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Full Length Article

Effect of Nitrogen Concentration on the Growth and Fatty Acid Content of *Mortierella alpina*

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Abstract

The effects of nitrogen sources and nitrogen concentration on the growth and fatty acid contents of the fungus *Mortierella alpina* were investigated in this study. The poorest nitrogen source (sodium nitrate) and the best nitrogen source (urea) from six nitrogen sources were selected and used for cultivation of *M. alpina* D36 in three nitrogen concentrations (0.05, 0.1 and 0.2 M) for 12 days. Dynamic changes in biomass (dry cell weight), yield, as well as fatty acid composition, proportion and content were determined during fermentation. The results showed that the growth of *M. alpina* was markedly influenced by nitrogen source and nitrogen concentration. With sodium nitrate as nitrogen source (low nitrogen), biomass was significantly higher than that from the medium- and the high-nitrogen concentrations, and the maximum biomass concentration was 6.42 g L^{-1}. However, with urea as nitrogen source, biomass concentration at the medium and high nitrogen concentrations were higher than that at low nitrogen concentration, and the maximum biomass concentration was 18.19 g L^{-1}. Besides, the effect of nitrogen concentration on fatty acid accumulation in *M. alpina* varied as a function of nitrogen source. Using sodium nitrate as nitrogen source, nitrogen concentration had very little effect on the content and yield of total fatty acids and polyunsaturated fatty acids (arachidonic acid and eicosapentaenoic acid). However, when urea was used as nitrogen source, the contents of fatty acids at low and medium nitrogen concentrations were significantly higher than those obtained at high nitrogen concentration. However, the fatty acid yield at low nitrogen concentration was not high due to lower biomass concentration. In conclusion, nitrogen source and nitrogen concentration greatly affect the growth and fatty acid accumulation in *M. alpina*, with medium urea concentration being the most conducive condition.

Keywords: Fatty acids; *Mortierella alpina*; Nitrogen concentration; Sodium nitrate; Urea

Introduction

Mortierella alpina, a fungus widely distributed in the soil, has a strong ability to produce lipids which account for up to 50% of its dry weight (Wang *et al.* 2013). At present, *M. alpina* is used commercially for the production of polyunsaturated fatty acids (PUFAs), especially arachidonic acid (Kikukawa *et al.* 2018). Besides, *M. alpina* produces many other polyunsaturated fatty acids, including linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid (Sakuradani 2010; Tang *et al.* 2018). These polyunsaturated fatty acids are very beneficial to human health,in that they are essential for normal physiological function (Wiktorowska-Owczarek *et al.* 2015; Madsen *et al.* 2019). Therefore, due to the oil-rich property and safety of *M. alpina* (Streekstra 1997; Nisha *et al.* 2009), it is considered a very promising producer of arachidonic acid, and it has attracted the attention of many researchers.

Although *M. alpina* has been used in the industrial production of arachidonic acid, optimization of fermentation conditions so as to increase yield is still the most direct and effective way for arachidonic acid production (Gu *et al.* 2018; Zhang *et al.* 2019). It has been proposed that when microorganisms are in a suitable environmental conditions with sufficient nutrition, various cellular metabolisms are active and in a state of balance. At this stage, cell metabolism mainly serves to promote cellular growth, while the lipid content in the cell is maintained at a low level. However, changes in environmental conditions impair the metabolic balance in the cell, a situation which is beneficial for the accumulation of some metabolites (Rattray 1984). Many factors affect the production of arachidonic acid through *M. alpina* fermentation. These factors include carbon source, nitrogen source, amount of dissolved oxygen, pH, and temperature. Nitrogen is a necessary nutrient element for the growth of microorganisms. Nitrogen source has significant effects on the growth of oleaginous microorganisms and the accumulation of lipids (Baky *et al.* 2020; Feng *et al.* 2020). Nitrogen limitation is a common strategy for inducing lipid synthesis in lipid-producing microorganisms (Janssen *et al.* 2019; Tossavainen *et al.* 2019). It has been shown that the yield of mono-unsaturated

fatty acids in *Saccharomyces cerevisiae* was higher under nitrogen-limited conditions than that under non-nitrogen limited conditions (Tang and Chen 2014). Previous studies have revealed that oil-producing microorganisms begin to accumulate vast amounts of lipids only when the nitrogen source is exhausted and the carbon source is sufficient (Granger *et al.* 1993; Raimondi *et al.* 2014).

At present, research on the effect of nitrogen sources on lipid accumulation in *M. alpina* focus mainly on the selection of nitrogen sources and the optimization of fermentation conditions (Lu *et al.* 2011; Asadi *et al.* 2018; Lu *et al.* 2019). Studies have shown that the available nitrogen sources for *M. alpina* are inorganic and organic nitrogen sources, and the direction and yield of metabolites are influenced by different types of nitrogen sources (Stressler *et al.* 2013; Yu *et al.* 2018). A study on the effects of eight nitrogen sources on the characteristics of fatty acids in *M. alpina* showed that organic nitrogen sources were more favorable for cell growth and total lipid accumulation, than inorganic nitrogen sources, with urea being the most economical nitrogen source for industrial production of arachidonic acid (Lu *et al.* 2011). However, there are limited reports on the effect of nitrogen concentration on lipid accumulation in *M. alpina*. Moreover, different strains of *M. alpina*may present distinct demands for nitrogen. In this study, the effects of six nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulfate, sodium nitrate, potassium nitrate, and urea) on the growth and fatty acid accumulation of *M. alpine* of *M. alpina* were investigated. *Mortierella alpine* D36 was fermented with sodium nitrate and urea as nitrogen sources under different nitrogen concentrations (0.05, 0.1 and 0.2 *M*). Dynamic changes in biomass concentration, nitrogen residue, fatty acid content, and yields of mycelia were measured during the fermentation. This could provide a reference for the industrial production of polyunsaturated fatty acids using *M. alpina*.

Materials and Methods

Fungal strain

Mortierella alpina D36 was previously isolated from the soil by the Laboratory of Food Microbiology, Yunnan Agricultural University, and identified using morphological and ITS sequences. This fungal strain was maintained on potato dextrose agar (PDA) slants at 4ºC.

Fermentation methods

The mycelia of *M. alpina* D36 were aseptically picked from the PDA slant medium and transferred to the inoculum medium comprised of glucose (30 g L^{-1}), yeast extract (6 g L^{-1},) KH_2PO_4 (3 g L^{-1}), $NaNO_3$(3 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}. The fungus was cultured with shaking at 175 rpm in Erlenmeyer flasks for 3 days at 20ºC. Then, 10–15 mycelium pellets were transferred to the fermentation medium containing glucose (50 g L^{-1}), KH_2PO_4 (3.8 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), and a specific nitrogen source (any of ammonium chloride, ammonium nitrate, ammonium sulfate, sodium nitrate, potassium nitrate, and urea). The amount of each nitrogen source was calculated based on 0.1 *M* nitrogen. Four replicates were carried out with each nitrogen source. The fungus was cultured in Erlenmeyer flasks for 12 days at 20ºC, with shaking at 175 rpm.

The formula of media

Different sodium nitrate concentrations were used to replace the nitrogen source in the above fermentation medium at concentrations of 4.25, 8.5 and 17 g L^{-1}. Similarly, the nitrogen source in the fermentation medium was replaced with urea at concentrations of1.5, 3.0and 6.0 g L^{-1}. The nitrogen concentrations in the medium were equivalent to 0.05 *M*, 0.1 *M*, and 0.2 *M*, respectively.

Determination of biomass concentration in medium

The mycelia from each fermentation medium were harvested using vacuum filtration, and washed three times with distilled water, followed by freeze-drying to a constant weight. Biomass concentration was expressed in terms of dry cell weight (DCW) per liter of fermentation medium.

Determination of nitrogen residue in media

The nitrogen content of the medium was determined using alkaline potassium persulfate digestion in combination with ultraviolet spectrophotometry (Sattayatewa *et al.* 2011).

Analysis of fatty acids

Dry mycelia (50 mg) were added to 1 mL of toluene, 2 mL of 1% (v/v) sulphuric acid in methanol, and 1 mL of heptadecanoic acid (1 mgmL^{-1} in hexane). The mixture was placed in a 50°C-water bath overnight. Then, 5 mL of 5% (w/v) NaCl was added, followed by two extractions with hexane. The hexane layer was evaporated with N_2, and the residue was dissolved in 1 mL of hexane for GC-MS analysis (Agilent 7890A/5975C). The GC-MS conditions and methods used were in line with those reported in a previous report (Gu *et al.* 2018). The quantity of each fatty acid component was calculated from the peak area on the chromatogram using $C_{17:0}$ as the internal standard.

Statistical analysis

Statistical analysis was performed with S.P.S.S. 19.0. Differences amongst multiple groups were determined using one-way analysis of variance (ANOVA), followed by Ducan's multiple range test. Values of $P < 0.05$ were taken as indicative of statistical significance.

Results

Effects of six nitrogen sources on biomass concentration of *M. alpina*

The nitrogen sources had significant effects on the biomass concentration of *M. alpina* (Fig. 1). The highest biomass concentration was 16.9 g L^{-1} when urea was used as the nitrogen source, followed by biomass concentration from use of ammonium nitrate (14.6 g L^{-1}). However, when the other four nitrogenous compounds were used as nitrogen sources, the biomass concentrations were all below 5 g L^{-1}, with sodium nitrate producing the lowest biomass concentration (2.6 g L^{-1}). Therefore, urea was the best nitrogen source for *M. alpina*, while sodium nitrate was the worst nitrogen source.

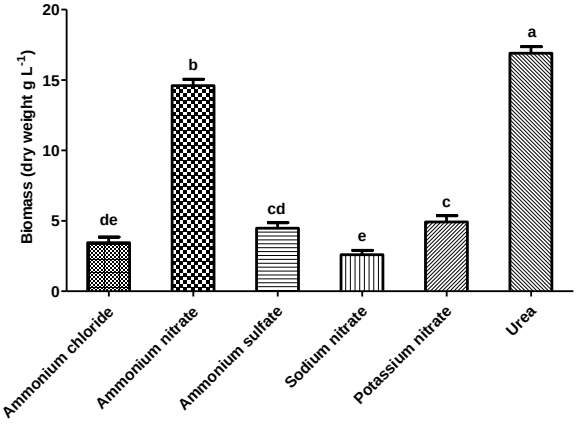

Fig. 1: Biomass concentration of *M. alpina* cultivated with different nitrogen sources. Data are expressed as mean ± SE of four replicates. Data with different letters differ significantly *(P* < 0.05)

Effect of nitrogen concentration on biomass of *M. alpina*

With sodium nitrate or urea as the nitrogen source, the biomass concentration of *M. alpina* was gradually increased time-dependently (Fig. 2). However, when sodium nitrate was used as the nitrogen source, there were no significant differences in fungal biomass under different nitrogen concentrations on the third day of fermentation. As the fermentation proceeded, *M. alpina* grew fastest under low sodium nitrate concentration (Fig. 2A). Furthermore, the highest biomass was 6.42 g L^{-1}, which was significantly higher than those under medium and high sodium nitrate concentrations. There was no statistical difference in biomass concentration between the medium and high concentrations at each time point of the fermentation, except for the 12th day.

Using urea as nitrogen source, the biomass values under the medium concentration condition on the 3rd, 6th, and 9th days of fermentation were significantly higher than those under the low and high concentrations (Fig. 2B). However, on the 12th day of fermentation, biomass under high urea concentration condition surpassed that under medium concentration condition, and reached the highest value of 18.19 g L^{-1}. Moreover, at the same fermentation time, the biomass resulting from use of organic nitrogen urea as the nitrogen source was significantly higher than that resulting from the use of inorganic nitrogen sodium nitrate as the nitrogen source (Fig. 2A, Fig. 2B). At the 12thday of fermentation, biomass concentration with urea as the nitrogen source was 2.83 times higher than that produced with sodium nitrate as nitrogen source. Therefore, urea was more suitable for the growth of *M. alpina* than sodium nitrate (Fig. 2).

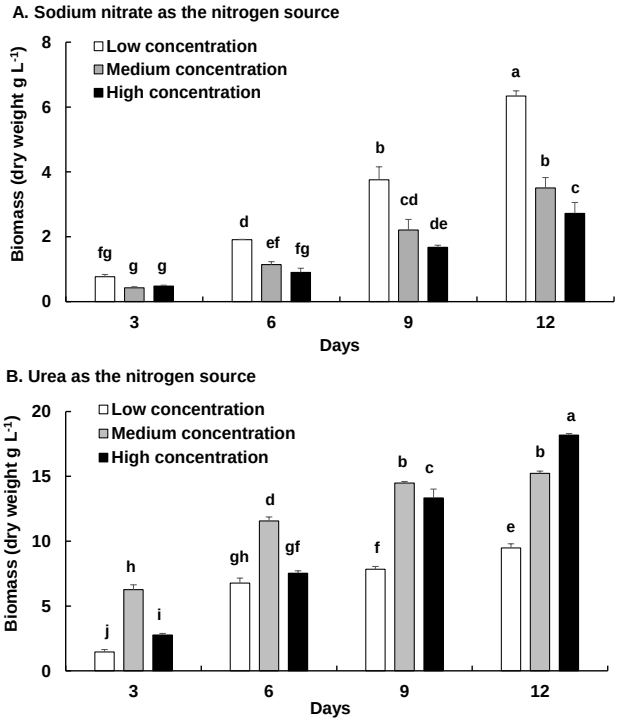

Fig. 2: Biomass concentration of *M. alpina* cultivated with different nitrogen concentrations during fermentation. (**A**) Sodium nitrate as nitrogen source; (**B**) urea as nitrogen source. Data are expressed as mean ± SE of four replicates. Data with different letters differ significantly *(P* < 0.05)

Residual nitrogen in fermentation media during the growth of *M. alpina*

In this study, nitrogen consumption by *M. alpina* depended on type of nitrogen source and nitrogen concentration in the media (Fig. 3). When sodium nitrate was the nitrogen source, nitrogen was slowly consumed by *M. alpina*. At the end of the fermentation, the nitrogen in the media with three concentrations of sodium nitrate (0.05, 0.1 and 0.2 *M*) was not depleted (Fig. 3A). Nitrogen consumption in the media with the low concentration of sodium nitrate was 0.035 *M*, which was 2.33 times higher than nitrogen consumption at the high concentration of sodium nitrate.

When urea was used as the nitrogen source to cultivate *M. alpina*, nitrogen content in the media decreased sharply with time (Fig. 3B). At the low urea concentration of 0.05 *M*, nitrogen in the media was depleted on the 9th day. At the medium urea concentration of 0.1 *M*, the nitrogen consumption was 0.093 *M* at the end of fermentation, whereas, at a high nitrogen concentration of 0.2 *M*, the nitrogen consumption was 0.14 *M* at the end of the fermentation. Correlation analyses showed that, irrespective of whether sodium nitrate or urea was used as the nitrogen source, the biomass concentration of *M. alpine* was significantly positively correlated with nitrogen consumption (Pearson correlation coefficient r = 0.816, n = 48, $P \leq 0.001$ for sodium nitrate; r = 0.903, n = 48, $P \leq 0.001$ for urea).

Effect of nitrogen concentration on the proportion of fatty acids in *M. alpina*

M. alpina D36 was cultured with different concentrations of nitrate or urea for 12 days. The fatty acid profiles of *M. alpina* cultured with different nitrogen concentrations are shown in Table 1. At different concentrations of the same nitrogen source, the fatty acid profiles did not differ much, except for the lack of lignoceric acid (C24:0) in the medium- and high-concentration sodium nitrate media. Monounsaturated fatty acid C24:1 was increased in mycelia when urea was used as the nitrogen source, when compared with sodium nitrate. Arachidonic acid was the most typical and the most abundant fatty acid in *M. alpina*.

With sodium nitrate as the nitrogen source, the level of arachidonic acid in total fatty acids fluctuated between 19.13 and 33.77%. At low sodium nitrate concentration, arachidonic acid level gradually decreased with fermentation time, but it was highest (27.54%) on the third day. Nevertheless, at the medium and high concentrations of sodium nitrate, the level of arachidonic acid first increased, and then decreased. Moreover, the arachidonic acid at the middle concentration of sodium nitrate reached a maximum level of 33.77% on the 6th day of fermentation. At the high concentration of sodium nitrate, the arachidonic acid level was only 19.55% on the 12th day of fermentation (Table 1).

With urea as nitrogen source, arachidonic acid level fluctuated between 22.02 and 34.32%. Unlike sodium nitrate, the levels of arachidonic acid at the low, medium, and high concentrations of urea slowly increased with fermentation time. Moreover, arachidonic acid level was higher at the low and medium concentrations of urea than at the high concentration of urea (Table 1).

The levels of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids in total fatty acids are shown in Fig. 4 (A–C). In most cases, polyunsaturated fatty acids in *M. alpina* accounted for more than 50% of total fatty acids, and even exceeded 60% at low urea concentration. Overall, nitrogen source and nitrogen concentration had no significant effects on the proportion of polyunsaturated fatty acids (Fig. 4C).

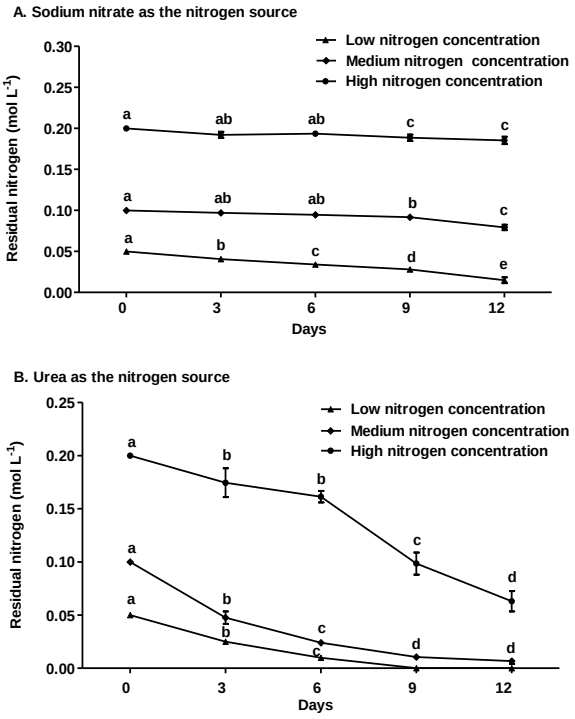

Fig. 3: Residual nitrogen in the fermentation media during the growth of *M. alpina.* (**A**) Sodium nitrate as the nitrogen source; (**B**) urea as the nitrogen source. Data with different letters differ significantly ($P < 0.05$)

Effect of nitrogen concentration on the fatty acid contents in *M. alpina*

Fatty acids contents in mycelia *M. alpina* cultivated under different nitrogen concentrations for 12 days are shown in Table 2. In most cases, the total fatty acid content (Fig. 5A) and the content of each fatty acid component (Table 2) increased gradually with increase in fermentation time. When sodium nitrate was used as the nitrogen source, nitrogen concentration had little effect on the levels of total fatty acids, polyunsaturated fatty acids, arachidonic acid, and eicosapentaenoic acid. However, when urea was used as nitrogen source, the contents of fatty acids at the low and medium nitrogen concentrations were significantly higher than those at the high concentration (Fig. 5A–D). Moreover, the fatty acid contents were significantly higher when urea was used as nitrogen source than when sodium nitrate was used as nitrogen source. Therefore, urea was more suitable for fatty acid production by *M. alpina* than sodium nitrate.

Effect of nitrogen concentration on the fatty acid yields in *M. alpina*

The effects of nitrogen source and nitrogen concentration on yields of fatty acids were significant (Fig. 6A–D). At the same nitrogen concentration, the yields of total fatty acids,

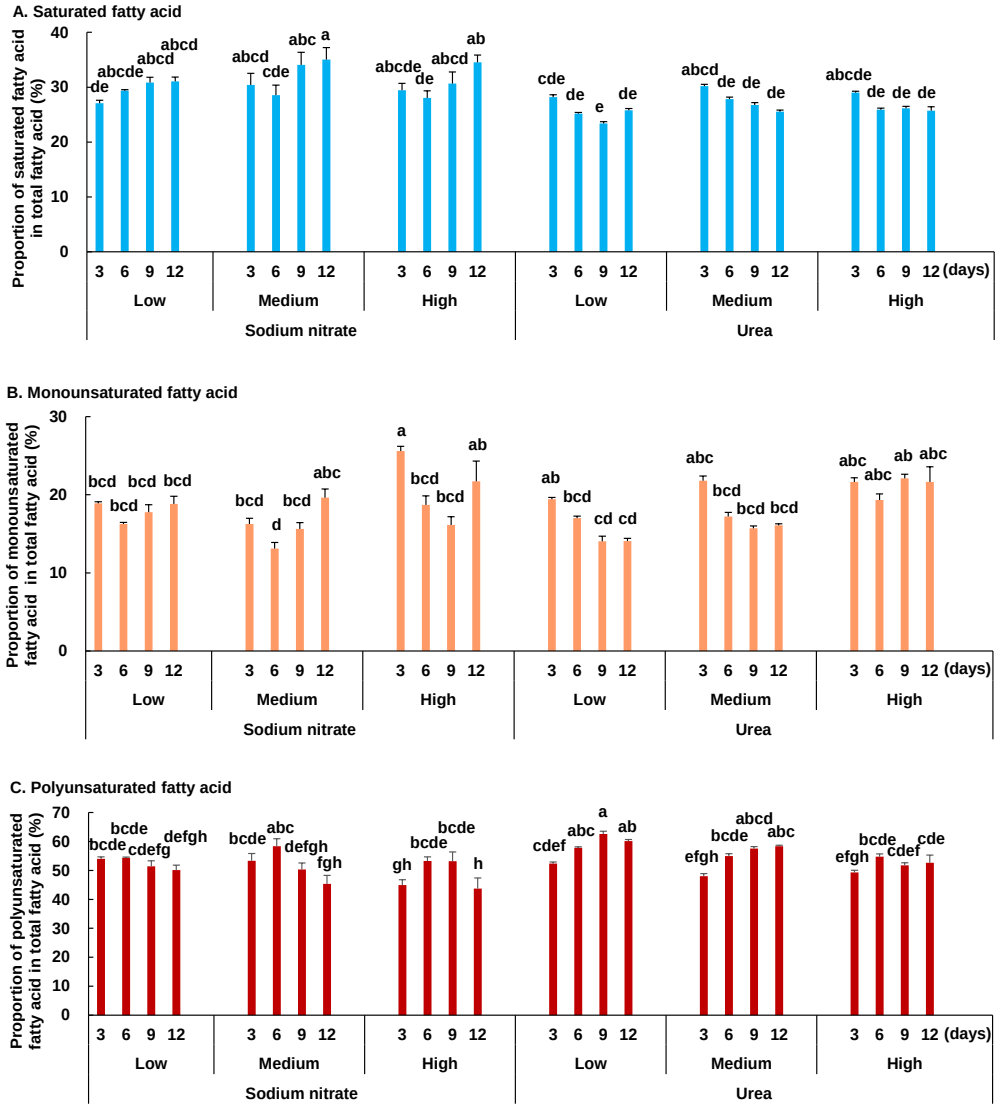

Fig. 4: Proportion of fatty acids in total fatty acids of *M. alpina* D36 cultivated at different nitrogen concentrations during fermentation. (**A**) Proportion of saturated fatty acids in total fatty acids; (**B**) Proportion of monounsaturated fatty acids in total fatty acids; (**C**) Proportion of polyunsaturated fatty acids in total fatty acids. Data with different letters differ significantly ($P < 0.05$)

polyunsaturated fatty acids, arachidonic acid, and eicosapentaenoic acid, increased gradually as the fermentation continued. Compared with sodium nitrate, the yield of fatty acids using urea as nitrogen source was significantly higher, mainly due to the much higher biomass concentration of *M. alpina* when urea was used as nitrogen source. With sodium nitrate as nitrogen source, the yield of fatty acids decreased with increasing nitrogen concentration, which was consistent with the trend in biomass concentration. The yield of arachidonic acid was 190.87 mg L^{-1} at low sodium nitrate concentration on the 12th day, which was 1.78 times higher than the corresponding yield at medium concentration, and 3.97 times higher than the yield at high concentration.

With urea as the nitrogen source, the yield of fatty acids was highest at the medium nitrogen concentration. On the 12th day, the yields of polyunsaturated fatty acids and arachidonic acid at the medium urea concentration were 1866.66 and 1090.97 mg L^{-1}, respectively, which were 1.53 and 1.66 times higher than the corresponding yields at low urea concentration, respectively. Moreover, the low and medium urea concentrations were conducive for the accumulation of eicosapentaenoic, another essential polyunsaturated fatty acid (Fig. 6D).

Discussion

Nitrogen sources are essential for microbial growth, but not all nitrogen sources are suitable for the growth and oil accumulation of *M. alpina*. This study compared the effects

Table 1: Levels of fatty acids of *M. alpina* D36 grown under different nitrogen concentrations during fermentation

Nitrogen source	Nitrogen concentration	Fermentation time (days)	Saturated fatty acids (%)					Monounsaturated fatty acids (%)			Polyunsaturated fatty acids (%)					
			$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{22:0}$	$C_{24:0}$	$C_{18:1}$	$C_{20:1}$	$C_{24:1}$	LA ($C_{18:2}$)	$C_{20:2}$	GLA ($C_{18:3}$)	DGLA ($C_{20:3}$)	ARA ($C_{20:4}$)	EPA ($C_{20:5}$)
Sodium nitrate	Low	3	1.09± 0.05bcd	15.83± 0.37bc	8.74± 0.25de	0.81± 0.27d	0.63± 0.12c	18.28± 0.31bcd	0.63± 0.21ab	-	5.65± 0.08b	0.80± 0.33c	6.98± 0.16b	8.66± 0.03bc	27.54± 0.52bc	4.37± 0.22bc
		6	1.00± 0.08cde	16.24± 0.19bc	10.07± 0.14cde	1.33± 0.05bc	0.75± 0.07b	15.52± 0.22cde	0.75± 0.04ab	-	4.95± 0.10cd	1.14± 0.06bc	5.79± 0.23bcd	8.28± 0.26bc	26.96± 0.48bc	7.23± 0.65a
		9	0.93± 0.05de	16.82± 0.73abc	10.79± 0.25cd	1.51± 0.05bc	0.79± 0.04b	17.04± 0.83cde	0.73± 0.13ab	-	4.35± 0.09efg	1.09± 0.12bc	5.13± 0.12cdef	7.95± 0.54c	26.03± 1.57bcd	6.84± 0.39a
		12	1.04± 0.02bcde	16.90± 0.60abc	10.69± 0.26bcd	1.45± 0.04bc	0.98± 0.06a	18.24± 1.03bcd	0.58± 0.05b	-	4.36 ±0.22efg	0.92± 0.06c	5.52± 0.37cde	8.38± 0.45bc	23.75± 1.82cde	7.19± 0.62a
	Medium	3	0.97± 0.05cde	17.60± 1.23abc	10.41± 0.75bcd	1.42± 0.11bc	-	15.72± 0.72cde	0.53± 0.02b	-	5.50± 0.19bc	1.15± 0.28bc	4.42± 0.28def	9.78± 0.51abc	29.40± 1.26b	3.20± 0.69c
		6	0.80± 0.07e	16.18± 1.00bc	10.05± 0.82cde	1.50± 0.12bc	-	12.39± 0.75f	0.71± 0.07ab	-	4.44± 0.14def	1.25± 0.11abc	4.45± 0.19def	9.92± 0.38ab	33.77± 1.72a	4.52± 0.53bc
		9	1.06± 0.07bcd	18.71± 1.24ab	12.19± 0.84ab	2.11± 0.19a	-	14.81± 0.86ef	0.82± 0.05ab	-	3.90± 0.22fgh	1.40± 0.15abc	4.15± 0.44ef	8.80± 0.89bc	27.80± 1.86bc	4.25± 1.26bc
		12	1.18± 0.07bc	19.48± 1.01a	12.19± 1.07ab	2.19± 0.11a	-	18.69± 1.07bc	0.94± 0.06a	-	3.69± 0.13h	1.80± 0.23a	4.04± 0.39f	8.77± 0.62bc	21.72± 1.74def	5.28± 0.63abc
	High	3	1.93± 0.08a	18.89± 0.60ab	8.32± 0.46e	0.32± 0.07e	-	25.49± 0.50a	0.09± 0.01c	-	6.80± 0.28a	0.13± 0.02d	9.46± 1.18a	6.14± 0.48d	19.13± 0.24f	3.30± 0.92c
		6	1.16± 0.01bcd	16.31± 0.61bc	9.54± 0.62cde	1.04± 0.14cd	-	17.99± 1.07bcde	0.71± 0.18ab	-	5.86± 0373b	1.39± 0.29abc	6.41± 0.46bc	9.00± 0.55bc	25.37± 1.21bcd	5.24± 0.63abc
		9	1.21± 0.11bc	15.32± 0.94c	11.59± 0.83ab	1.45± 0.16bc	-	14.61± 0.91def	0.79± 0.13ab	-	4.96± 0.10de	1.29± 0.05abc	4.47± 0.03def	11.39± 0.45a	25.98± 2.08bcd	7.09± 0.26a
		12	1.26± 0.09b	18.42± 1.04abc	13.11± 0.41a	1.76± 0.13ab	-	21.00± 2.63b	0.71± 0.04ab	-	3.86± 0.16gh	1.54± 0.18abc	3.95± 0.73f	9.12± 1.04bc	19.55± 2.28ef	5.72± 0.50ab
Urea	Low	3	1.58± 0.09b	15.24± 0.21a	8.38± 0.25b	1.11± 0.04f	1.89± 0.19e	17.56± 0.22a	0.40± 0.05e	1.46± 0.02a	5.31± 0.36ab	0.55± 0.05e	7.85± 0.24b	8.77± 0.24bc	22.02± 0.52c	7.88± 0.68c
		6	0.92± 0.03c	12.35± 0.17cd	8.04± 0.11bc	1.30± 0.03de	2.58± 0.11d	15.24± 0.27c	0.66± 0.22a	1.64± 0.32a	6.38± 0.91a	0.87± 0.14d	6.31± 0.13cd	9.60± 0.14a	27.74± 0.36b	9.66± 0.40b
		9	0.66± 0.01e	11.64± 0.21d	6.37± 0.17fg	1.35± 0.01cd	3.37± 0.07c	12.10± 0.64e	0.49± 0.01bcd	1.46± 0.03a	3.11± 0.03d	1.27± 0.04bc	4.78± 0.07g	8.87± 0.10bc	32.55± 1.13a	11.98± 0.31a
		12	0.49± 0.02f	13.66± 0.14b	6.16± 0.16gh	1.45± 0.04c	4.04± 0.13b	12.25± 0.33de	0.49± 0.01bcd	1.34± 0.06abc	3.56± 0.13cd	1.38± 0.08bc	4.27± 0.03h	7.82± 0.17d	32.46± 0.96a	10.64± 0.39ab
	Medium	3	1.55± 0.07b	16.19± 0.20a	9.21± 0.19a	1.26± 0.06de	1.99± 0.11e	20.39± 0.56a	0.56± 0.01a	0.84± 0.08e	5.00± 0.19b	1.00± 0.07d	8.18± 0.23ab	9.09± 0.13bc	20.83± 1.40c	3.90± 0.42ef
		6	0.88± 0.04c	13.60± 0.38b	7.77± 0.16cd	1.58± 0.01b	4.03± 0.16b	15.92± 0.58bc	0.58± 0.01a	0.73± 0.06e	3.90± 0.08c	1.22± 0.03c	5.94± 0.13de	9.22± 0.03ab	31.17± 0.89ab	3.84± 0.36f
		9	0.83± 0.02cd	12.95± 0.40bc	6.94± 0.03e	1.61± 0.03b	4.44± 0.14ab	14.29± 0.31cde	0.54± 0.02abc	0.89± 0.05e	3.72± 0.07c	1.33± 0.10bc	5.34± 0.06f	8.87± 0.08bc	32.75± 1.12a	5.53± 0.44de
		12	0.69± 0.06de	13.40± 0.37b	5.85± 0.13h	1.41± 0.02c	3.40± 0.77c	14.42± 0.20cd	0.47± 0.01cde	1.20± 0.07de	3.52± 0.14de	1.45± 0.06ab	4.65± 0.10gh	7.89± 0.03d	34.32± 0.52a	6.51± 0.84cd
	High	3	1.72± 0.06a	15.83± 0.28a	8.52± 0.22b	1.21± 0.02ef	1.73± 0.06e	19.84± 0.58a	0.40± 0.03e	1.41± 0.06ab	5.52± 0.10a	0.61± 0.13e	8.46± 0.26a	7.77± 0.33d	20.67± 0.68c	6.29± 0.90cd
		6	0.93± 0.06c	12.62± 0.25bcd	8.33± 0.09b	1.42± 0.04c	2.59± 0.12d	17.55± 0.70b	0.56± 0.03ab	1.23± 0.03bcd	3.64± 0.11d	1.23± 0.07bc	6.56± 0.16c	9.70± 0.15a	28.37± 0.97b	5.26± 0.76def
			0.92± 0.04c	12.67± 0.29bcd	7.44± 0.12d	1.59± 0.03b	3.51± 0.06c	20.41± 0.56a	0.57± 0.02a	1.11± 0.04d	3.56± 0.05cd	1.58± 0.02a	5.74± 0.07ef	8.86± 0.12bc	28.40± 0.83b	3.47± 0.28f
		12	0.65± 0.02e	11.83± 0.65d	6.71± 0.24ef	1.72± 0.07a	4.82± 0.23a	20.22± 2.02a	0.61± 0.01a	0.82± 0.10e	3.97± 0.15c	1.65± 0.02a	6.00± 0.25de	8.57± 0.19c	28.33± 2.49b	4.10± 0.73ef

"-" Indicates 'not detected'. Data are expressed as mean ± SE of four replicates. Values with different letters differ significantly ($P < 0.05$)

of six nitrogen sources on the growth of *M. alpina* D36, and found that the organic nitrogen urea was the most beneficial for the growth of mycelia, followed by an inorganic nitrogen i.e. ammonium nitrate, while the most unfavorable nitrogen source was sodium nitrate. Moreover, urea was more conducive to accumulation of fatty acids in *M. alpina* than sodium nitrate. These results are consistent with the findings of other researchers who reported that organic nitrogen sources were more conducive for *M. alpina* growth and oil accumulation than inorganic nitrogen sources (Lu *et al.* 2011; Nisha and Venkateswaran 2011). Singh and Ward (1997) used 1% (w/v) corn steep instead of sodium nitrate, for cultivation of *M. alpine,* and found that the production of arachidonic acid improved significantly. Similar results were found in other organisms. Certik *et al.* (1999) compared lipogenesis and activities of lipogenic enzymes in the fungus *Cunninghamella echinulata* as a function of different inorganic and organic nitrogen sources, and found that organic nitrogen enhanced lipid accumulation. A study on the effects of sodium nitrite, sodium nitrate, urea, and ammonium chloride on the growth and lipid accumulation of marine algae *Desmodesmus* spp. WC08 showed that the use of urea produced the best growth and lipid accumulation of *Desmodesmus* spp. WC08 (Luo *et al.* 2016).

In the present study, nitrogen concentration had little effect on the proportions of fatty acids in *M. alpina*. However, it significantly affected the contents and yields of total fatty acids, polyunsaturated fatty acids, arachidonic acid, and eicosapentaenoic acid. When urea was used as nitrogen source, the contents and yields of total fatty acids,

Table 2: Contents of fatty acids in *M. alpina* D36 grown at different nitrogen concentrations during fermentation

Nitrogen source	Nitrogen concentration	Fermentation time	Saturated fatty acids (mg g^{-1})					Monounsaturated fatty acids (mg g^{-1})			Polyunsaturated fatty acids (mg g^{-1})					
			$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{22:0}$	$C_{24:0}$	$C_{18:1}$	$C_{20:1}$	$C_{24:1}$	LA ($C_{18:2}$)	$C_{20:2}$	GLA ($C_{18:3}$)	DGLA ($C_{20:3}$)	ARA ($C_{20:4}$)	EPA ($C_{20:5}$)
Sodium nitrate	Low	3	0.81± 0.04c	12.05± 0.79cd	6.54± 0.44d	0.61± 0.21c	0.26± 0.02c	13.17± 0.99bc	0.47± 0.16def	-	4.23± 0.31b	0.62± 0.29e	5.24± 0.42bcd	6.51± 0.53c	20.78± 2.04bc	3.31± 0.38efg
		6	1.11± 0.07bc	18.20± 0.25abcd	11.29± 0.18abc	1.49± 0.06b	0.85± 0.09b	17.39± 0.28abc	0.84± 0.06bc	-	5.55± 0.05a	1.27± 0.06bc	6.50± 0.33bc	9.27± 0.27abc	30.21± 0.43ab	8.12± 0.80ab
		9	1.10± 0.14bc	19.56± 1.33abcd	12.65± 1.15abc	1.77± 0.15b	0.92± 0.07b	19.78± 1.24abc	0.82± 0.05bcd	-	5.15± 0.58ab	1.31± 0.22bc	6.06± 0.68bcd	9.55± 1.47abc	31.23± 4.83ab	8.01± 0.86ab
		12	1.32± 0.10abc	21.47± 1.50abc	13.61± 1.01ab	1.83± 0.07b	1.25± 0.07a	23.30± 2.48ab	0.73± 0.06bcde	-	5.50± 0.18a	1.17± 0.09bcd	7.00± 0.37ab	10.65± 0.80ab	30.30± 2.46ab	9.18± 1.08a
	Medium	3	0.58± 0.03c	10.34± 0.70d	6.11± 0.41d	0.83± 0.08c	-	9.77± 0.71c	0.33± 0.01f	-	3.52± 0.37b	0.78± 0.14de	2.95± 0.36d	6.52± 0.64c	19.09± 2.08bc	2.46± 0.36g
		6	1.04± 0.10bc	20.44± 1.60abc	12.73± 1.36abc	1.70± 0.07b	-	15.58± 1.25bc	0.85± 0.12bc	-	5.56± 0.13a	1.40± 0.05bc	5.31± 0.23bcd	11.75± 0.30a	41.08± 2.82a	5.06± 0.62cdef
		9	1.36± 0.04abc	24.40± 1.24ab	15.75± 0.74a	2.73± 0.22a	-	19.17± 0.92abc	1.06± 0.09a	-	5.06± 0.33ab	1.83± 0.19b	5.41± 0.63bcd	11.49± 1.35a	36.30± 3.52a	5.61± 1.69bcde
		12	1.63± 0.04a	27.02± 1.70a	14.24± 1.83ab	3.03± 0.11a	-	26.16± 2.47a	1.30± 0.07a	-	5.17± 0.48ab	2.54± 0.39a	5.75± 0.93bcd	12.39± 1.67a	30.42± 3.66ab	7.51± 1.35abc
	High	3	1.33± 0.10abc	12.64± 1.07cd	5.88± 0.81d	0.43± 0.05c	-	17.43± 1.82abc	0.45± 0.10def	-	4.01± 0.55b	0.91± 0.22cde	7.18± 1.07a	4.52± 1.07c	13.76± 1.39c	2.86± 0.29g
		6	1.14± 0.04bc	16.03± 0.46bcd	9.38± 0.49bcd	1.02± 0.11bc	-	17.69± 0.91abc	0.69± 0.13cde	-	5.77± 0.31a	1.35± 0.21bc	6.34± 0.56bc	8.86± 0.57abc	25.09± 1.81bc	5.20± 0.71cdef
		9	1.38± 0.27abc	17.61± 1.61bcd	13.69± 1.60ab	1.72± 0.23b	-	17.18± 1.85abc	0.92± 0.12bc	-	5.39± 0.23ab	1.49± 0.16bc	5.18± 0.49bcd	12.29± 0.93a	27.62± 2.51bc	7.34± 0.38abcd
		12	1.14± 0.09bc	16.63± 1.29bcd	11.91± 1.05abc	1.58± 0.12b	-	18.69± 1.36abc	0.66± 0.09cde	-	3.55± 0.43b	1.40± 0.20bc	3.71± 0.80d	8.52± 1.48abc	18.29± 3.39c	5.20± 0.59cdef
Urea	Low	3	1.21± 0.07cd	11.86± 1.18de	6.53± 0.69f	0.87± 0.12g	1.51± 0.32de	13.62± 1.22gh	0.31± 0.05cd	1.14± 0.13def	4.08± 0.28bcd	0.43± 0.28e	6.06± 0.45fg	6.84± 0.24e	17.12± 1.75f	6.24± 1.15de
		6	1.12± 0.04cde	15.08± 0.64cd	9.82± 0.40de	1.60± 0.10ef	3.17± 0.24d	16.41± 2.35fg	0.84± 0.32ab	1.61± 0.50cd	7.75± 0.49a	1.05± 0.15cd	7.69± 0.21cde	11.72± 0.45cd	33.92± 1.76de	11.87± 1.01bc
		9	1.21± 0.03cd	21.54± 1.16b	11.77± 0.63ab	2.48± 0.10bc	6.22± 0.21bc	22.36± 1.56cde	0.90± 0.04a	2.71± 0.15a	5.75± 0.28abcd	2.35± 0.14b	8.82± 0.31abc	16.36± 0.10a	60.01± 2.51b	22.19± 1.41a
		12	1.04± 0.03efg	29.06± 1.28a	13.10± 0.69a	3.08± 0.17a	8.57± 0.39a	26.05± 1.28bc	1.04± 0.07a	2.84± 0.08a	7.06± 0.51ab	2.91± 0.10a	9.10± 0.42ab	16.64± 0.77a	69.15± 4.10a	22.66± 1.42a
	Medium	3	0.93± 0.02fgh	9.82± 0.48ef	5.59± 0.29fg	0.77± 0.07g	1.21± 0.10e	12.39± 0.83gh	0.34± 0.02cd	0.51± 0.05g	3.02± 0.12cd	0.61± 0.07de	4.95± 0.17gh	5.52± 0.29ef	12.66± 1.15f	2.36± 0.27f
		6	1.10± 0.05def	17.03± 0.52c	9.73± 0.30de	1.98± 0.09de	5.08± 0.39c	19.92± 0.60def	0.71± 0.02ab	0.92± 0.09efg	4.88± 0.16abcd	1.53± 0.09c	7.44± 0.20de	11.57± 0.51cd	39.22± 2.49cd	4.38± 0.53ef
		9	1.44± 0.10a	22.52± 2.07b	12.01± 0.83ab	2.78± 0.21ab	7.69± 0.55ab	24.80± 2.06bcd	0.93± 0.10a	1.53± 0.12cd	6.45± 0.52abc	2.33± 0.34b	9.25± 0.69a	15.36± 1.05ab	56.29± 1.71b	9.65± 1.21cd
		12	1.42± 0.04ab	28.11± 2.65a	12.17± 0.72ab	2.95± 0.20a	7.08± 1.65ab	30.13± 2.33ab	0.98± 0.09a	2.49± 0.19ab	7.30± 0.36ab	3.03± 0.31a	9.68± 0.58a	16.49± 1.25a	71.52± 4.84a	13.89± 2.51b
	High	3	0.86± 0.04h	7.97± 0.53f	4.27± 0.22g	0.61± 0.05g	0.88± 0.08e	9.93± 0.40h	0.20± 0.03d	0.72± 0.07fg	2.77± 0.13d	0.32± 0.08e	4.23± 0.18g	3.93± 0.37f	10.43± 0.88f	3.22± 0.60ef
		6	0.92± 0.04gh	12.58± 0.65de	8.32± 0.53e	1.43± 0.13f	2.61± 0.28de	17.40± 0.42efg	0.55± 0.02bc	1.26± 0.06def	3.63± 0.22cd	1.22± 0.05c	6.55± 0.45ef	9.71± 0.72d	28.46± 2.54e	5.30± 0.99ef
		9	1.27± 0.05bc	17.49± 0.56c	10.28± 0.37cd	2.20± 0.10cd	4.85± 0.18c	28.22± 1.35ab	0.79± 0.04ab	1.53± 0.04cd	4.92± 0.12abcd	2.18± 0.08b	7.94± 0.31bcd	12.24± 0.36c	39.15± 0.68cd	4.81± 0.51ef
		12	1.04± 0.06efg	18.95± 1.43bc	10.74± 0.61abc	2.75± 0.18ab	7.70± 0.41ab	32.45± 3.70a	0.97± 0.05a	1.31± 0.14de	6.35± 0.31abc	2.64± 0.13ab	9.61± 0.62a	13.74± 0.81bc	45.17± 3.67c	6.49± 1.04de

"-" Indicates 'not detected'. Data are expressed as mean ± SE of four replicates. Values with different letters differ significantly ($P < 0.05$)

polyunsaturated fatty acids, and arachidonic acid at the low and medium nitrogen concentrations were higher than those at the high nitrogen concentration. These findings are consistent with the results obtained in a previous study in which the effects of low and high nitrogen concentrations on the growth and lipid accumulation of two oleaginous micro algae were investigated (Wu *et al.* 2015). The results showed that the levels of total lipids, neutral lipids, and total fatty acids produced by these two microalgae at low nitrogen concentration were significantly higher than those produced at the high nitrogen concentration. In the present study, using sodium nitrate as the sole nitrogen source, fungal biomass and total fatty acids, polyunsaturated fatty acids, and arachidonic acid yields decreased with increasing nitrogen concentration, indicating that a high concentration of nitrate could inhibit the growth and fatty acid accumulation of *M. alpina*. Using urea as nitrogen source, the nitrogen was completely consumed or nearly consumed at the low and medium nitrogen concentrations on the 9th and 12th days, at which time the mycelia of *M. alpina* grew slowly. However, the fungal biomass increased rapidly at the high nitrogen concentration on the 9th and 12th days, due to sufficient nitrogen content in the media. In other words, the low and medium urea concentrations were not conducive for the growth of *M. alpina* at the later stages of fermentation. It is likely that under nitrogen-restricted conditions, *M. alpina* initiated a stress-resistant physiological response which resulted in inhibition of mycelial protein synthesis, thereby decreasing the levels of enzymes required for mycelial growth and metabolism.

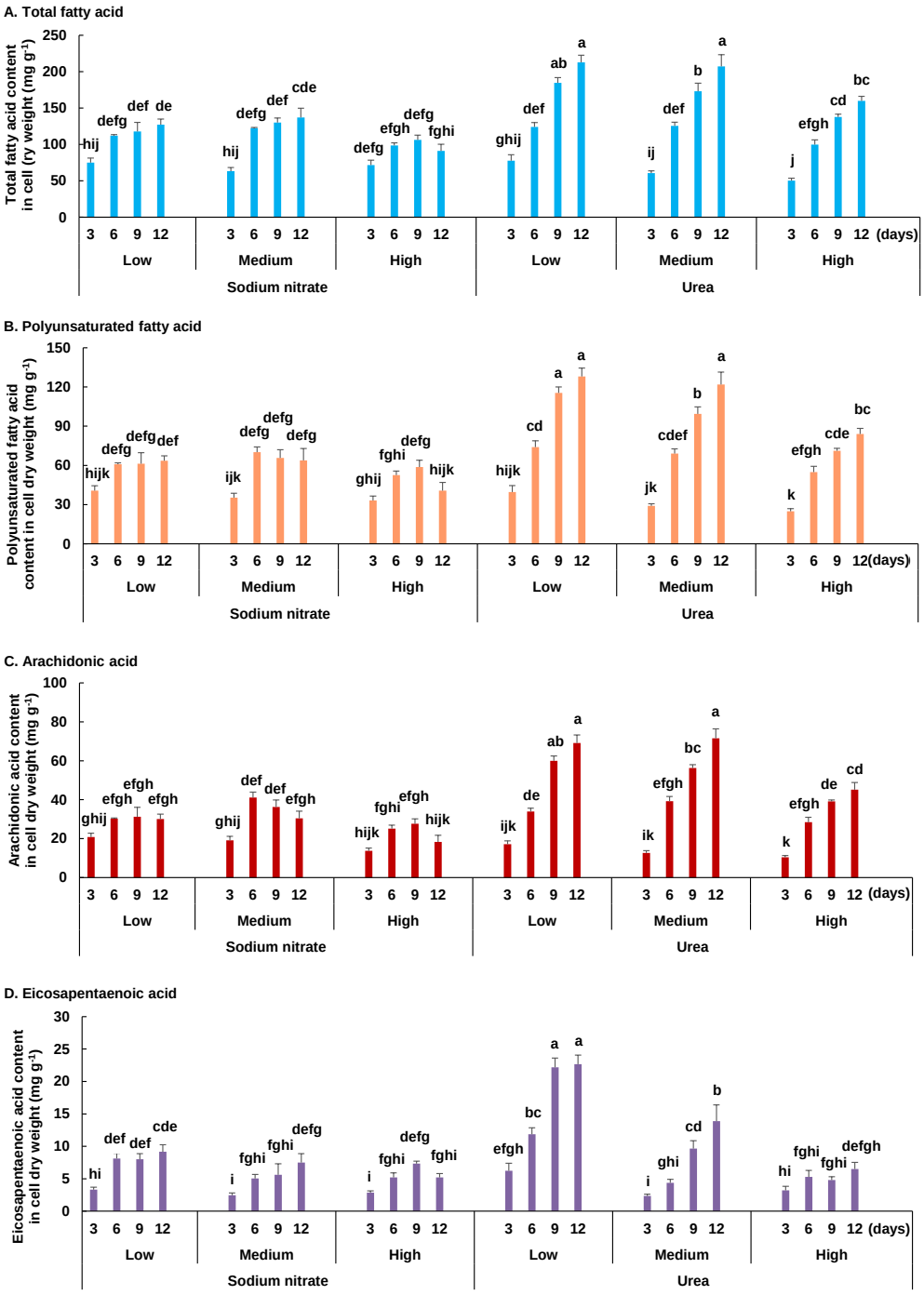

Fig. 5: Fatty acid contents of cell dry weight (mg g^{-1}) in *M. alpina* D36 cultivated with different nitrogen concentrations during fermentation. (**A**) Total fatty acid contents of cell dry weight; (**B**) polyunsaturated fatty acid contents of cell dry weight; (**C**) arachidonic acid contents of cell dry weight; (**D**) eicosapentaenoic acid contents of cell dry weight. Data with different letters differ significantly ($P < 0.05$)

It is noteworthy that the biomass of *M. alpine* reached its maximum on the 12th day of fermentation at the high urea concentration, but the contents of total fatty acids, polyunsaturated fatty acids, and arachidonic acid were significantly lower than the corresponding contents at the low and medium urea concentrations. These results are consistent with the findings in some studies which showed that oleaginous yeasts and molds accumulated high levels of lipids in nitrogen-restricted media (Arous *et al.* 2016; Janssen *et al.* 2019; Tossavainen *et al.* 2019). The primary

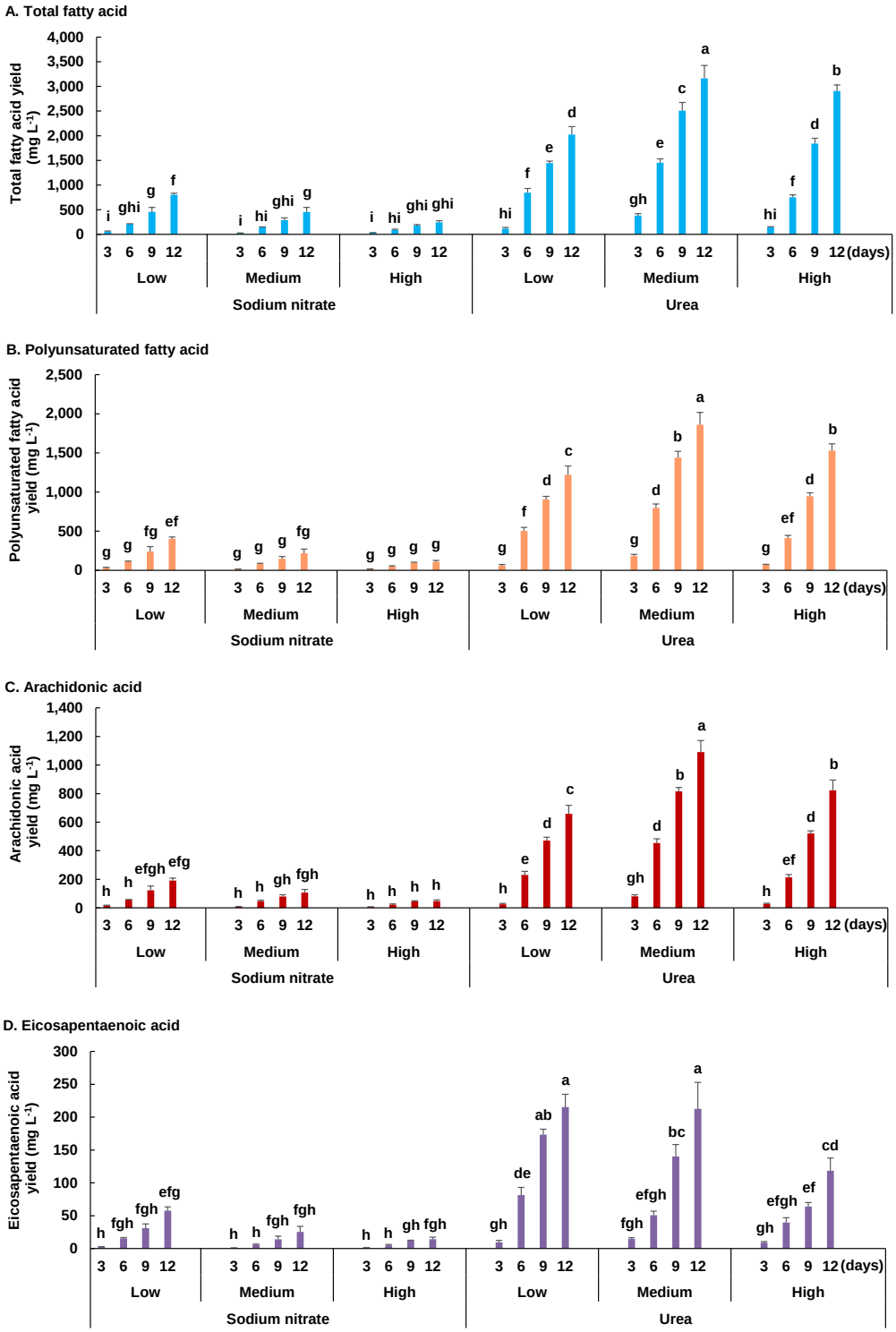

Fig. 6: Fatty acid yields at different nitrogen concentrations during fermentation. (**A**) Total fatty acid yield; (**B**) polyunsaturated fatty acid yield; (**C**) arachidonic acid yield; (**D**) eicosapentaenoic acid yield. Data with different letters differ significantly ($P < 0.05$)

function of a nitrogen source is to enhance the synthesis of proteins and nucleic acids. When the nitrogen source is limited, the synthesis of intracellular proteins and nucleic acids is blocked, and the tricarboxylic acid cycle is impeded, thereby suppressing cell proliferation and causing changes in metabolic pathways. Thus, the carbon flow in the media is channeled in the direction of lipid synthesis. A previous study showed that when a bioengineered strain of

Saccharomyces cerevisiae was cultivated under nitrogen-limited conditions, the yields of monounsaturated fatty acids were higher than the corresponding yields under sufficient nitrogen conditions (Tang and Chen 2014). Moreover, citrate level in the *S. cerevisiae* under limited nitrogen condition was much higher than that under ample nitrogen limited condition. The accumulated citrate in cells is cleaved by ATP-citrate lyase (ACL) to acetyl-CoA, thereby providing a key substrate for fatty acid synthesis.

The oil-producing performance of *M. alpina*is closely related not only to the fatty acid content of the fungal mycelia, but also to the biomass concentration. Low biomass reduces the yield of fatty acids. Therefore, this study also used the volumetric productivity of each fatty acid component to evaluate the fatty acid production performance of *M. alpina*. The results showed that with sodium nitrate as the nitrogen source, there was little change in the fatty acid content of *M. alpina*, but its biomass concentration was significantly lower, when compared with biomass with urea as the nitrogen source. As a result, the volumetric productivities of total fatty acids, polyunsaturated fatty acids, and arachidonic acid were very low when sodium nitrate was used as the nitrogen source. Therefore, urea is more suitable for the growth and accumulation of fatty acids in *M. alpina* than sodium nitrate. In this study, with urea as nitrogen source, low nitrogen concentration (0.05 *M*) was beneficial to the accumulation of fatty acids, but it was not conducive for the growth of *M. alpina*. Sufficient nitrogen at high concentration (0.2 *M*) promoted the growth of *M. alpina*, but inhibited the accumulation of fatty acids in mycelia. However, biomass concentration and fatty acid production of *M. alpina* were higher under medium concentration of urea (0.1 *M*). Generally, the inverse relationship between biomass concentration and lipid accumulation is the most significant technical bottleneck faced when using *M. alpina* to produce lipids. If the nitrogen demand can be accurately controlled, the biomass concentration and lipid content of *M. alpina* can be increased, so that the yields of polyunsaturated fatty acids, especially arachidonic acid, can be maximized.

Conclusion

The effects of nitrogen sources and nitrogen concentrations on the mycelial growth and fatty acid accumulation of *M. alpina* were determined in this study. The results suggest that the biomass concentration, and contents and yields of total fatty acids, polyunsaturated fatty acids, and arachidonic acid are regulated when *M. alpine* is grown under different nitrogen sources and concentrations. In this study, urea was more beneficial to the growth and fatty acid accumulation of *M. alpine* than sodium nitrate, and the biomass and fatty acid yields at the medium urea concentration were relatively high. However, the mechanism underlying the effect of nitrogen source and its concentration on the growth and fatty acid synthesis of *M. alpine* is unclear. This needs further research.

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Author Contributions

Lingfei Li designed the study and revised the manuscript. Lingfei Li and Na Jin performed the experiments and analyzed the data. Lingfei Li wrote the first draft of the manuscript.

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