewater treatment by the oleaginous yeast *Rhodosporidium toruloides* were investigated using synthetic wastewater with the composition similar to the mixture of real distillery and domestic wastewater. When Mg^{2+} was supplemented as $MgCl_2$ at 200 mg/L, both lipid production and wastewater treatment were enhanced. Compared to control without Mg^{2+} supplement on day 5, the biomass and lipid production increased from 6.48±0.69 g/L and 0.96±0.13 g/L to 7.78±0.58 g/L and 1.35±0.02 g/L, respectively. The removal efficiencies for COD and TP also increased from 72.09±3.32% and 19.57±2.51% to 79.20±1.13% and 23.19±3.07%, respectively. These increases were considered mainly due to the important role of Mg^{2+} in lipid synthesis, phosphate transfer, and carbohydrate metabolism. In comparison to Mg^{2+}, the effect of Na^+ depended on the supplement form. When 200 mg/L Na^+ were provided as NaCl, maximum lipid production was 2.66±0.11g/L while only 1.98±0.63g/L when Na^+ provided as Na_2SO_4. For a better lipid accumulation and wastewater treatment performance, the optimal concentrations of both Mg^{2+} and Na^+ were considered around 200 mg/L.

Keywords: Biolipid, Oleaginous yeast, Trace elements, Wastewater treatment

1 INTRODUCTION

According to the International Energy Outlook [1], the world energy consumption will rise 4-9% between 2019 and 2030, and the renewable energy like solar or nuclear energy will become the leading source of primary energy consumption by 2050. From the perspective of environmental protection and the resource recovery strategy, many countries have actively promoted the development of some environmentally friendly energy source that can be regenerated and cleaned. As a high-quality diesel substitute, biodiesel has drawn increasing attention worldwide due to its important strategic significance for sustainable economic development, promoting energy substitution, and reducing environmental pressure and pollution. Biodiesel is defined as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats.

Using oleaginous microorganisms to produce biodiesel is a good alternative way to the traditional biodiesel productions, based on the economic and environmental consideration. Oleaginous microorganisms such as fungi, yeast, microalgae, and bacilli can be used for the lipid and biodiesel production [2, 3]. Our previous studies show the potential use of yeast *Rhodosporidium toruloides* to treat distillery wastewater while simultaneously generating/accumulating lipids for the biodiesel production purpose under both sterile and non-sterile conditions, in shake flasks or open-bubble-column reactor [4, 5]. However, only a very few studies focus on the effects (stimulatory/inhibitory) of microelements on the lipid production by *R. toruloides*.

Considering the presence of various microelements in the industrial wastewater, even though some previous studies show the effect of microelements (such as iron, zinc, magnesium, and sodium) on lipid production by microalgae and yeast [6, 7], very few studies have been done on the dual purpose process of microbial lipid production simultaneously with effective wastewater treatment, with the addition of microelements. Hence, this study is to explore the potential stimulatory effect of microelements supplement on microbial lipid production and wastewater treatment using oleaginous yeast.

Sodium ions are the major cation in the extracellular fluid that aids metabolism, and can maintain turgor pressure in microbes. In addition, sodium ion is essential in microbial growth as well as commonly present in industrial wastewater including distillery wastewater and distillery spent wash, with the average range of

61-211 mg/L [8, 9]. On the other hand, magnesium ion is also essential to the basic nucleic acid chemistry of organisms due to the interaction between phosphate and magnesium ions. Also, many trace metal ions like magnesium are cofactors of enzymes involved in many metabolic activities.

2 MATERIALS AND METHODS

2.1 Strain, Medium, and Synthetic Wastewater

Yeast strain *Rhodosporidium toruloides* AS 2.1389 used was purchased from the China General Microbiological Culture Collection Center. The strain was subcultured on YPD agar and stored at 4°C, and seed culture was prepared in YPD liquid medium [10]. The ingredients of synthetic wastewater were dextrose (46 mg/L), yeast extract (19 mg/L), $(NH_4)_2SO_4$ (3.6 mg/L), $K_2HPO_4•3H_2O$ (6 mg/L), corresponding to COD (65,000 mg/L), TN (2,450 mg/L), TP (1,150mg/L), NH_3-N (790 mg/L), and pH 3.7, similar to real distillery wastewater [5]. The synthetic wastewater prepared originally contained around 27.23 mg/L of Na^+ and 11.96 mg/L of Mg^{2+}.

2.2 Experimental Setup

The oleaginous yeast *R. toruloides* grown on the YPD agar plate was transferred to 150 mL flasks containing 30 mL fresh YPD medium using the inoculating loop. The flasks were cultured at 30°C, 200 rpm for 48 h for seed propagation. Synthetic wastewater was mixed with DI water (deionized water) at a certain proportion (1:1.5, v/v) [10, 11]. Before two microelements supplemented, the initial concentrations of sodium and magnesium ions in the mixed synthetic wastewater were measured by atomic absorption spectroscopy (AAS) and set as a background. Both cations were supplemented at 0, 200, 400, and 600 mg/L, and for the possible effect of anions associated, Na^+ was supplied as NaCl and Na_2SO_4, and Mg^{2+} as $MgCl_2•6H_2O$ and $MgSO_4•7H_2O$. After 30 mL of mixed synthetic wastewater were added to flasks and autoclaved, seeds with the initial cell density of $2x10^7$ cells/mL were inoculated and cultured at 30°C, 200 rpm for 5 days. Samples were collected in every 24 h and centrifuged for the analyses of COD, TN, TP, and ammonia nitrogen in supernatants and the biomass and lipid production in cell pellets. All the experiments were conducted in duplicates.

2.3 Analytical Methods

Standard Methods [12] were used to measure COD, TP, TN, and ammonia-nitrogen, using Hach reagents and Hach reactor DR/2800 (Hach Company, Loveland, Colorado). The pH was measured using pH meter (S220 seven compact, Mettler Toledo). The concentrations of trace elements (Mg^{2+}, Na^+) in the synthetic wastewater were measured by AAS. The biomass produced was dry weight of cell pellets after centrifuged (4,000 rpm, 10 min) and cell pellets washed twice using distilled water and then dried at 60°C until the weight remained constant. The total lipids were measured following Bligh and Dyer [13] with modifications described by Ling et al. [10]. The biolipid was trans-methylated with 1 mL of 2% (v/v) H_2SO_4-mathanol solution for each sample using water bath at 70℃ for 1 h. After *n*-hexane was used to extract FAMEs (fatty acid methyl esters), the upper layer was washed with DI water before measurement. The biodiesel composition was analyzed by GC-MS (Thermo Scientific, TRACE 1300-ISQ QD system) equipped with TR-FAME column [14].

For the statistical significance of results, the t-test (paired samples test) conducted using software SPSS was applied to compare the lipid production and wastewater treatment results from sodium and magnesium supplement with control group.

3 RESULTS AND DISCUSSION

In general, both sodium and magnesium supplements showed positive impact on lipid production (Fig. 1). When magnesium was supplemented as $MgCl_2$ at a relatively lower concentration (200 mg/L), lipid production increased significantly ($p=0.003<0.05$), achieving maximum lipid production (1.36±0.10 g/L) on day 3, compared to no significant increases observed at higher concentrations (400 and 600 mg/L). Oleaginous yeast is known to gain most of lipid accumulation in early stationary phase [15] and in case of *R. toruloides*, it started from day 3 [10]. The increase in lipid production from magnesium supplement was mainly contributed by the increase in biomass production (Figs 2a and 2b). On day 3 with 200 mg/L Mg^{2+} supplemented as $MgCl_2$, biomass production increased from 6.48±0.69 to 7.78±0.58 g/L, while lipid content increased from 13.20±1.01 to 15.99±0.12%, compared to no supplement. Similar trend was also observed on day 5 with the magnesium supplement regardless of the supplement form. The increases in biomass and lipid production have also been reported. As a cofactor of more than 300 enzymes and a crucial ion in nucleic acid synthesis for yeast growth and metabolisms [16], 125.6±7.2% increase in biomass was reported when 1 g/L $MgSO_4$ supplied [17]. Dombek et al. [18] also observed increase in yeast biomass after supplying magnesium. Magnesium is also an important cofactor for enzymes related to lipid production, like malic enzyme in transdehydrogenase cycle during fatty acid biosynthesis [19], and could also induce the activity of acetyl CoA carboxylase enzyme [20]. Apart from the lipid and biomass production, magnesium supply also promoted wastewater treatment especially for the COD and TP removal. The highest increases in COD and TP removal were observed when 200 mg/L Mg^{2+} were supplied in $MgCl_2$, from 72.09±3.32 and 19.57±2.51% to 79.20±1.13 and 23.19±3.07%, respectively, in 5 days (Figs 2a-c). To achieve the optimal in both wastewater treatment and lipid production, samples harvested on day 5 showed 29 and 44% increases in COD and TN removal while with similar lipid production (1.36±0.10 g/L on day 3 and 1.35±0.02 g/L on day 5), compared to when harvested on day 3. These increases in COD and TP removal were considered due to the important role of magnesium in phosphate transfer and carbohydrate metabolism [21, 22].

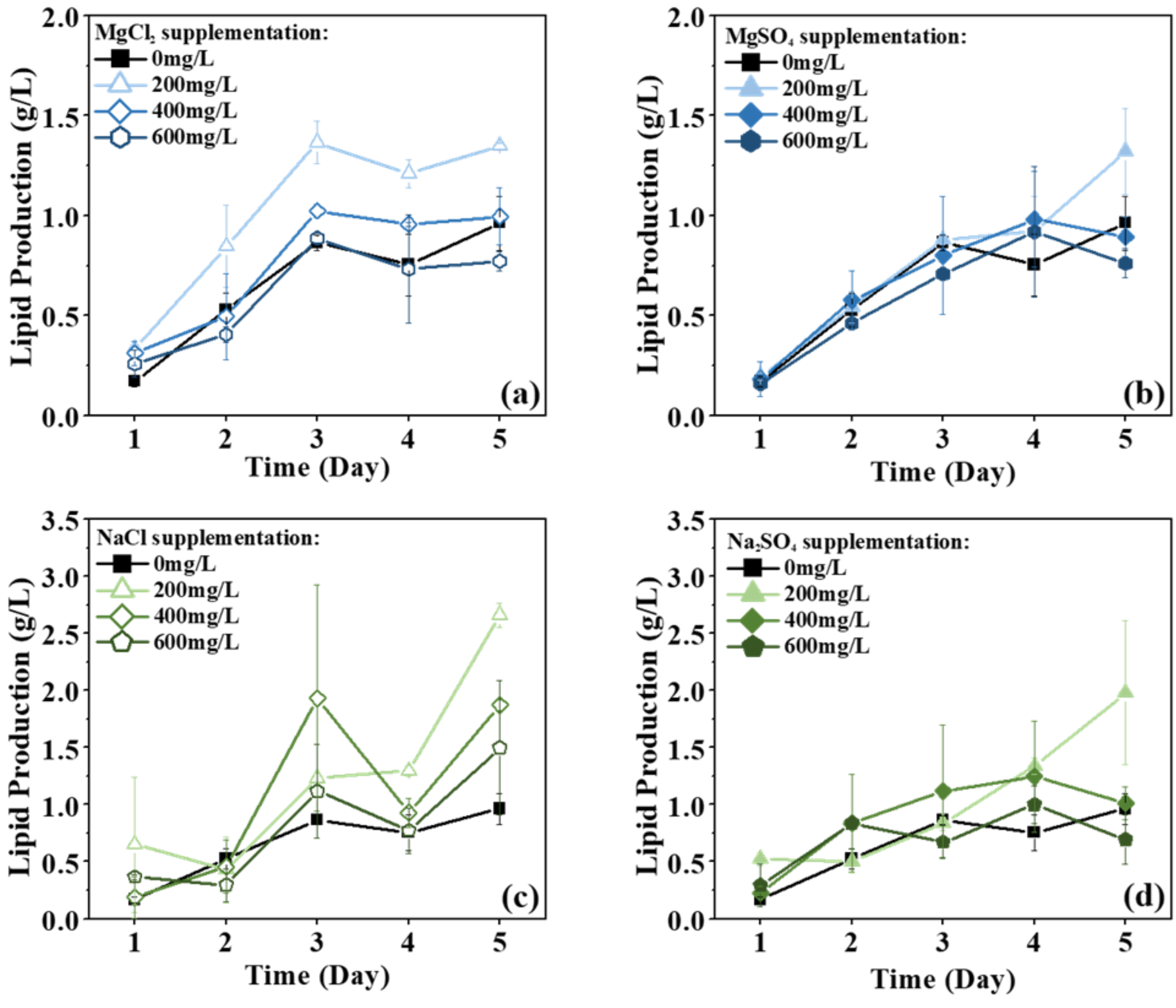

Figure 1. Lipid Production for Sodium and Magnesium Supplement

When sodium was supplemented as NaCl at 200 mg/L, lipid production increased and maximum lipid production (2.66±0.11 g/L) was achieved on day 5 (Fig. 1c), almost the double compared to the magnesium supplement. In comparison, however, when sodium supplemented as NaCl, no obvious impact was shown at 200 mg/L Na^+ while higher concentrations (400 and 600 mg/L Na^+) showed inhibitory to biomass production (Figs 2d-f). On the other hand, a slight increase in biomass production was observed when sodium supplemented as Na_2SO_4 at 200 and 400 mg/L. When NaCl was supplemented, it resulted in stimulating more lipid content which subsequently led to higher lipid production and this might be because

NaCl increased the salinity of the medium. Increasing salinity has been reported to have a positive impact on lipid content and a negative impact on biomass production [23]. A low concentration of salt (NaCl) can increase lipid production while letting the cells adapt to the salinity [24], in accordance with the results from supplement of 200 mg/L sodium ion in NaCl in this study. When sodium was supplemented as NaCl, compared to Na_2SO_4, both biomass production and lipid content were increased as well as COD and TN removal (Figs 2e and 2f). The Na_2SO_4 supplement increased COD removal slightly, while the NaCl supplement at 200 mg/L Na^+ increased TN removal significantly ($p=0.03<0.05$).

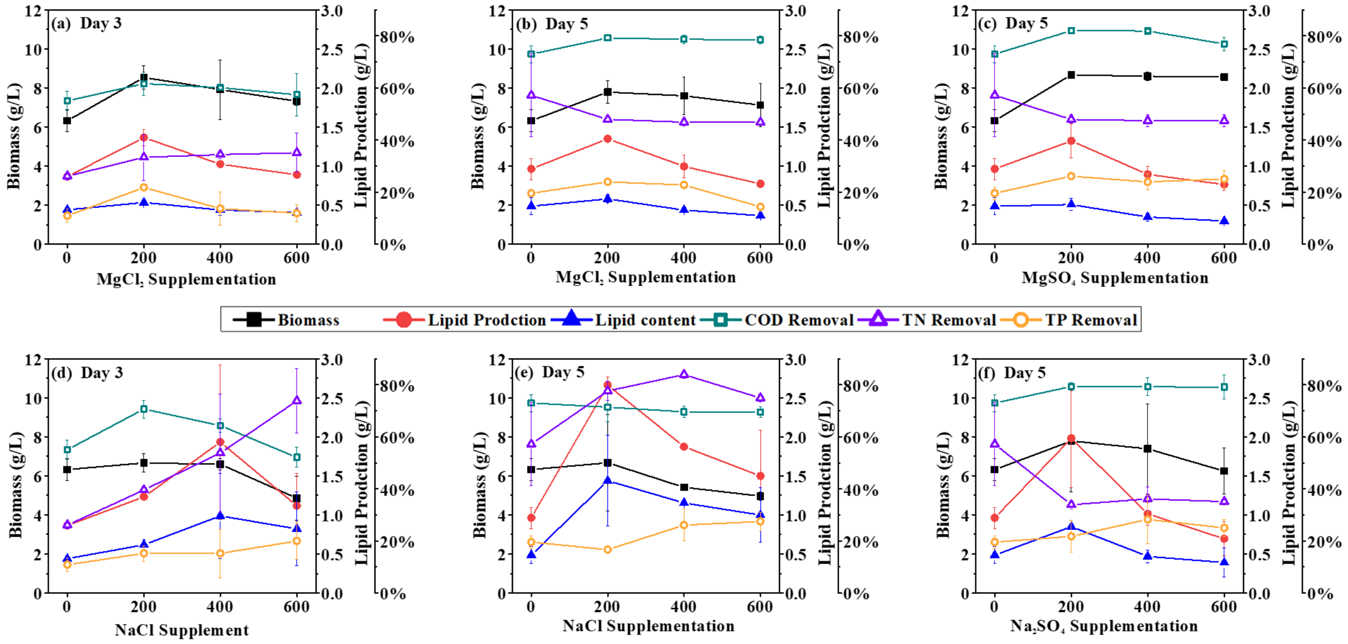

Figure 2. Effects of Sodium and Magnesium Supplement on Lipid Production and Wastewater Treatment

Both sodium and magnesium supplement showed no obvious impact on lipid composition. Composition of biodiesel obtained after with and without sodium and magnesium supplement is shown Table 1. The dominant components were oleic acid methyl ester (C18:1), linoleic acid methyl ester (C18:2), and palmitic acid methyl ester (16:0), together accounting for over 80% of total FAMEs detected. Biodiesel rich in oleic acid methyl ester (almost 50% detected) is known favoured, providing suitable cetane number, cold-flow parameters, and viscosities [25]. Magnesium supplement also increased the total unsaturation degree from 100.17 to 108.86 while sodium supplement increased the unsaturation degree to 103.45. This increases were mainly contributed by the increase of linoleic acid methyl ester content. The unsaturation degree of biodiesel would further affect its quality such as cetane number and iodine value. The biodiesel would not meet the EN 14214 standard [26] in cetane number and iodine value if the degree of unsaturation larger than 137 [27], while the unsaturation degree of biodiesel from sodium and magnesium supplement in this study is still within the suitable range. On the other hand, the linolenic acid methyl ester (C18:3) content was within the EN 14214 standard requirements (below 12%), regardless of sodium or magnesium supplement. Supplement of sodium and magnesium also showed decrease of lignoceric acid methyl ester (C24:0) content and increase in stearic acid methyl ester (C18:0), further meaning decrease in overall chain length. Chain length is considered another important factor that impacts biodiesel quality [15, 25] and shorter chain length generally leads to better cold weather performance of biodiesel [28].

Table 1. Lipid Composition (%) with or without Sodium or Magnesium Supplement

	C16:0	C17:1	C18:0	C18:1	C18:2	C18:3	C24:0	Others	Degree of Unsaturation
No Addition	11.80±0.05	1.33±0.03	3.77±0.55	47.81±0.16	21.77±0.15	3.81±0.15	4.96±0.27	4.58±0.12	100.17
Mg^{2+} 200 mg/L ($MgCl_2$)	10.37±0.37	0.93±0.01	4.88±0.34	47.25±0.38	27.13±0.51	3.20±0.18	3.01±0.20	4.17±0.22	108.86
Na^+ 200 mg/L (NaCl)	11.13±0.71	1.21±0.28	4.72±0.62	49.22±4.45	23.55±4.46	3.00±0.19	3.64±0.96	4.74±0.83	103.45

4 CONCLUSION

For the enhanced lipid production simultaneously with wastewater treatment by the oleaginous yeast strain *R. toruloides*, trace elements sodium and magnesium are proposed to be supplemented at 200 mg/L, as forms of NaCl and $MgCl_2$, respectively. The enhancement by magnesium is considered due to its important role in lipid synthesis, cells growth and metabolism, and increase of salt content in medium. Supplementation of sodium and magnesium also led to slight increase in unsaturation degree and decrease in chain length of biodiesel.

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