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ORIGINAL PAPER

Improving arachidonic acid accumulation in *Mortierella alpina* through B-group vitamin addition

Yan Zeng · Xiao-Jun Ji · Shu-Mei Chang · Zhi-Kui Nie · He Huang

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Abstract To improve the arachidonic acid (ARA) accumulation in *Mortierella alpina*, a mixed B-group vitamin addition strategy was developed. The ARA titer reached up to 10.0 g/L, 1.7-fold of the control. At the same time, the highest specific activities of key enzymes involved in ARA biosynthesis, including malic enzyme, glucose-6-phosphate dehydrogenase and ATP: citrate lyase, were 63.3, 38.6 and 53.7% higher than the control, respectively. The possible vitamin triggered improved ARA accumulation mechanism was thus elucidated that B-group vitamins could function as the cofactors of the key enzymes involved in ARA biosynthesis, or precursors for the formation of NADPH and acetyl-CoA which were crucial for ARA synthesis, and strengthened the related metabolic flux.

Keywords Arachidonic acid · B-group vitamins · Biosynthesis · Enzyme activity · *Mortierella alpina*

Introduction

Arachidonic acid (5, 8, 11, 14-*cis*-eicosatetraenoic acid, ARA) belongs to ω-6 class polyunsaturated fatty acids (PUFAs). It plays an important role as structural components of membrane phospholipids, especially in the brain and retina [1]. It is also a direct precursor of eicosanoids, which are important modulators and mediators of a variety of essential biological processes [2]. Owing to its unique biological properties, ARA has been widely applied in medicine, pharmacology, cosmetics, food industry, agriculture, and the other fields [3].

The filamentous fungus, *Mortierella alpina*, is thought to be the most prominent ARA producer [4–8]. During the past decades, the effects of medium composition on ARA production by *M. alpina* have been widely investigated. Glucose was tested as the optimal carbon source for ARA production [9]; while yeast extract or soybean meal, was suggested to be the suitable nitrogen source [10]. In addition, minerals such as phosphorus, potassium, sulfur, calcium, sodium, iron, and magnesium were tested as the major inorganic constituents for the fungus growth. However, some other micro-nutritional factors such as B-group vitamins, which might have a great contribution on the ARA production, have not been investigated. In the previous study, it showed that the microbial cell growth and some metabolite accumulation could be improved by adding additional B-group vitamins into the culture, which has been applied successfully in the fermentative production of lactic acid [11, 12], pyruvic acid [13], and lovastatin [14]. The mechanisms have also been clarified due to the strengthening of the key enzyme activities involved in the metabolic pathway triggered by the vitamin addition.

In the present study, a B-group vitamin addition strategy was developed for efficient ARA production by *M. alpina*. The possible vitamin-strengthen ARA accumulation mechanism was further elucidated through analyzing the activities of key enzymes involved in ARA biosynthesis.

Materials and methods

Microorganism

M. alpina ATCC 16266 which was obtained from American Type Culture Collection was used in the present study.

Y. Zeng · X.-J. Ji · S.-M. Chang · Z.-K. Nie · H. Huang (✉)
State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, No. 5 Xinmofan Road, Nanjing 210009, People's Republic of China
e-mail: biotech@njut.edu.cn

It was maintained on potato dextrose agar slants at 4 °C and transferred every 3 months.

Culture media and cultivation methods

Inoculum medium (g/L): glucose 30.0, yeast extract 6.0, KH_2PO_4 3.0, $NaNO_3$ 3.0, and $MgSO_4 \cdot 7H_2O$ 0.5. Inoculums 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 250-mL baffled flasks containing 50 mL production medium were inoculated at 10% (v/v) and incubated on a shaker for 7 days at 23 °C and 130 rpm. Fermentation medium (g/L): glucose 80.0, yeast extract 11.0, KH_2PO_4 3.8, $NaNO_3$ 3.4, and $MgSO_4 \cdot 7H_2O$ 0.5. All the media were sterilized at 115 °C for 30 min except for the vitamin solution, which was sterile-filtered with 0.2 μm cellulose acetate membrane. The solutions of complex nitrogen sources were sterilized separately.

Analytical methods

Glucose was measured by a biosensor with glucose oxidase electrode (SBA-40C; Institute of Biology, Shandong Academy of Sciences, China). Dry cell weight (DCW) determination was performed by harvesting the culture broth, after which the cells were separated by filtration through filter paper, washed with distilled water, and then dried at 65 °C to constant weight. The methods of total lipids (TLs) extraction, fatty acid methyl esters (FAMEs) preparation and FAMEs analysis were the same as those used in the previous studies [15, 16]. Degree of unsaturation of fatty acids was calculated as the sum of the product of concentration (%, w/w) and the number of unsaturated double bonds of each fatty acid as follows [17].

$$\begin{aligned}\text{Degree of unsaturation} = {} & 1\,(\%\text{ of monoene}) \\ & + 2\,(\%\text{ of diene}) + 3\,(\%\text{ of triene}) + 4\,(\%\text{ of tetraene}) \\ & + 5\,(\%\text{ of pentaene}) + 6\,(\%\text{ of hexaene})\end{aligned}$$

Preparation of cell extracts and enzyme activity assay

The cells were harvested by centrifugation and washed with distilled water. Samples of the culture filtrate were retained for separate analysis (stored at −20 °C if necessary). The fungus was then suspended in 400 mM Tris-HCl buffer (pH 7.4) containing 20% (w/v) glycerol, 1 mM benzamidine and 1 mM DTT, and disrupted by ultrasonic disrupter for 20 min on the ice. The resulting homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was then used to determine the enzyme activity. The activities of malic enzyme (EC 1.1.1.40, ME), glucose-6-phosphate dehydrogenase (EC1.1.1.49, G6PDH), and ATP: citrate lyase (EC 4.1.3.8, ACL) were determined by using continuous spectrophotometric assay to test the oxidation or reduction of NADPH at 340 nm at 25 °C according to the previous study [16]. One unit of enzyme activity (U) was defined as the formation of the quantities amount of NADPH per minute, specific activity (U/mg protein) was defined as the unit of activity/mg of protein. Total protein concentration was determined by means of Bradford [18] using bovine serum albumin as a standard.

Results and discussion

Effect of individual B-group vitamin addition on ARA production

As shown in Fig. 1, high levels of TLs and ARA concentration were obtained with the addition of thiamin, nicotinamide, D-pantothenate and pyridoxine. The results also showed that thiamin and nicotinamide were the optimum additives suitable for lipid accumulation, while adding D-pantothenate and pyridoxine was more effective for the fungal cell growth. Generally, almost all the tested B-group vitamins in a suitable concentration could enhance the ARA production.

When the concentration of added thiamin was 0.1 g/L, the TLs achieved the maximum, while the thiamin concentration of 0.05 g/L resulted in the maximal value of ARA concentration (7.6 g/L). Obviously, the favorable thiamin concentration for lipid accumulation and ARA synthesis were inconsistent. However, when the thiamin concentration was 0.05 g/L, the ARA percentage over the total fatty acids was 40.1%, higher than the value (36.2 %) at the thiamin concentration of 0.1 g/L (Table 1). As the ARA percentage is a key factor governing the final lipid product quality, the proper concentration of thiamin for efficient ARA rich lipid production was 0.05 g/L. Similarly, the proper added nicotinamide, D-pantothenate and pyridoxine concentration should be controlled at 0.15, 0.20 and 0.10 g/L.

Effect of complex B-group vitamin addition on ARA production

A mixture of B-group vitamins (0.05 g/L thiamin, 0.15 g/L nicotinamide, 0.20 g/L D-pantothenate, and 0.10 g/L pyridoxine) was used to illustrate their roles on the ARA production. Time courses of cell growth, TLs production and glucose consumption were shown in Fig. 2. DCW with complex B-group vitamin addition was higher than the control at all time, reaching the maximum value of 33.5 g/L, 8.1% higher than that of the control. As for residual glucose, it decreased slowly in the early period, when the

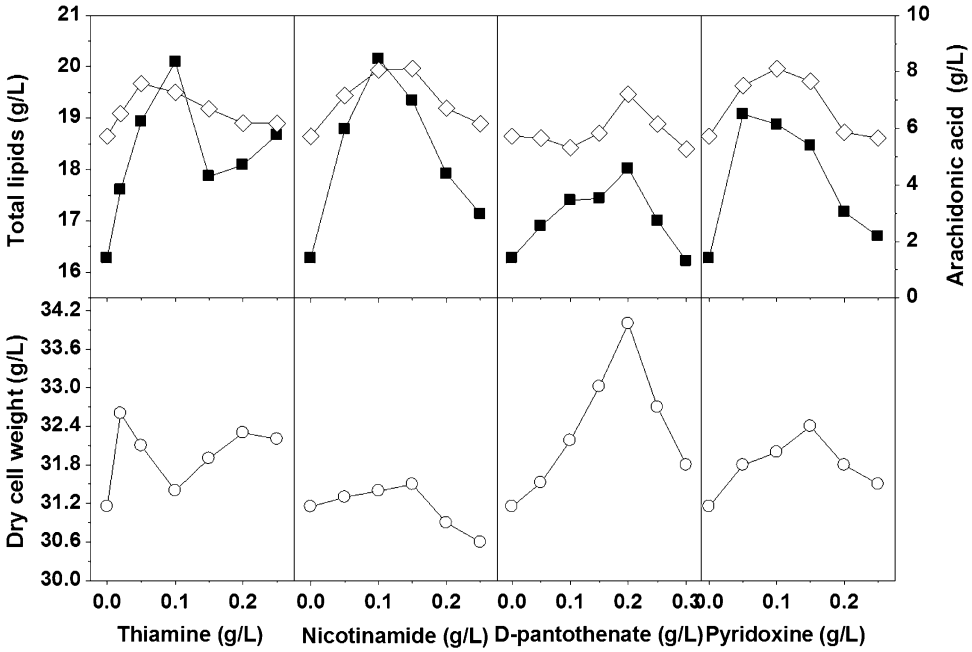

Fig. 1 Effect of adding different B-group vitamins on arachidonic acid production. Dry cell weight (*unfilled circle*); total lipids (*filled square*); arachidonic acid (*unfilled diamond*)

Table 1 The arachidonic acid percentage over the total fatty acids with different concentration of B-group vitamin addition

	Arachidonic acid percentage (%)					
B-group vitamins (g/L)	0.0	0.05	0.10	0.15	0.20	0.25
Thiamin	35.2	40.1	36.2	37.5	34.2	33.1
Nicotinamide	35.0	38.2	40.0	42.1	37.5	36.0
D-pantothenate	35.1	33.5	30.6	33.4	40.0	36.2
Pyridoxine	35.2	39.4	43.0	41.5	34.1	33.9

cell growth entered the exponential phase, glucose concentration decreased quickly. At 144 h, the glucose in the culture with the addition of complex B-group vitamins was nearly depleted while the glucose concentration of the control was 9.0 g/L. This indicated that complex B-group vitamin addition accelerated the glucose consumption rate. As far as the TLs were concerned, lipid content gradually increased with time as expected after inoculation. The value of TLs concentration with B-group vitamin addition was higher than that of the control during 48–120 h. However, the value showed an apparently decreasing tendency in the later period, with the amount nearly parallel to that of the control. The reason might be that the rate of glucose consumption with B-group vitamin addition was high and residual glucose decreased to 0 at 144 h, ahead of the control. In order to support the activity of series of desaturases which played key roles in ARA biosynthesis, the fungus started to utilize the accumulated lipids as the carbon source. This negative phenomenon might be avoided using fed-batch strategy to maintain the glucose concentration at a proper level in the future.

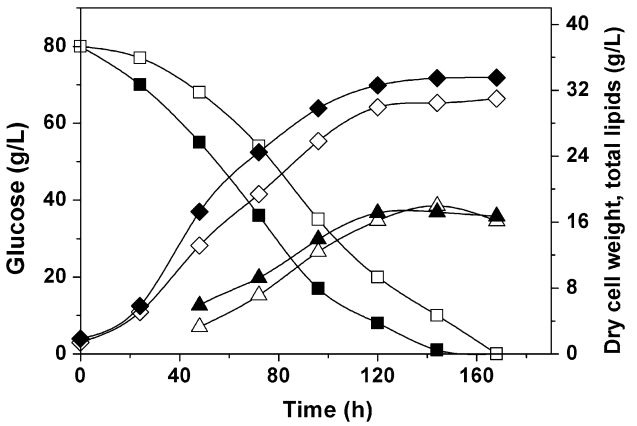

Fig. 2 Time courses of cell growth, glucose consumption and total lipids accumulation during batch cultivation of *Mortierella alpina* between the complex B-group vitamin addition strategy and the control. Dry cell weight (DCW) with B-group vitamin addition (*filled diamond*), DCW of the control (*unfilled diamond*); residual glucose with B-group vitamin addition (*filled square*), residual glucose of the control (*unfilled square*); total lipids (TLs) with B-group vitamin addition (*filled triangle*), TLs of the control (*unfilled triangle*)

The changes of ARA concentration, lipid content of biomass (TLs/DCW, %), and ARA content of lipid (ARA/TLs, %) between the complex vitamin-addition strategy and the control were presented in Fig. 3. In the early stage (48–72 h), the TLs/DCW in the vitamin addition strategy was higher than that of the control; however, the tendency was opposite later. The possible reason might be that B-group vitamins were assimilated sequentially by the ARA producing fungus. The thiamin and nicotinamide, which were found to be more effective for lipid accumulation, might be the assimilated prior to the

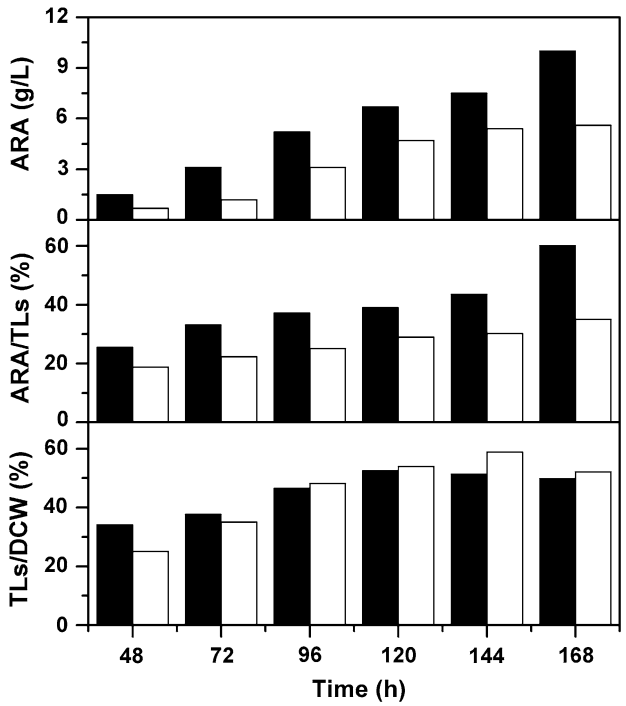

Fig. 3 Time courses of arachidonic acid (ARA) concentration, lipid content of biomass (TLs/DCW), ARA content of lipid (ARA/TLs) during ARA fermentation between the complex B-group vitamin addition strategy (*filled rectangle*) and the control (*unfilled rectangle*)

D-pantothenate and pyridoxine which were more effective for fungus cell growth. As the parameter of TLs/DCW was associated with both cell growth and lipid accumulation, the above phenomenon was thus explained by the fact that the TLs were highly accumulated faster than the DCW increase.

It should also be noticed that ARA/TLs with B-group vitamin addition increased steadily during the period from 1 to 6 days, then reached up to 60% on the 7th day drastically, which was remarkable and 1.7-fold of the control. At the end of the fermentation, TLs in B-group vitamin addition experiment were not different from the control. It was speculated that other fatty acids were largely converted into ARA. Because ARA concentration was dependent on TLs and ARA/TLs, the increase of ARA/TLs resulted in a higher ARA concentration.

Effect of complex B-group vitamin addition on fatty acid composition

During the fermentation process, an interesting phenomenon was found that the extracted lipids from the fungus cultured with B-group vitamin addition had better fluidity, compared with the control (data not shown). It was possibly demonstrated that B-group vitamin addition favored the formation of PUFAs which have lower melting point than the saturated fatty acids. The fatty acid composition and the degree of unsaturation were shown in Table 2. There were significant differences between the B-group vitamin addition experiment and the control about the unsaturation degree and fatty acid composition. The results showed that the fatty acids mainly contained palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and arachidonic acid (C20:4). It was observed that the addition of B-group vitamins could affect the degree of unsaturation obviously. The value of unsaturation degree was 2.64 with an increase of 43% compared with the control. In this case, the ARA/TLs increased at the expense of other fatty acids especially saturated fatty acids (palmitic acid and stearic acid) and monounsaturated fatty acid (oleic acid).

Effect of complex B-group vitamin addition on the activities of the key enzymes involved in ARA biosynthesis

Activities of three key enzymes involved in ARA biosynthesis, i.e. ME, G6PDH, and ACL, were measured every 24 h. In order to accumulate large quantities of fatty acids, NADPH and acetyl-CoA must be supplied sufficiently and continuously. It is evident that ME is the main source of NADPH for lipid biosynthesis and desaturation [19]. NADPH could also be generated through the hexose monophosphate pathway (HMP), in which G6PDH was the key enzyme [20]. There were also some results that the formation of acetyl-CoA in oleaginous microorganisms has been attributed to the presence of ACL [16, 21].

Figure 4 showed the enzyme activity difference between the B-group vitamin addition strategy and the control during the fermentation. The highest ME, G6PDH and ACL activity of the B-group vitamin addition strategy was 1,500, 5,526

Table 2 Comparison of fatty acid composition between the complex B-group vitamin addition strategy and the control

	Fatty acid composition (%)							Degree of unsaturation
	C16:0	C18:0	C18:1	C18:2	C18:3	C20:4	Others	
I	15.1	16.9	13.6	7.8	5.4	35.0	6.2	1.85
II	10.2	11.8	7.0	4.3	2.8	60.1	3.8	2.64

I the control, with no B-group vitamin addition

II with complex B-group vitamin addition

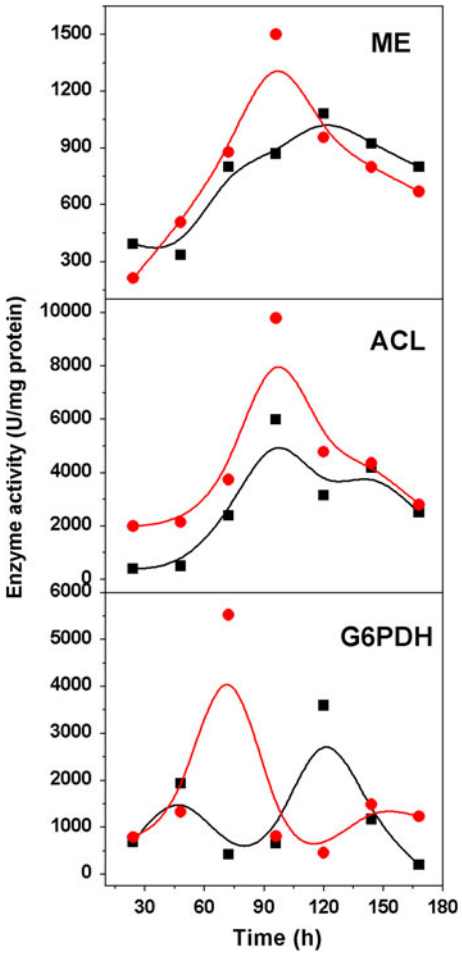

Fig. 4 Time courses of key enzyme activity during arachidonic acid fermentation by *Mortierella alpina* between the complex B-group vitamin addition strategy (*filled circle*) and the control (*filled square*). *ME* malic enzyme, *ACL, ATP* citrate lyase, *G6PDH* glucose-6-phosphate dehydrogenase

and 9,800 U/mg protein, 38.6, 53.7 and 63.3% higher than the control. It was found that G6PDH activity was only 20% of the ME activity, which indicated that the production of NADPH was largely depended on the ME activity. It was consistent with the previous conclusion [22], claiming that ME specifically provides the NADPH for the fatty acid biosynthesis to function and no other NADPH-generating enzyme will suffice. In addition, the time when ACL activity with complex B-group vitamin addition achieved the peak was ahead about 48 h. In this case, the higher ACL activity would generate more acetyl-CoA for fatty acid synthesis, which would be more effective for ARA accumulation.

Possible mechanism of ARA production improvement triggered by the vitamin addition

Acetyl-CoA and NADPH are two kinds of very important factors governing the PUFAs accumulation in oleaginous microorganisms [21]. In order to achieve efficient PUFA accumulation, two conditions should be satisfied simultaneously [23]. One is a continuous supply of acetyl-CoA directly in the cytosol of the cell as a necessary precursor for the fatty acid synthetase, and the other is a sufficient supply of NADPH as the essential reductant used in the fatty acid biosynthesis. According to the metabolic pathway of ARA biosynthesis in *M. alpina*, the formation of acetyl-CoA is facilitated by the presence of ACL. The production of NADPH for the fatty acid biosynthesis is dependent on ME and G6PDH [22].

All the four vitamins tested in the present study could more or less strengthen the supply of the two important factors governing the ARA accumulation. Thiamin, which was the cofactor of transketolase (EC 2.2.1.1) [24], might reinforce the HMP and thus enhance the rate of NADPH formation. Moreover, thiamin could act as the cofactor of pyruvate dehydrogenase complex (PDH), which catalyzed the rate-limiting step for acetyl-CoA formation. As for nicotinamide, it was the precursor of NAD, a cofactor of glycolysis [25]. Along with PDH and CoA, NAD could function as the cofactor catalyzing the reaction from pyruvate to acetyl-CoA, the precursor for the fatty acid synthesis. Nicotinamide is also the substrate of NADPH, another key factor for ARA biosynthesis. In the case of D-pantothenate, on one hand, it was the component of CoA which was the cofactor for the reaction from pyruvate to acetyl-CoA, on the other hand, the thioester derivative of CoA was acetyl-CoA, another important precursor for fatty acid synthesis [21]. Furthermore, the pyridoxine was the cofactor for some amino acid transaminases [26], which could catalyze the organic acids to form related amino acids. This process would be beneficial for transferring the citrate emerged in the tricarboxylic acid (TCA) cycle from the mitochondria to the cytosol, the flux of the transhydrogenase cycle serving for NADPH generation was thus strengthened, and the ARA biosynthesis triggered by this vitamin was thus fulfilled. To sum up, different B-group vitamins could act as cofactors of key enzymes or as precursors for the formation of NADPH and acetyl-CoA which are crucial for ARA synthesis.

Conclusions

In this study, B-group vitamin addition strategy was developed to improve ARA accumulation in *M. alpina*. The vitamin supplementation could not only increase the ARA titer, but also increase the fungus growth rate and in turn enhance the final biomass. At last, the maximum ARA concentration with this method reached up to 10.0 g/L. This strategy, which could supply the precursors for ARA biosynthesis continuously, proved to be an effective

method for economical ARA production. The idea developed in this paper could be applied to the other similar oleaginous microorganisms to achieve high related PUFA accumulation.

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