Fermentation of *Phelinus baumii* MK818502 for polysaccharides and flavonoids production using defatted silkworm pupa hydrolysates as a nitrogen source

Zong-Nan Li[1], Wen-Jing Li[1], Jin-Zheng Wang[1], Shuai You[1, 2], Jun Wang[1, 2,*], Fu-An Wu[1, 2*]

[1] School of Biotechnology, Jiangsu University of Science and Technology, 212018 Zhenjiang, China;
[2] Sericultural Research Institute, Chinese Academy of Agricultural Sciences, 212018 Zhenjiang, China.
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Presenting author email: wangjun@just.edu.cn, fuword@163.com

Abstract: The hydrolysates of defatted silkworm pupa were prepared from abandoned silkworm pupa to culture *Phelinus baumii* MK818502. The ratio of raw material dried discard pupa to hydrolysates products was 1: 0.37 and the product nitrogen content was 3.18%. The optimum culture conditions for *P. baumii* cultivation were obtained, fermentation time was 5 d. defatted silkworm pupa hydrolysates content was 32 g/L, temperature was 26 ℃, pH=7 and the revolving speed was 120 rpm. Fermentation experiment confirmed that the substrate consumption of reducing sugar accounted for 75.61% of the total reducing sugar and nitrogen consumption accounted for 35.19% of the total nitrogen content. Active metabolites production experiment showed that extracellular polysaccharides concentration was 7.71 g/L, and the extracellular flavonoid concentration was 0.4 g/L. Compared with other nitrogen sources, the low-cost hydrolysates nitrogen content was 0.044 g/L with great fermentation performance, mycelia biomass reached to 0.36 g/L and produced 0.376 g/L polysaccharides, 0.22 g/L flavonoids.

Keywords: Defatted silkworm pupa hydrolysates, nitrogen source, *P. baumii*, biomass, extracellular polysaccharides, extracellular flavonoids, intracellular polysaccharides, intracellular flavonoids, nitrogen content.

Abbreviations:
DSPH: Defatted silkworm pupa hydrolysates EP: extracellular polysaccharides EF: extracellular flavonoids IP: intracellular polysaccharides IF: intracellular flavonoids DNS: 3, 5-Dinitrosalicylic acid OD: optical density

Introduction

China is a large sericulture producer who produces a large number of silkworm pupae which were discarded from silk reeling industry every year. Silkworm pupae are rich in oil [1], protein [2] and other trace elements. The random disposal of discarded pupae will not only cause environmental pollution, but also cause waste of resources. After extracting silkworm pupa oil, the defatted silkworm pupae which is rich in high nutritional value protein is used in food [3], feed[4], medicine[2] and other industries. The defatted silkworm pupa has high protein content, its amino acids provide nitrogen source for microorganisms [5]. Other nutrients contained in it also promote the accumulation of fungi mycelia biomass [6]. Therefore, under the situation of high fermentation cost, it is economically feasible and environmentally friendly to use defatted silkworm chrysalis protein as an alternative nitrogen source to cultivate fungi with extremely high medicinal value.

Medical fungi have been widely studied in recent years, *Phellinus baumii* is a medical fungus that ranks first in the field of immunotherapy for the treatment of cancer [7]. Cultivation of the *Phellinus baumii* has great potential for disease treatment. The fermentation of *P. baumii* produced bioactive metabolites such as polysaccharides, flavonoids, triterpenoids, proteins and amino acids [8]. The content of total sugar of *P. baumii* was 56% and reducing sugar was about 10% [9]. Reasonable selection of nitrogen source would effectively promote the growth of *P. baumii* and accumulation of protein metabolites. Traditional high cost nitrogen source were insatiable in *P. baumii* culture, so one kind of substitutable low-price nitrogen soure is silkworm pupa hydrolysates. The survival of *P. baumii* and silkworm pupas are both related to mulberry tree. The mulberry tree's active ingredients which accumulated in DSPH would be affinitive and efficient absorbed.

Some recent studies utilized the alternative nitrogen source for culture organisms, the nitrogen index was showed in table 1. Rice bran [10] can be used as bacterial cellulose medium alternative nitrogen source with a maximum nitrogen index of 0.71%. Chicken feather peptone [11] can be used in the production of pigment. Seaweed *Palmaria palmata* hydrolysised with xylanase [12] could obtain essential amino acids. Anaerobically digested dairy manure [13] was selected as nitrogen source to plant corn. Fermented soybean residue could produce a solution rich in free amino nitrogen [14]. The fishmeal wastewater that rich in nitrogen [15] was used to culture the *Bacillus subtilis* A3 with production of γ-polyglutamic acid (γ-PGA). White clover [16] could be an alternative protein source for monogastrics.

Table 1 The alternative nitrogen source in recent studies, the table included materials, processing methods, products, nitrogen indexes, cultured organisms and references.

Alternative nitrogen material	Processing method	Nitrogen product	Nitrogen index (%)	Cultured organism	Reference
Rice bran	heating	crude protein	0.24 - 0.71	*Acetobacter xylinum*	[10]
Waste feathers	hydrolysis neutralization	chicken feather peptone	67.2	*M. purpureus* ATCC16365	[11]
Red seaweed	enzymatic hydrolysis	proteins	2 - 8.8	-	[12]
Anaerobically digested dairy manure	anaerobically digesteddairy	amino acid	0.25 - 0.42	*Zea mays*	[13]
Soybean residue	solid-state fermentation	amino nitrogen	18	*Aspergillus oryzae* TISTR 3087	[14]
Fishmeal wastewater	-	γ-polyglutamic acid	0.19 - 0.2	*Bacillus subtilis* A3	[15]
White clover	acid precipitation	amino acids	8.35 - 20.28	*Trifolium repens L.* and *Taconic, Denmark*	[16]
DSPH	enzymatic hydrolysis	silkworm pupa protein	3.18±0.1	*P. baumii* MK818502	This study

Material and methods

Material preparation

Silkworm pupae were acquired from Xinyuan Silk Group Co., Ltd, Nantong, Jiangsu province, China. The pupae had been collected from water-reeled silkworm and stored under -20 ℃. Oven-heating method dried the pupae at 60 ℃ for 18 h until both of surface and inside of the pupae lost moisture. Mill crushed the pupae into powder, mesh number range of the powder is between 60 and 200. The pupae powder was put in a brown reagent bottle and d with petroleum ether (boiling range 60~90 ℃), the ratio of pupae powder to petroleum ether ≈ 1:1 (V: V). Ultrasonic (Power 200 W, frequency 28 kHz) treated the mixture at 60 ℃ for 2 h and shook up every 20 minutes [17]. The mixture was filtered, dregs and petroleum ether were separated. Ether solution contained silkworm pupa oil that could produce biodiesel, rotary evaporator method obtained crude silkworm pupa oil at a temperature of 60 ℃. After evaporator, 1.5 L petroleum ether gained 150 mL pupa oil that weighted 131.60 g. Air-drying pupa dregs were collected as hydrolysis ingredient, 0.544 kg pupa dregs were got from 1.5 L pupa powder which weighted 0.83 kg. The preparation work concentrated on silkworm pupa degreasing process.

Defatted silkworm pupa hydrolysates

50 g dried silkworm pupa dregs mixed with 1 L distilled water in shake flask, autoclaving in a high-pressure steam sterilizer at 121° C for 20 min and waited it cold to room temperature. pH meter adjusted the mixture pH value to 7.0 and added dispase (enzyme activity ratio: 50 U/mg, dosage: 5% weight percent of pupa dregs). After being treated in a 150 r /min water bath shaker at 55 ° C for 4 h, centrifuged the compound at 8000 rpm for 2 min to obtain pupa hydrolysate. After lyophilized for 48 h, powdery solid defatted silkworm pupa hydrolysates (DSPH) were obtained. The processing technique was repeated for 3 times, the weights and nitrogen contents of the DSPH were recorded to measure the production quality.

Strain and culture media

The *Phelinus baumii* MK818502 was preserved by BioControl & BioRefi laboratory, Biotechno logy College, Jiangsu University of Science and Technology, China. The strain was cultivated in culture plate with enrichment potato dextrose agar (EPDA). EPDA medium composition: 200 g/L potato (boil and extract filtrate), 20 g/L dextrose, 20 g/L agar, 3 g/L KH_2PO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L vitamin B, 1 L distilled water, under natural pH. Liquid medium was used for single factor optimization experiments, the components were similar to EPDA without agar and added nitrogen source. Fermentation medium ingredients were identical to liquid medium exclude potato.

Culture conditions optimization

The *P. baumii* MK818502 single colony cultured in EPDA plates at 25 ℃ for a week until the yellow hyphae grow over the surface of the plate medium. The growth curve of the strain was obtained by weighing the collected mycelia in fermenter culture. Sterile inoculating shovel chipped 0.5 x 0.5 cm^2 EPDA cultured new-growth mycelia that placed on the edge of the single colony, the mycelia was inoculated in 200 mL shake flask which contained 100 mL sterilization liquid medium. After inoculation, 4 parts single factor experiment were made in air bath shaker for 5 days. The single factor optimizations comprised DSPH concentration, temperature, pH and revolutions per minute (rpm). The DSPH were divided into 9 concentration gradients: 4 g/L, 8 g/L, 12 g/L, 16 g/L, 20 g/L, 24 g/L, 28 g/L, 32 g/L and 36 g/L. The different mass concentration hydrolysates added in liquid medium as nitrogen source. Culture temperature was 25 ℃ and revolution value was 160 rpm. At the end of the experiment, the mycelia and ferment liquid were collected. Relevant indicators were determined to obtain the optimum nitrogen source concentration of the medium. The single factor experimental temperatures were 20 ℃, 22 ℃, 24 ℃, 26 ℃, 28 ℃ and 30 ℃. The medium used 20 g/L DSPH, other conditions were same to DSPH single factor experiment. The optimum culture temperature was determined by measuring relevant indicators. During the pH single factor experiment, pH was adjusted to 6 stages: 5, 5.5, 6, 6.5, 7 and 7.5. Single factor revolutions were set at 100 rpm, 120 rpm, 140 rpm, 160 rpm, 180 rpm and 200 rpm. Medium composition was same to temperature experiment, shaker temperature was 26 ℃. Other ascertainment factors were 20 g/L DSPH and revolution 120 rpm. Every single factor gradient did three replicates. After culturing, the culture solution was filtered through. Vacuum pump filtered the culture solution by aperture 12.5 μm filter paper to collect hyphae and liquid. The liquid was collected in 10mL centrifuge tube. The mycelia was washed by distilled water for 3 times and lyophilized for 24 h. Semi-micro balance (0.1 mg) weighted the mass of the mycelia. Hyphae was grinded into powder, 1 mg powder dissolve in 2 ml centrifuge tube with pure water. Ultrasound wave processed the mixture at 60 ℃ for 2 hours and centrifuged at 12,000 rpm for 2 min to gain the supernatant as the test solution. The test solutions and culture liquid were determined extracellular/intracellular polysaccharides (EP/IP) and extracellular/intracellular flavonoids (EF/IF). The hyphal mass and active metabolite contents of *P. baumii* were analyzed quantitatively to confirm the optimum fermentation DSPH concentration, culture temperature, rotation speed and pH.

Bioreactor fermentation of P. baumiiy using DSPH

Medium consumption study used a 3 L bioreactor and working volume was 2 L. The culture media used fermentation medium composition: 20 g/L dextrose, 20 g/L DSPH, 3 g/L KH_2PO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L vitamin B and 2 L distilled water, pH value was adjusted to 6.0 before sterilization. Inoculum was cultured in no-nitrogen ferment medium in shaker for 5 days until the mycelia pellet appeared. 0.2 L inoculum was pumped in bioreactor and recorded time as the start of fermentation. Reactor heating blanket kept temperature at 26 ℃. The medium pH value was controlled at 6.0 ± 0.1 by feeding 1 M NaOH. The speed of agitation was 120 rpm. Sampling time was every 12 h during 5 days total fermentation time. The carbon and nitrogen contents of the fermentation broth were measured, the carbon to nitrogen ratio was calculated, and the substrate consumption value was determined.

Fed batch fermentation with controlled DSPH feeding

The bioreactor ferment conditions had similarity settings to consumption experiment. Continuous supplement DSPH were fed to keep 20 g/L nitrogen source. Culture temperature, revolution, pH, inoculum and ferment time were same to the consumption study. Sampler drew nearly 10 mL fermentation liquid every 12 h then filtrated the liquid to isolate mycelia and fermentation broth. Dry mycelia biomass, extracellular polysaccharides (EP) and extracellular flavonoids (EF) were measured in each treatment and drew the fermentation generation kinetic curve.

Fermentation kinetics fitting

The fermentation kinetics fitting of *P. baumii* MK818502 included mycelia growth, substrate consumption and active products generation. Logistic equation is a typical S-shaped curve, which is suitable for fitting the growth rule of mycelia in the process of *P. baumii* batch fermentation. Substrate consumption fitting equations contained consume of reducing sugar and nitrogen. The substrate consumption contained reducing sugar and nitrogen. Substrate consumption model is a theoretical model based on material balance. The product formation includes EP and EF. Luedeking-equation could be used to describe the product synthesis and cell growth.

Nitrogen source fermentation comparison

Comparison experiment selected three kinds of common organic NITROGEN SOURCE: tryptone, soybean peptone and yeast powder, then compared the fermentation performance with DSPH. Every king

of nitrogen source was weighted 0.01g and put into 10mL centrifuge tube with 10ml pure water. Quantitative Analysis of four nitrogen contents determined the concentrations of four nitrogen source adding in the fermentation medium. Other culture conditions were same as the consumption kinetics experiments. Dynamic changes of biomass accumulation by different nitogen were identified through the fermentation process. Morphological characters of four kinds of liquid hyphae were compared by microscope (400 ×) then evaluate the economic cost of DSPH as an alternative nitrogen source.

Polysaccharides assay

The *P. baumii* mycelia secreted EP into the fermentation broth during the growth process. The polysaccharides mixed with the glucose in fermentation broth, so it is difficult to measure the extracellular polysaccharides directly. Determination method was using extracellular total sugar content minus extracellular reducing sugar content. Phenol sulfuric acid method [18] determined total sugar. 0.2 g $C_6H_{12}O_6$ added water in 1 L constant volume bottle to a volume of 1 L. 0 mL, 0.4 mL, 0.8 mL, 1.2 mL, 1.6 mL and 2 mL 0.2 g/L $C_6H_{12}O_6$ added respectively into test tube and complemented pure water to 2 mL in test tube mixed with 1 ml of 6% C_6H_5OH. 5 mL 98% H_2SO_4 was added in mixture and shook up then let stand for 30 min. After reaction, the liquid added into 96-well costar plate, microplate reader measured sample absorbance at optical density (OD) 490 nm [19]. Phenol sulfuric acid method measured the OD value. $C_6H_{12}O_6$ concentration and OD value formed total sugar standard curve and fitting equation. The total sugar contents of the samples were calculated according to the fitting equation. 20 μL fermentation broth added pure water to 2 ml (diluted 100 times), then treated the sample and tested OD value. Extracellular total sugar contents were calculated by the fitting equation. Mycelia aqueous solution total sugar contents were determined by the same method without dilution. 3, 5-Dinitrosalicylic acid (DNS) method [20] measured concentration of reducing sugar. DNS reagent: 10 ± 0.1 g 3, 5-Dinitrosalicylic acid mixed with 0.6 L pure water, added 10 g NaOH gradually into the water and stirred in a 50 ℃ water bath. 200 g seignette salt, 2 g phenol and 5 g Na_2SO_3 were added in order to the solution. After all reagents dissolved to clarify and cooled to room temperature, then pure water was added to a constant volume of 1 L [21]. The filtered liquid was stored in a brown bottle and left in the dark for 7 d, then the DNS reagent could be used. 0 mL, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL 0.2 g/L $C_6H_{12}O_6$ added pure water respectively to 0.5 mL. The diluent mixed with 0.5ml DNS. Boiling water bath treated the sample for 5 min and left the liquid cooled down to room temperature. 4mL distilled water mixed with the reaction fluid and measured the light absorption value was detected at wavelength 540 nm. Standard curve equation was determined according to glucose concentration and light absorption value. Fermentation broth was diluted 50 times to 0.5 mL and measured, reducing sugar of mycelia aqueous solution was determined without dilution. The polysaccharides content was equal to the total sugar content minus the reducing sugar content.

Flavones assay

Flavonoid color solution was constituted with 0.4 g H_3BO_3, 0.5 g $C_2H_3NaO_2$ and 50 mL 70% C_2H_6O [22]. Rutin standard curve: 20 mg of rutin standard substance was weighted in 50 ml constant volume bottle with 70% C_2H_6O constant volume, 4 mg/ mL rutin standard solution was obtained. 0-1 mL liquid was divided averagely to 11 stages, the corresponding volume of standard solutions were added to the test tube and supplemented 70% C_2H_6O to make up to 5 mL. The solution was used right after it was ready. The absorbance was measured at OD385 after adding 5 mL color solution. Fitting equation was obtained according to the rutin curve. Sample treatment: mycelia mixed with 55% C_2H_6O by a ratio of 1: 20 (g/mL), ultrasound assisted extraction treated the mixture at 70 ℃ for 1.5 h. 0.2mL sample liquid (without dilution) added 70% C_2H_6O to 5 mL and repeat the procedure.

Nitrogen determination method

Ninhydrin colorimetry method was used to determine the nitrogen contents of samples. Ninhydrin solution: 0.6 g ninhydrin (recrystallized) mixed with 15 mL n-propanol, 30 mL n-butanol, 60 mL ethylene glycol and 9 mL acetate buffer solution (pH 4.54). The solution stored in brown laboratory bottle under 4 ℃ and the shelf life is 10 days [23]. Glycine standard curve: 0.1 g dry glycine added pure water to a constent volume of 1 L. 0.2 mL, 0.4 mL, 0.6 mL 0.8 mL, 1 mL, 1.2 mL, 1.4 mL, 1.6 mL, 1.8 mL and 2 mL glycine liquid added pure water to 2 mL. The mixture added 0.1 mL 1% ascorbic acid and 1 mL ninhydrin solution was treated by 15 min boiling water bath and cooled to room temperature [24]. 5 ml of 40% ethanol was added in the liquid and mixed well. The light absorption value was detected at wavelength 570 nm. The standard curve and fitting equation were determined according to the absorption value and sample concentration. The contents of nitrogen were measured and calculated after the fermentation broth was diluted 100 times, and the contents of nitrogen were determined directly by hyphae aqueous solution.

Results and discussion

Extraction rate and properties of DSPH

Three batches of dried silkworm pupa powder were used to prepare DSPH, the productivity was 54.67%, 52.41% and 53.96%. The nitrogen contents of the three batches of hydrolysate were 3.11%, 3.19% and 3.25%. The average production of 1 kg dried silkworm chrysalis was 0.37 kg hydrolysate with 3.18%nitrogen content. The extracted concentrated hydrolysate of defatted silkworm chrysalis was viscous liquid, and the freeze-dried hydrolysate of defatted silkworm chrysalis was crispy brown solid (Figure1), which could be used after being mashed.

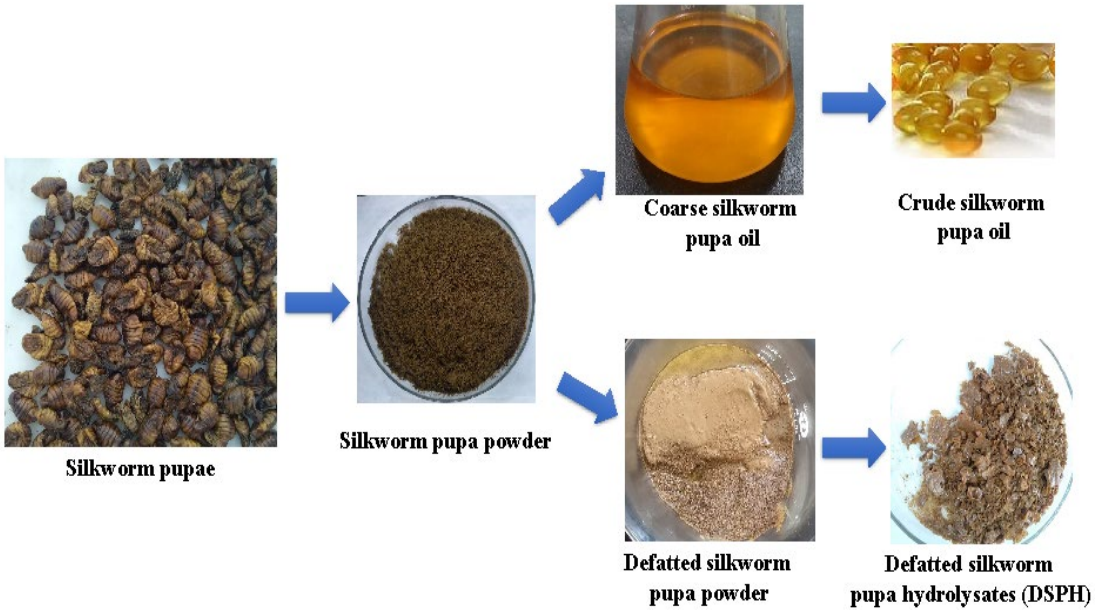

Fig. 1. Defatting and hydrolysis progress of silkworm pupae. The abandoned silkworm was shattered into silkworm pupae powder. Coarse silkworm oil and defatted silkworm pupa powder were obtained by petroleum ether extraction. The oil could make capsules. DSPH was obtained after enzyme treatment and freeze-dried for 48 hours.

The growth curve of P. baumii MK818502

The strain MK818502 used in the experiment was identified as belonging to *P. baumii*. The growth curve in Fig. 2 was determined by dynamic culture of 50ml liquid in bioreactor. The biomass increased rapidly to 0.138 g/L during 12 -48 h after fermentation, and slowly to 0.18 g/L during 48-108 h fermentation. After 120 h of fermentation, mycelia biomass accumulated to 0.26 g/L, and mycelia biomass remained basically unchanged from 120 h to 144 h and began to decline after cultured for 144 h. The mycelia mass decreased to 0.174 g/L in 156-168 h cultivation. The experimental results showed that at the beginning of fermentation, the fermentation broth had sufficient nutrients, and the mycelia grew rapidly. With the stable growth of the mycelia, it reached to maximum value in the logarithmic phase for a period of time. Nutrient consumption and metabolic waste increased, the biomass gradually decreased. According to variation tendency of the growth curve, the culture time was selected for 120 h.

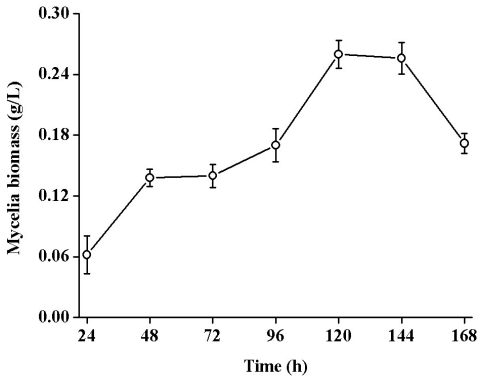

Fig. 2. Growth curve of the *P. baumii* MK818502. The biomass of the mycelia changed with culture time.

DSPH concentration

DSPH with 4-36 g/L were selected to cultivate *P. baumii* MK818502 for five days. Mycelia mass change was not dramatically (Fig. 3a), the biggest mycelia content was 0.392 g/L with 20 g/L DSPH added. During the fermentation process, the maximum EP content was 1.012 g/L when DSPH was 32 g/L. Following DSPH concentration increased, the contents of EF increased either (Fig. 3c). When 32 g/L DSPH was added into medium, the maximum EF content was 0.386 g/L. The EP was 0.856 g/L and EF was 0.262 g/L in 20 g/L DSPH substrate. With 36 g/L DSPH added, the extracellular active products began to decline (Fig. 3b and 3c), possibly because the growth of mycelia was inhibited by the high concentration of nitrogen in culture medium, and the secreted extracellular active products also decreased [25]. The contents of intracellular active products were under 0.03 g/L. Both of the IP and IF had the largest content under 20 g/L DSPH, the maximum IP was 0.029 g/L and the maximum IF was 0.026 g/L. The percentages of EP in total polysaccharides contents were from 96.7% to 99.7%, and the percentage of EF in total flavonoids were from 91% to 98.9%. Based on the changing of extracellular and intracellular products, the concentration of DSPH was determined to be 32 g/L.

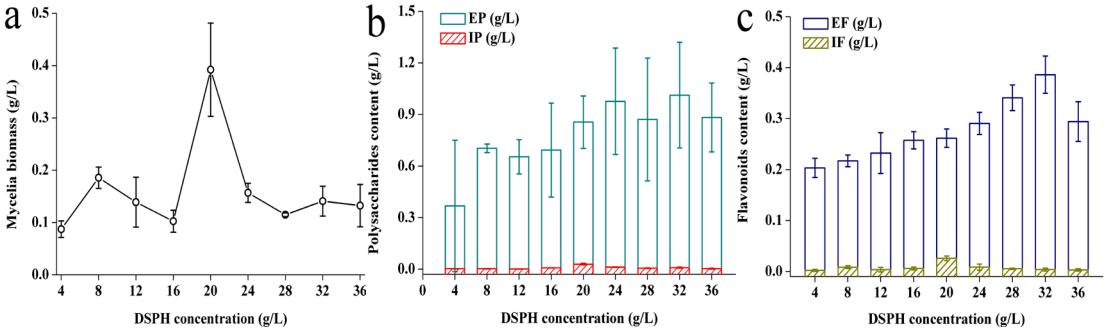

Fig. 3. Changes of related metabolites in DSPH single factor experiment. With different medium DSPH concentration, mycelia biomass (a) was determined. EP and IP contents were shown in Fig. b. Fig. c showed EF and IF contents (c). The mycelia cultured at 25 ℃, under pH 6.5, and the revolution speed of the shaker was 160 rpm.

Temperature

The *P. baumii* is a kind of shade-loving fungus whose growth is significantly affected by temperature. Single factor experimental results were showed in Fig. 4. When the temperature was 20 ℃ to 26 ℃, the mycelial biomass of *P. baumii* MK818502 kept rising and reached to the maximum value 0.826 g/L at 26 ℃ (Fig. 4a). The mycelia biomass decreased gradually from 28 ℃ to 30 ℃ cultivation environment, the biomass fell to minimum value 0.147 g/L at 30 ℃. The polysaccharides contents were determined in Fig. 4b. The highest EP content was 7.607 g/L at 26 ℃ while the lowest EP content was 0.943 g/L under 24 ℃ culture condition. IP contents increased continuously from 20 ℃ to 26 ℃ and reached to maximum value 0.007 g/L at 26 ℃. Between 28 ℃ to 30 ℃ culture temperature, the IP contents declined to the minimum value 0.001 g/L, maybe the increasing temperature restrained mycelial growth and produced lower polysaccharides [26]. The EF contents were between 0.228 g/L to 0.295 g/L (Fig. 4c), the maximum EF concentration was under 26 ℃. The IF contents from 20 ℃ to 26 ℃ were on rise and reached to maximum value 0.030 g/L. The IF content decreased to 0 g/L at 30 ℃ fermentation temperature. It was speculated that the high culture temperature might inhibit the synthesis of flavonoids from *P. baumii*. EF percentage was from 90.73% to 100%, and the percentage reached to highest value at 26 ℃. EP contents of total polysaccharides were from 99.74% to 99.97 % and IP under 26 ℃ cultivation was 0.1%. Above the experimental results, the culture temperature was selected at 26 ℃.

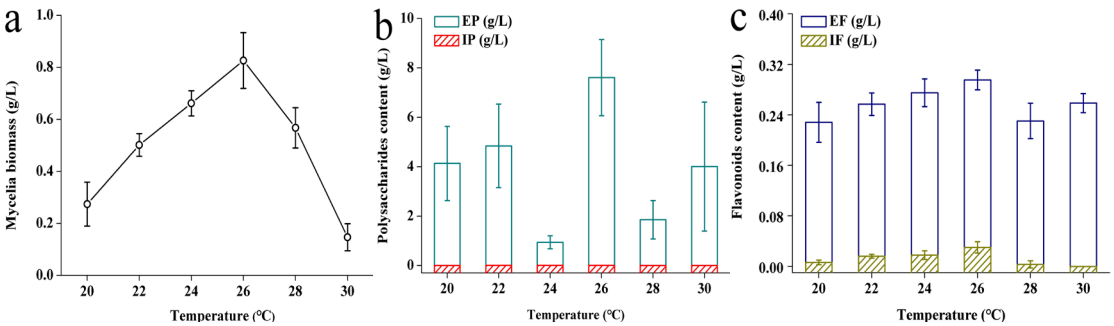

Fig. 4. Changes of related metabolites in temperature single factor experiment. Mycelia biomass (a), the contents of EP, IP (b) and EF, IF (c) were tested at different temperature. 32g/L DSPH was added into the medium. The cultivation temperature was 25 ℃ and shaker revolution speed was 160 rpm.

pH

As a saprophytic fungus, *P. baumii* generally likes to grow in an acidic environment [27], and the pH single-factor experiment determined the suitable pH for the growth of the strain (Fig. 5). Mycelia biomass increased from pH 5.0 to 5.5 and reached to the maximum value 0.237 g/L (Fig. 5a). Then the biomass decreased gradually with the increasing pH value, and reached to the minimum value 0.125 g/L at pH 7.5, when pH=6.5, the biomass was 0.175 g/L. The EP contents between pH 5.0 to 5.5 were from 2.012 g/L to 3.588 g/L. The highest EP content was 5.956 g/L when the medium pH was 7.0. From pH 5 to 6, the concentration of IP increased continuously to the maximum value 0.007 g/L. Then the IP content decreased to the minimum value 0.004 g/L at pH 7.5. The EF contents between pH 5.0 to 6.0 declined to 0.12 g/L, and then reached to the maximum content 0.48 g/L at pH 7.5 (Fig. 5b). The maximum IF content was 0.03 g/L at pH 5.5 and reached to minimum value 1 mg/L at pH 7.5. The reason for such experimental results may be that with the increasing of pH value, mycelia autolysis increased extracellular active products, mycelia biomass and intracellular active products were reduced. The percentage of EP in total polysaccharides ranged from 99.72% to 99.93%, with the largest proportion at pH 6.5. The mass percentage of EF in total flavonoids was from 84.68% to 99.76%, the percentage was largest at pH 6.5. Thus, the suitable pH was pH 7.

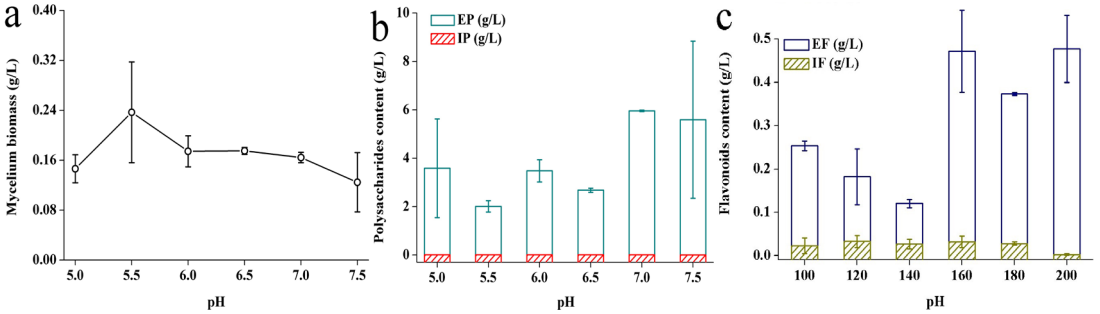

Fig. 5. Changes of related metabolites in pH single factor experiment. The medium was added 32 g/L DSPH at pH 6.5, and the shaker temperature was 26 ℃ with 160 rpm. Mycelia mass (a), EP and EF contents (b), IP and IF contents (c) were measured at different pH ferment condition.

Revolutions per minute

The conditions of dissolved oxygen were limited and the single factor of rotation speed mainly affected the biomass accumulation and the changing of active metabolites of mycelia (Fig. 6). Mycelia biomass presented a slow decline trend with the increasing of rotation speed (Fig. 6a); the maximum mycelia biomass was 0.238 g/L at 100 rpm and the minimum mycelia biomass at 180 rpm was 0.133 g/L. Revolution speed from 100 to 120 rpm, the EP contents rose to the maximum 7.278 g/L (Fig. 6b). From 140 to 180 rpm, the EP content declined to minimum 1.123 g/L at 180 rpm. The highest IP content was 0.012 g/L at 100 rpm, and the lowest IP concentration was 0.6 mg/L at 200 rpm. Between 100 to 120 rpm, EF contents were high and the maximum was 0.195 g/L at 100 rpm (Fig. 6c). When the revolution increased from 160 to 200 rpm, the EF contents were low and the minimum was 0.016 g/L at 160 rpm. The highest IF content was 0.009 g/L at 120 rpm and the lowest content was 0.8 mg/L at 180 rpm. Such results may be caused by the increasing rotation speed made mycelia wrap to form a tight mycelia pellet, then extracellular secretions of polysaccharides and flavonoids were reduced. Then the excessive rotation speed injured mycelia and decreased the polysaccharides release. The percentage of EP mass in total polysaccharides was from 99.16% to 99.98%. The proportion of EF in total flavonoids was from 69.76% to 99.28%. Based on the experiment results, the compatible culture speed was determined to be 120 rpm.

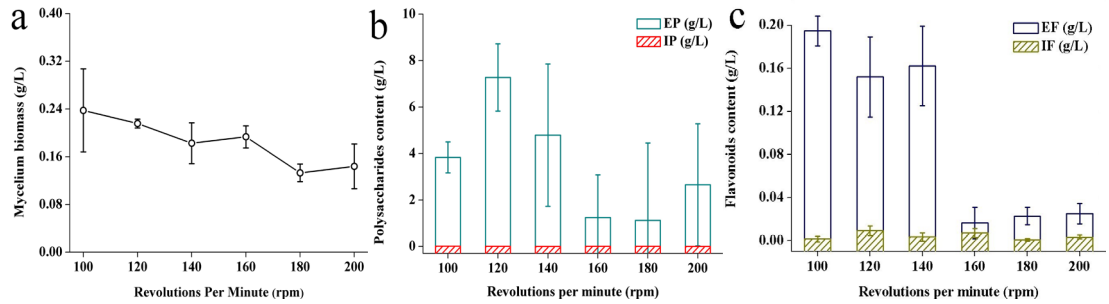

Fig. 6. Changes of related metabolites in RPM single factor experiment. Mycelia biomass (a), EP, EF (b), IP, IF (c) contents were tested at different rpm value. The DSPH content in medium was 32 g/L; the cultivation temperature was 26 ℃ and the culture medium pH was 7.

Substrate consumption and active product generation

2 L bioreactor added nitrogen source DSPH 20 g/L, under the condition of the culture temperature was 26 ℃, pH=6.5, rotating speed 120 RPM for culture, the reducing sugar and nitrogen consumption were determined. In the process of culture, the consumption of reducing sugar was slower at the beginning and accelerated after 72 h (Fig. 7c). The reducing sugar content in starter was 19.68 g/L and reached to 15.31 g/L after fermented for 72 hours that accounting for 22.21% of the total reducing sugar content. The reducing sugar consumption speed increased from 72 h to 120 h, and the reducing sugar contents decreased to the lowest value of 4.80 g/L at 120 h. The whole fermentation process consumed 29.76 g of reducing sugar which accounting for 75.61% of the total reducing sugar. The total consumption of the DSPH showed a decreasing trend. The nitrogen content was 0.64g /L at the beginning of fermentation and reached to 0.55g/L at 72 h. From 0 to 72 h, and 0.17 g NS was consumed which accounting for 26.51% of the total nitrogen content. The rate of nitrogen consumption was accelerated from 72 to 120 h, nitrogen content in 120 h was 0.41 g/L, from 72 to 120 h nitrogen consumption accounted for 21.93% of the total nitrogen content. The nitrogen consumption in the whole fermentation process was 0.45g, accounting for 35.19% of the total nitrogen content. The fermenter was used to test the contents of EP [28] and EF by continuous feeding [29]. At the starting point of fermentation, the content of EP was 8 mg/L. During 0 to 48 h of fermentation time, the contents of EP increased slowly. At 48 h, the content of EP in the fermentation broth was 0.46 g/L. From 48 to 72 h, the accumulation rate of EP in the fermentation broth was accelerated [30], the EP was 1.25 g/L at 72 h. From 72 to 120 h, the EP increased rapidly, the EP content was 7.71 g/L at 120 h. The mass of total EP was 15.4 g. The contents of EF increased slowly from 0 to 72 h, and EF accumulation increased rapidly from 72 to 120 h. At the beginning of fermentation, the EF content was 0.013 g/L, and the EF content accumulated to 0.07g/L at 72 h which accounted for 13.78% of the total flavonoids content. The content of EF reached a maximum of 0.40 g/L after 120 h fermentation, and the flavonoid output during the whole fermentation process was 0.78g.

Dynamic fitting

Logistic equation [31] is often used to describe the growth of fungi, which can be expressed as formula 1. When t=0, $C_x=C_{x,0}$, formula 1 can be integrated into formula 2 or 3. Meaning of the symbol in equations were C_x: mycelia biomass, $C_{x,max}$: maximum mycelial biomass, $C_{x,0}$: initial mycelia biomass, Volume biomass, μ_m: maximum specific growth rate of bacteria, t: fermentation time. μ_m was calculated as the slope of the curve(y=-3.2696+0.0393x) with t as the X-axis and $In(\frac{C_x}{C_{x,max}-C_x})$ as the Y-axis. $C_{x,max}$ was 0.6 g/L, $C_{x,0}$=0.0220 g/L. By substituting these data into equation 3, the growth kinetics model of mycelia (formula 4, R^2=0.9568) was obtained. The biomass of mycelia simulated by this model and the experimental value were combined well at 0-108 h after fermentation. There is a big error at 120 h, mainly because the influencing factors of this stage are relatively complex. The average error of the mycelia growth model was 4.3%, indicating that the selected model could better reflect the mycelia growth in the process of *P. baumii* batch fermentation.

The production of extracellular products from *P. baumii* belongs to the semi-growth coupling type, and luedeking-piret equation (formula 5) can be used to describe the relationship between cell growth and products synthesis. The products were EP and EF. EP selected as an example, equation 1 substituted into equation 5, after integration formula 6 was obtained. In formula 6, C_p was the concentration of EP and $C_{p,0}$ was initial concentration of EP, α and β were model parameters. During steady st ate, $\frac{dC_x}{dt}=0$, $C_x=C_{max}$, it can be obtained from formula 5 that $\beta=\left(\frac{dC_p}{dt}\right)_{st}/C_{x,max}$, β=0.3643 by calculation. Formula 6 could be written as formula 7, $A(t)=C_x(t)-C_{x,0}$, $B(t)=\left(\frac{C_{x,max}}{\mu_m}\right)In\left(\frac{C_{x,max}-C_{x,0}+C_{x,0}e^{\mu_m t}}{C_{x,max}}\right)$. μ_m=0.0393 h^{-1}, $C_{x,max}$=0.6 g/L, $C_{x,0}$=0.0220 g/L, β=0.3643. The plot was drawn as A (t) was the X-axis and $[C_p(t)-C_{p,0}-\beta B(t)]$ was the Y-axis, the fitting curve was y=1.5749-54.1012x (R^2=0.9965) and the slope α=-54.1012 was obtained. The kinetic model of EP growth (formula 8, R^2=0.9272) was obtained by substituting relevant data into formula 6. The kinetic equation of EF formation (formula 9, R^2=0.9521) was fitted similarly. Formula 8 and 9 fitted the experimental values well, indicating that the consumption kinetic model can well describe the situation of EP and EF synthesis of *P. baumii*.

Substrate consumption model is a theoretical model based on material balance, the substrate consumption rate can be expressed by formula 10. The experiment mainly studied the consumption model of reducing sugar (as an example) and nitrogen. $Y_{x/s}$ was the mycelia biomass accumulated by consuming 1 g sugar, $Y_{p/s}$ was the amount of EP obtained by consuming 1g reducing sugar. K_e represented the cell maintenance coefficient and reducing sugar concentration (g/L) marked as C_s. Equation 5 was substituted into equation 10, then equation 11 and simplified equation 12 were obtained.

In equation 12, $b_1 = \frac{\beta}{Y_{p/s}} + K_e$, $b_2 = \frac{1}{Y_{x/s}} + \frac{\alpha}{Y_{p/s}}$, when t=0, C_s=$C_{s,0}$. If mycelia growth was stable, $\frac{dC_x}{dt} = 0$, C_x=C_{max}, formula 12 caused $b_1 = \left(\frac{dC_s}{dt}\right)_{st} / C_{x,max}$, the value of b_1 was calculated to 0.5195. Integral formula 12 combined with formula 1 and got formula 13 which could be written as $C_s(t)$=$C_{s,0}$-$b_2A(t)$-$b_1B(t)$; $A(t)$=$C_x(t)$-$C_{x,0}$, $B(t)$= $\left(\frac{C_{x,max}}{\mu_m}\right) In\left(\frac{C_{x,max}-C_{x,0}+C_{x,0}e^{\mu_m t}}{C_{x,max}}\right)$. The curve was fitted with A (t) as the X-axis and [$C_{s,0}$-C_s (t)-b_1 B (t)] as the Y-axis, formula was obtained as y=-0.35+4.8169x (R^2=0.9715), b_2 equaled to the slope value 4.8169. The kinetic model of fermentation sugar consumption (formula 14, R^2=0.9418) was gained by substituting the calculated and measured data into equation 13. Similarly, the kinetic model of nitrogen consumption (formula 15, R^2=0.8733) was fitted. The fitting curves of fermentation substrate consumption were compared with the measured value. The average relative error between the calculated value and the experimental value of the reducing sugar consumption model was 5.82%, indicating that the model could reflect the sugar consumption changing well with time in the fermentation process of *P. baumii*. Relative error of the nitrogen consumption model was 12.67%, the fitting degree was general, which may because the complex nitrogen metabolism process affected the fitting model. Logistic equation and luedeking-piret equation were used to establish the kinetic models of biomass accumulation, extracellular product synthesis and substrate consumption in *P. baumii* fermentation process. Product collection and continuous feeding time can be predicted by the models. The fitting degree was greater than 94%, which have reference significance for the actual fermentation production.

$$\frac{dC_x}{dt} = \mu_m \left(1 - \frac{C_x}{C_{x,\max}}\right) \tag{1}$$

$$\mu_m t = In\left(\frac{C_{x,\max}}{C_{x,0}} - 1\right) + In\left(\frac{C_x}{C_{x,\max} - C_x}\right) \tag{2}$$

$$C_x(t) = \frac{C_{x,\max} e^{\mu_m t} C_{x,0}}{C_{x,\max} - C_{x,0} + e^{\mu_m t} C_{x,0}} \tag{3}$$

$$C_x(t) = \frac{0.0132 e^{0.0393t}}{0.578 + 0.022 e^{0.0393t}} + 0.0085 \tag{4}$$

$$\frac{dC_p}{dt} = \alpha\left(\frac{dC_x}{dt}\right) + \beta C_x \tag{5}$$

$$C_p(t) = C_{p,0} + \alpha[C_x(t) - C_{x,0}] + \beta\left(\frac{C_{x,\max}}{\mu_m}\right) In\left(\frac{C_{x,\max} - C_{x,0} + C_{x,0} e^{\mu_m t}}{C_{x,\max}}\right) \tag{6}$$

$$C_p(t) = \alpha A(t) + \beta B(t) + C_{p,0} \tag{7}$$

$$C_p(t) = 1.1968\text{-}54.1012 C_x(t) + 5.5618 In(0.9633 + 0.0367 e^{0.0393t}) \tag{8}$$

$$C_f(t) = 0.0643\text{-}2.3380 C_x(t) + 0.2397 In(0.9633 + 0.0367 e^{0.0393t}) \tag{9}$$

$$-\frac{dC_s}{dt} = \frac{1}{Y_{x/s}}\left(\frac{dC_s}{dt}\right) + \frac{1}{Y_{p/s}}\left(\frac{dC_p}{dt}\right) + K_e C_x \tag{10}$$

$$-\frac{dC_s}{dt} = \frac{1}{Y_{x/s}}\left(\frac{\beta}{Y_{x/s}} + K_e\right) C_x + \left(\frac{1}{Y_{x/s}} + \frac{\alpha}{Y_{p/s}}\right)\left(\frac{dC_x}{dt}\right) \tag{11}$$

$$\frac{dC_s}{dt} = -b_1 C_x - b_2\left(\frac{dC_x}{dt}\right) \tag{12}$$

$$C_s(t) = C_{s,0} - b_2[C_x(t) - C_{x,0}] - b_1\left(\frac{C_{x,\max}}{\mu_m}\right) In\left(\frac{C_{x,\max} - C_{x,0} + C_{x,0} e^{\mu_m t}}{C_{x,\max}}\right) \tag{13}$$

$$C_s(t) = 19.7868 - 4.8169 C_x(t) - 7.9359 In(0.9633 + 0.0367 e^{0.0393t}) \tag{14}$$

$$C_n(t) = 0.6424 - 0.1707 C_x(t) - 0.0947 In(0.9633 + 0.0367 e^{0.393t}) \tag{15}$$

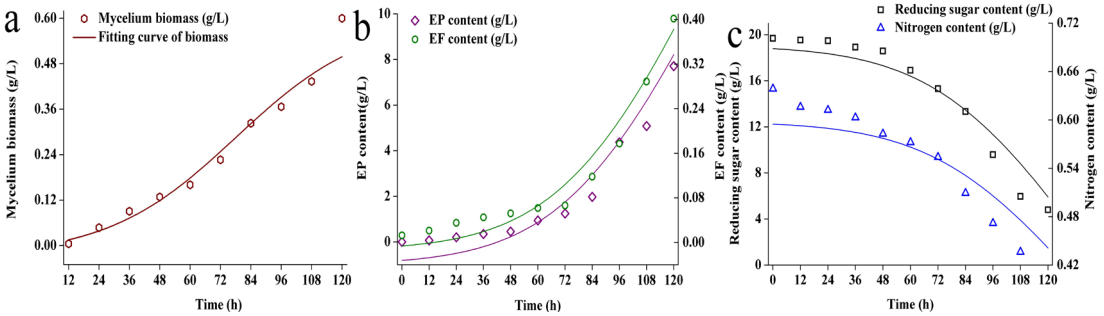

Fig. 7. During the substrate consumption experiment, 20 g/L glucose and 20 g/L DSPH were added in the medium. The contents of reducing sugars and nitrogen were determined, and the fitting curves were obtained by the logistic function in origin (a). The culture process controlled temperature at 26 ℃, rotation speed was 120 rpm and pH=6.5. The product generation experiment maintained glucose and nitrogen concentration by continuous feeding, other conditions were same. Contents of EP and EF were measured during fermentation time, and the data was analyzed to gain the fitting curves (b). Similarly, reducing sugar and nitrogen consumption model were obtained (c).

Nitrogen source contrast

The DSPH compared with three common organic sources: tryptone, soy peptone and yeast powder in bioreactor fermentation system. 1mg/mL of four nitrogen source of were configured, the nitrogen contents of the them were 0.044 g/L, 0.062 g/L, 0.156 g/L and 0.142 g/L, its nitrogen contents ratio was 1 : 1.43 : 3.58 : 3.26. The commonly used NS were 5 g/L, so 5 g/L DSPH was chosen, according to the same nitrogen content ratio, 3.49 g tryptone, 1.40 g soy peptone and 1.54 g yeast powder were added respectively into the liquid medium, and the culture was conducted in four batches using the bioreactor. The main evaluation index of NS was the accumulation of mycelia biomass that showed in Fig. 8. 50mL of medium was sampled every 24 hours and obtained freeze-dried mycelia after centrifugation, the dry weight of mycelia was weighed. At the beginning 24 h of fermentation, mycelia biomass accumulated continuously, the maximum biomass was 0.26 g/L by using DSPH as nitrogen source then increased to 0.32 g/L at 48 h. After fermented for 48 h, tryptone culture mycelia accumulated to 0.077 g/L, yeast powder cultured mycelia biomass was 0.32 g/L and soybean peptone culture mycelia was 0.15 g/L. With the increase of culture time, mycelia quality decreased to a certain extent. The mycelia mass of DSPH decreased from 0.3 g/L at 72h to 0.22 g/L at 96 h. Tryptone kept biomass 0.77 g/L at 72 h, then rose to 0.088 g/L at 96 h. Soybean peptone decreased to 0.136 g/L at 72 h and yeast cultured mycelia similarly reduced to 0.216 g/L at 72 h. In the next stage of fermentation, mycelia quality continued to increase. When the mycelia cultured by DSPH for 96-120h, mycelia biomass increased and accumulated to a maximum value of 0.36 g/L at 120 h. Mycelia fermentation with tryptone increased to 0.088 g/L at 96 h and determined the maximum biomass of 0.11 g/L at 120 h. The biomass of the mycelia that cultured by soybean peptone increased to 0.221 g/L at 96 h, and reached to the maximum 0.168 g/L at 120 h. The mycelia biomass cultivated by yeast powder reached to the maximum 0.221 g/L at 96 h and decreased to 0.211 g/L after 120 h fermentation. Thus the biomass accumulation of DPSH in all fermentation periods was better than that of other nitrogen sources. Mycelia microscopical (400times magnification, scale 20 μm) characterization showed that four NS cultured *P. baumii* MK818502 liquid mycelia were yellow and white transparent (Fig. 9).

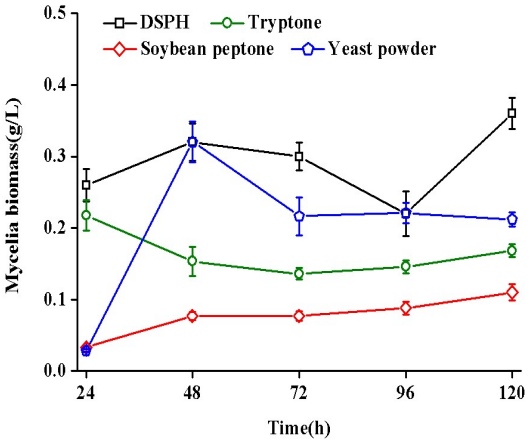

Fig. 8. The fermentation properties of four NS (5 g/L DSPH, 3.49 g/L tryptone, 1.4 g/L soybean peptone and 1.54 g/L yeast powder) which had same nitrogen contents were compared. The change curve of *P. baumii* mycelia biomass with four kinds of NS adding in medium.

Compared with the other three strains, the mycelia cultured by DSPH were longer and straighter (Fig. 9a), the mycelia grew orderly with fewer branches, and the thickness of mycelia were uniform. The mycelia in tryptone culture varied greatly in thickness (Fig. 9b), and the growth of mycelia was more scattered and branching. The mycelia in soybean peptone culture also had great difference in thickness and scattered growth, and the shape of mycelia was curved (Fig. 9c). The liquid mycelia in yeast culture was thicker and more uniform (Fig. 9d), and the state of mycelia was regular. The Based on the above results, it can be concluded that NSPH is an excellent alternative NS, which was beneficial to the growth and biomass accumulation.

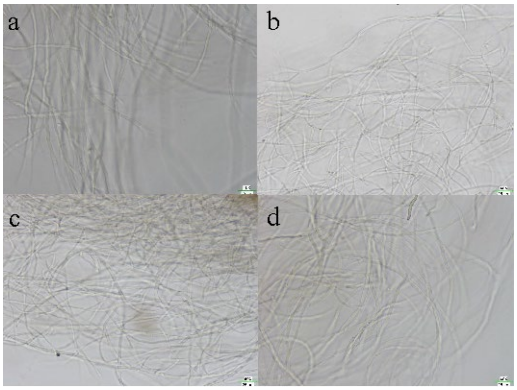

Fig. 9. After fermentation, the liquid mycelia of DSPH (a), tryptone (b), soybean peptone(c) and yeast powder (d) were collected to compare mycelia morphology qualitatively. The magnification of the microscope was 400 times.

Economic analysis for DSPH

As commonly used organic NS, 250 g price of the four NS was tryptone: 30-45 ¥, soybean peptone: 50-150 ¥ and yeast powder: 50-160 ¥. The cost of producing 0.25 kg DSPH included the price of silk reeling pupae eliminated by industrial production was 6 ¥, and the cost of 5% dispase (50g) is 18 ¥. The electricity price is 0.52 ¥/kW·h, the cost of water bath enzymatic hydrolysis was 2.1 ¥, the working cost of the lyophilization was 14.2 ¥ and the loss cost of related equipment was 1.9 ¥. Therefore, the total production cost of 250 g DSPH was about 42.2 ¥. If treatment technology of silkworm pupa is put into large-scale industrial production, DSPH production costs will be further reduced. The prices of common organic nitrogen source were compared with DSPH in Table 3. The DSPH costed half price of tryptone which had lowest price in the table. Combined with the results, DSPH prepared from silkworm pupa had a great economic value.

Table 2 The nitrogen sources economic analysis was shown. The table included organic nitrogen name, total nitrogen content, purity, specification, price, unit price and manufacturer. Unit price of this experiment was also shown.

Organic nitrogen source	Total nitrogen content (%)	Purity	Specification (g)	Price (¥)	Unit price (¥/g)	Manufacturer
Tryptone	13.5±1.5	BR	500	95	0.19	Sinopharm Chemical Reagent Co., Ltd
Soybean peptone	≥8	BR	250	91	0.36	Sinopharm Chemical Reagent Co., Ltd
Yeast extract	10.9	BR	500	100	0.2	Thermo Fisher Scientific
Corn steep liquor	4	LR	540	130	0.24	Shanghai Fangqi Metrologic Instruments
Cottonseed powder	5	FMB	1000	200	0.2	Shaanxi Chengrui Biotechnology Co., Ltd
Malt extract	0.6	BR	25	60	2.4	Thermo Fisher Scientific
Casein	15±0.5	CP	250	85	0.34	Sinopharm Chemical Reagent Co., Ltd
DSPH	3.18±0.1	FMB	500	42.2	0.08	This study

DSPH fermentation effect

To verify the effect of DSPH as an alternative nitrogen source for *Phellinus* cultivation, different NS fermentation products were compared in Table 3. All the studies in the table were cultured *Phellinus* genus fungi in different species. With 5 g/L DSPH added into liquid medium in bioreactor and cultured *P. baumii* for 5 d, the biomass was 0.36 g/L. Cultivation time was not long enough, so the mycelia biomass did not accumulate to the maximum and there are some differences between different species. However, it produced 0.376 g/L polysaccharides which was the highest content among the 6 kinds of NS;

meanwhile, the flavonoid content was 0.22 g/L. Based on the above results, it can be concluded that DSPH is a good nitrogen source to promote the production of polysaccharides from *P. baumii*.

Table 3 The fermentation effect of 6 kings of nitrogen source. The addition amount, culture time of fermentation process, mycelia biomass, EP produce, EF concentration and references were listed in the table. All the studies used *Phellinus* genus fungi and fermented in liquid medium. The DSPH in this study also listed in the bottom of the table to compare with other NS.

Nitrogen source selection	Additon (g/L)	Culture time(d)	Mycelia biomass (g/L)	Polysaccharides production (g/L)	Flavonoids Production (g/L)	Reference
Bran	5	8	6.886	0.020	0.007	[32]
Yeast extract	5	5	0.85	0.102	-	[33]
Soybean meal	5.7	7	-	0.177	0.11	[34]
Tryptone	5	7	5	-	0.33	[35]
Yeast extract	5	14	1.1	-	0.30	[36]
DSPH	5	5	0.36	0.376	0.22	This study

Conclusions

This study innovatively used DSPH as an alternative NS to improve a series of active metabolites of the medicinal fungus *P. baumii* MK818502. By single factor optimization experiment the optimal DSPH content was determined to 32 g/L. Determination of culture temperature (26 ℃), pH (7) and rotation speed (120rpm) were the conditions for the fermentation experiment. The maximum values mycelia biomass was 0.707 g/L, EP was 1.54 g/L and EF was 0.078 g/L. The kinetic models could be used for reference in the industrial production of polysaccharides flavonoids and bath feeding substrates. By contrast with other NS, the DSPH biomass accumulated to 0.36 g/L, the cost of production was 42.2 ¥/250g and produced 0.376 g/L EP which was maximum in NS comparison. In conclusion, DSPH is a great nitrogen source to *ferment P.baumii* and have broad application prospects in biology.

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