Original Article
Enhanced polyunsaturated fatty acids production in Mortierella alpina by SSF and the enrichment in chicken breasts

Shengli Yang1* and Hui Zhang2*
1The College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou, People’s Republic of China; 2Physical and Chemical Test Center, Zhejiang Institute of Quality Inspection Science, Hangzhou, China

Abstract

Background: Distiller’s dried grains with solubles (DDGS) and soybean meal were used as the substrates for the production of polyunsaturated fatty acids (PUFA) in solid-state fermentation (SSF) by Mortierella alpina. These fermented products were fed to laying hens. PUFA enrichment from chicken breasts was studied.

Methods: The maximum productivity of PUFA was achieved under optimized process condition, including 1% w/w yeast extract as additive, an incubation period of 5 days at 12°C, 10% v/w inoculum level, 75% moisture content, and pH 6.0. The hens were then fed with ration containing soybean DDGS, rapeseed oil, soybean oil, and peanut oil. The control group was fed with basal ration.

Results: Under the optimal condition, M. alpina produced total fatty acids (TFA) of 182.34 mg/g dry substrate. It has better mycelial growth when soybean meal was added to DDGS (SDDGS). PUFA fermentation product increased with higher soybean meal content. The addition of 70% soybean meal to DDGS substrate yielded 175.16 mg of TFA, including 2.49 mg eicosapentaenoic acid (EPA) and 5.26 mg docosahexaenoic acid (DHA). The ratios of ω-6/ω-3 found in chicken breasts fat were all lower than that found in control by 36.98, 31.51, 18.15, and 12.63% for SDDGS, rapeseed oil, soybean oil, and peanut oil, respectively.

Conclusions: This study identified an optimized SSF process to maximize PUFA productivity by M. alpina as the strain. This PUFA-enriched feed increased the PUFA contents as well as the proportions of ω-6 and ω-3 in chicken breasts and liver.

Keywords: PUFA; DDGS; Mortierella alpina; soybean meal; solid-state fermentation; chicken breasts; enrichment

Received: 27 December 2015; Revised: 28 August 2016; Accepted: 11 September 2016; Published: 14 October 2016

Fat acids are the major energy source and reservoir for animals to grow and survive (1–3). However, not all the fatty acids are equal. Dietary saturated fat intake has been associated with increased risk of cardiovascular disease. As substitutes of saturated fatty acids, polyunsaturated fatty acids (PUFA) provide many health benefits (4, 5), for example, a reduction in low-density lipoprotein or bad cholesterol and an increase in high-density lipoproteins or good cholesterol (6). Moreover, PUFA contain essential fatty acids like ω-3 and ω-6, which the body need but are not able to synthesize. Eicosapentaenoic acid (EPA), found in fatty cold-water fish and fish oil supplements along with docosahexaenoic acid (DHA) (7), is one of the several ω-3 fatty acids used by the body. Omega-3 fatty acids help lower the risk of heart disease. Increased intake of EPA has beneficial effects on coronary heart disease, high blood pressure, and inflammatory disorders, such as rheumatoid arthritis (8). Most people in the Western countries do not get enough omega-3 fatty acids in their diet, which is one of the reasons that PUFA production has become a prominent research focus.

Microorganisms have been used in PUFA production in recent years (9–15). Several Mortierella strains are reported (16–18). The most common Mortierella species are M. alpina (19, 20) and M. isabellina (21, 22). Among the large amounts of published information on microbial PUFA production, EPA (23–28), arachidonic acid (AA) (29–32), and γ-linolenic acid (GLA) (33, 34) are the most popular targets.

Agricultural coproducts such as soybean meal, distiller’s dried grains with solubles (DDGS), rice bran, and wheat bran are abundantly produced. Both soybean meal and DDGS are good substrates for enzymes to produce oil. Being economically competitive, soybean meal and DDGS were used as the base substrate in the present study to produce PUFA using M. alpina by solid-state fermentation (SSF).

Adding lipids in feed has been in place for more than 50 years, since 1953. Lipids improve the energy density of
the diet and provide essential fatty acids for poultry. Lipids also improve the palatability and processing properties of feed. Essential fatty acids can inhibit the inflammation, promote brain development, inhibit tumor growth, as well as can reduce the plasma triglyceride and cholesterol levels to inhibit thrombosis and atherosclerosis (35).

Fermentation using agricultural coproducts as a substrate can produce a variety of unsaturated fatty acids. PUFA, especially EPA and DHA deposition in poultry, are very important as we essentially need them for our health. The objectives of this study were to determine the parameters for the growth of the fungi selected and to examine the PUFA changes after fermentation. This study at the current stage is a proof of concept in nature. This PUFA-enriched feed increased the PUFA contents as well as the proportions of ω-6 and ω-3 in chicken breasts and liver.

Materials and methods

Microorganisms

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

Seed culture and inoculum preparation

The fungi were cultivated on PD agar containing 2.0% agar and incubated at 20°C for 7 days until complete sporulation. The spores from the agar were suspended in 15% glycerol. The suspension was used as inoculums (107 spores/mL).

Solid-state fermentation

Two miniliter spore suspensions of *M. alpina* were transferred to shake flasks (250 mL) containing 50-mL PD medium. Flasks were shaken at 20°C and 150 rpm for 24 h. Inoculum (10% v/w) was transferred to 40 g (as is) substrate in a 250-mL Erlenmeyer flask. The moisture contents of soybean meal and DDGS were adjusted to 75% and the pH to 6.0 before inoculation. The suspension was used as inoculums (107 spores/mL).

Optimization of process parameters

SSF was conducted to optimize various process parameters influencing PUFA production, including incubation temperature (10, 12, 15, 20, and 25°C), incubation time, initial moisture content of the substrate (60, 65, 70, 75, and 80%), inoculation volume (5, 8, 10, 12, 15, and 20%), and initial pH (5, 5.5, 6, 6.5, 7, 7.5, and 8). All treatments were performed in triplicate.

Effect of carbon source on DDGS SSF

DDGS was supplemented with different carbon sources (1% w/w) such as glucose, maltose, fructose, sucrose, and starch to study their effects on PUFA production. The substrate moisture content was 75% and pH was 6.0. All samples were incubated for 5 days at 20°C and then at 12°C for an additional 5 days. All treatments were performed in triplicate.

Effect of nitrogen source on DDGS SSF

Given the poor growth of the fungi on DDGS, nitrogen sources (1% w/w), such as ammonium sulfate, urea, peptone, casein, and yeast extract, were added into DDGS to examine their effect on PUFA production. All treatments were performed in triplicate.

Effect of soybean meal addition on DDGS SSF

The moisture contents of soybean meal and DDGS were adjusted to 75%. Soybean meal was added at various concentrations (0, 30, 40, 50, 60, and 70%, as is) to the DDGS substrate to enhance the PUFA production. The substrate pH was adjusted to 6.0, and fermentation was conducted for 5 days at 20°C and then at 12°C for an additional 5 days. All treatments were performed in triplicate.

Test animals and feeds

Sixty healthy 52-week-old laying hens were selected and divided into four treatment groups based on weights. Each treatment was repeated five times, with four laying hens for each repetition. Single-factor completely randomized design was adopted in the test. Test Groups 1, 2, 3, and 4 were fed with a recipe containing soybean DDGS (SDDGS), 3% rapeseed oil, 3% soybean oil, and 3% peanut oil, respectively. The control group was fed with base recipe. The test period was 45 days.

Sample collection and analytical methods

During the study period, eggs were collected and counted; the weight of the eggs and the ration consumption were determined. This study was approved by the Animal Ethics Committee in Zhejiang University (Institutional Animal Welfare and Ethics Committee in Zhejiang University, China). At the end of the test period, the laying hens were sacrificed by cervical dislocation, and their liver and breast were obtained for later use.

PUFA analysis

Fermented flour (1 g) was soaked in 30 mL of chloroform/methanol (2:1, v/v) supplemented with 1.5 mL sulfuric acid. Mixture was refluxed for 1 h and reaction was stopped by adding 5 mL deionized water. The lipid phase was then dried. Fatty acid methyl esters were extracted using hexanes and analyzed using capillary gas chromatography (GC; Hewlett-Packard model 5890 series II) equipped with flame ionization detector. The conditions used for GC were as follows: injection temperature, 230°C; detector temperature, 230°C, oven temperature was programmed from 130 to 220°C with a heating rate of 10°C/min. The column used was a supelco sp-2330
(Bellefonte, PA) capillary column, 15 m (length) × 0.25 nm (i.d.) × 0.2 μm (film thickness).

**Statistical analyses**
All treatments were repeated as described under each experiment. ANOVA was performed using SAS and the least significant difference mean comparison was used to compare treatment mean differences at *p* = 5%.

**Results**

**PUFA accumulation by SSF with M. alpina using DDGS and soybean meal as substrates**
As shown in Table 1, each gram of soybean meal substrate yielded 58.75 mg of total fatty acids (TFA), including 0.91 mg EPA, 2.10 mg DHA, 23.65 mg linoleic acid (LA), and 2.29 mg α-linolenic acid (ALA) after a 10-day incubation. As shown in Table 2, each gram of DDGS yielded only 0.42 mg EPA and 0.95 mg DHA, in comparison. This also indicates that soybean meal may be a better nitrogen source for *M. alpina* than DDGS. Therefore, the addition of soybean meal to DDGS may help improve SSF performance.

**Effect of incubation temperature and time on PUFA content of DDGS SSF**
The maximum PUFA yield was obtained at 12°C; however, the microbial growth was better at 20°C. The strains hardly synthesized EPA and DHA when the temperature exceeded 20°C. The detailed results are presented in Table 3. A decrease in the PUFA yield was observed when the incubation temperature was higher or lower than 12°C, indicating that 12°C is more advantageous for PUFA production by *M. alpina*. Although a higher temperature was beneficial to mycelial growth, some adverse effects on the metabolic activities were nevertheless observed. Therefore, we designed a two-phase incubation process with programmed temperatures, that is, 5-day preincubation at 10°C and an additional incubation period (2, 3, 4, 5, 6, and 7 days) at 12°C. The maximum PUFA yield was obtained after 5-day preincubation at 20°C followed by another 5-day incubation at 12°C (Table 4).

**Effect of initial substrate moisture content on PUFA content**
A high PUFA yield was attained with an initial moisture level of 75% (Fig. 1a). The moisture content of the SSF substrate affects dissolved oxygen and, consequently, the biosynthesis and secretion of products (36). Low moisture content decreases the solubility of nutrients in the substrate. Also, low degree of swelling and low water activity limit the growth of microorganisms (37). This explains low PUFA content at low moisture content (Fig. 1a). An increase in moisture level is believed to reduce substrate porosity, thus limiting oxygen transfer (38). Therefore, the moisture content of the medium should be controlled within a suitable range. The most suitable moisture content of the medium for PUFA production of *M. alpina* was 75%.

**Effect of inoculation volume on DDGS SSF and PUFA content**
The inoculation volume significantly affected fermentation, considering the maximum PUFA production was obtained at 10% (v/w) inoculum compared with other higher inoculation volumes. The results are presented in Fig. 1b. A lower inoculum level may give insufficient biomass, causing reduced product formation, whereas a higher inoculum level may produce too much biomass, leading to poor product formation.

**Effect of initial pH on DDGS SSF and PUFA content**
The pH level is another important factor affecting the growth and product yield during SSF. Each microorganism has an optimal pH and pH range for its growth and activity. The initial pH values were adjusted with the addition of HCl or NaOH to 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5 to

**Table 1.** Polyunsaturated fatty acids accumulation from *M. alpina* for SSF on soybean meal after 10-day incubation

| PUFA name | PUFA formation (original), mg/g dry substrate | PUFA formation (fermented), mg/g dry substrate |
|-----------|-----------------------------------------------|-----------------------------------------------|
| 16:0      | 1.33 ± 0.17                                   | 9.96 ± 0.82*                                  |
| 18:0      | 0.50 ± 0.04                                   | 2.63 ± 0.23*                                  |
| 18:1      | 1.63 ± 0.24                                   | 12.91 ± 1.18*                                 |
| 18:2      | 2.01 ± 0.21                                   | 23.65 ± 2.84*                                 |
| 18:3      | 0.27 ± 0.02                                   | 2.29 ± 0.33*                                  |
| 20:5      | 0.00                                          | 0.91 ± 0.04*                                  |
| 22:6      | 0.00                                          | 2.10 ± 0.22*                                  |
| Total     | 6.08                                          | 58.75                                         |

PUFA, polyunsaturated fatty acids; SSF, solid-state fermentation.

*p* < 0.005 compared with unfermented group.

**Table 2.** Polyunsaturated fatty acids accumulation from *M. alpina* for SSF on DDGS after 10-day incubation

| PUFA name | PUFA formation (original), mg/g dry substrate | PUFA formation (fermented), mg/g dry substrate |
|-----------|-----------------------------------------------|-----------------------------------------------|
| 16:0      | 14.81 ± 1.08                                  | 15.48 ± 1.40                                  |
| 18:0      | 2.00 ± 0.19                                   | 2.32 ± 0.18*                                  |
| 18:1      | 25.70 ± 2.32                                  | 25.50 ± 2.06                                  |
| 18:2      | 57.46 ± 4.88                                  | 56.42 ± 5.71                                  |
| 18:3      | 2.11 ± 0.24                                   | 2.43 ± 0.28                                   |
| 20:5      | 0.00                                          | 0.42 ± 0.03*                                  |
| 22:6      | 0.00                                          | 0.95 ± 0.08*                                  |
| Total     | 105.16                                        | 108.32                                        |

DDGS, distiller’s dried grains with solubles; PUFA, polyunsaturated fatty acids; SSF, solid-state fermentation.

*p* < 0.005 compared with unfermented group.

Enhanced polyunsaturated fatty acids production

Citation: Food & Nutrition Research 2016, 60: 30842 - http://dx.doi.org/10.3402/fnr.v60.30842

(page number not for citation purpose)
evaluate the effects of the initial pH value of the solid substrate on PUFA synthesis. Microbial growth achieved a high rate in the pH range of 5–7.5 and decreased dramatically when the pH falls out of this range. Maximum PUFA production was obtained at pH 6.0 (Fig. 1c).

Effect of additional carbon source on DDGS SSF and PUFA content
The substrate moisture content was maintained at 75%, the pH at 6.0, and the inoculation volume at 10% (v/w). All samples were initially incubated for 5 days at 20°C and then at 12°C for an additional 5 days. PUFA production was assayed for the control with DDGS alone as the substrate. Very low PUFA were obtained. The TFA obtained were about 103.52 mg/g dry substrate, composing of 0.42 mg EPA and 0.95 mg DHA, during the 10-day incubation. The addition of different sugars (1% w/w) into DDGS resulted in better PUFA production, the highest was obtained with starch, TFA obtained were about 103.52 mg/g dry substrate, composing of 0.42 mg EPA, 72.38 mg LA, 3.54 mg ALA, and 6.05 mg DHA, after the 10-day incubation. Peptone yielded the second highest PUFA yield, followed by casein. Furthermore, organic nitrogen sources were significantly better than inorganic nitrogen sources, indicating that organic nitrogen sources may be more suitable for the growth of M. alpina and PUFA formation.

Effect of additional nitrogen source on DDGS SSF and PUFA content
The nitrogen source or nitrogen availability is an important factor in microbial growth and enzyme production. Since DDGS led to poor fungal growth, different nitrogen sources were added into DDGS to further optimize SSF efficiency. Figure 2a shows the differences in PUFA contents produced with various nitrogen sources. The results showed that PUFA production in nitrogen-supplemented DDGS increased remarkably in comparison with the control. Among these nitrogen sources, the addition of yeast extract yielded the highest PUFA, about 182.34 mg/g dry substrate including 2.84 mg EPA, 79.03 mg LA, 7.78 mg ALA, and 6.05 mg DHA, after the 10-day incubation. Peptone yielded the second highest PUFA yield, followed by casein. Furthermore, organic nitrogen sources were significantly better than inorganic nitrogen sources, indicating that organic nitrogen sources may be more suitable for the growth of M. alpina and PUFA formation.

Effect of soybean meal addition in DDGS on PUFA content
PUFA production was relatively low in DDGS with poor growth. The strains grew better when soybean meal was added into DDGS. The addition of soybean meal increased remarkably in comparison with the control. Among these nitrogen sources, the addition of yeast extract yielded the highest PUFA, about 182.34 mg/g dry substrate including 2.84 mg EPA, 79.03 mg LA, 7.78 mg ALA, and 6.05 mg DHA, after the 10-day incubation. Peptone yielded the second highest PUFA yield, followed by casein. Furthermore, organic nitrogen sources were significantly better than inorganic nitrogen sources, indicating that organic nitrogen sources may be more suitable for the growth of M. alpina and PUFA formation.

### Table 3. Effect of incubation temperature on PUFA from M. alpina for SSF on DDGS

| Temp. (°C) | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:5 | 22:6 | Total |
|-----------|------|------|------|------|------|------|------|-------|
| 10        | 15.35 ± 1.32b | 2.17 ± 0.28b | 24.16 ± 2.59b | 57.43 ± 4.66b | 5.28 ± 0.24bc | 0.32 ± 0.03b | 0.74 ± 0.05b | 109.26b |
| 12        | 15.89 ± 1.44b | 2.51 ± 0.17b | 24.33 ± 2.31bc | 58.04 ± 5.27b | 2.58 ± 0.26b | 0.41 ± 0.04b | 0.93 ± 0.08b | 111.69b |
| 15        | 14.78 ± 1.39b | 2.22 ± 0.21bc | 24.03 ± 2.48d | 57.49 ± 5.06b | 2.58 ± 0.24b | 0.28 ± 0.03b | 0.66 ± 0.07b | 109.14b |
| 20        | 15.37 ± 1.52b | 2.32 ± 0.22b | 24.58 ± 2.34a | 56.42 ± 4.77a | 2.43 ± 0.21b | 0.02 ± 0.01c | 0.15 ± 0.01c | 107.56a |
| 25        | 15.28 ± 1.48b | 2.03 ± 0.19d | 24.22 ± 2.26bc | 57.63 ± 5.13b | 2.34 ± 0.16e | 0.00 ± 0.06e | 0.06 ± 0.01c | 107.21c |

DDGS, distiller's dried grains with solubles; PUFA, polyunsaturated fatty acids.

Mean SD, n = 3. Means in the same column that do not share alphabetic superscript show significant difference at 0.05 level according to Duncan’s multiple range test.

*Means in the same column per parameter with different letters are significantly different at P < 0.05 by Tukey’s range test.

### Table 4. Effect of incubation time on PUFA from M. alpina for SSF on DDGs

| Time (days) | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:5 | 22:6 | Total |
|-------------|------|------|------|------|------|------|------|-------|
| 2           | 14.87 ± 1.25a | 2.04 ± 0.22a | 25.67 ± 2.47b | 56.88 ± 5.28b | 2.16 ± 0.19b | 0.09 ± 0.01a | 0.32 ± 0.03a | 107.42a |
| 3           | 14.99 ± 1.39a | 2.26 ± 0.23ab | 25.59 ± 2.27b | 57.19 ± 5.09a | 2.13 ± 0.24b | 0.14 ± 0.01a | 0.51 ± 0.03a | 108.29a |
| 4           | 15.72 ± 1.51b | 2.01 ± 0.25b | 25.48 ± 2.26b | 56.07 ± 4.97b | 1.96 ± 0.16b | 0.27 ± 0.01b | 0.76 ± 0.04b | 110.58b |
| 5           | 16.02 ± 1.47c | 2.28 ± 0.26ab | 25.74 ± 2.31a | 56.18 ± 5.17a | 2.49 ± 0.22c | 0.45 ± 0.02c | 0.92 ± 0.05c | 111.68a |
| 6           | 15.87 ± 1.45c | 2.31 ± 0.18a | 24.93 ± 2.29a | 57.04 ± 4.83ab | 2.36 ± 0.20ab | 0.41 ± 0.02ab | 0.87 ± 0.06ab | 110.86a |
| 7           | 15.26 ± 1.43c | 2.26 ± 0.21ab | 24.27 ± 2.34a | 56.13 ± 5.28a | 2.05 ± 0.17a | 0.31 ± 0.02a | 0.65 ± 0.05ab | 108.43c |
added to DDGS, as mentioned early. PUFA production also increased with increasing soybean meal content. PUFA production from fermented DDGS with different soybean meal concentrations is presented in Fig. 2c. A positive relation between PUFA and soybean meal percentage in DDGS was observed. The addition of 70% soybean meal to the DDGS substrate yielded 175.16 mg of TFA, including 2.49 mg EPA, 75.94 mg LA, 7.52 mg ALA, and 5.26 mg DHA for 10-day incubation.

PUFA enrichment and proportion in chicken breasts and liver
The changes in the enrichment and proportion of PUFA in chicken breasts and liver are shown in Table 5. In the test groups fed with SDDGS and vegetable oil-added ration, the PUFA contents in the chicken breasts and liver were higher than those in the control group (p < 0.05). Table 5 shows that the proportions of ω-6 and ω-3 in chicken breasts in Test Groups 1, 2, 3, and 4 were 36.98, 31.51, 18.15, and 12.63% lower than that of the control group, respectively. The content of ω-6 in Test Group 3 was significantly different from that of the control group (p < 0.01). In addition, the proportions of both in liver and in the test groups were slightly higher than that of the control group, with the values for Test Groups 1, 2, and 3 higher by 0.76, 0.50, and 0.31%, respectively. A significant difference in the ω-6 content in the liver was observed among the test groups (p < 0.05). No difference was found in the ω-6 content in chicken breasts among Test Groups 1, 2, and 4. The ω-3 content in the liver of Test Group 1 was significantly different from that of the control group (p < 0.01). Furthermore, the ω-3 content was significantly different among the test groups (p < 0.05).
Table 5. PUFA enrichment and proportion in chicken breasts and liver

| Group                        | Liver  |                      |         | Chicken breasts |                      |         |
|------------------------------|--------|----------------------|---------|-----------------|----------------------|---------|
|                              | ω-6 (g/100g TFA) | ω-3 (g/100g TFA) | Proportion | ω-6 (g/100g TFA) | ω-3 (g/100g TFA) | Proportion |
|------------------------------|---------|----------------------|-----------|-----------------|----------------------|-----------|
| 1 (SDDGS)                    | 24.50 ± 2.62a | 4.01 ± 0.64a | 6.11:1*  | 23.70 ± 3.14a | 0.94 ± 0.33a | 25.21:1* |
| 2 (3% rapeseed oil)          | 22.70 ± 2.87b | 3.88 ± 0.71b,c | 5.85:1b  | 22.40 ± 2.72c | 0.63 ± 0.45b | 30.68:1b |
| 3 (% soybean oil)            | 22.30 ± 1.93b | 3.94 ± 0.43b | 5.66:1i  | 22.90 ± 2.46b | 0.52 ± 0.17c | 44.04:1c |
| 4 (3% peanut oil)            | 21.20 ± 2.46c | 3.98 ± 0.35b | 5.33:1   | 22.80 ± 2.91b,c | 0.46 ± 0.25c | 49.56:1d |
| Control group                | 17.40 ± 2.22 | 3.25 ± 0.62 | 5.35:1   | 19.90 ± 2.78 | 0.32 ± 0.28 | 62.19:1  |

PUFA, polyunsaturated fatty acids; SDDGS, soybean distiller's dried grains with solubles; TFA, total fatty acids.

Mean SD, n = 3. Means in the same column that do not share alphabetic superscript show significant difference at 0.05 level according to Duncan’s multiple range test.

Means in the same column per parameter with different letters are significantly different at P < 0.05 by Tukey’s range test.

**Discussion**

Nitrogen source supplement could provide additional nutrients for microbial growth and consequently increase PUFA production. Ben-Amotz (39) indicated that high concentrations of nitrogen source could stimulate EPA production. Nitrogen content of the solid substrate could be used to improve PUFA production by SSF using DDGS as the substrate. Some researchers have suggested that different strains have different preferences for either inorganic or organic nitrogen for growth and PUFA production, although organic nitrogen sources are usually used for PUFA production (40). In our studies, PUFA production was higher when organic nitrogen sources were used (Fig. 2b and c). Furthermore, organic nitrogen sources were proven more effective than inorganic nitrogen sources in enhancing cell growth and PUFA production. Thus, the soybean meal and DDGS mixture showed good potential as base substrate for PUFA production with *M. alpina*. Sajbidor et al. (41) and Jang et al. (42) reported that carbon/nitrogen ratio of solid substrate was very important in PUFA production.

PUFA production of the fungi was significantly influenced by the moisture content of substrate in SSF. The moisture content in SSF substrate affects dissolved oxygen and thus the biosynthesis and secretion of PUFA. Low moisture content causes a reduction in the solubility of nutrients in the substrate and a low degree of swelling (37), and low water activity limits the growth of microorganisms (43). Lekha and Lonsane (44) also indicated that high moisture contents decreased the porosity and the gas exchange, induced the loss of particle structure and the production of stickiness, reduced the gas volume, and enhanced the aerial mycelium formation. Jang et al. (45) reported that the initial moisture content of solid substrate ranging from 60 to 65% was good for PUFA production. Moisture content at 70–75% favored the production of ω-6 series PUFA; while moisture content at 60–65% was good for the production of ω-3 series PUFA. Therefore, we determined that the moisture content of 75% is most suitable for PUFA production in our system (Fig. 1).

The suitable range of ratios between ω-6 and ω-3 recommended by WHO are 1:1–8:1 (46), that by USA are 4:1–15:1 (47), respectively. However, with more demand for ω-3 due to health concerns, this ratio needs to be further reduced (48, 49). This study shows that the proportion of both in the liver was within the suitable range. The proportion of these two fatty acids in chicken breasts was obviously beyond the suitable range. The result indicates that, after adding either of the three vegetable oils and SDDGS, the increment in ω-6 content was higher than that of ω-3. This observation may be related to the different LA contents in the three vegetable oils and SDDGS. These fatty acids, after being absorbed through the intestines of domestic birds, are transported to the liver, and go into the tissue. During this period, palmitic acid, saturated fatty acids can be converted into linoleic and linolenic acids through desaturase and chain extension, while stearic acid can be converted into oleic and LAs. Therefore, after adding vegetable oil and SDDGS into the feed, the proportions of ω-6 and ω-3 in the liver tended to stabilize. Furthermore, the proportions of ω-6 and ω-3 in chicken breasts fed by SDDGS were more close to the suitable range, indicating that SDDGS as a feed for chicken breasts is more helpful in balancing ω-6 and ω-3 in chicken breasts than vegetable oil.

The balance between ω-6 and ω-3, which is very important for stable internal environment and normal growth, can reduce the incidence of cardiovascular diseases and other chronic diseases, and is conducive to mental health (50–54). Both of them cannot be converted in but can interact with the body (55–57). Therefore, the focus should be given on their proportion while their adequate supply is ensured. Many experiments have verified that regulated feed was feasible to produce poultry products with high ω-3 and controlled ratio of ω-6 and ω-3 (58). These studies provided a reliable basis for further investigation and production of poultry products rich in ω-3.
Conclusions
In this study, soybean meal and DDGS were successfully utilized as SSF substrates for PUFA production by *M. alpina*. Higher biomass and PUFA yield were achieved using DDGS supplemented with soybean meal compared with DDGS alone. PUFA production can also be optimized by added carbon and nitrogen sources, especially organic nitrogen, for the growth of *M. alpina* and PUFA production. SSF enrichment of PUFA is feasible using *M. alpina* as the starting strain. The product from this SSF process improved the laying rate, increased the PUFA contents in chicken breasts and liver, and enhanced the proportions of ω-6 and ω-3. The optimized SSF process verified is a unique process to maximize PUFA yield by *M. alpina*.

Acknowledgements
Great appreciation is given to all members of our laboratory for their enthusiastic participation in the research, as well as Wang Guang and Michael Pu for their editorial help.

Conflict of interest and funding
The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

References
1. Jain JL, Jain S, Jain N. Fundamentals of biochemistry. India: Chand (S.) & Co Ltd; 2005. pp. 565.
2. Yngvar O. Resources for fish feed in future mariculture. Aquacult Environ Interact 2011; 1: 187–200.
3. Annison EF, Bryden WL. Perspectives on ruminant nutrition and metabolism. I. Metabolism in ruminant tissues. Nutr Res Rev 1999; 12: 147–77.
4. Ciftci O, Cetin A, Aydin M, Kaya K, Oguz F. Fish oil contained in eicosapentaenoic acid and docosahexaenoic acid, attenuates testicular and spermatological damage induced by cisplatin in rats. Andrologia 2014; 46: 1161–8.
5. Yoshida H, Ito K, Sato R. Clinical relevance of decreased ratios of serum eicosapentaenoic acid/arachidonic acid (AA) and docosahexaenoic acid/AA to impaired arterial stiffness. Int J Cardiol 2014; 177: 517–9.
6. Siri-Tarino PW, Krauss RM, Sun Q, Hu FB. Saturated fatty acids and risk of coronary heart disease: modulation by replacement nutrients. Curr Atheroscler Rep 2010; 12: 384–90.
7. James GM, Jerry K, Bradley R, James CL, Paul R, Ross Z. Biochemical characterization of polysaturated fatty acid synthesis in Schizochytrium: release of the products as free fatty acids. Plant Physiol Bioch 2009; 47: 472–8.
8. Jeffery LE, Calder P, Raza K. Plasma levels of polysaturated omega-3-eicosapentaenoic acid are associated with anti-TNF response in rheumatoid arthritis and inhibit the etanercept driven rise in Th17 cell differentiation in vitro. Immun 2014; 143: 143.
9. Ashwini T, Ramin A, Ajay KD, Uday SA, Janusz AK. Extraction and transesterification of polysaturated fatty acids from *Mortierella* in using supercritical fluid: experimental and kinetic studies. Asia Pac J Chem Eng 2014; 9: 507–18.
10. Eiji S, Akinori A, Sakaya S, Jun O. Metabolic engineering for the production of polysaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S–4. J Biosci Bioeng 2013; 116: 417–22.
11. Hao G, Chen H, Wang L, Gu Z, Song Y, Zhang H, et al. Role of malic enzyme during fatty acid synthesis in the oleaginous fungus *Mortierella alpina*. Appl Environ Microbiol 2014; 80: 2672–8.
12. Jung IS, Lovitt RW. Integrated production of long chain polysaturated fatty acids (PUFA)-rich Schizochytrium biomass using a nutrient supplemented marine aquaculture wastewater. Aquacult Eng 2010; 43: 51–61.
13. Nisha A, Venkateswaran G. Effect of culture variables on myceial arachidonic acid production by *Mortierella alpina*. Food Bioproc Tech 2011; 4: 232–40.
14. Spencer DS, Roberto EA, Kevin TB, Andrew WN. Use of raw glycerol to produce oil rich in polysaturated fatty acids by a thraustochytrid. Enzyme Microb Technol 2011; 48: 267–72.
15. Sun Q, Liu J, Zhang Q, Qing X, Gary D, Li X. Characterization of three novel desaturases involved in the delta-6 desaturation pathways for polysaturated fatty acid biosynthesis from *Phytophthora infestans*. Appl Microbiol Biotechnol 2013; 97: 7689–97.
16. Jin MJ, Huang H, Xiao AH, Gao Z, Liu X, Peng C. Enhancing arachidonic acid production by *Mortierella alpina* ME-1 using improved mycelium aging technology. Bioprocess Biosyst Eng 2009; 32: 117–22.
17. Worapol J, Tsunehiro A, Rieko T, Kazuhiro I, Seiji K, Kazuhisa O. Purification and characterization of intracellular lipase from the polysaturated fatty acid-producing fungus *Mortierella alliuca*. N Biotechnol 2011; 28: 158–64.
18. Yu AQ, Zhu JC, Zhang B, Xing LJ, Li MC. Effects of different carbon sources on the growth, fatty acids production, and expression of three desaturase genes of *Mortierella alpina* ATCC 16266. Curr Microbiol 2011; 62: 1617–22.
19. Ganesan V, Govindarajulu V. Production and enhancement of omega-3 fatty acid from *Mortierella alpina* CFR-GV15: its food and therapeutic application. BioMed Res Int 2014; 2014: 9–17.
20. Hiroshi K, Eiji S, Shigenobu K, Si-Bum P, Akinori A, Jun S, et al. Characterization of a trifunctional fatty acid desaturase from oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast expression system. J Biosci Bioeng 2013; 116: 672–6.
21. Gao D, Zeng J, Yu X, Dong T, Chen S. Improved lipid accumulation by morphology engineering of oleaginous fungus *Mortierella isabella*. Biotechnol Bioeng 2014; 111: 1758–66.
22. Zhang J, Hu B. Microbial lipid production from corn stover via *Mortierella isabella*. Appl Biochem Biotechnol 2014; 174: 574–86.
23. Annali J, Alf B, Willem HVZ. The production of eicosapentaenoic acid by representatives of the genus *Mortierella* grown on brewers’ spent grain. Biologia 2009; 64: 871–6.
24. Fu Y, Fan X, Li X, Wang H, Chen H. The Desaturase OPIN17 from *Phytophthora infestans* converts arachidonic acid to eicosapentaenoic acid in CHO cells. Appl Biochem Biotechnol 2013; 171: 975–88.
25. Sakuradani E, Akinori A, Jun O, Sakayu S. Improved production of various polysaturated fatty acids through filamentous fungus *Mortierella alpina* breeding. Appl Microbiol Biotechnol 2009; 84: 1–10.
26. Snehak KA, Rafael AG, When ZY. Use of biodiesel-derived crude glycerol for producing eicosapentaenoic acid (EPA) by the fungus *Pythium irregulare*. J Agric Food Chem 2009; 57: 2739–44.
27. Wu L, Charles LR, Wen Z. The safety assessment of *Pythium irregulare* as a producer of biomass and eicosapentaenoic acid for use in dietary supplements and food ingredients. Appl Microbiol Biotechnol 2013; 97: 7579–85.
28. Zhang R, Zhu Y, Ren L, Zhou P, Hu J, Yu L. Identification of a fatty acid Δ6-desaturase gene from the eicosapentaenoic acid-producing fungus Pythium splendens RBB-5. Biotechnol Lett 2013; 35: 431–8.

29. Alpettiyil N, Navin KR, Govindarjulu V. Optimization of media components for enhanced arachidonic acid production by Mortierella alpine under submerged cultivation. Biotechnol Bioproc Eng 2011; 16: 229–37.

30. Jin MJ, Huang H, Xiao AH, Zhang K, Liu X, Li S, et al. A novel two-step fermentation process for improved arachidonic acid production by Mortierella alpine. Biotechnol Lett 2008; 30: 1087–91.

31. Lio JY, Wang T. Pythium irregulare fermentation to produce arachidonic acid and dihomo-gamma-linolenic acid from soybean processing co-products as substrates. Appl Biochem Biotechnol 2011; 164: 979–90.

32. Hou CT. Production of arachidonic acid and dihomo-γ-linolenic acid from glycerol by oil-producing filamentous fungi, Mortierella in the ARS culture collection. J Ind Microbiol Biotechnol 2008; 35: 501–6.

33. Xian M, Kang YJ, Yan JC. Production of linolenic acid by Mortierella isabellina grown on octadecane. Curr Microbiol 2002; 44: 141–4.

34. Das UN. Essential fatty acids: biochemistry, physiology and pathology. Biotechnol J 2006; 1(4): 420–39.

35. Lu JM, Peng C, Ji XJ, You JY, Cong LL, Ouyang PK, et al. Fermentation characteristics of Mortierella alpine in response to different nitrogen sources. Appl Biochem Biotechnol 2011; 164: 595–611.

36. Laukevics JJ, Apsite AF, Viesturs US, Tengerdy RP. Steric hindrance of growth of filamentous fungi in solid substrate fermentation of wheat straw. Biotechnol Bioeng 1985; 27: 1687–91.

37. Feniksova RV, Tikkimirova AS, Rakheeva EE. Conditions for fermentation of selected species of microalgae with emphasis on lipids. J Biochem 1995; 30: 305–9.

38. Ben-Amotz A, Tornabene TG, Thomas WH. Chemical profile of selected species of microalgae with emphasis on lipids. J Phycol 1985; 21: 72–81.

39. Devyani S, Rupali M, Ramchandra G, Sanjay N, Harisukkar AM. Production of polyunsaturated fatty acids in recombinant Lipomyces starkeyi through submerged fermentation. Bioprocess Biosyst Eng 2015; 38: 1407–14.

40. Sajbidor J, Dobronova S, Certik M. Arachidonic acid production by Mortierella sp. S-17: influence of C/N ratio. Biotechnol Lett 1990; 12: 455–6.

41. Jang HD, Lin YY, Yang SS. Effect of culture media and conditions on polyunsaturated fatty acid production by Mortierella alpina. Bioresour Technol 2005; 96: 1633–44.

42. Zandrazil F, Brunert H. Investigation of physical parameters important for solid-state fermentation of straw by white rot fungi. Eur J Appl Microbiol Biotechnol 1981; 11: 183–8.

43. Lekha PK, Lonsane BK. Comparative titres, location and properties of tannin acyl hydrolase produced by Aspergillus niger PKL104 in solid state, liquid surface and submerged fermentation. Process Biochem 1994; 29: 497–503.

44. Jang HD, Lin YY, Yang SS. Polyunsaturated fatty acid production with Mortierella alpina by solid substrate fermentation. Bot Bull Acad Sin 2000; 41: 41–8.

45. Joint WHO/FAO expert consultation. In: World Health Organization, ed. Diet, nutrition and the prevention of chronic diseases. Geneva, Switzerland: World Health Organization; 2003, p. 89.

46. Institute of Medicine. In: Institute of Medicine, ed. Food and Nutrition Board. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. Washington, DC: National Academies Press; 2002, pp. 769–70.

47. Lands WEM, Libelt B, Morris A, Kramer NC, Prewitt TE, Bowen P, et al. Maintenance of lower proportions of n-6 eicosanoid precursors in phospholipids of human plasma in response to added dietary n-3 fatty acids. Biochim Biophys Acta 1992; 1180: 147–62.

48. Ramsden CE, Faurot KR, Zamora D, Suchindran CM, Macintosh BA, Gaylord S, et al. Targeted alteration of dietary n-3 and n-6 fatty acids for the treatment of chronic headaches: a randomized trial. Pain 2013; 154: 2441–51.

49. Jang HD, Lin YY, Yang SS. Effect of culture media and conditions on polyunsaturated fatty acid production by Mortierella alpine. Biotechnol Lett 1990; 12: 455–6.

50. Gorsini C, Bremme O, Loria Kohen V. Importance of a balanced omega 6/omega 3 ratio for the maintenance of health: nutritional recommendations. Nutr Hosp 2011; 26: 323–9.

51. Zhang R, Zhu Y, Zhou Q, Chen CG. The relationships between erythrocyte membrane n-6 to n-3 polyunsaturated fatty acids ratio and blood lipids and C-reactive protein in Chinese adults: an observational study. Biomed Environ Sci 2011; 24: 234–42.

52. Yamashita T, Oda E, Sano T, Yamashita T, Ijiri Y, Giddings JC, et al. Varying the ratio of dietary n-6/n-3 polyunsaturated fatty acid alters the tendency to thrombosis and progress of atherosclerosis in apoE−/−LDLRe−/− double knockout mouse. Thromb Res 2005; 116: 393–401.

53. Lucas M, Mirzaei F, O’Reilly EJ, Pan A, Willett WC, Kawachi I, et al. Dietary intake of n-3 and n-6 fatty acids and the risk of clinical depression in women: a10-y prospective follow-up study. Am J Clin Nutr 2011; 93: 1337–43.

54. Lands B, Lamoreaux E. Describing essential fatty acid balance as 3–6 differences rather than 3/6 ratios. Nutr Metab 2012; 9: 46–54.

55. Hibbeln JR, Nieminen LR, Blasbalg TL, Riggs JA, Lands WE. Healthy intakes of n-3 and n-6 fatty acids: estimations considering world wide diversity Internet. Am J Clin Nutr 2006; 83(6 Suppl): 1483S–93.

56. Kartikasari LR, Hughes RJ, Geier MS, Makrides M, Gibson RA. Dietary alpha-linolenic acid enhances omega-3 long chain polyunsaturated fatty acid levels in chicken tissues. Prostaglandins, Leukot Essent Fatty Acids 2012; 87: 103–9.