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ORIGINAL RESEARCH PAPER

A novel two-step fermentation process for improved arachidonic acid production by *Mortierella alpina*

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Abstract A novel two-step fermentation process was developed to enhance arachidonic acid (ARA) production by *Mortierella alpina* ME-1 in a 5 l fermentor. Agitation speed and aeration rate were adjusted from 180 to 40 rpm and from 0.6 to1 vvm, respectively, after 5 days cultivation, to decrease physical damage to the mycelia and to extend the stationary phase. Moreover, 3% (w/v) and 2% (w/v) ethanol were fed after 5 and 7 days cultivation, respectively, to enhance ARA content of total lipid. Eventually, an ARA yield of 19.8 g/l was achieved, which was 1.7 times higher than that of a one-step fed-batch cultivation.

Keywords Arachidonic acid · Fermentation · Lipid production · Polyunsaturated fatty acid · *Mortierella alpina*

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Introduction

Arachidonic acid (ARA; 5,8,11,14-*cis*-eicosatetraenoic acid) is a major constituent and plays the role of maintaining membrane fluidity in some biological cells (Higashiyama et al. 2002). As a precursor of prostaglandins, thromboxane, prostacyclin, and leucotrienes, ARA has various physiological functions (Gill and Valivety 1997; Horrobin and Huang 1987), and has applications in medicine, pharmacology, cosmetics, the food industry, agriculture and other fields (Eroshin et al. 2000).

Although animal liver, fish oil and egg yolk are well known as sources of ARA (Gill et al. 1997; Ratledge 2004), *Mortierella* fungi seems to be the most prominent source (Sakuradani et al. 2004). During the past 20 years, the effect of cell morphology, dissolved O_2 concentration and medium compositions, including carbon, nitrogen sources and mineral addition, on ARA productivity were widely investigated and known as the key factors (Higashiyama et al. 1998; Park et al. 1999; Koike et al. 2001). However, most of those works limited the fermentation period to 6–8 days as the fungus is physically fragile (Higashiyama et al. 2002) with both shaking and agitation inhibiting or even damaging the growth of mycelia during the stationary phase of fermentation which, in turn, inhibits the synthesis of fatty acids and shortens the stationary phase. On the other hand, the stationary phase itself is an excellent phase for fatty acids, especially ARA, synthesis (Jin et al. 2007). Therefore, the present investigation

highlights the use of the stationary phase to enhance ARA production by developing a two-step fermentation system of *M. alpina*. The first step was similar to conventional fermentation but in the second step the agitation was adjusted to an extremely low level to decrease physically damage and extend the period of stationary phase.

Changes of the lipid composition in the presence of ethanol have been investigated in several microorganisms other than *M. alpina*, showing an increase in the ratio of unsaturated to saturated fatty acids (D'Amore and Stewart 1987; Teixeira et al. 2002; Jones 1989). Moreover, ARA is the main unsaturated fatty acid in *M. alpina*. Accordingly, ethanol was introduced in the second step of the two-step fermentation system to further enhance ARA content of the lipid.

Materials and methods

Microorganism

Mortierella alpina ME-1 was obtained by UV mutation of the original ATCC 16266 strain at 20 W for 15 min. It generates a lot of yellow agglomerations on mycelia when cultured on slant for 8 days.

Culture conditions

The inoculum medium contained (g/l): glucose 30; yeast extract 6; KH_2PO_4 3; $NaNO_3$ 3; $MgSO_4 \cdot 7H_2O$ 0.5. 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. The 5 l fermentor containing 3 l production medium was inoculated at 10% (v/v), and incubated at 23°C, aeration rate 0.6 vvm, and agitation speed 180 rpm with pH uncontrolled.

Analytical methods

The dinitrosalicylic acid method was used to assay the glucose content. Dry cell weight (DCW) concentration was determined gravimetrically. Total lipids were extracted with chloroform/methanol (2:1, v/v) following the method of Bligh and Dyer (1957). Fatty acids were methylated by BF_3 in methanol (Metcalfe and Schmitz 1961). Fatty acid methyl esters and ethanol were determined by GC–MS and GC (Jin et al. 2007; Hu et al. 2007).

Results and discussion

One step fed-batch cultivation

Time courses of cell growth, glucose consumption, and total lipid and ARA biosynthesis of *M. alpina* ME-1 during one-step fed batch cultures are shown in Fig. 1 and fatty acids profiles of lipids are presented in Table 1. The biomass reached its maximal value (33.5 g/l) after 7 days. The mycelial morphology of *M. alpina* ME-1 was maintained in a fluffy pellet, which is suggested to be suitable for ARA production (Park et al. 1999). Lipid reached 18.9 g/l and ARA 11.7 g/l after 8.5 days. Then, however, the biomass declined (see Fig. 1) with a certain amount of glucose still remaining. At the same time the mycelia became fragmented, possibly caused by a high shear stress. This fragility of the biomass may thus explain the decreased biomass.

Two-step cultivation with feeding glucose

A high shear stress clearly disrupted the mycelia in the stationary phase of the one-step fed-batch

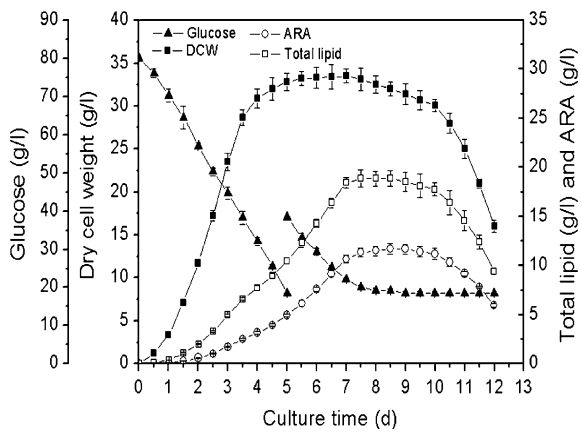

Fig. 1 Profiles of cell growth, glucose consumption, and total lipid and ARA production during one-step fed-batch cultivation of *M. alpina* ME-1 with feeding 2% (w/v) glucose and 0.3% (w/v) $NaNO_3$ after 5 days. Each datum is the mean value of three identical samples

Table 1 Fatty acyl profiles of lipids, lipid content of biomass and cell dry weight harvested at the time of ARA yield reaching its maximum during fermentation[a]

	Cell dry weight (g/l)	Lipid in biomass (%, w/w)	Fatty acyl content of total lipid (%, w/w)						
			16:0	18:0	18:1	18:2	18:3	20:4	Others
One-step fed-batch cultivation[b]	33.5	59	11	12	6	4	1	62	4
Two-step cultivation[c]	35.5	65	9	11	6	3	1	65	5
Two-step cultivation[d]	37.2	67	6	11	4	2	1	72	4
Two-step cultivation[e]	38.3	69	6	12	3	1	0	75	3

[a] Cells were grown in a 5 l fermentor with 3 l working volume containing (g/l): glucose 80, YE 11, KH_2PO_4 3.8, $NaNO_3$ 3.4, and $MgSO_4 \cdot 7H_2O$ 0.5; and incubated at 23°C, aeration rate 0.6 vvm, and agitation speed 180 rpm with pH uncontrolled

[b] Additional 2% (w/v) glucose and 0.3% (w/v) $NaNO_3$ were fed after 5 days (see Fig. 1)

[c,d,e] Agitation speed and aeration rate were adjusted to 40 rpm and 1 vvm, respectively, after 5 days with feeding additional 0.3% (w/v) $NaNO_3$ and [c] 2% (w/v) glucose (see Fig. 2); [d] 5% (w/v) ethanol (see Fig. 3); [e] 3% (w/v) ethanol and adding 2% (w/v) ethanol after 7 days (see Fig. 4)

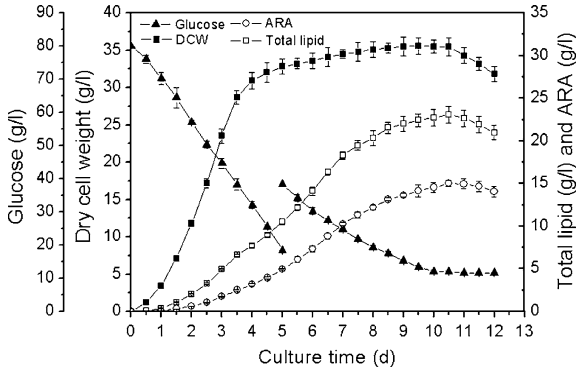

Fig. 2 Profiles of cell growth, glucose consumption, and total lipid and ARA production during two-step cultivation of *M. alpina* ME-1 with agitation speed and aeration rate adjusted from 180 to 40 rpm and from 0.6 to 1 vvm, respectively and feeding 2% (w/v) glucose and 0.3% (w/v) $NaNO_3$ after 5 days. Each datum is the mean value of three identical samples

cultivation (see Fig. 1). Thus a two-step cultivation system was introduced, in which the agitation speed was reduced to 40 rpm and aeration rate was adjusted to 1 vvm in the second phase (see Fig. 2). Compared with the one-step fed batch cultivation, physically damage was reduced and the mycelia disruption did not begin until 10.5 days. As a result, the total lipid content of biomass increased to 65%, and the ARA yield now was 15 g/l.

Two-step cultivation with feeding ethanol

To achieve a higher ARA content, 5% (w/v) ethanol instead of glucose was fed after 5 days (see Fig. 3). The final ARA content of total lipid was increased to 72% and ARA yield was 17.9 g/l after 11 days. Ethanol in its metabolism might generate additional reducing power, NADPH, (Sijtsma et al. 2005) for the various desaturases needed to produce ARA and which may be a limiting factor for ARA production.

During the stationary phase with a low concentration of nitrogen source, the carbon flux through the glycolytic pathway was decreased (Jin et al. 2007), leading to a relative low glucose consumption (see Fig. 2). When ethanol was used as a carbon source, it avoided the glycolytic pathway and the consumption of carbon source was improved (see Fig. 3). In addition, ethanol could be converted directly to

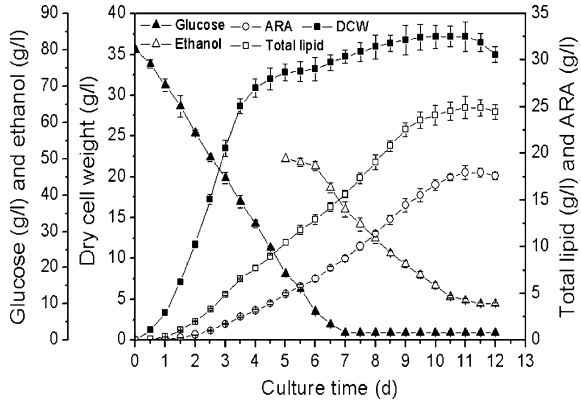

Fig. 3 Profiles of cell growth, glucose consumption, and total lipid and ARA production during two-step cultivation of *M. alpina* ME-1 with agitation speed and aeration rate adjusted from 180 to 40 rpm and from 0.6 to 1 vvm, respectively and feeding 5% (w/v) ethanol and 0.3% (w/v) $NaNO_3$ after 5 days. Each datum is the mean value of three identical samples

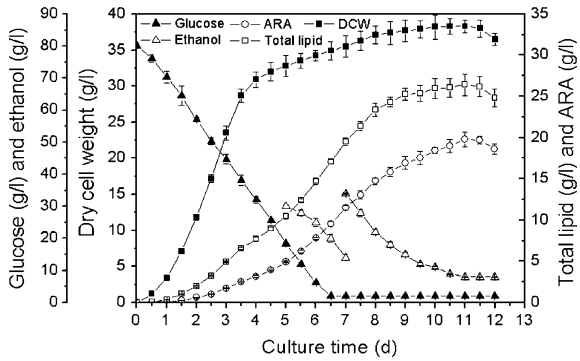

Fig. 4 Profiles of cell growth, glucose consumption, and total lipid and ARA production during two-step cultivation of *M. alpina* ME-1 with agitation speed and aeration rate adjusted from 180 to 40 rpm and from 0.6 to 1 vvm, respectively and feeding 3% (w/v) ethanol with 0.3% (w/v) $NaNO_3$ and 2% (w/v) ethanol after 5 and 7 days, respectively. Each datum is the mean value of three identical samples

acetyl-CoA and provided additional reducing power NADPH, which is needed for lipogenesis (Sijtsma et al. 2005). Therefore, a higher fatty acids production rate (see Fig. 3) was achieved. Eventually, the total lipid content of biomass was enhanced to 67%.

During the initial 24 h after feeding 5% (w/v) ethanol, there was a slight inhibition on biomass synthesis (see Fig. 3). To maximize the final product concentration, the effect of substrate inhibition was removed by first feeding 3% (w/v) ethanol after 5 days fermentation and adding 2% (w/v) ethanol at the 7th day (Fig. 4). Eventually, ARA content of total lipid was enhanced to 75% and ARA accumulated up to 19.8 g/l after 11 days cultivation, which was 1.7 times higher than that of one-step fed batch cultivation and was also higher than the previously reported yields (Singh and Ward 1997; Hwang et al. 2005).

Conclusions

A novel two-step fermentation process was developed to enhance arachidonic acid (ARA) production by *M. alpina* ME-1 in a 5 l fermentor. In the first step, the agitation speed and aeration rate were maintained to 180 rpm and 0.6 vvm, respectively. During the second step, the agitation speed was reduced to 40 rpm and aeration rate was adjusted to 1 vvm to reduce the physically damage caused by agitation to the mycelia and extend the period of stationary phase, which is an excellent phase for fatty acids, especially ARA, synthesis. Moreover, ethanol was introduced to enhance ARA content of total lipid by feeding 3% (w/v) and 2% (w/v) ethanol after 5 and 7 days cultivation, respectively. Eventually, an ARA yield of 19.8 g/l, which constituted 75% of total lipid, was achieved after 11 days cultivation. It was 1.7 times higher than that of one-step fed-batch cultivation. The relatively high ARA yield displays the potential of using the two-step fermentation process in developing bioprocess for commercial production of ARA.

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