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Fermentation Characteristics of *Mortierella alpina* in Response to Different Nitrogen Sources

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Abstract The fermentation characteristics of *Mortierella alpina* were investigated in response to various nitrogen sources. Influences on nitrogen source and glucose uptake rate, mycelial morphology of *M. alpina*, and pH of medium in relation to different nitrogen sources were discussed. Effects of different nitrogen sources on cell growth, fatty acid composition, arachidonic acid (ARA), and total lipid concentration were also evaluated. It revealed that the maximum nitrogen source uptake ratio was obtained when corn steep liquor was used as nitrogen source. When yeast extract was used as the sole nitrogen source, glucose was completely exhausted at the end of fermentation. The maximum dry cell weight obtained from medium with yeast extract as nitrogen source had the highest total lipid concentration. Sodium nitrate was the favorable nitrogen source for ARA accumulation, and the highest ARA percentage in total fatty acids was obtained, 35.9%. Urea was identified as the favorable nitrogen source for ARA production, the highest ARA concentration obtained from urea was 5.8 g/l. Compared with inorganic nitrogen sources, organic nitrogen compounds are favorable for both cell growth and total lipids accumulation.

Keywords Arachidonic acid · *Mortierella alpina* · Nitrogen source · Fermentation characteristics

Introduction

Polyunsaturated fatty acids (PUFAs) have been more attractive recently as a result of their biological activities and clinical effects, they have been more attractive recently due to their biological activities and clinical effects [1–4]. Arachidonic acid (cis-5, 8, 11, 14-eicosatetraenoic acid, ARA), as a representative ω-6 PUFA, is important as a natural constituent of biological membranes and a precursor for numerous eicosanoids such as prostaglandins, thromboxanes, and leukotriens [5]. There has recently been increasing

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interest in the production of ARA because of its unique biological activities [6–8]. Since *Mortierella alpina* is widely used for ARA production, this organism was selected for a study of the biochemical basis of ARA formation [9]. The major obstacle for large-scale ARA production by *M. alpina* is the relatively high cost, which is expected to be solved by increasing the dry cell weight (DCW) while keeping relatively high ARA percentage in total fatty acids and reducing the fermentation cost.

Nitrogen sources are very important chemical compounds in the cultivation of *M. alpina*. They can use a wide variety of nitrogen sources; however, not all nitrogen sources support cell growth and ARA production equally well. Compared with other inorganic nitrogen sources, sodium nitrate and potassium nitrate as the sole nitrogen source showed a high cell growth, total PUFAs, and ARA production [10]. Replacement of sodium nitrate with corn steep liquor (CSL, 1% w/v) significantly improved the ARA production of *M. alpina* ATCC 32222 [11]. Most of the previous works have proven that yeast extract was favorable for cell growth, but not for ARA production by *M. alpina*, and it was not an effective nitrogen resource for large-scale industrial ARA production because of its relatively high cost [12, 13].

Influences of nitrogen sources on oleaginous microorganisms have been widely investigated. Sodium nitrate cannot only stimulate cell growth but also be considered as the favorable nitrogen source for lipid accumulation of *Neochloris oleoabundans* [14]. It has also been reported that certain oleaginous *yeast* accumulated more lipid when an organic nitrogen source rather than an inorganic nitrogen source was used [15, 16]. Urea and sodium nitrate as the sole nitrogen source, respectively, could increase the intracellular ergosterol content, but sodium nitrate inhibited the growth of yeast considerably; CSL, yeast extract, and ammonium sulfate could prompt cell growth, but reduced intracellular ergosterol content [17].

The diversity of available nitrogen sources in the fermentation medium is important both for cell growth and ARA accumulation in *M. alpina*. However, there are few systematic studies reported in the literature regarding the application of different nitrogen sources for ARA production. It is therefore of both academic and practical relevance to investigate the effects of different nitrogen sources on the cell growth, total lipid, and ARA production. In this paper, we investigated nitrogen source and glucose uptake rate, mycelia morphology and the pH of medium under the condition of using different nitrogen sources, and the cell growth, ARA percentage in total fatty acids, fatty acids composition, total lipids, and ARA concentration of *M. alpina* in response to different nitrogen source were also discussed. This study was useful for how to select nitrogen sources for efficient ARA production.

Materials and Methods

Microorganism

M. alpina ME-AA01 (CCTCC M 207067), preserved in the China Center for Type Culture Collection (CCTCC), was used in the present study. It was maintained on potato dextrose agar (PDA) slants at 4 °C and transferred every 3 months.

Culture Media and Cultivation Methods

Inoculum medium (g/l): glucose 30; yeast extract 6; KH_2PO_4 3; $NaNO_3$ 3; $MgSO_4 \cdot 7H_2O$ 0.5. Fermentation medium (g/l): glucose 100; KH_2PO_4 3.8; $MgSO_4 \cdot 7H_2O$ 0.5. The single nitrogen source was: sodium nitrate, potassium nitrate, ammonium nitrate, ammonium

chloride, ammonium nitrate, yeast extract, CSL, and urea. On an equivalent nitrogen basis, other nutrients concentration in culture medium in each test was the same. The media were autoclaved at 115 °C for 30 min. Inocula were prepared in 250 ml baffled flasks containing 50 ml medium. The culture was grown for 3 days at 25 °C with shaking at 120 rpm. Then 5 l bioreactor (New Brunswick Scientific, USA) containing 3 l fermentation medium was incubated at 25 °C, agitation speed 120 rpm, and pH 6.0. To investigate the pH of cultivation medium under different nitrogen sources, the pH was first set as 6.0 and was not controlled during the fermentation process. The cultivation time in the experiments ranged from 0 to 168 h as indicated. Samples were taken periodically for analysis.

Analytical methods

DCW was determined gravimetrically. Glucose concentration was measured by a biosensor with glucose oxidase electrode (Institute of Biology, Shandong Academy of Sciences SBA-40 C). Total lipids were extracted with chloroform/methanol (2:1, v/v) following the method of Bligh and Dyer [18]. Fatty acids were methylated by boron trifluoride in methanol according to the method of Metchalfe and Schmitz [19]. Fatty acid methyl esters were determined using a gas chromatography–mass spectrometry Thermo Finnigan TRACE DSQ [20]. The degree of unsaturation (Δ/mol) in the lipid fraction was calculated by the method of Kates [21], according to the formula: Δ/mol=[1.0 (% monoene)+2.0 (% diene)+3.0 (% triene)+4.0 (% tetraene)+5.0 (% pentaene)]/100. The nitrogen concentration in media was determined using alkaline potassium persulfate digestion and ultraviolet spectrophotometer according to the China Standard GB 11894-89 method.

Results

Effects of Different Nitrogen Sources on the Uptake Ratio of Nitrogen and Glucose

Single-factor experiments with triplets were carried out to investigate the nitrogen source uptake ratio (residual nitrogen source concentration to initial nitrogen source concentration ratio,%) and glucose uptake ratio (residual glucose concentration to initial glucose concentration ratio,%) by *M. alpina* cultured with different organic nitrogen sources (CSL, yeast extract and urea) and different inorganic nitrogen sources (sodium nitrate, potassium nitrate, ammonium sulfate, ammonium nitrate, and ammonium chloride). Figure 1 shows nitrogen source and glucose uptake ratio in relation to different nitrogen sources. The nitrogen source uptake ratio with organic nitrogen sources was higher than that of inorganic nitrogen sources. The maximum nitrogen uptake ratio (75.2%) was obtained when CSL was used as the sole nitrogen source; however, its glucose uptake ratio was only 37.3%, lowest among three tested organic nitrogen sources (yeast extract, CSL, and urea). The maximum glucose uptake ratio (100%) was obtained using yeast extract as the sole nitrogen source, indicating that glucose was exhausted at the end of fermentation. The difference in nitrogen uptake rate among sodium nitrate, potassium nitrate, ammonium sulfate, ammonium nitrate, and ammonium chloride was small, varying from 34.0% to 32.4%. However, there was significant difference in glucose uptake ratio among five tested inorganic nitrogen sources (sodium nitrate, potassium nitrate, ammonium sulfate, ammonium nitrate, and ammonium chloride), and the glucose uptake ratio of three ammonium salts (ammonium nitrate, ammonium chloride, and ammonium sulfate) was lowest among the tested nitrogen sources. These results suggest that, with different

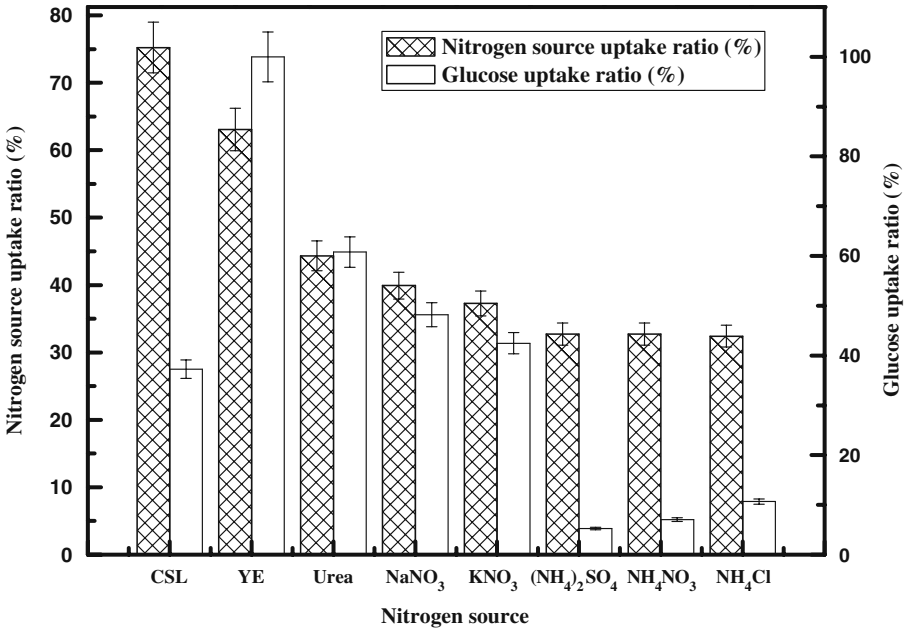

Fig. 1 Nitrogen source uptake rate and glucose uptake ratio by *M. alpina* ME-AA01 with different nitrogen sources. *CSL* Corn steep liquor, *YE* yeast extract, $NaNO_3$ sodium nitrate, KNO_3 potassium nitrate, $(NH_4)_2SO_4$ ammonium sulfate, NH_4NO_3 ammonium nitrate, NH_4Cl ammonium chloride

nitrogen compounds as the sole nitrogen source, nitrogen source depletion was not in parallel with glucose depletion.

Effects of Different Nitrogen Sources on Cell Growth, ARA, and Total Lipid Production

Dynamic courses of cell growth and ARA percentage in total fatty acids obtained with different nitrogen sources are shown in Fig. 2. According to Fig. 2a, it was obvious that yeast extract, urea, CSL, sodium nitrate, and potassium nitrate could bring positive effect on cell growth, and the DCW kept increasing throughout the cultivation process. When yeast extract was used as the sole nitrogen source, the fungi grew faster in the early stage of cultivation, the DCW increased rapidly during the first 72 h, and then reached peak (29.8 g/l) after 168 h, which was 1.2 and 1.6 times of that obtained from urea and CSL, respectively. The DCW obtained from urea increased slowly for the first 120 h and then more rapidly reached 25.7 g/l after 168 h. When sodium nitrate was used as the sole nitrogen source, the DCW increased to 17.5 g/l after 168 h, which was higher than that obtained from potassium nitrate (15.8 g/l). On the other hand, the DCW obtained from ammonium salts nearly did not increase during the whole fermentation process. The least DCW obtained with ammonium nitrate was only 0.7 g/l. According to the dynamic analysis of growth curve of *M. alpina* with different nitrogen sources, the final DCW obtained from organic nitrogen sources was higher than that obtained from inorganic nitrogen sources, and yeast extract was considered as the most favorable nitrogen source for cell growth of *M. alpina*.

Different kinds of nitrogen sources not only affect cell growth but also affect ARA accumulation. As shown in Fig. 2b, the ARA percentage in total fatty acids obtained from yeast extract, urea, CSL, sodium nitrate, and potassium nitrate kept increasing throughout

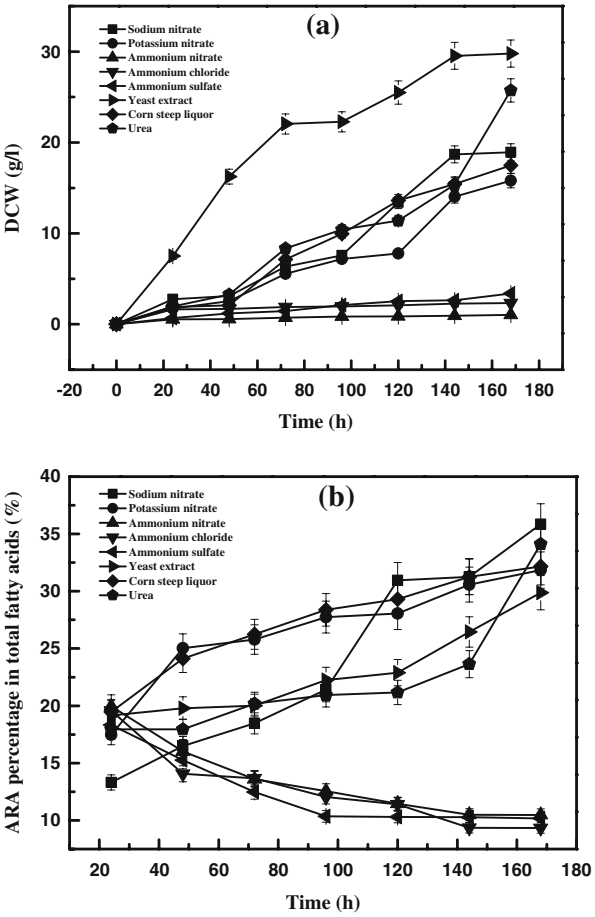

Fig. 2 Cell growth (**a**) and ARA percentage in total fatty acids (**b**) of *M. alpina* ME-AA01 cultivated with different nitrogen sources

the whole cultivation process. The ammonium salts brought negative effect on ARA percentage in total fatty acids and showed a decreasing tendency throughout the whole cultivation process. The ARA percentage in total fatty acids obtained from sodium nitrate increased rapidly after 120 h and then slowly reached to peak (35.9%) after 168 h, which was 3.1, 3.8, and 3.5 times of that obtained from ammonium nitrate, ammonium chloride, and ammonium sulfate, respectively. When urea was used as the sole nitrogen source, the ARA percentage in total fatty acids slowly increased to 34.1% after 168 h, which was 1.1 times of that obtained from yeast extract and CSL, respectively. These results suggested that sodium nitrate was the favorable nitrogen source for ARA accumulation by *M. alpina*.

Table 1 illustrates the fatty acids composition obtained with different nitrogen sources. When sodium nitrate was used as the sole nitrogen source, the proportion of dihomo-γ-1inolenic acid (C20:3) in total fatty acids were lowest compared with other fatty acids. The maximum proportion of γ-linolenic acid (C18:3) in total fatty acids (29.2%) was obtained from ammonium sulfate, followed by ammonium nitrate, 29.1%. The highest degree of unsaturation of fatty acids obtained from sodium nitrate was 2.0, which indicated that using sodium nitrate as the sole nitrogen source could achieve a reasonably good degree of unsaturation. When ammonium chloride was used as the sole nitrogen source, the proportion of stearic acid (C18:0) was higher than that obtained from other nitrogen

Table 1 Fatty acid composition of *M. alpina* ME-AA01 cultured with different nitrogen sources

Nitrogen source	Fatty acids composition (%)							Degree of unsaturation (Δ/mol)
	C16:0	C18:0	C18:1	C18:3	C20:3	C20:4	Others	
$NaNO_3$	13.0	7.8	16.5	14.0	1.2	35.9	11.6	2.0
KNO_3	14.3	7.1	15.9	11.3	1.1	31.8	18.5	1.8
NH_4NO_3	4.2	25.2	10.4	29.1	1.2	11.5	18.4	1.5
NH_4Cl	3.0	29.7	11.9	28.3	1.4	9.4	16.3	1.4
$(NH_4)_2SO_4$	3.2	29.5	11.0	29.2	1.5	10.4	15.2	1.5
YE	22.6	22.9	26.4	3.9	2.3	21.4	0.5	1.3
CSL	18.0	6.5	20.2	0.5	2.4	32.2	20.2	1.6
Urea	4.3	4.2	16.5	1.2	0.8	34.1	38.9	1.7

C16:0 Palmitic acid, *C18:0* stearic acid, *C18:1* oleic acid, *C18:3* γ-linolenic acid, *C20:3* dihomo-γ-linolenic acid, *C20:4* arachidonic acid

sources. The lowest degree of unsaturation of fatty acids was obtained from ammonium chloride (1.4). The proportion of ARA in total fatty acids obtained from ammonium salts was obviously lower than that of other fatty acids, and the degree of unsaturation of fatty acids was also obviously lower than that of other nitrogen sources. Using yeast extract as the sole nitrogen source, the proportion of palmitic acid (C16:0), C18:0 and oleic acid (C18:1) in total fatty acids were relatively higher than other fatty acids, and the proportion of ARA in fatty acids was obviously lower than that obtained from sodium nitrate, potassium nitrate, CSL, and urea. The proportion of C18:3 and C20:3 in total fatty acids were lower than that of other fatty acids when urea was used as the sole nitrogen source.

ARA and total lipid concentration of *M. alpina* cultivated with different nitrogen sources are shown in Fig. 3. The maximum ARA concentration (5.8 g/l) was obtained with urea, which was 1.3 and 1.5 times of that obtained from yeast extract and CSL, respectively. Among five tested inorganic nitrogen sources (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium sulfate, and ammonium chloride), the maximum ARA concentration (3.9 g/l) was obtained from sodium nitrate, which was 6.7, 7.2, and 7.2 times of that obtained from ammonium nitrate, ammonium chloride, and ammonium sulfate, respectively. Figure 3 shows that the total lipids concentration obtained from organic nitrogen sources was higher than that obtained from inorganic nitrogen sources. The maximum total lipid concentration (21.6 g/l) was obtained from yeast extract, which was 1.2 and 1.7 times of that obtained from urea, and CSL, respectively. The difference in total lipids between sodium nitrate and potassium nitrate was small, varying from 12.2 to 11.7 g/l. The total lipids concentration obtained from three tested ammonium salts was obviously lower than that of other tested nitrogen sources, the lowest total lipids concentration was obtained from ammonium sulfate, 4.1 g/l. These results demonstrated that the ammonium salts was not favorable for lipid synthesis and caused the lowest total lipids concentration.

Mycelial Morphology and Medium pH in Response to Different Nitrogen Sources

The morphology of a culture is considered to affect enzymes and secondary metabolites, and the morphology of *M. alpina* could vary between circular pellet and radial filamentous forms during cultivation process. It has been reported that changes in morphology were affected by, for example, culture media and environmental parameters such as pH, aeration,

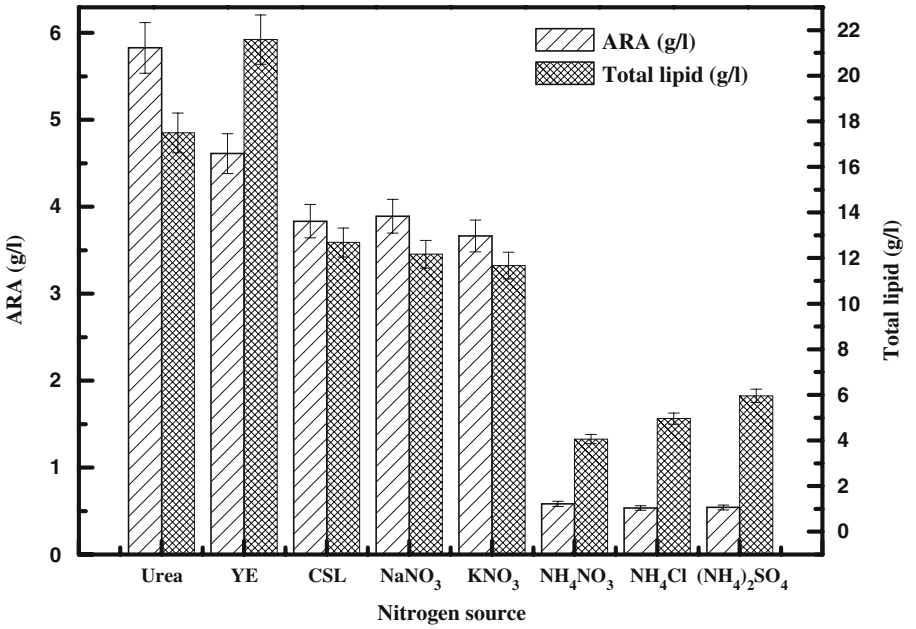

Fig. 3 ARA (**a**) and total lipids (**b**) concentration of *M. alpina* ME-AA01 cultivated with different nitrogen sources. *$NaNO_3$* Sodium nitrate, *KNO_3* potassium nitrate, *NH_4NO_3* ammonium nitrate, *NH_4Cl* ammonium chloride, *$(NH_4)_2SO_4$* ammonium sulfate, *YE* yeast extract, *CSL* corn steep liquor

and stirring speed [22, 23]. The circular pellet type was obtained in the cases of using sodium nitrate, potassium nitrate, yeast extract, CSL, and urea as nitrogen source, respectively. On the other hand, radial filamentous form of mycelia was obtained from ammonium nitrate, ammonium chloride, and ammonium sulfate (Fig. 4).

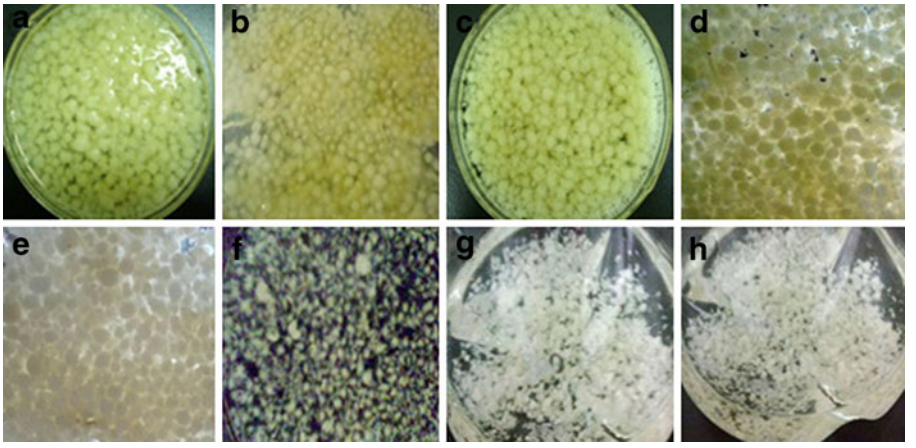

Fig. 4 Morphology of *M. alpina* ME-AA01 in cultures using yeast extract (**a**), corn steep liquor (**b**), urea (**c**), sodium nitrate (**d**), potassium nitrate (**e**), ammonium nitrate (**f**), ammonium chloride (**g**), and ammonium sulfate (**h**) as nitrogen source, respectively

Figure 5 shows the pH profiles of the culture in response to different nitrogen sources. There was little difference in the initial pH among five tested inorganic nitrogen sources (sodium nitrate, potassium nitrate, ammonium chloride, ammonium sulfate, and ammonium nitrate). When sodium nitrate and potassium nitrate were used as the sole nitrogen source, the pH kept increasing during the whole period of cultivation, and the final pH were 7.5 and 7.7, respectively. However, the pH of the ammonium salts significantly decreased after 24 h. With ammonium nitrate as the sole nitrogen source, the pH almost did not change from 24 to 168 h, and the finial pH was 2.8. When ammonium chloride and ammonium sulfate were used as the sole nitrogen source, the pH was constant from 24 to 120 h, and significantly decreased from 120 to 144 h, respectively, then nearly kept constant until the end of fermentation, and the final pH was 2.2 and 2.3, respectively.

When urea was used as the sole nitrogen source, the pH significantly decreased during the first 48 h, and then slowly increased to 5.7 till the end of fermentation. The initial pH of medium containing yeast extract was lower than that of urea; the pH significantly decreased for the first 72 h, increased rapidly from 96 to 120 h, then slowly increased to 5.0 after 168 h. With CSL as the sole nitrogen source, the initial pH of medium was lowest among three tested organic nitrogen sources, then pH slowly decreased from 0 to 72 h and increased to 6.3 till the end of cultivation.

Discussion

Sodium nitrate and potassium nitrate were selected to represent nitrate. Ammonium nitrate, ammonium chloride, and ammonium sulfate were selected to represent ammonia. Yeast extract and CSL are common organic nitrogen sources for microorganism fermentation. Both of them can act not only as the nitrogen sources but also as amino acid, vitamin, growth factor, and salt [17]. On an equivalent nitrogen basis, urea is cheaper than yeast extract and CSL. In addition, its nitrogen content is higher than that of yeast extract and CSL. It would be interesting to further investigate the effect of other nitrogen sources on cell growth, ARA, and total lipid production of these fungi.

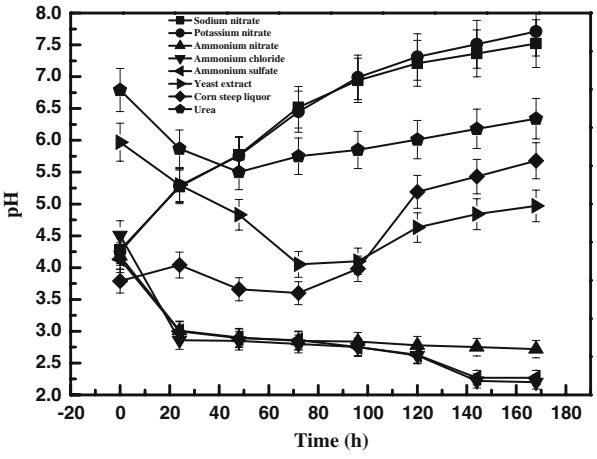

Fig. 5 pH of cultivation medium under different nitrogen sources

The DCW and total lipids obtained from organic nitrogen source were higher than that obtained from inorganic nitrogen source, suggesting that *M. alpina* hardly assimilate inorganic nitrogen source and require amino acid or protein for cell growth and total lipid accumulation. It has been known that organic nitrogen source has a higher stimulating effect on the lipid synthesis in some oleaginous *yeast* as inorganic nitrogen source [24]. Organic nitrogen source substrates of a complex chemical structure supported an intensive lipid accumulation, and the nature of the nitrogen source influences the amount and the composition of *M. alpina* CCF 185 lipid produced [25].

ARA is not a nitrogenous compound and its content in cell is mainly influenced by nitrogen source. Nitrogen sources are essential chemical compounds for *M. alpina* cell growth, for they require amino acid and protein to obtain a certain amount of DCW [13]. Lipid accumulation is only favored by a fixed range of carbon to nitrogen (C/N) ratio, and C/N ratio also influences ARA production in the culture of *M. alpina* [26]. The optimal C/N ratio for ARA production was around 15–20 in a culture of *M. alpina* CBS 754.68 [27]. When the C/N ratio was higher than 20, cell growth was inhibited and ARA decreased due to nitrogen limitation [13]. In our study, the glucose concentration was fixed and constant, the nitrogen source concentration of different nitrogen source medium was equivalent to each other, and the C/N ratio was approximately 19.8. However, the DCW, total lipid, and ARA concentration were obviously different among these tested nitrogen sources. It was clear that this effect was not caused by C/N ratio.

As mentioned above, glucose uptake ratio and ARA percentage in total fatty acids obtained from yeast extract, urea, CSL, sodium nitrate, and potassium nitrate were higher than that obtained from ammonium salts. These results suggest that, under exhaustion of glucose from the medium, ARA synthesis occurred at the expense of the other fatty acids in the intracellular lipid pool. Intensive ARA accumulation was accompanied by a decrease in the amounts of saturated fatty acids and unsaturated ARA precursors. This result was in accordance with previous study [28]. The difference in nitrogen source uptake rate among the five tested inorganic nitrogen sources was small. It could be assumed that the lowest DCW, ARA, and total lipids concentration obtained from ammonium salts were not caused by nitrogen source uptake ratio. It has been known that the cell stopped growing mainly because of carbon source limitation and, consequently, inefficient energy source and carbon source needed for cell growth [29]. The glucose uptake ratio of three tested ammonium salts was lower than that of other tested nitrogen sources, and it seems to be reasonable to assume that the lower glucose uptake ratio could be the factor that inhibited cell growth of *M. alpina*. The cell growth was related to glucose depletion. The higher glucose uptake ratio was, the higher DCW could be obtained at the end of fermentation. In the presence of sufficient nitrogen source, ARA and total lipid concentration were dependent on glucose assimilation. From above results, nitrogen source uptake ratio of organic nitrogen sources was higher than that of inorganic nitrogen sources, and both DCW and total lipids concentration obtained from organic nitrogen sources were higher than that of inorganic nitrogen sources. These phenomena could be explained as follows: firstly, nitrogen is the most commonly reported nutritional limiting factor triggering lipid accumulation, it hardly assimilated inorganic nitrogen sources for cell growth [13]; secondly, the depletion of nitrogen in the medium evokes a decrease in the intracellular concentration of AMP, an activator of isocitrate dehydrogenase, and, as a consequence, an increase in the contents of citrate and isocitrate in the cells; citrate is converted by adenosine triphosphate: citrate lyase to oxaloacetate and acetyl-CoA, the latter is used for the synthesis of fatty acids [29].

It has been proven that nitrogen source affected mycelial morphology and ARA production of *M. alpina* CBS 754.68 [30]. Compared with filamentous form obtained from ammonium nitrate, ammonium chloride, and ammonium sulfate, small pellets were obtained from other tested nitrogen sources (yeast extract, CSL, urea, sodium nitrate, and potassium nitrate) that exhibited considerably higher productivity of ARA, which results from gas dispersion, mass and heat transfer, and homogenization [31, 32].

Throughout cultivation process, the pH of medium containing sodium nitrate and potassium nitrate increased slowly, and the pH values were maintained in the range of 4.3–7.5 and 4.3–7.7, respectively. This might be due to the neutral pH in the nitrate salt amendment. An acidic pH was observed in ammonium salts supplementation, the metabolism of these three kinds of ammonium salts can contribute to the acidification of the medium. The pH of yeast extract and urea medium firstly decreased and then increased to neutral throughout fermentation. The pH drop at first might be due to the accumulation of organic acid [33].

In conclusion, the present study provides some fermentation characteristics in relation to different nitrogen sources. Nitrogen source uptake ratio and fatty acid compositions of *M. alpina* with different nitrogen sources could provide valuable information as to how to effectively apply nitrogen sources and select nitrogen sources to enhance ARA concentration. Although it is suggested that yeast extract could enhance DCW and total lipids accumulation by *M. alpina*, the ARA percentage in total fatty acids was lower than that obtained from urea, sodium nitrate, and potassium nitrate, resulting in lower ARA concentration. Because of its high cost, it may not be suitable for large-scale industrial ARA production. When sodium nitrate was used as the sole nitrogen source, the ARA percentage in total fatty acids was highest, but the DCW and total lipids concentration were lower than that obtained from yeast extract and urea. So the ARA concentration was lower than that obtained from yeast extract and urea. When urea was used as the sole nitrogen source, there was little difference in the ARA percentage in total fatty acids from sodium nitrate. The DCW and total lipids concentration obtained from urea were significantly higher than that obtained from sodium nitrate, and urea showed the highest ARA concentration and ARA proportion in total fatty acids. According to our calculation, when urea was used as the sole nitrogen source, the cost was lowest among these tested nitrogen source; therefore, it may be an economic nitrogen resource for large-scale industrial ARA production.

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References

1. Calder, P. C. (2001). Polyunsaturated fatty acids, inflammation and immunity. *Lipids, 36*, 1007–1024.
2. Fontani, G., Corradeschi, F., Felici, A., Alfatti, F., Migliorini, S., & Lodi, L. (2005). Cognitive and physiological effects of w-3 polyunsaturated fatty acid supplementation in healthy subjects. *European Journal of Clinical Investment, 35*(11), 691–699.
3. Maclean, C. H., Newberry, S. J., Mojica, W. A., Khanna, P., Issa, A. M., Suttorp, M. J., et al. (2006). Effects of omega-3 fatty acids on cancer risk: A systematic review. *Journal of the American Medical Association, 295*(4), 403–415.

4. Siddiqui, R. A., Shaikh, S. R., Sech, L. A., Yount, H. R., Stillwell, W., & Zaloga, G. P. (2004). Omega 3-fatty acids: Health benefits and cellular mechanisms of action. *Mini Reviews in Medicinal Chemistry, 4* (8), 859–871.
5. Gill, I., & Valivety, R. (1997). Polyunsaturated fatty acid: Part 1. Occurrence, biological activities and applications. *Trends in Biotechnology, 15*(10), 401–409.
6. Glomset, J. A. (1985). Fish fatty acids and human health. *The New England Journal of Medicine, 312*, 1353–1354.
7. Phillipson, B. E., Rothrock, D. W., Conor, W. E., Harris, W. S., & Illingwort, D. R. (1985). Reduction of plasma lipids, lipoproteins and apolipoproteins by dietary fish oils in patients with hypertriglyceridemia. *The New England Journal of Medicine, 312*, 1210–1216.
8. Wong, S. H., Nestel, P. J., Trimble, R. P., Storer, G. B., Illman, I. J., & Topping, D. L. (1984). The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. *Biochimica et Biophysica Acta, 792*(2), 103–109.
9. Certik, M., & Shimizu, S. (2000). Kinetic analysis of oil biosynthesis by an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4. *Applied Microbiology and Biotechnology, 54*(2), 224–230.
10. Chang, C. C., & Chen, H. C. (1994). Arachidonic acid production by *Mortierella alpina*. *Journal of the Chinese Agricultural Chemical Society, 32*, 395–405.
11. Singh, A., & Ward, O. P. (1997). Production of high yields of arachidonic acid in a fed-batch system by *Mortierella alpina* ATCC 32222. *Applied Microbiology and Biotechnology, 48*(15), 1–5.
12. Bajpai, P. K., & Bajpai, P. (1992). Review: Arachidonic acid production by microorganisms. *Biotechnology and Applied Biochemistry, 15*(1), 1–10.
13. Jang, H. D., Lin, Y. Y., & Yang, S. S. (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*. *Bioresource Technology, 96*(15), 1633–1644.
14. Li, Y. Q., Horsman, M., Wang, B., Wu, N., & Lan, C. Q. (2008). Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Applied Microbiology and Biotechnology, 81*(4), 629–636.
15. Steinerm. (1959). The utilization of amino and amide nitrogen by *Endomycopsis vernalis* and other yeasts. *Symposia of the Society for Experimental Biology, 13*, 177–192.
16. Witter, B., & Steinerm, D. (1974). Die lipide von endomycopsis cernalis bei verschiedener stickstoff-ernahrung. *Archives of Microbiology, 101*, 321–335.
17. Shang, F., Wen, S. H., Wang, X., & Tan, T. W. (2006). Effect of nitrogen limitation on the ergosterol production by fed-batch culture of *Saccharomyces cerevisiae*. *Journal of Biotechnology, 122*(3), 285–292.
18. Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*(8), 911–917.
19. Metchalfe, L. D., & Schmitz, A. R. A. (1961). The rapid preparation of fatty acid esters for gas chromatographic analysis. *Analytical Chemistry, 33*(3), 363–372.
20. Jin, M. J., Huang, H., Zhang, K., Yan, J., & Gao, Z. (2007). Metabolic flux analysis on arachidonic acid fermentation. *Journal of Chemical Engineering Chinese University, 21*(2), 316–321.
21. Kates, M., & Baxter, R. M. (1962). Lipid composition of mesophilic and psychrophilic yeasts as influenced by environmental temperature. *Canadian Journal of Biochemistry and Physiology, 40*, 1213–1227.
22. Metz, B., & Kossen, N. W. F. (1977). The growth of molds in the form of pellets — a literature review. *Biotechnology and Bioengineering, 19*(6), 781–799.
23. Solomons, G. L. (1975). In J. Smith & D. Berry (Eds.), *The filamentous fungi, Vol 1. Industrial mycology* (pp. 249–264). London: E. Arnold.
24. Evanas, T., & Ratledge, C. (1984). Phosphofructokinase and the regulation of the flux of carbon from glucose to lipid in the oleaginous yeast *Rhodospovidium tovuloides*. *Journal of General Microbiology, 130*, 1693.
25. Stredansk , S., & Šajbidojr, J. (1993). Influence of carbon and nitrogen sources on the lipid accumulation and arachidonic acid production by *Mortierella alpina*. *Acta Biotechnologie, 13*(2), 185–191.
26. Chen, H. C., Chang, C. C., & Chen, C. X. (1997). Optimization of arachidonic acid production by *Mortierella alpina* Wuji-H4 isolate. *Journal of the American Oil Chemists' Society, 74*(5), 569–578.
27. Koike, Y., Cai, H. J., Higashiyama, K., Fujikawa, S., & Park, E. Y. (2001). Effect of consumed carbon to nitrogen ratio on mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*. *Journal of Bioscience and Bioengineering, 91*(4), 382–389.
28. Eroshin, V. K., Satroutdinov, A. D., Dedyukhina, E. G., & Chistyakova, T. I. (2000). Arachidonic acid production by *Mortierella alpina* with growth-coupled lipid synthesis. *Process Biochemistry, 35*(10), 1171–1175.

29. Zhu, M., Yu, L. J., Li, W., Zhou, P. P., & Li, C. Y. (2006). Optimization of arachidonic acid production by fed-batch culture of *Mortierella alpina* based on dynamic analysis. *Enzyme and Microbial Technology, 38*(6), 735–740.
30. Park, E. Y., Koikjz, Y., Higashiyama, K., Fujikawa, S., & Okabe, M. (1999). Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*. *Journal of Bioscience and Bioengineering, 88*(1), 61–67.
31. Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawa, S., & Shimizu, S. (1998). Effects of mineral addition on the growth morphology of an arachidonic acid production by *Mortierella alpina* 1S-4. *Journal of the American Oil Chemists' Society, 75*(12), 1815–1819.
32. Vardar, F. (1983). Problems of mass transfer and momentum transfer in large fermenters. *Process Biochemistry, 18*(5), 21–23.
33. Matsushima, K., Hayakawa, M., Ito, M., & Stimada, K. (1981). Features of the proteolytic enzyme system of hyper-acid productive and non-acid-productive fungi. *The Journal of General and Applied Microbiology, 27*, 423–426.