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ORIGINAL PAPER

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An inexpensive medium for production of arachidonic acid by *Mortierella alpina*

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Abstract The production of arachidonic acid was studied in the fungus *Mortierella alpina* using an inexpensive medium. Glucose derived from maize starch hydrolysate was the sole carbon source and defatted soybean meal and sodium nitrate were the nitrogen sources. Optimal arachidonic acid yield (1.47 g l^{-1}) was observed at a glucose concentration of 100 g l^{-1}. Various treatments of defatted soybean meal to extract soluble nitrogen nutrients were evaluated. Alkali extract was the most effective for arachidonic acid production. A mixture of soybean alkali-extract protein and sodium nitrate was an excellent nitrogen source for fungal growth, lipid accumulation, and arachidonic acid production. A maximum yield of 1.87 g arachidonic acid l^{-1} was obtained with a soybean protein concentration of 4.6 g l^{-1} and a sodium nitrate concentration of 2.3 g l^{-1}.

Keywords Arachidonic acid · *Mortierella alpina* · Maize starch hydrolysate · Defatted soybean meal · Combined nitrogen source

Introduction

Arachidonic acid (AA, 5,8,11,14-*cis*-eicosatetraenoic acid), a member of the ω-6 class of polyunsaturated fatty acids, has various physiological functions and plays an important role in infant nutrition. AA is an essential fatty acid in human nutrition and a biogenetic precursor of the biologically active prostaglandins and leukotrienes [8]. Furthermore, as a component of mature human milk, AA is necessary for the neurological and neurophysiological development of both term [4] and preterm infants [3]. Many expert organizations, including FAO/WHO [7], recommended that infant formula be supplemented with AA, with the result that AA is now one of the ingredients in many brands of infant formula.

The conventional commercial sources of AA are animal viscera, e.g. pig liver. AA supplemented to infant formula is from egg-yolk lipids, which also contain other components such as cholesterol and phosphorus. Adding substantial amounts of egg-yolk lipids to formula could lead to nutritional imbalances. Thus, there is increasing interest in the microbial production of AA, which has a higher yield and contains fewer impurities. The filamentous fungus *Mortierella alpina*, belonging to the Phycomycetes class, has been identified as a promising producer of AA [6].

In developing a large-scale fermentation process for AA, it is necessary to utilize inzexpensive medium components to lower the cost. At present, most studies have used glucose and yeast extract as carbon and nitrogen sources [13]. However, these are expensive and may make the product less economically competitive. There have been a number of efforts to use cheaper sources of carbon and nitrogen for polyunsaturated fatty acid production. Lindberg and Hansson [9] examined the use of rape meal, cocos expeller and beet molasses as nitrogen and carbon sources, on γ-linolenic acid production. Chaudhuri et al. [5] determined that mustard meal was a good substrate for production of AA-rich oil. However, little work has been done on utilizing starch hydrolysate and combinations of cheaper inorganic and organic nitrogen sources as potential substrates for the production of fungal oil rich in AA.

Maize starch and defatted soybean meal are abundant farm products in China. As low-cost materials, they are already used in many industrial fermentation processes. Soy flour has been used as a nitrogen source by adding it directly to the medium for AA production [14]. However, since soy flour contains insoluble materials, and lipid in fungal cells is an intracellular product,

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adding soy flour directly to the medium increases the difficulty of separating biomass from soybean solids. The objective of this study was to investigate the feasibility of using maize starch hydrolysate as a sole carbon source and a combination of soybean meal and sodium nitrate as a nitrogen source for cell growth, and lipid and AA productivity by the filamentous fungus *Mortierella alpina*.

Materials and methods

Microorganisms

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

Preparation of maize starch hydrolysate

The starch hydrolysate was prepared by enzymatic hydrolysis of 1 kg maize starch in 4 l water. One ml heat-resistant amylase(Wuxi Genencor Bioengineering, Wuxi, China; 20,000U ml^{-1}) was added for gelatinization at 105°C for 10 min, then at 95°C for liquefaction for 1 h. The temperature was lowered to 60°C and 2 g glucoamylase (Wuxi Genencor Bioengineering; 50,000U g^{-1}) was added for saccharification for 24 h. The yield was 15–20% dextrose equivalents in the filtrate.

Treatment of defatted soybean meal

Defatted soybean meal was purchased at a local market. Three treatment methods were used. Acidic hydrolysis was carried out using 6 mol HCl l^{-1} (3 ml per 1 g meal) at 121°C for 4 h. For alkali extraction, a mixture of defatted soybean flour and water (1:10) was boiled for 30 min and the pH was maintained at 8 by addition of 2 mol NaOH l^{-1}. Water extraction was done in the same way as alkali extraction but without pH adjustment. After treatment, all mixtures were filtered and the pHs were adjusted to 6.0. Filtrates were then frozen and used as required.

Culture conditions

The inoculum medium contained (g l^{-1}): glucose 30; yeast extract 5; $K_2HPO4{\cdot}3H_2O$ 4; $CaCl_2{\cdot}2H_2O$ 0.1; $MgSO_4{\cdot}7H_2O$ 0.5. Inocula were prepared in 500-ml flasks containing 200 ml medium. The culture was grown for 2.5 days at 25°C with shaking at 120 rpm. Then 250-ml flasks containing 50 ml production medium were inoculated at 5%v/v and incubated on a shaker 7 days at 25°C for at 120 rpm. The production medium consisted of (g l^{-1}): $K_2HPO4{\cdot}3H_2O$ 4; $CaCl_2{\cdot}2H_2O$ 0.1; $MgSO_4{\cdot}7H_2O$ 0.5. The concentrations of maize starch hydrolysate and the nitrogen sources in the medium were varied as described below. The pH of the medium was adjusted to 6.0 before it was autoclaved.

Analytical methods

The dinitrosalicylic acid (DNS) method was used to assay the glucose content of maize starch hydrolysate [12]. The protein concentration of defatted soybean meal filtrate was assayed by Lowry's method [10]. The dry weight of biomass was determined by filtration of the fungal cell culture, washing the cells with water three times, and drying them at 80°C for 12 h. Total lipids were extracted following the method of Bligh and Dyer[2]. Fatty acids were methylated by boron trifluoride in methanol according to the method of Metcalfe and Schmitz [11] .Fatty acid methyl esters were determined using a gas chromatograph HP 5890 Series II (Hewlett-Packard) equipped with a 10% DEGS/Chromosorb packed column (National Chromatographic R.and A. Center, Dalian, China; 2 m×2 mm) and a flame ionization detector. The carrier gas was nitrogen with a flow rate of 33 ml/min. The temperatures of the oven, injection and detector were 200, 220 and 240°C, respectively. AA methyl ester was identified by comparing its retention time with that of an authentic standard obtained from Sigma (St. Louis, Mo., USA).

Results and discussion

Effect of concentration of maize starch hydrolysate on arachidonic acid production

The glucose concentration of maize starch hydrolysate was varied between 50 and 150 g l^{-1}(Table1). Dry biomass and AA yield (1.47g l^{-1}) were optimal when the medium contained 100 g glucose l^{-1}.

The lipid content in dry biomass increased with increasing glucose concentration, indicating that a high glucose concentration is beneficial to lipid synthesis in this fungus. Dry biomass was low at low glucose concentrations because glucose was exhausted (residual sugar after fermentation was undetectable when the glucose concentration was 50 g l^{-1}). The decreased biomass yield obtained when the glucose concentration exceeded 100 g l^{-1}, might be due to the increased osmotic pressure in the medium and the presence of unconsumed glucose. The amounts of residual sugar for initial glucose concentrations of 120 and 150 g l^{-1} were 35 and 63 g l^{-1}, respectively. This would not only represent a material waste but also a potent pollution source that would make downstream treatment difficult. Considering that changes in the lipid content of biomass and of

Table 1 Effect of glucoseconcentration (derived from maize starch hydrolysate) on biomass, total lipid,and arachidonic acid production. Mean values are given of duplicate samples (plusand minus standard error); yeast extract (8g l^{-1}) was used as nitrogen source. *AA* Arachidonic acid, *TL* total lipids, *DW* dry weight of biomass

Glucose concentration (g l^{-1})	DW (g l^{-1})	TL/DW (%, w/w)	TL (g l^{-1})	AA/TL (%, w/w)	AA (g l^{-1})
50	14.4 ± 0.6	27.8 ± 0.5	4.0 ± 0.2	22.4 ± 0.2	0.90 ± 0.05
80	21.8	34.7 ± 0.8	7.6 ± 0.1	17.6 ± 0.4	1.33 ± 0.06
100	22.5 ± 0.3	37.3 ± 0.3	8.4	17.5 ± 0.4	1.47 ± 0.03
120	21.7 ± 0.4	37.5 ± 0.4	8.1 ± 0.2	17.4 ± 0.5	1.42 ± 0.07
150	20.6 ± 0.2	38.1 ± 0.2	7.8 ± 0.2	17.4 ± 0.3	1.36 ± 0.06

AA in lipid were not significant between glucose concentrations of 100 and 150 g l^{-1}, AA yield was mainly determined by dry biomass. A glucose concentration of 100 g l^{-1} was optimal for AA production, and the residual sugar was only 6 g l^{-1}.

Effect of various treatments of defatted soybean meal on growth, lipids, and arachidonic acid production

Various methods were tested for their ability to release soluble nitrogen nutrients efficiently from defatted soybean meal. Acid hydrolysate, alkali extract, and water extract, used as nitrogen sources, were compared with yeast extract for their effects on cell growth, lipid yield, and AA yield (Fig. 1). Among the three treatment methods, alkali extraction was the most effective in enhancing biomass, lipids in biomass, and lipids and AA production. No significant differences in the AA content of lipids were observed as a result of different treatment methods. Although the nitrogen in water and alkali extracts was present as protein, water extract was not as good as alkali extract. Since the isoelectric point of the main soy protein is at pH4.5 [1], it could be extracted more effectively under alkaline conditions. Acid hydrolysate was the poorest nitrogen source for lipid production in biomass (providing nearly one-third that of other treatment methods). Acid hydrolysate of soy protein comprises mainly amino acids, since during the preparation process soy protein was hydrolyzed to amino acids. It seems that *Mortierella alpina* prefers soy protein to amino acids derived from soy.

A combination of organic nitrogen sources (soybean protein) and an inorganic nitrogen source (sodium nitrate) was used for the improved production of AA. The biomass and AA contents in lipids were higher when using combined nitrogen sources (Figs.1 and 2). A combination of soybean alkali extract and nitrate was the best nitrogen source for biomass and AA content. Although alkali extract of soybean meal as sole nitrogen source was inferior to yeast extract, when combine with nitrate it was more effective than yeast extract in enhancing cell growth and AA production .

Optimization of the combined nitrogen sources, soybean meal alkali extract and sodium nitrate, for arachidonic acid production

The soybean meal protein and sodium nitrate concentrations in the medium were varied in the range of 0.6–12 g l^{-1} and 0.2–4 g l^{-1}, respectively. As shown in Tab. 2, biomass produced in test no.1 was the lowest (12.9 g l^{-1}), although the overall nitrogen level used was not low, suggesting that cell growth was limited by inorganic nitrate and promoted by organic nitrogen. The biomass in test no. 3, which contained the lowest overall nitrogen level, was also poor (17.5 g l^{-1}). This may have been caused by an unsuitable C/N ratio. Therefore, a low nitrogen content is unfavorable for cell growth and results in a low level of AA production. The lipid:biomass ratio decreased with increasing concentrations of soybean meal alkali extract. Likewise, the AA content of lipids also decreased with increasing concentrations of soybean meal alkali extract, except in test No.1 in which growth was poor.

AA production reached 1.60 g l^{-1} when 6.0 g protein l^{-1} with 2.0 sodium nitrateg l^{-1} were used as nitrogen sources, and 1.57 g l^{-1} when 1.5 g protein l^{-1} with 4.0 g sodium nitratel^{-1} were used as nitrogen sources. There was no significant difference between these two tests in AA production. The concentrations of protein and sodium nitrate were further optimized as shown in Tab. 3. Cell growth increased with increased protein concentration

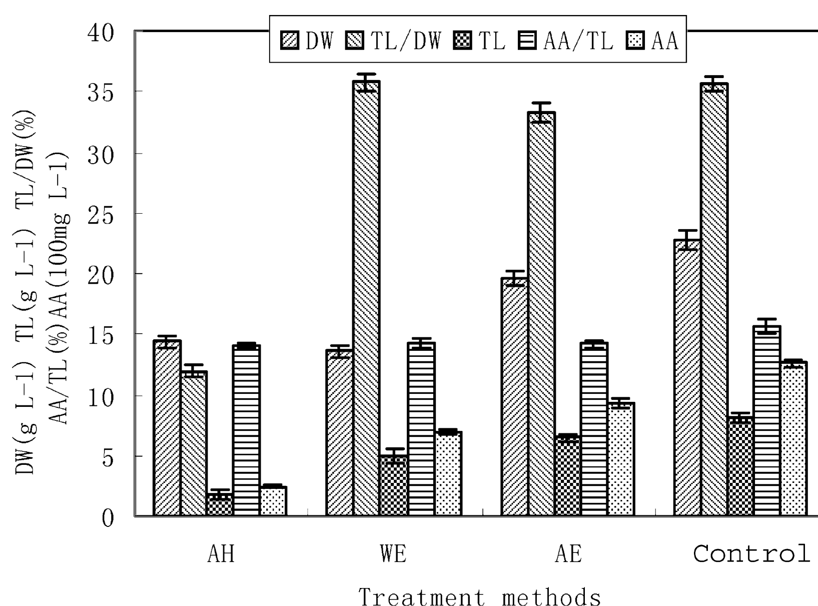

Fig. 1 Effects of various treatments of defatted soybean meal on growth, lipids, and AA production. Mean values are given of duplicate samples. *AH* Acid hydrolysate, 2% defatted soybean meal equivalent; *WE* water extract, 2% defatted soybean meal equivalent; *AE* alkali extract, 2% defatted soybean meal equivalent. Yeast extract (8g l^{-1}) was used as control; the glucose concentration of maize starch hydrolysate was 100g l^{-1}. *AA* Arachidonic acid, *TL* total lipids, *DW* dry weight of biomass

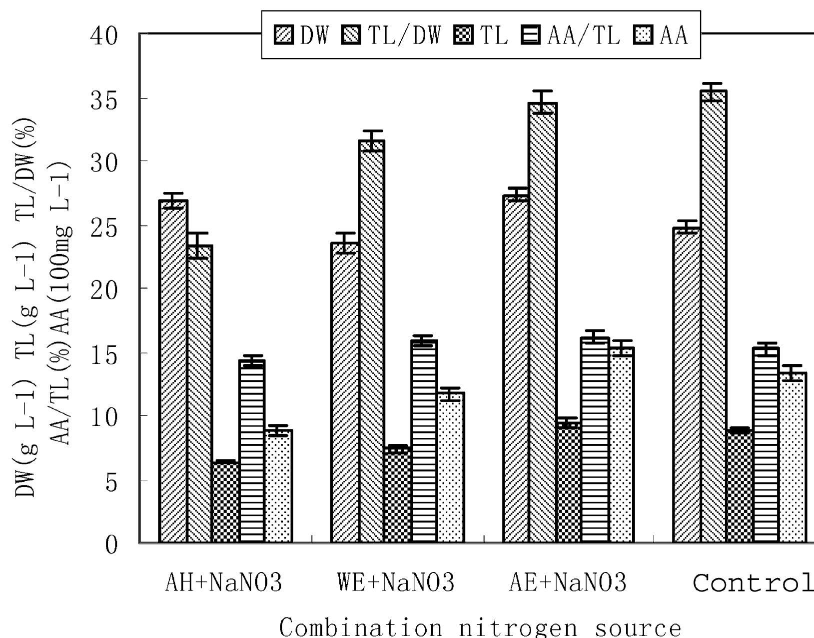

Fig. 2 Effects of combined nitrogen sources of various treatments of defatted soybean meal and inorganic nitrate on growth, lipids, and AA production. AH, WE, AE were equivalent to 1% defatted soybean meal; the concentration of $NaNO_3$ was 3g l^{-1}; 8g l^{-1} yeast extract was used as a control. The glucose concentration of maize starch hydrolysate was 100g l^{-1}. *AA* Arachidonic acid, *TL* total lipids, *DW* dry weight of biomass

Table 2 Arachidonic acid production on combination nitrogen sources of protein from soybean meal alkali extract and sodium nitrate. Mean values are given of duplicate samples (±standard error). Yeast extract (8g l^{-1}) was used as control. Glucose concentration of maize starch hydrolysate was 100g l^{-1}. *AA* Arachidonic acid, *TL* total lipids, *DW* dry weight of biomass

Test no.	Protein concentration (g l^{-1})	Sodium nitrate (g l^{-1})	Overall N (g l^{-1})	DW (g l^{-1})	TL/DW (%, w/w)	TL (g l^{-1})	AA/TL (%, w/w)	AA (g l^{-1})
1	0.6	3.0	0.53	12.9 ± 0.6	42.7 ± 0.4	5.5 ± 0.3	17.0 ± 0.2	0.93 ± 0.06
2	1.5	4.0	0.75	24.8 ± 0.8	34.6 ± 0.4	8.6 ± 0.4	18.2 ± 0.4	1.57 ± 0.10
3	3.0	0.5	0.27	17.5 ± 0.4	34.5 ± 0.5	6.0 ± 0.2	18.0 ± 0.6	1.10 ± 0.05
4	6.0	2.0	0.71	29.5 ± 0.8	31.1 ± 0.6	9.2 ± 0.4	17.4 ± 0.5	1.60 ± 0.12
5	9.0	0.2	0.60	26.7 ± 0.3	29.9 ± 0.5	8.0 ± 0.2	15.2 ± 0.4	1.22 ± 0.05
6	12.0	1.0	0.92	28.1 ± 0.5	21.7 ± 0.6	6.1 ± 0.3	12.3 ± 0.2	0.75 ± 0.05
7	Control		0.72	21.6 ± 0.6	36.2 ± 0.4	7.8 ± 0.3	15.2 ± 0.2	1.19 ± 0.06

Table 3 Optimization of combination nitrogen sources for arachidonic acid production. Mean values are given of duplicate samples (plus and minus standard error). The glucose concentration of maize starch hydrolysate was 100g l^{-1}. *AA* Arachidonic acid, *TL* total lipids, *DW* dry weight of biomass

Protein concentration (g l^{-1})	Sodium nitrate (g l^{-1})	DW (g l^{-1})	TL/DW (%, w/w)	TL (g l^{-1})	AA/TL (%, w/w)	AA (g l^{-1})
1.5	4.0	23.8 ± 0.5	36.2 ± 0.6	8.6 ± 0.3	18.6 ± 0.6	1.60 ± 0.11
2.9	3.7	25.1 ± 0.6	35.9 ± 0.4	9.0 ± 0.3	18.6 ± 0.4	1.67 ± 0.10
3.75	3.0	26.8 ± 0.6	35.5 ± 0.8	9.5 ± 0.4	18.0 ± 0.4	1.72 ± 0.10
4.6	2.3	31.2 ± 0.7	34.7 ± 0.6	10.8 ± 0.3	17.3 ± 0.6	1.87 ± 0.12
6.0	2.0	29.3 ± 0.4	33.6 ± 0.7	9.8 ± 0.4	17.1 ± 0.2	1.68 ± 0.08

while the lipid content of biomass and the AA content of lipids generally decreased with increased protein concentration, as previously shown. The highest productivities (31.2 g biomassl^{-1} , 10.8 g lipids l^{-1}, 1.87g AA l^{-1}) were achieved when a combination of 4.6 g soybean protein l^{-1} and 2.3 g sodium nitrate l^{-1} was used as the nitrogen source. Therefore, the present results show that a combination nitrogen sources consisting of defatted soybean meal protein and inorganic nitrate are good substitutes for yeast extract in the production of AA.

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