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Optimization of submerged culture for biomass and polysaccaharide of *Pleurotus ostreatus* BPPTCC 6017 using response surface methodology

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ABSTRACT

Aims: Polysaccharide of *Pleurotus ostreatus* is one of the fungal polysaccharide which has been widely studied, produced by extracting the fruiting body. An alternative method for producing polysaccharide of *P. ostreatus* directly from the mycelia instead of the fruiting body is through submerged culture. This study was aimed to determine the optimum submerged culture conditions for producing biomass and intracellular polysaccharide of the oyster mushroom.
Methodology and results: *P. ostreatus* BPPTCC 6017 was collected from traditional mushroom farm in West Java, Indonesia. Submerged fermentation was conducted in 1000 mL medium (2 L flask). Four variables were tested: temperature, pH, agitation, and fermentation time, using central composite design of the response surface methodology. Mycelial biomass produced, was extracted to obtain water-soluble and alkali-soluble polysaccharide. Experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis and also analysed by appropriate statistical methods. The 3-D response surface plots derived from the mathematical models were applied to determine the optimum conditions: temperature 27.89 °C, initial pH medium 5.49, agitation 124.08 rpm, and fermentation time 11.44 days. The predicted results of the models were 33.75 g/L mycelia, 0.33 g/L water-soluble polysaccharide, and 0.64 g/L alkali-soluble polysaccharide. Those results were then verified on the optimum conditions, and produced 32.00±1.25 g/L mycelia, 0.29±0.01 g/L water-soluble polysaccharide and 0.60±0.02 g/L alkali-soluble polysaccharide, were close to the theoretical predictions.
Conclusion, significance and impact study: The present study was a first effort to assess and obtain the optimum conditions for producing the biomass and polysaccharides of the strain *P. ostreatus* BPPTCC 6017 using submerged fermentation.

Keywords: Oyster mushroom, *Pleurotus ostreatus* BPPTCC 6017, polysaccharide, optimum condition, central composite design, submerged culture

INTRODUCTION

Mushroom polysaccharides have emerged as important bioactive substances in recent years, on account of their medicinal and therapeutic properties (Wasser, 2002; Zhang *et al*., 2007; Liu *et al*., 2009; Rop *et al*., 2009; Sun and Liu, 2009). Reshetnikov *et al*. (2001) have listed 650 species and intraspecific taxa from 182 genera of higher Hetero- and Homo-basidiomycetes which contain pharmacologically active polysaccharides which can be derived from the fruiting body, culture mycelium and culture broths. Oyster mushroom (*Pleurotus*) is a widely known edible mushroom used as a source of food and pharmaceutical products (Gregori *et al*., 2007; Novaes *et al*., 2007; Gern *et al*., 2008; Synytsya *et al*., 2009). Compounds such as lectins, polysaccharides, polysaccharide-peptides, polysaccharide-protein complexes derived from the oyster mushroom showed activity as immuno-modulators, antitumor, antiviral, anti-inflammatory, antibiotic, antimutagenic, antineoplastic, antioxidant, antilipidemic, hypoglycemic, hypotensive, hypocholesterolemic, hepatoprotective and anti-ageing (Sarangi *et al*., 2006; Gregori *et al*., 2007; Novaes *et al*., 2007; Tong *et al*., 2009; Patel *et al*., 2012). The oyster mushroom contains a polysaccharide called pleuran, which acts as a Biological Response Modifier (Karacksonyi and Kuniak, 1994; Bohn and BeMiller, 1995; Smith *et al*., 2002).

Mushroom polysaccharides are commonly extracted from the fruiting body. Traditionally, growing mushrooms on wooden logs are cultivated in a green house under controlled temperature and humidity. This method has many disadvantages, such as long term cultivation up to several months, easily contaminated by other microbes.

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As an alternative method, culture in submerged fermentation to produce mycelium has been proven as a promising way to produce mushroom polysaccharides in large quantity and relatively short time (Ishikawa *et al*., 2001; Gregori *et al*., 2007; Maftoun *et al*., 2013). Submerged fermentation for producing fungal polysaccharides, has increased in recent decades, because produced more uniform mycelia, more reproducible, compact space, less time, and lower the risk of contamination, easier scale-up and downstream process (Gregori *et al*., 2007; Pokhrel and Ohga, 2007, Gern *et al*., 2008; Refaie *et al*., 2009; Papaspyridi *et al*., 2010).

Many optimization studies have been reported to produce fungal polysaccharides. Sugihara and Humfeld (1954), Hadar and Cohen-Arazi (1986) reported a submerged culture of *Pleurotus ostreatus* mycelia to produce high biomass and the chemical constituents produced are similar to the fruiting bodies. Manu-Tawiah and Martin (1987) reported that the minerals content in the mycelium and in the fruiting bodies were comparable as the results of submerged fermentation of *P. ostreatus* concerning various carbon and nitrogen sources. Gregori *et al*. (2007) reported a liquid medium that produces the highest polysaccharide from the *Pleurotus* spp. containing the C/N ratio of 40, pH = 5.5 and incubation temperature of 25 °C. Kim *et al*. (2005) reported that the pH of the liquid medium is a critical fermentation factor, but often overlooked. Gern *et al*. (2008) reported that the maximum productivity of biomass and polysaccharide of *P. ostreatus* (POP) through the liquid fermentation obtained from the medium containing yeast extract 5 g/L and glucose 40 g/L. Wang *et al*. (2005) used a response surface methodology to determine the optimal conditions for production of water-soluble polysaccharides of the culture broth of *Pleurotus citrinopileatus*.

Response surface methodology (RSM) is a mathematical and statistical technique for designing experiments, building models, evaluating the effects of factors and searching optimum condition of factors for desirable responses. RSM is an effective tool when many factors and interactions affect desired response (Montgomery, 2001). White oyster mushroom has been widely grown by farmers in Indonesia. This mushroom was collected in BPPT Culture Collection. The optimum submerged culture conditions of *P. ostreatus* BPPTCC 6017 to produce biomass and polysaccharide has not yet available. The present study conducted to obtain the optimum conditions for producing biomass and POP in submerged culture using RSM. Employing a central composite design (CCD) to study the effects of the pH medium, agitation speed, temperature, and fermentation time on the mycelial biomass and POP.

MATERIALS AND METHODS

Microorganism

Pleurotus ostreatus BPPTCC 6017 used in this study was obtained from BPPT Culture Collection (BPPTCC, Jakarta, Indonesia). The strain was maintained on potato dextrose agar (PDA) slant. The slant was incubated at 26 °C-29 °C, for 3-5 days, then stored at 4 °C, and sub-cultured every 2 months.

Inoculum preparation and submerged culture

The inoculum was prepared by mixing 1 g of dry mycelia in 100 mL of sterile medium. The seed culture was grown in a 500 mL flask containing 100 mL of modified medium of Liu *et al*. (2010) (potato dextrose broth 20 g/L, yeast extract 3 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, and KH_2PO_4 1 g/L) at 28±2 °C, 150 rpm of agitation for 7 days. Flask culture experiments were performed in 2 L flask containing 1000 mL medium after inoculating with 100 mL seed culture. The mycelium was harvested by centrifugation at 8000 ×*g* for 15 min. After three repeated washings with distilled water, the mycelial pellets were lyophilized for later experiments (Hadar and Cohen-Arazi, 1986; Kim *et al*., 2005; Pokhrel and Ohga, 2007; Dong *et al*., 2009; Luo *et al*., 2009; Liu *et al*., 2010).

Experimental design and analysis

Response surface methodology was used to determine the influence of four independent variables and the optimum conditions of the biomass and intracellular polysaccharide of *P. ostreatus* (POP) production. The effects of the variables: fermentation temperature (X_1), initial pH medium (X_2), agitation speed (X_3), and fermentation time (X_4) on the biomass and POP production were investigated. Each variable was coded at five levels and six central points (Table 1). A central composite design (CCD) was arranged for fitting model, in total 30 experimental units, consisting of 2^4 (16) factorial experimental units, 8 units starting point, and 6 replicates center point (Table 2). The data of dry weight of biomass, water-soluble and akali-soluble POP were plotted on a second-order polynomial equation as follows:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 \quad (1)$$

Notes:

Y	:	Response
a_0	:	Constants
a_1, a_2, a_3, a_4	:	Coefficient of linear effect of each factor
a_{12},a_{13},a_{14}, a_{23}, a_{24}, a_{34}	:	Coefficient of interaction between factor
a_{11}, a_{22}, a_{33}, a_{44}	:	Coefficient of quadratic effect of each factor

The significance of each coefficient was determined using the *F*-test and *p*-value. The corresponding variables would be more significant if the absolute *F*-value becomes greater and the *p*-value becomes smaller. All analysis were aided with Stat-Ease Design Expert 7.1.5. (Stat-Ease Inc. Minneapolis, USA).

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Table 1: Independent variables and their levels used in the response surface design.

Independent variables	Range and level of variable				
	Start point (-α)	Low level (-1)	Center level (0)	High level (+1)	Start point (+α)
Temperature (°C)	24	26	28	30	32
pH	4.5	5.0	5.5	6.0	6.5
Agitation (rpm)	75	100	125	150	175
Fermentation time (days)	6	8	10	12	14

Extraction of POP

Extraction of water-soluble and alkali-soluble POP as described by Mizuno (Wasser, 2002), Yap and Ng (Smith *et al*., 2002), and Synytsya method (Synytsya *et al*., 2009) with a few modification. About 200 g of wet mycelia of *P. ostreatus* was blended. The homogenates were added with 3 times volume of distilled water and then heated to boil for 3 h. The extract was filtered to separate the filtrate and residue. The obtained filtrate was then added with 3 times the volume of 80% ethanol, placed at 4 °C for 24 h, while the residue was stored for the extraction of alkali-soluble POP. After 24 h, the filtrate was centrifuged at 4 °C, 6000 x*g* for 10 min, and the precipitate obtained lyophilized to give water-soluble POP. The residues of the water-soluble POP extraction was weighed, then 1 liter of 80% ethanol (1:10 v/v) was added, stirred for 3 h, and filtered. The obtained filtrate was discarded, and the insoluble parts collected. Then 1 N NaOH (1:10 v/v) was added, stirred for 2 h, and filtered. The obtained filtrate neutralized by adding 2 M acetic acid, was centrifuged at 6000 x*g*, 4 °C for 10 min. The precipitate washed with distilled water, ethanol, and finally with distilled water. The precipitate obtained was freeze-dried to give alkali-soluble POP.

Analysis of POP with high performance liquid chromatography (HPLC)

Polysaccharide of *P. ostreatus* (POP) was analyzed by HPLC using Shimpack Collumn SCR 101 C. Ultrapure water was used as the mobile phase with a flow rate of 1.0 mL/min., temperature 8 °C, volume of the sample 20 µL. The product was identified using a refractive index detector (RID) (Wood *et al*., 1991; Johansson, 2006). Curdlan was used as the standard.

RESULTS AND DISCUSSION

RSM model fitting

Response surface methodology is more advantageous than the single parameter optimization in that it saves time, space, and raw material. In this present study, optimization of the submerged fermentation conditions comprised four factors: temperature, pH, agitation, and fermentation time. RSM was employed to investigate the effect and interaction of the individual factors on the biomass and intracellular polysaccharides of *P. ostreatus* (POP) yields. All 30 of the designed experiments were conducted for optimizing the four variables in CCD. Table 2 shows the experimental conditions and the results of biomass, water- and alkali-soluble POP yields according to the factorial design.

Multiple regression analysis was used to analyze the data obtained and a second-order polynomial equation was derived from regression analysis as follows:

Mycelial biomass response :

$$Y= 32{,}65 + 0{,}53X_1 + 0{,}32X_2 - 0{,}32X_3 + 2{,}95X_4 + 0{,}49\ X_1X_2 - 6{,}25 \times 10^{-4}\ X_1X_3 - 0{,}96\ X_1X_4 + 0{,}94\ X_2X_3 - 0{,}35\ X_2X_4 + 0{,}24\ X_3X_4 - 3{,}13\ X_1^2 - 1{,}68\ X_2^2 - 2{,}69\ X_3^2 - 1{,}98\ X_4^2 \quad (2)$$

Water-soluble POP response :

$$Y= 0{,}32 + 4{,}58 \times 10^{-3}X_1 + 2{,}08 \times 10^{-3}X_2 - 5{,}42 \times 10^{-3}X_3 + 0{,}028X_4 + 3{,}13 \times 10^{-3}X_1X_2 - 1{,}88 \times 10^{-3}X_1X_3 - 9{,}38 \times 10^{-3}X_1X_4 + 5{,}63 \times 10^{-3}X_2X_3 - 4{,}38 \times 10^{-3}X_2X_4 + 6{,}25 \times 10^{-4}X_3X_4 - 0{,}03X_1^2 - 0{,}016X_2^2 - 0{,}027X_3^2 - 0{,}02X_4^2 \quad (3)$$

Alkali-soluble POP response:

$$Y= 0{,}63 + 5{,}42 \times 10^{-3}X_1 + 0{,}012X_2 - 2{,}92 \times 10^{-3}X_3 + 0{,}05X_4 + 8{,}13 \times 10^{-3}\ X_1X_2 + 1{,}87 \times 10^{-3}X_1X_3 - 0{,}024X_1X_4 + 3{,}13 \times 10^{-3}\ X_2X_3 - 0{,}016X_2X_4 + 5{,}63 \times 10^{-3}X_3X_4 - 0{,}06X_1^2 - 0{,}03X_2^2 - 0{,}05X_3^2 - 0{,}037X_4^2 \quad (4)$$

In the equations, Y represents biomass and POP yields (g/L); X_1, X_2, X_3, and X_4 represent the independent variable of temperature, pH, agitation, and fermentation time, respectively.

Analysis of variance (ANOVA) was used to find out the adequacy of the model (Table 3). The model F-values of 13.12, 13.51, and 16.39 for biomass, water-soluble POP, and alkali-soluble POP respectively (Table 3) implied that the models were significant at a high confidence level. The model *p*-value of < 0.0001 also showed that there was only a 0.01% chance that the model *F*-value could occur due to noise. The fitness of the model was examined by determination coefficient, gave the R^2 = 0.925 for biomass, R^2 = 0.927 for water-soluble POP, and R^2 = 0.939 for alkali-soluble POP. The R^2 values indicated that the sample variation of more than 92.5%, 92.7%, and 93.9% were attributed to the

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Table 2: CCD matrix of the four variables in coded and uncoded units for optimum conditions of mycelial biomass, water-soluble and alkali-soluble POP yields.

No	Experimental Design	Coded Variable				Uncoded Variable				Response Yield (dry weight)		
		X_1	X_2	X_3	X_4	T (°C)	pH	Agit. (rpm)	Time (day)	Mycelial Biomass (g/L)	Water-soluble POP (g/L)	Alkali-soluble POP (g/L)
1	Factorial (2^n)	-1	-1	-1	-1	26	5	100	8	19.57	0.19	0.38
2		1	-1	-1	-1	30	5	100	8	24.05	0.24	0.40
3		-1	1	-1	-1	26	6	100	8	18.08	0.18	0.36
4		1	1	-1	-1	30	6	100	8	21.67	0.22	0.50
5		-1	-1	1	-1	26	5	150	8	19.12	0.19	0.38
6		1	-1	1	-1	30	5	150	8	16.25	0.16	0.35
7		-1	1	1	-1	26	6	150	8	18.67	0.19	0.40
8		1	1	1	-1	30	6	150	8	26.25	0.24	0.48
9		-1	-1	-1	1	26	5	100	12	27.00	0.27	0.50
10		1	-1	-1	1	30	5	100	12	25.57	0.25	0.46
11		-1	1	-1	1	26	6	100	12	26.64	0.27	0.54
12		1	1	-1	1	30	6	100	12	25.12	0.25	0.44
13		-1	-1	1	1	26	5	150	12	25.57	0.25	0.52
14		1	-1	1	1	30	5	150	12	26.56	0.26	0.52
15		-1	1	1	1	26	6	150	12	26.78	0.25	0.52
16		1	1	1	1	30	6	150	12	26.19	0.24	0.48
17	*Starting point* (2 x n)	-α	0	0	0	24	5.5	125	10	19.21	0.18	0.35
18		α	0	0	0	32	5.5	125	10	20.49	0.20	0.40
19		0	- α	0	0	28	4.5	125	10	25.20	0.25	0.49
20		0	α	0	0	28	6.5	125	10	26.14	0.26	0.53
21		0	0	- α	0	28	5.5	75	10	22.98	0.22	0.45
22		0	0	α	0	28	5.5	175	10	20.27	0.20	0.40
23		0	0	0	- α	28	5.5	125	6	18.20	0.18	0.36
24		0	0	0	α	28	5.5	125	14	30.74	0.30	0.59
25	*Center point* (2 + n)	0	0	0	0	28	5.5	125	10	35.45	0.35	0.66
26		0	0	0	0	28	5.5	125	10	34.44	0.34	0.63
27		0	0	0	0	28	5.5	125	10	32.21	0.32	0.64
28		0	0	0	0	28	5.5	125	10	30.76	0.30	0.62
29		0	0	0	0	28	5.5	125	10	32.74	0.32	0.61
30		0	0	0	0	28	5.5	125	10	30.29	0.30	0.60

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Table 3: Analysis of variance of response surface quadratic model for the mycelial biomass and polysaccharides of *P. ostreatus* (POP).

Source	DF	Mycelial biomass				Water-soluble POP				Alkali-soluble POP			
		SS	MS	*F*-value	*p*-value	SS	MS	*F*-value	*p*-value	SS	MS	*F*-value	*p*-value
Model	14	538.86	38.49	13.12	< 0.0001	$7.09x10^{-2}$	$3.30x10^{-3}$	13.51	< 0.0001	$2.49x10^{-1}$	$1.78x10^{-2}$	16.39	< 0.0001
X_1	1	22.20	22.20	1.72	0.2098	$5.04x10^{-4}$	$5.04x10^{-4}$	1.34	0.2644	$7.04x10^{-4}$	$7.04x10^{-4}$	0.65	0.4333
X_2	1	12.43	12.43	0.60	0.4489	$1.04x10^{-4}$	$1.04x10^{-4}$	0.28	0.6059	$3.50x10^{-3}$	$3.50x10^{-3}$	3.23	0.0926
X_3	1	3.09	3.09	0.63	0.4408	$7.04x10^{-4}$	$7.04x10^{-4}$	1.88	0.1907	$2.04x10^{-4}$	$2.04x10^{-4}$	0.19	0.6708
X_4	1	219.24	219.24	52.68	< 0.0001	$1.87x10^{-2}$	$1.87x10^{-2}$	49.88	< 0.0001	$5.90x10^{-2}$	$5.90x10^{-2}$	54.33	< 0.0001
$X_1 X_2$	1	0.37	0.37	0.98	0.3379	$1.56x10^{-4}$	$1.56x10^{-4}$	0.42	0.5284	$1.06x10^{-3}$	$1.06x10^{-3}$	0.97	0.3397
$X_1 X_3$	1	0.0065	0.0065	0.00	0.9990	$5.63x10^{-5}$	$5.63x10^{-5}$	0.15	0.7040	$5.63x10^{-5}$	$5.63x10^{-5}$	0.05	0.8230
$X_1 X_4$	1	11.67	11.67	3.70	0.0736	$1.41x10^{-3}$	$1.41x10^{-3}$	3.75	0.0719	$9.51x10^{-3}$	$9.51x10^{-3}$	8.75	0.0098
$X_2 X_3$	1	10.92	10.92	3.58	0.0781	$5.06x10^{-4}$	$5.06x10^{-4}$	1.35	0.2634	$1.56x10^{-4}$	$1.56x10^{-4}$	0.14	0.7098
$X_2 X_4$	1	1.34	1.34	0.50	0.4893	$3.06x10^{-4}$	$3.06x10^{-4}$	0.82	0.3804	$3.91x10^{-3}$	$3.91x10^{-3}$	3.60	0.0773
$X_3 X_4$	1	0.77	0.77	0.23	0.6360	$6.25x10^{-6}$	$6.25x10^{-6}$	0.02	0.8990	$5.06x10^{-4}$	$5.06x10^{-4}$	0.47	0.5052
X_1^2	1	148.93	148.93	67.71	< 0.0001	$2.84x10^{-2}$	$2.84x10^{-2}$	75.78	< 0.0001	$1.05x10^{-1}$	$1.05x10^{-1}$	97.01	< 0.0001
X_2^2	1	3.87	3.87	19.40	0.0005	$6.97x10^{-3}$	$6.97x10^{-3}$	18.58	0.0006	$2.19x10^{-2}$	$2.19x10^{-2}$	20.12	0.0004
X_3^2	1	73.40	73.40	49.87	< 0.0001	$2.03x10^{-2}$	$2.03x10^{-2}$	54.06	< 0.0001	$6.72x10^{-2}$	$6.72x10^{-2}$	61.83	< 0.0001
X_4^2	1	101.64	101.64	26.96	0.0001	$1.06x10^{-2}$	$1.06x10^{-2}$	28.35	< 0.0001	$3.75x10^{-2}$	$3.75x10^{-2}$	34.53	< 0.0001
Residual	15	164.20	10.95			$5.63x10^{-3}$	$5.04x10^{-4}$			$1.63x10^{-2}$	$1.09x10^{-3}$		
Lack of Fit	10	163.46	16.35	0.96	0.5549	$3.54x10^{-3}$	$3.54x10^{-4}$	0.85	0.6148	$1.40x10^{-2}$	$1.40x10^{-3}$	2.99	0.1191
Pure error	5	0.73	0.15			$2.08x10^{-3}$	$1.26x10^{-5}$			$2.33x10^{-3}$	$4.67x10^{-4}$		
Cor Total	29	703.05				$7.65x10^{-2}$				$2.66x10^{-1}$			
		R^2 =	0.925			R^2 =	0.927			R^2 =	0.939		
		R^2 adj. =	0.854			R^2 adj. =	0.858			R^2 adj. =	0.881		
		α =	5%			α =	5%			α =	5%		

DF, degree of freedom; SS, sum of square; MS, mean square

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variables. It also indicated a high correlation between the experimentally observed and predicted values.

The lack of fit of *p*-value for biomass, water-soluble POP, and alkali-soluble POP were found to be 0.55, 0.61, and 0.32, respectively, which implied that the lack of fit were insignificant relative to the pure error due to noise. The insignificant lack of fit made the model fit. The results suggested that the proposed experimental design was suitable for optimization of biomass, water-soluble POP, and alkali-soluble POP within the range of variables employed.

The interactions and optimum levels of the variables were determined by plotting the response surface curves. These response surface curves were the representation of the regression equations 2 – 4. The three dimension response surface plots provided a visual intepretation of the interaction between two factors and facilitated the location of optimum experimental conditions. There were six response surface plots generated from four variables for each response yield (biomass, water-soluble POP, and alkali-soluble POP). Figures 1, 2, and 3 represent the effect of two variables and their reciprocal interaction on the yields of biomass, of the water-soluble POP, and of alkali-soluble POP at the constant another two variables. Figures 1A, 2A, and 3A represent the effects of fermentation temperature, initial pH medium, and their reciprocal interaction on the yields of biomass, water-soluble POP, and alkali-soluble POP respectively, at a constant agitation speed of 125 rpm and fermentation time of 10 days. At the designed range of initial pH medium from 5.0 to 6.0, the yields increased with the increasing of temperature, and reached the peak value at 27.82 °C, then decreased from 27.82 °C to 32 °C.

Figures 1B, 2B, and 3B represent the effects of fermentation temperature, agitation speed, and their reciprocal interaction on the yields, at a constant initial pH medium of 5.5 (0 level) and fermentation time of 10 days (0 level). At the designed range of temperature from 25 °C to 30 °C, the yields increased with the increasing of agitation speed, and reached the peak value at 124.08 rpm. Agitation speed higher than 124.08 rpm decreased the yields. Figures 1C, 2C, and 3C represent the effects of fermentation temperature, fermentation time, and their reciprocal interaction on the yields, at a constant of another two factors. At the designed range of fermentation temperature, the yields increased with the increasing of fermentation time, and reached the peak at 11.44 days. Figures 1D, 2D, and 3D represent the effects of initial pH medium, agitation speed and their reciprocal interaction on the yields, at a constant of fermentation temperature of 28 °C (0 level) and fermentation time of 10 days (0 level). At the designed range of initial pH medium from 5.0 to 6.0, the highest yields produced at the agitation speed of 124.08 rpm, and decreased with the increasing of agitation speed more than 124.08 rpm.

Figures 1E, 2E, and 3E represent the effects of initial pH medium, fermentation time and their reciprocal interaction on the yields, at a constant fermentation temperature (0 level) and agitation speed (0 level). At the designed range of fermentation time from 8 days to 12 days, the highest yields obtained from the initial pH medium of 5.49. Figures 1F, 2F, and 3F represent the effects of agitation speed, fermentation time and their reciprocal interaction on the biomass, water-soluble POP, and alkali-soluble POP respectively, at a constant fermentation temperature of 28 °C (0 level) and initial pH medium 5.5 (0 level). At the designed range of agitation speed from 100 rpm to 150 rpm, the yields increased with the increasing of fermentation time, and reached maximum yields at 11.44 days.

Numerical optimization was performed to get the optimum conditions which produce a maximum mycelial biomass, water-soluble POP, and alkali-soluble POP. By adjusting the operating variables (temperature, pH, agitation, and fermentation time) within the experimental range, and set the maximum yields been expected, the optimum conditions generated. The optimum conditions suggested by numerical optimization were as follows: temperature 27.89 °C; pH 5.49; agitation 124.08 rpm, for 11.44 d incubation. The predicted maximum yields of biomass was 33.75 g/L, of water-soluble POP was 0.33 g/L, and of alkali-soluble POP was 0.64 g/L, with the desirability 92.1% (Table 4).

Table 4: Summary of response yields of mycelial biomass, water-soluble and alkali-soluble POP, based on the DOE optimization, theoretical prediction, and validation of the model.

Response yields of	Average response yields (g/L)		
	Mycelial Biomass	Water-soluble POP	Alkali-soluble POP
DOE optimization	25.07±5.13	0.24±0.05	0.49±0.09
Theoretical prediction	33.75	0.33	0.64
Validation of the model	32.00±1.35	0.29±0.01	0.60±0.02

Experimental validation of the optimized conditions

In order to confirm the model adequacy and the results from an analysis of the response surface, four additional experiments were conducted under the optimum conditions. The summary of response yields based on the design of experiment (DOE) optimization, theoretical prediction, and validation of the model showed in Table 4. The theoretical predictions of the yield of biomass, water-soluble POP, and alkali-soluble POP were 33.75 g/L, 0.33 g/L, and 0.64 g/L respectively. The yields of biomass, water-soluble POP, and alkali-soluble POP of DOE optimization were 25.0±5.13 g/L, 0.24±0.05 g/L, and 0.49±0.09 g/L respectively. After conducting the validation of optimized condition suggested by RSM, the yields of biomass, water-soluble POP and alkali-soluble POP were 32.00±1.35 g/L, 0.29±0.01 g/L, and 0.60±0.02 g/L respectively. There was 21.66 % increase in biomass yield, water-soluble POP 17.24 %, and alkali-soluble POP

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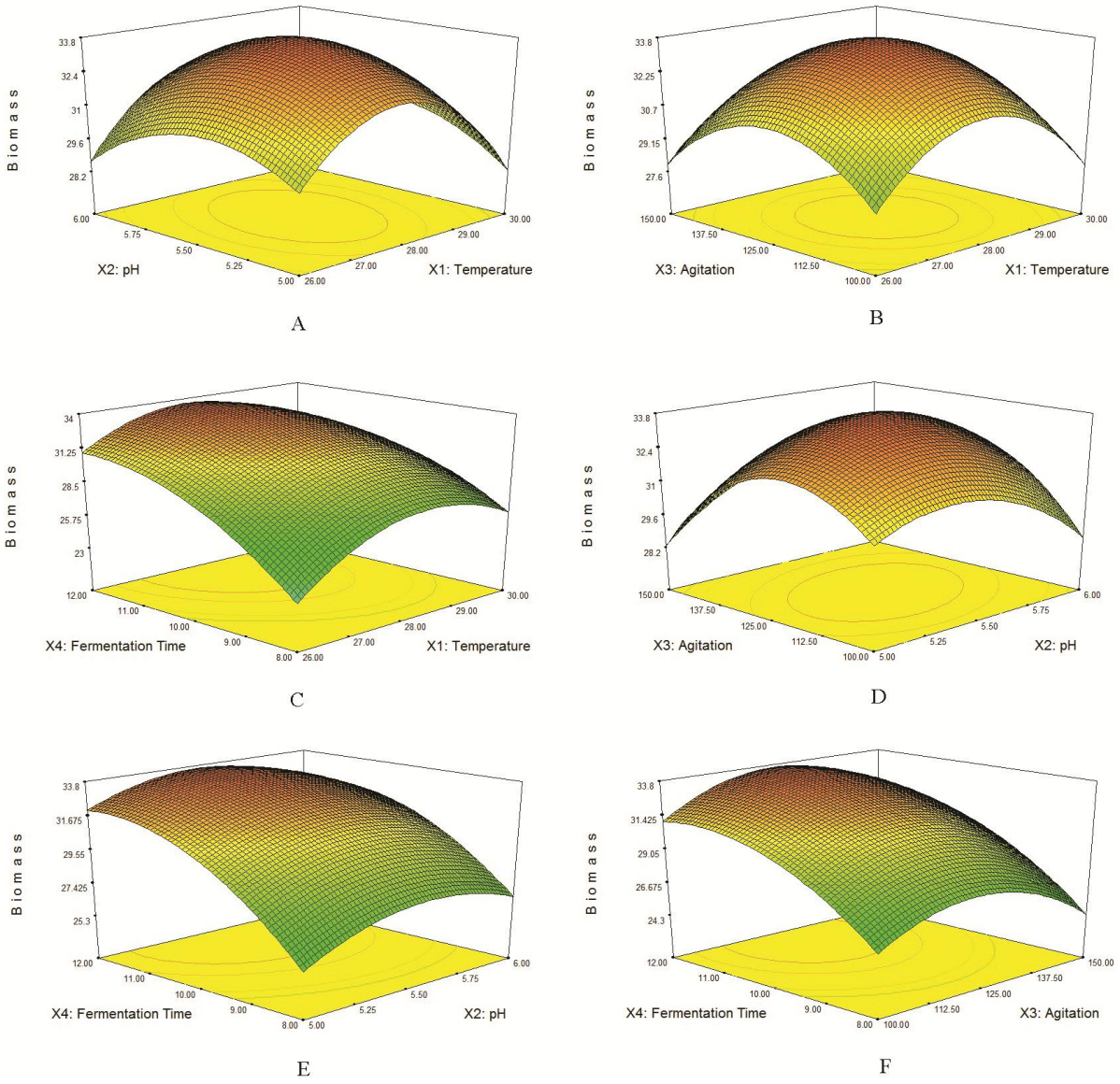

Figure 1: Three-dimensional response surface plots representing the effect of two variables and their reciprocal interaction on the yield of biomass of *P. ostreatus* BPPTCC 6017 at the constant another two variables.

A, Effect of interaction of temperature and initial pH; B, Effect of interaction of temperature and agitation; C, Effect of interaction of temperature and fermentation time; D, Effect of interaction of initial pH and agitation; E, Effect of interaction of initial pH and fermentation time; F, Effect of interaction of agitation and fermentation time.

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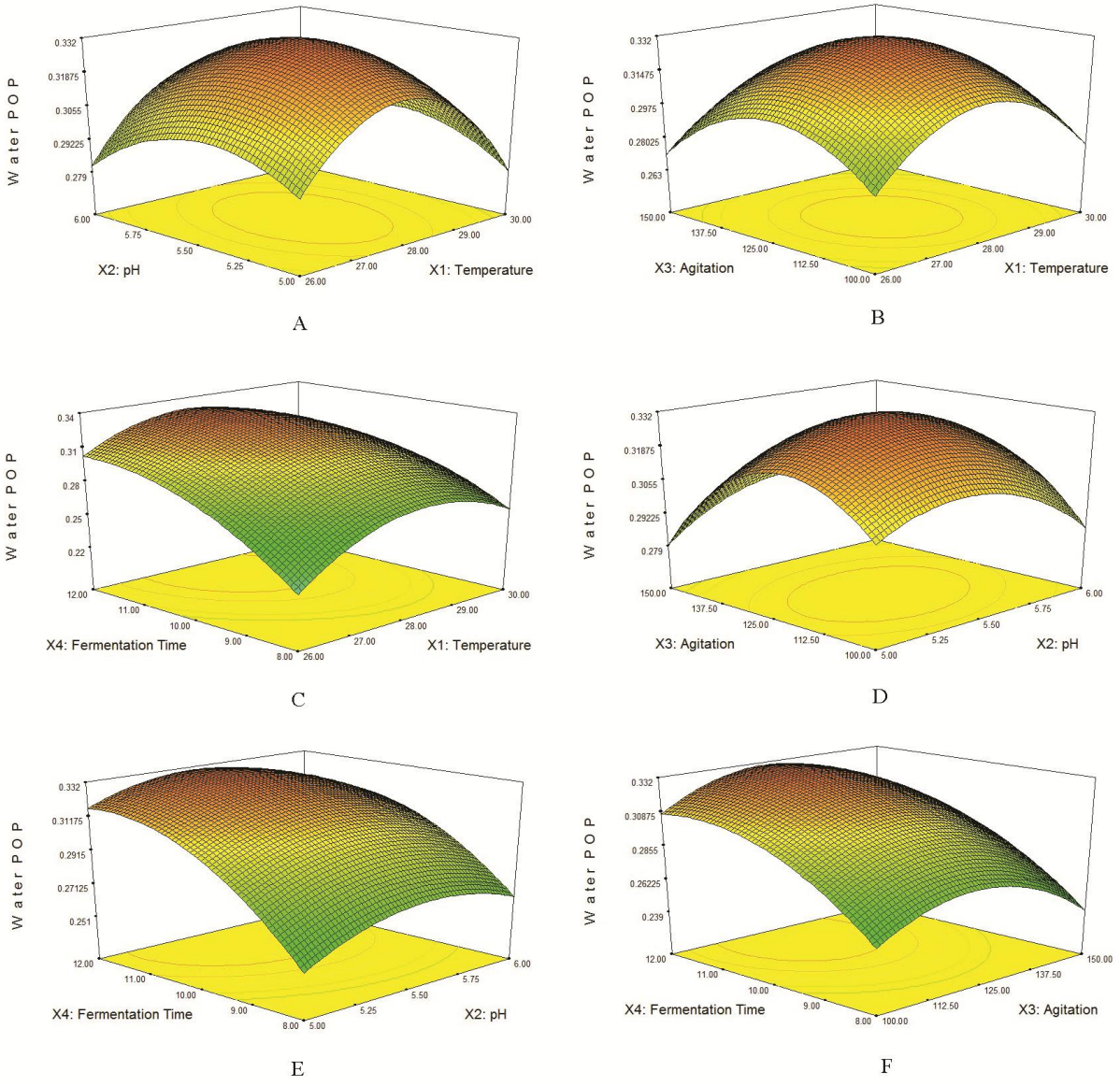

Figure 2: Three-dimensional response surface plots representing the effect of two variables and their reciprocal interaction on the yield of intracellular water-soluble POP production at the constant another two variables.

A, Effect of interaction of temperature and initial pH; B, Effect of interaction of temperature and agitation; C, Effect of interaction of temperature and fermentation time; D, Effect of interaction of initial pH and agitation; E, Effect of interaction of initial pH and fermentation time; F, Effect of interaction of agitation and fermentation time.

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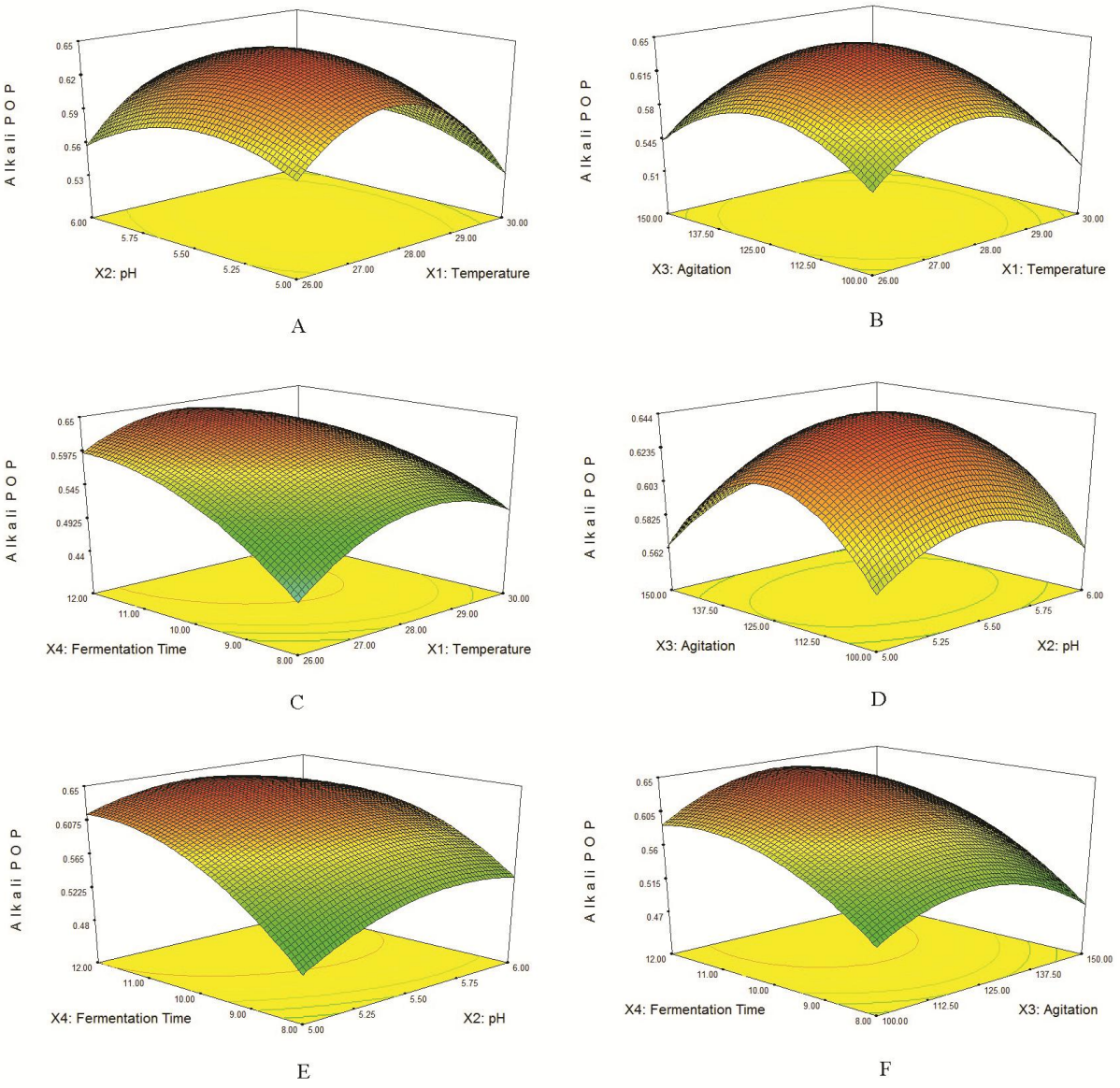

Figure 3: Three-dimensional response surface plots representing the effect of two variables and their reciprocal interaction on the yield of intracellular alkali-soluble POP production at the constant another two variables.

A, Effect of interaction of temperature and initial pH; B, Effect of interaction of temperature and agitation; C, Effect of interaction of temperature and fermentation time; D, Effect of interaction of initial pH and agitation; E, Effect of interaction of initial pH and fermentation time; F, Effect of interaction of agitation and fermentation time.

of 18.33%. The results obtained were close to the theoretical predictions.

Composition of medium used in this present study was comparable with medium BSM (Lavi *et al*., 2006; 2010) and medium Liu *et al*. (2010). Culture medium in this study using potato dextrose broth, instead of potato and glucose, which is more simple, reliable and reproducible when using formulated potato dextrose broth, than extracting potato dices. Yeast extract was chosen as the nitrogen source in this present study because it was proven better than other nitrogen sources, such as: ammonium sulfate, nitrate, pepton, asparagine, or corn steep liquor. Manu-Tawiah and Martin (1987) reported that the best production of *P. ostreatus* biomass was reached with yeast extract as a nitrogen source.

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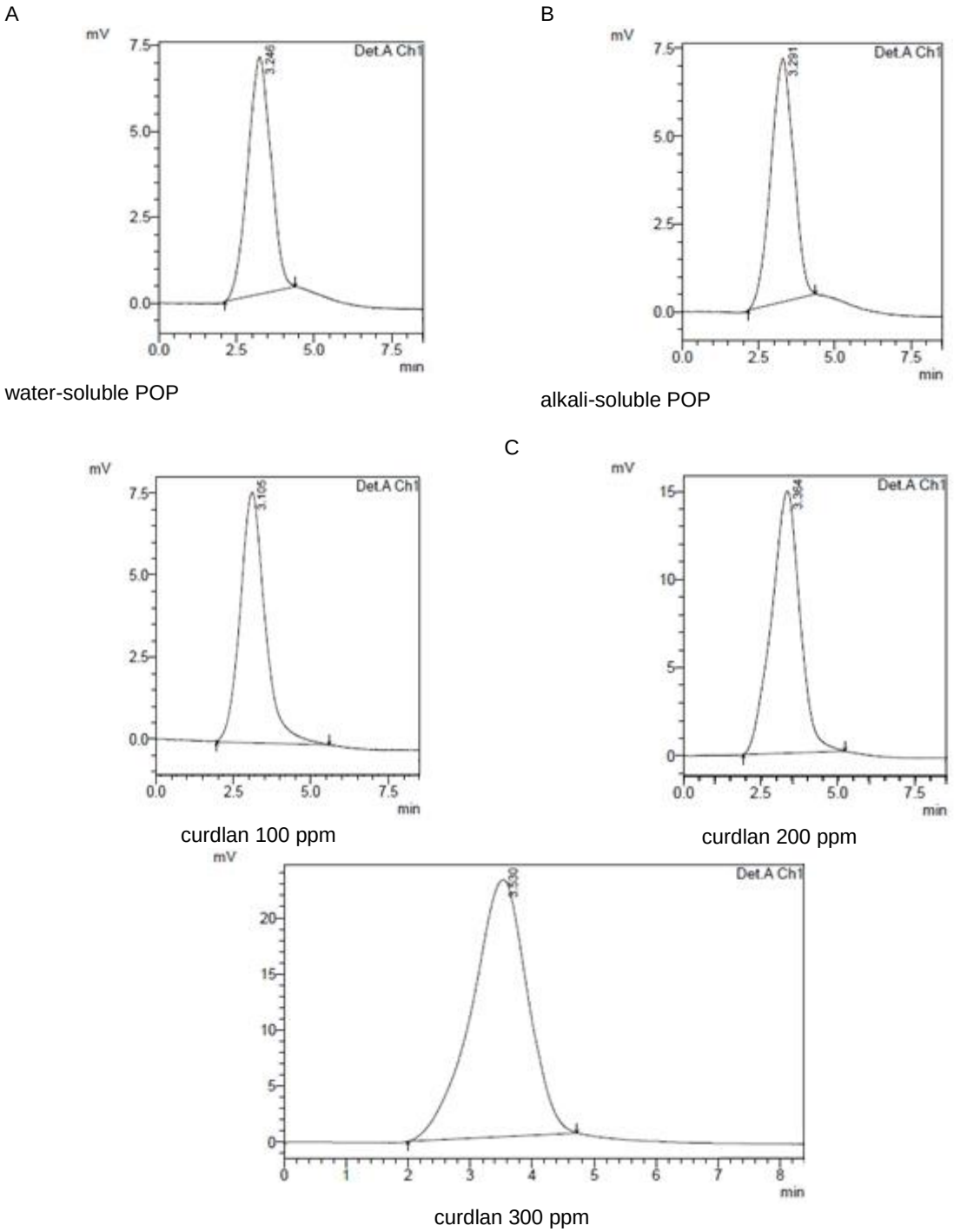

Figure 4: HPLC chromatogram of water-soluble POP. A, alkali-soluble POP; B and C, HPLC chromatogram of standard curdlan 100, 200, and 300 ppm.

Optimum temperature condition obtained from this study was 27.89 °C, comparable with previous published reports: 25 °C (Wang *et al*., 2005; Refaie *et al*., 2009; Liu *et al*., 2010; Devi *et al*., 2013), 28 °C (Hadar and Cohen-Arazi, 1986), 28±1 °C (Manu-Tawiah and Martin, 1987), and 30 °C (Gern *et al*., 2008). Papaspyridi *et al*. (2010) also conducted a submerged culture to produce biomass of *P. ostreatus* ATHUM 4438 at 28±2 °C. This result

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temperature was agreed with Brown (1988) that fungal microorganisms are mesophilic in character with their optimum temperature for growth occuring in the range 25-40 °C. The result indicated that to obtain the maximum growth of mycelia and POP of *P. ostreatus* BPPTCC 6017, the temperature of 27.89 °C was needed.

The pH of medium affected the response yields of mycelial biomass and POP. It agreed with Kim *et al.* (2005) that the pH of medium is very important but is often a neglected environmental factor. Many investigators claimed that the different morphology of mycelium under different initial pH value was the critical factor in biomass accumulation and metabolite formation. The medium pH may affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis. In the present study, maximum mycelial biomass and intracellular polysaccharide of *P. ostreatus* were obtained in cultures grown at an initial pH 5.49. It has been reported as well that many kinds of mushroom have more acidic pH optimum for mycelial growth and polysaccharide production during their submerged cultures (Kim *et al*. 2005).

Mycelial biomass produced in the present study, formed small pellets. In submerged fermentation, *P. ostreatus* forming pellet-like colonies (Sugihara and Humfeld, 1954; Hadar and Cohen-Arazi, 1986). As described by Brown (1988), fungi can grow as small hyphal fragments or can develop a pelleted growth form. In pelleted form, densely packed mycelium forms small spherical particles which are visible to the naked eyes. The factors influencing pellet formation are complex and a wide variety of parameters including: form of inoculum, kind of strain, the degree and nature of mechanical agitation, composition and pH medium. Marquez-Rocha *et al*. (1999) reported that the growth of *P. ostreatus* in liquid fermentation is affected by aeration and agitation. The maximum specific growth rate of *P. ostreatus* decreased by 15 % when aeration was increased from 1 to 1.5 vvm and decreased 28 % when the agitation was increased from 200 to 400 rpm. Also reported was a decrease in the size of mycelial pellets, in line with increased aeration and agitation, which may be caused by damage of the stability of mycelial pellets.

The optimum fermentation time of this study (11.4 d) comparable with the previous published reports: 4 d (Hadar and Cohen-Arazi, 1986), 5 d (Lavi *et al*., 2006), 8-14 d (Gern *et al*., 2008), 12-15 d (Refaie *et al.,* 2009), 6 d (Liu *et al*., 2010), and 21 d (Devi *et al*., 2013). Maximum biomass production occured at this period of the logarithmic phase. Brown (1988) stated that the production of biomass occurs in the unlimited growth phase (logarithmic phase) of a batch culture. This result suggested that to obtain the maximum biomass and POP of *P. ostreatus* BPPTCC 6017 in a batch submerged fermentation only 11.44 incubation days was needed

Biomass of *P. ostreatus* produced in this study (32.00±1.35 g/L) was comparable with results of Gern *et al*. (2008) and Papaspyridi *et al.* (2010), which produced 20.49±1.83 g/L and 39.2 g/L respectively. Results of this present study were also comparable with the the optimal condition to produce 0.56 mg/mL water-soluble polysaccharides of *P. citrinopileatus,* with the initial pH medium of 5.5, agitation at 100 rpm, incubated at 25 °C for 21 d (Wang *et al*., 2005). Results showed that production of intracellular polysaccharides was fully associated with the biomass production. The highest biomass product also presents the highest yield of intracellular polysaccharides. The optimum conditions of submerged culture of *P. ostreatus* BPPTCC 6017 to produce biomass was also to produce the highest yield of intracellular polysaccharides.

Result of HPLC analysis of POP showed that water and alkali-soluble of POP had one peak, with retention time 3.246 and 3.291 min respectively. The retention time of standard curdlan (100, 200, and 300 ppm) showed at 3.105, 3.364, and 3.530 min respectively. This result means that intracellular polysaccharide contained curdlan like molecule (Figure 4).

In conclussion, by conducting this present study, the optimum submerged culture conditions for producing biomass and polysaccharide of a white oyster mushroom widely grown by farmers in Indonesia are achieved. It offers the possibility to produce biomass and POP in a compact space, shorter time, and with fewer chances of contamination. Optimum conditions of submerged fermentation to produce mycelial biomass and polysaccharides of *P. ostreatus* BPPTCC 6017 were: temperature of 27.89 °C, initial pH medium of 5.49, agitation speed of 124.08 rpm, and 11.44 d of fermentation time. This optimum condition will be helpful for further scale-up study for producing biomass and polysaccharide of *P. ostreatus* BPPTCC 6017 using large-scale batch bioreactor.

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