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Exobiopolymer production of *Phytocordyceps* sp. BCC 2744 and *Akanthomyces pistillariiformis* BCC 2694; optimization and scale-up

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Abstract

Production of exobiopolymers by *Phytocordyceps* sp. BCC 2744 and *Akanthomyces pistillariiformis* BCC 2694, interleukin-8 (IL-8) inducers, were optimized using experimental design. Biological and physiological properties of these exobiopolymers are attractive to use as wound-dressing material, so that it is very interesting to increase its production. *Phytocordyceps* sp. BCC 2744 and *Akanthomyces pistillariiformis* BCC 2694 were cultivated on different carbon and nitrogen sources and the best carbon and nitrogen sources for exobiopolymer production of *Phytocordyceps* sp. BCC 2744 were glucose and peptone, respectively, and 0.88 g/L exobiopolymer was obtained. Higher exobiopolymer (1.82 g/L) was obtained on glucose and meat extract by *Akanthomyces pistillariiformis* BCC 2694. After the effects of 4 variables were studied using a two-level fractional design, glucose and peptone concentration were the most influential parameters on exobiopolymer production of *Phytocordyceps* sp. BCC 2744. Lower exobiopolymer production was obtained in the medium supplemented with 5-Fluorouracil and vitamin solution at high level (10 mM and 3mL/L, respectively). The highest exobiopolymer production was obtained on 60 g/L glucose and 20 g/L peptone and 2.32 g/L exobiopolymer was produced. About 4.0 g/L exobiopolymer production in a 20 L bioreactor was obtained. At least 4 variables; medium type, nitrogen sources, glucose, and nitrogen concentration were applied on a two-level factorial design of biomass and exobiopolymer production of *Akanthomyces pistillariiformis* BCC 2694. Models obtained from the multiple regression are significant. At high level of glucose (60 g/L), phosphate medium, and 20 g/L meat extract, higher exobiopolymer production and about 2.54 g/L was obtained. About 4.5 g/L exobiopolymer production in 20 L bioreactor was obtained.

Keywords : *Exobiopolymer, Phytocordyceps, Akanthomyces, general factorial design, enthomopathogenic fungi.*

1. Introduction

Exobiopolymer from a group of entomopathogenic fungi are attractive due to its biological and physical properties (Methacanon et al., 2005). They have been used in many applications such as, antitumor and immunomodulating agents (Peng et al., 2003), bioactive ingredients (Yaman, 2001), and wound dressing material (Madla et al., 2004) etc. The application was defined by its structure composition and molecular sizes of biopolymer (Methacanon et al., 2005). Furthermore, other groups of fungi were also reported to produce exobiopolymer (Carbonero et al., 2001, Kremer et al., 1999, Madla et al., 2004, Methacanon et al., 2005, Kim et al., 2002b, 2003a, b, c, Park et al., 2001, 2002a, b, Xu et al., 2003) and basidiomycetes were mostly reported to produce exobiopolymer (Chen et al., 2007, Kim et al., 2002a, Kim et al, 2006, Tang et al., 2002). Most exobiopolymer produced were used for other specific purposes such as food and biocoating materials (McNeely et al., 1973), prebiotics (Su et al., 2007), and as bioactive compounds (Xu et al., 2006, Cui et al., 2003) etc.

Exobiopolymer from Phytocordyceps sp. BCC 2744 is a potent wound dressing material due to its biological and physiological properties. It is biocompatible, non-cytotoxic, and strong inducer of interleukin-8 (IL-8) (>2.43 ng/mL produced by normal human dermal fibroblast cells), a cytokine responsible for enhancing wound healing process (Madla et al., 2004, Methacanon et al., 2005). Molecular weight of this exobiopolymer from Phytocordyceps sp. BCC 2744 are 9162 and 9.04 kDa, water and DMSO soluble (Methacanon et al., 2005). Its structure composes of a (1→3)-β-D-glucan backbone substitute at O-6 with side chains of (1→6)-β-D-pyranosyl units, with high branching structure (Methacanon et al., 2005). Furthermore, these exobiopolymer compositions are arabinose 2.84 %, mannose 8.02 %, galactose 6.23 %, and glucose 57.94 % (Methacanon et al., 2005). With these biological and physiological properties, it is attracted to increase production of this exobiopolymer using factorial experimental design and scale up to bioreactor.

2. Materials and Methods

Fungal strain

Phytocordyceps sp. BCC 2744 and Akanthomyces pistillariiformis BCC 2694 are entomopathogenic fungi isolated from a limacodid pupa (Khaoyai National Park of Thailand) and from an adult moth (Namnao National Park of Thailand), respectively, which were collected, identified, and isolated by Dr. Nigel L. Hywel-Jones of the BIOTEC. These fungi are deposited in the BIOTEC Culture Collection (BCC), Thailand.

Inoculum

The fungi were initially grown on potato dextrose agar (PDA) at 25 °C for 5-7 days. The agar block (1 cm^3) containing mycelia was cut into small pieces and then transferred to 25 mL potato dextrose broth (PDB) in a 250 mL Erlenmeyer flask and incubated at 25 °C on a rotary shaker at 200 rpm for 5-7 days.

Fermentation condition

About 10 percent (v/v) of the seed culture was transferred into the 50 mL liquid medium [sugar 20 g/L, nitrogen source 10 g/L, NaCl 0.2 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4$ 0.14 g/L, and trace solution 1 mL (trace solution composed of the following per litre; $ZnSO_4.H_2O$ 14.3 g, $CuSO_4.5H_2O$

2.5 g, $NiCl_2.6H_2O$ 0.5 g, and $FeSO_4.H_2O$ 13.8 g)] in a 250 mL Erlenmeyer flask and incubated at 25 °C on a rotary shaker at 200 rpm for 7 days. The medium used in 20 L fermentor (Marubishi Co., Ltd., Pathum Thani, Thailand) for Phytocordyceps sp. BCC 2744 with a working volume of 16 L was as follow; glucose 60 g/L, peptone 20 g/L, NaCl 0.2 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4$ 0.14 g/L, trace solution 1 mL/L. The medium used for Akanthomyces pistillariiformis BCC 2694 was as follow; glucose 60 g/L, meat extract 20 g/L, NaCl 0.2 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4$ 0.14 g/L, trace solution 1 mL/L. Agitation 200 rpm, aeration 1 vvm, and pH was not controlled.

Biomass determination

About 30-40 mL of culture broth was drawn from the bioreactor. The culture broth was then centrifuge at 10,000 g for 10 min and supernatant was removed. The fresh mycelium was then washed with 20-30 mL normal saline and the biomass content was determined by filtering samples through Whatman No1 filter paper. The filter cakes were washed with distilled water and dried at 105–110 °C for 24–48 h until a stable weight was achieved. Culture filtrate was subjected to sugar analysis and exobiopolymer extraction.

Exobiopolymer precipitation and purification

Culture filtrate was mixed with four volumes of 95% ethanol, stirred vigorously, and stored at 20 °C for at least 12 h. The precipitated polymer was recovered by centrifugation at 10,000 g for 20 min and the supernatant was discarded. The polymers were then lyophilized until dried. During purification step, the polymer was re-dissolved in distilled water and any insoluble materials were discarded by centrifugation at 10,000 g for 20 min. The supernatant was dialyzed (molecular weight cut off 2,000 Da, Spectrum Laboratories, Inc., USA) against 4 L of distilled water for 24 h and lyophilized until completely dried.

Sugar determination

For measurement of sugars, supernatant was centrifuged at 10,000 g for 10 min and filtered through a 0.22 mm filter paper. The filtrate was subjected to HPLC analysis using a Sugar-Pak column (Waters, MA, USA). The mobile phase was water at a flow rate of 0.6 mL min^{-1} and column temperature was 90 °C. Sugars were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA, USA).

Experimental design

General factorial design was used to find the best carbon and nitrogen sources. About 20 g/L carbon and 10 g/L nitrogen sources were used. After obtaining the best carbon and nitrogen sources, a two-level factorial design was applied on 4 selected factors influencing exobiopolymer production with 3 center points and duplicates. All four factors are as follow for quantitative optimization of Phytocordyceps sp. BCC 2744; 5-fluorouracil, peptone concentration, glucose concentration, and vitamin. For A. pistillariiformis BCC 2694, four factors include; medium, nitrogen source, glucose and nitrogen concentration, were applied in quantitative optimization. A two-level factorial design of 2^n was used and factors influencing exobiopolymer were analysed based on ANOVA statistical analysis using the same software. The software Design Expert (Version 7.0.b1.1, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis, and linear model building. The optimal fermentation conditions for enhanced yield

of exobiopolymer were obtained by solving the regression equation and also by analyzing the interaction using the same software.

3. Results and Discussion

Effects of carbon and nitrogen sources on exobiopolymer production

Phytocordyceps sp. BCC 2744 was cultivated on different carbon and nitrogen sources using general factorial design, in which biomass and exobiopolymer were analysed. The highest biomass production (19.35 g/L) was obtained on sucrose and peptone combined with yeast extract as carbon and nitrogen sources, respectively (Table 1). The highest exobiopolymer production (0.88 g/L) was obtained on glucose and peptone as a sole carbon and nitrogen sources, respectively. Sucrose was favor for biomass production but soluble starch was a poor carbon source for biomass production. Galactose, mannose, soluble starch, and sucrose gave lower amount of exobiopolymer. Malt extract was not favor for both biomass and exobiopolymer production. Glucose and peptone were the best carbon and nitrogen sources, respectively, which were used for further two-level factorial design to find the effects of other influential variables on exobiopolymer production.

Table 1. Biomass and exobiopolymer production by *Phytocordyceps* sp. BCC 2744 on different carbon and nitrogen sources using general factorial design.

Carbon source	Nitrogen source	Biomass (g/L)	Exobiopolymer (g/L)
Galactose	Corn steep liquor	NE	NE
	Malt extract	3.76	0
	Peptone	15.13	0.63
	Yeast extract	12.49	0.35
	Peptone + Yeast extract	-	-
Glucose	Corn steep liquor	5.81	0
	Malt extract	1.88	0
	Peptone	16.84	0.88
	Yeast extract	16.46	0.55
	Peptone + Yeast extract	16.39	0.32
Mannose	Corn steep liquor	7.17	0
	Malt extract	2.03	0
	Peptone	16.12	0.57
	Yeast extract	16.74	0.51
	Peptone + Yeast extract	15.47	0
Soluble starch	Corn steep liquor	6.87	0
	Malt extract	NE	NE
	Peptone	11.83	0.17
	Yeast extract	9.62	0
	Peptone + Yeast extract	11.80	0
Sucrose	Corn steep liquor	7.38	0
	Malt extract	NE	NE
	Peptone	18.91	0
	Yeast extract	17.60	0
	Peptone + Yeast extract	19.35	0.60

NE= No evaluation

Exobiopolymer production by *A. pistillariiformis* BCC 2694 was studied on different carbon and nitrogen sources using general factorial design. The highest biomass yield (13.14 g/L) was obtained on galactose and yeast extract as a sole carbon and nitrogen source, respectively (Table 2). The highest exobiopolymer was obtained on glucose and meat extract (1.82 g/L). The fungus produced high biomass but low exobiopolymer yields. Galactose, mannose, soluble starch, and sucrose were preferable for biomass growth but not for exobiopolymer production. Among all nitrogen sources, peptone (except on glucose) and corn steep liquor were not favor for exobiopolymer production. Thus, lower exobiopolymer production was obatined on the combination of peptone and yeast extract as nitrogen sources. Furthermore, exobiopolymer was produced at lower concentration on yeast extract. This is clearly shown that biomass yield had inverse relationship with exobiopolymer production.

Exobiopolymer production using two-level factorial design

At least 4 variables; 5-fluorouracil concentration, peptone concentration, glucose concentration, and vitamin, were applied to a two-level factorial design and their effects were analyzed using ANOVA statistics. Lower biomass production was obtained in 20 mM 5-fluorouracil and it affected negatively biomass production of *Phytocordyceps* sp. BCC 2744 (equation 1). Glucose had positive effect on biomass production and higher biomass production was obtained on 60 g/L glucose. Positive interaction between peptone and glucose concentration was achieved on biomass production.

Biomass (g/L)= 9.37+0.93A+1.81B +0.085C+0.24D-0.30AB+0.32AC +0.037AD+0.34BC+3.0E3BD +0.13CD…….(1)

Exobiopolymer (g/L)=0.76-0.13A+0.11B+0.31C-0.25D -0.033AB-0.022AC+0.029AD +0.16BC-0.097BD-0.13CD…….(2)

When A= 5-Fluorouracil, B= peptone concentration, C= glucose concentration, and D=vitamin

This fungus produced high biomass but low exobiopolymer production on all medium composition. Positive effect of glucose on exobiopolymer production was revealed (equation 2) and lower exobiopolymer production was obtained on medium supplemented with 3 mL/L vitamin solution. The positive interaction between peptone and glucose concentration on exobiopolymer was also achieved similar to biomass production but at a lower positive coefficient. The highest biomass production (13.36 g/L) was obtained on 20 g/L glucose, 20 g/L peptone, and 3 mL/L vitamin solution (Table 3). The highest exobiopolymer production (2.32 g/L) was obtained on 60 g/L glucose and 20 g/L peptone. This showed that the condition for exobiopolymer production by *Phytocordyceps* sp. BCC 2744 were as follows; glucose 60 g/L, peptone 20 g/L, NaCl 0.2 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4$ 0.14 g/L, and trace solution 1 mL/L. This condition was used for exobiopolymer production in 20 L bioreactor.

Variables; medium composition, nitrogen sources, glucose concentration, and nitrogen concentration (Table 4),

influencing exobiopolymer production by *A. pistillariiformis* BCC 2694 were applied on two-level fractional factorial design and all effects were analysed. The highest exobiopolymer concencentration (2.54 g/L) was obtained on phosphate medium, 20 g/L meat extract, and 60 g/L glucose (Table 4). The highest biomass yield (23.06 g/L) was obtained on phosphate medium, 20 g/L yeast extract, and 60 g/L glucose. Nitrogen concentration had the higher positive effect on biomass (equation 3) and exobiopolymer production (equation 4). Glucose concentration had positive interaction with nitrogen concentration on biomass and biopolymer production (equation 3 and 4, respectively).

Biomass
(g/L)=14.64+0.50A+0.21B+5.65C
+0.92D-0.058AB+0.38AC+0.042AD
+0.36BC-0.071BD+0.71CD……..(3)

Biopolymer (g/L)=1.39+0.028A-0.073B
+0.13C+0.15D-0.14AB+0.036AC
+0.037AD-0.15BC-0.17BD
+0.063CD……..…..(4)

When A= medium, B= nitrogen source, C= glucose concentration, and D=nitrogen concentration

This is clearly shown that higher exobiopolymer production by *A. pistillariiformis* BCC 2694 was obtained on 20 g/L meat extract and 60 g/L glucose.

Exobiopolymer production in 20 L bioreactor

The production of exobiopolymer by *Phytocordyceps* sp. BCC 2744 was carried out in a 20 L bioreactor to investigate the possibility of scaling up to a larger scale. Figure 1 shows the exobiopolymer production on 60 g/L glucose and 20 g/L peptone, specific growth rate of (μ) 0.011 h^{-1}, biomass yield (Y_{SX}) of 0.53 g/g glucose, exobiopolymer yield (Y_{XP}) of 0.18 g/g biomass (Y_{SP} of 0.11 g/g sugar), and exobiopolymer production rate (q_p) of 0.09 g/L/d (Table 5) were obtained. The highest exobiopolymer concentration of 4.0 g/L at 292 h was obtained. This was almost two times higher than in shake flask.

Using 60 g/L glucose and 20 g/L meat extract, a specific growth rate (μ) of *A. pistillariiformis* BCC 2694 of 0.005 h^{-1} was obtained, with biomass yield (Y_{SX}) of 0.34 g/g glucose, exobiopolymer yield (Y_{XP}) of 0.46 g/g biomass (Y_{SP} of 0.28 g/g sugar), and exobiopolymer production rate (q_p) of 0.13 g/L.d (Table 5). The highest exobiopolymer concentration (4.5 g/L) was obtained at 241 h (Figure 2). High production of exobiopolymer obtained from this optimization process shows promise in scaling up for industrial needs.

Exobiopolymers produced by ascomycetes have been described from *Cordyceps militaris* (Kim *et al*., 2003), the maximum production of 10.3 g/L in a 5-L bioreactor was obtained. *Paecilomyces japonica* was reported to produce exobiopolymer at the maximum yield of 8 g/L in a 2.5-L jar bioreactor (Bae *et al*. 2000). The exobiopolymer production by *Phytocordyceps* sp. BCC 2744 and *A. pistillariiformis* BCC 2694 were lower than other published data but the structure (glucan), molecular weight (8.3 and 9.04 kda for *Phytocordyceps* sp. BCC 2744 and *A. pistillariiformis* BCC 2694, respectively) of these exobiopolymers produced were different and lead to different applications compared to other organism reported (Madla *et al*., 2005). With these specific properties, exobiopolymers produced from these two fungi can be further applied in pharmaceutical and medical uses.

Table 2. Biomass and exobiopolymer production by *A. pistillariiformis* BCC 2694 on different carbon and nitrogen sources using general factorial design.

Carbon source	Nitrogen source	Biomass (g/L)	Exobiopolymer (g/L)
Galactose	Corn steep liquor	NE	NE
	Meat extract	NE	NE
	Peptone	13.04	0
	Yeast extract	**13.14**	0.42
	Peptone + Yeast extract	NE	NE
Glucose	Corn steep liquor	5.50	0.47
	Meat extract	9.12	1.82
	Peptone	9.75	1.16
	Yeast extract	7.72	0.65
	Peptone + Yeast extract	10.67	0.88
Mannose	Corn steep liquor	5.89	0.56
	Meat extract	NE	NE
	Peptone	8.60	0.54
	Yeast extract	8.77	0.65
	Peptone + Yeast extract	9.30	0.70
Soluble starch	Corn steep liquor	5.17	0.78
	Meat extract	NE	NE
	Peptone	7.80	0
	Yeast extract	7.40	0
	Peptone + Yeast extract	8.63	0
Sucrose	Corn steep liquor	6.09	0
	Meat extract	NE	NE
	Peptone	10.71	0
	Yeast extract	11.73	0.50
	Peptone + Yeast extract	12.04	0.76

NE= No evaluation

Table 3. Biomass and exobiopolymer production by *Phytocordyceps* sp. BCC 2744 affected by different factors and levels using two-level fractional factorial design.

Run	5-Fluorouracil (mM)	Peptone conc. (g/L)	Glucose conc. (g/L)	Vitamin (mL/L)	Biomass (g/L)	Exobiopolymer (g/L)
1	0	10	20	0	9.00	0.78
2	0	10	20	0	9.09	0.79
3	10	10	20	0	7.33	0.35
4	10	10	20	0	6.30	0.26
5	0	20	20	0	11.69	0.64
6	0	20	20	0	11.44	0.72
7	10	20	20	0	9.75	0.46
8	10	20	20	0	8.78	0.48
9	0	10	60	0	7.60	1.21
10	0	10	60	0	7.25	1.03
11	10	10	60	0	6.23	0.80
12	10	10	60	0	5.74	1.16
13	0	20	60	0	11.90	1.86
14	0	20	60	0	12.79	2.32
15	10	20	60	0	12.23	2.08
16	10	20	60	0	8.87	1.16
17	0	10	20	3	8.44	0.49
18	0	10	20	3	8.48	0.53
19	10	10	20	3	8.33	0.41
20	10	10	20	3	5.51	0.35
21	0	20	20	3	13.36	0.28
22	0	20	20	3	12.76	0.22
23	10	20	20	3	9.23	0.22
24	10	20	20	3	9.01	0.15
25	0	10	60	3	7.87	0.56
26	0	10	60	3	7.81	0.58
27	10	10	60	3	6.76	0.46
28	10	10	60	3	9.17	0.56
29	0	20	60	3	12.78	1.05
30	0	20	60	3	12.56	1.19
31	10	20	60	3	10.49	0.50
32	10	20	60	3	11.19	0.58
33	5	15	40	1.5	8.42	0.36
34	5	15	40	1.5	8.34	0.42
35	5	15	40	1.5	8.74	0.52

Table 4. Biomass and exobiopolymer production by *A. pistillariiformis* BCC 2694 affected by different factors and levels using the two-level fractional factorial design.

Run	Medium	Nitrogen source	Glucose (g/L)	Nitrogen (g/L)	Biomass (g/L)	Exobiopolymer (g/L)
1	Phosphate- limited medium	meat extract	20	10	8.74	0.63
2	Phosphate medium	meat extract	20	10	9.14	1.18
3	Phosphate- limited medium	yeast extract	20	10	8.42	1.59
4	Phosphate medium	yeast extract	20	10	8.83	1.28
5	Phosphate- limited medium	meat extract	60	10	17.35	1.40
6	Phosphate medium	meat extract	60	10	18.52	1.39
7	Phosphate- limited medium	yeast extract	60	10	18.55	1.39
8	Phosphate medium	yeast extract	60	10	20.22	1.08
9	Phosphate- limited medium	meat extract	20	20	9.24	1.48
10	Phosphate medium	meat extract	20	20	9.39	1.42
11	Phosphate- limited medium	yeast extract	20	20	9.06	1.36
12	Phosphate medium	yeast extract	20	20	9.05	1.11
13	Phosphate- limited medium	meat extract	60	20	20.14	1.66
14	Phosphate medium	meat extract	60	20	22.87	**2.54**
15	Phosphate- limited medium	yeast extract	60	20	21.61	1.40
16	Phosphate medium	yeast extract	60	20	**23.06**	1.34
17	Phosphate- limited medium	meat extract	40	15	14.47	1.76
18	Phosphate medium	meat extract	40	15	15.59	1.57
19	Phosphate- limited medium	yeast extract	40	15	14.98	1.64
20	Phosphate medium	yeast extract	40	15	16.54	1.56
21	Phosphate- limited medium	meat extract	40	15	14.65	2.04
22	Phosphate medium	meat extract	40	15	15.21	1.73
23	Phosphate- limited medium	yeast extract	40	15	15.84	1.81
24	Phosphate medium	yeast extract	40	15	15.66	1.54
25	Phosphate- limited medium	meat extract	40	15	14.83	1.93
26	Phosphate medium	meat extract	40	15	14.83	1.53
27	Phosphate- limited medium	yeast extract	40	15	15.25	1.62
28	Phosphate medium	yeast extract	40	15	14.99	1.59

Table 5. Specific growth rate (μ), biomass yield (Y_{SX}), exobiopolymer yield on biomass (Y_{XP}), exobiopolymer yield on sugar (Y_{SP}), and exobiopolymer production rate (q_p) of *Phytocordyceps* sp. BCC 2744 and *A. pistillariiformis* BCC 2694 cultivated in 20-L bioreactor.

Fungi	m (h^{-1})	Y_{SX} (g dw/g sugar)	Y_{XP} (g/g dw)	Y_{SP} (g/g sugar)	q_p g/L.d
Phytocordyceps sp. BCC 2744	0.011	0.53	0.18	0.11	0.09
pistillariiformis BCC 2694	0.005	0.34	0.46	0.28	0.13

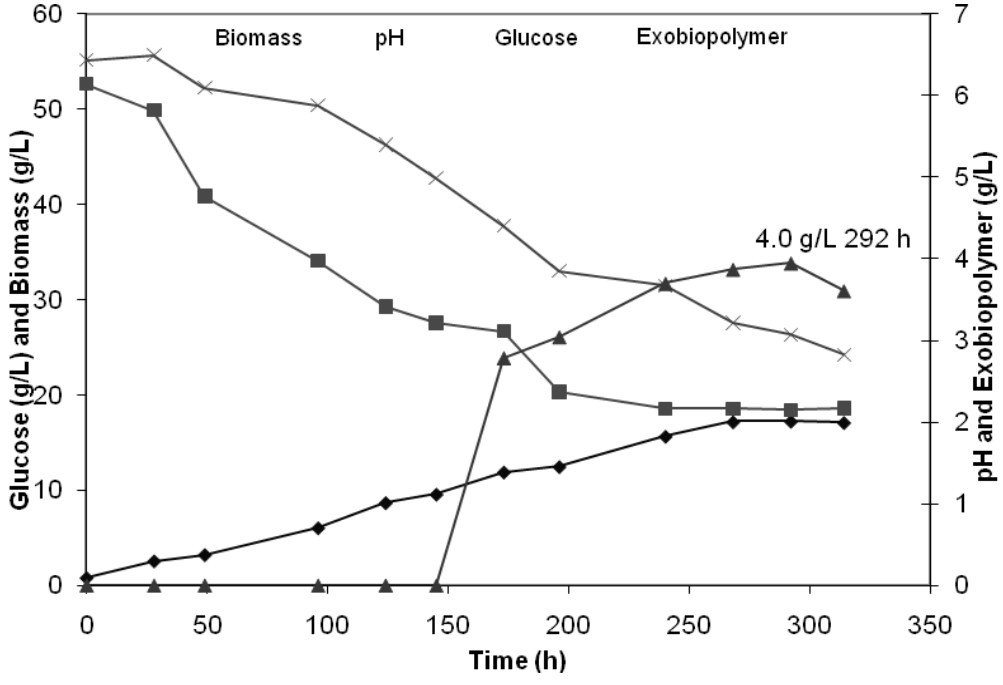

Figure 1. Time profile of growth and exobiopolymer production of *Phytocordyceps* sp. BCC 2744 on 60 g/L glucose and 20 g/L peptone in a 20-L bioreactor.

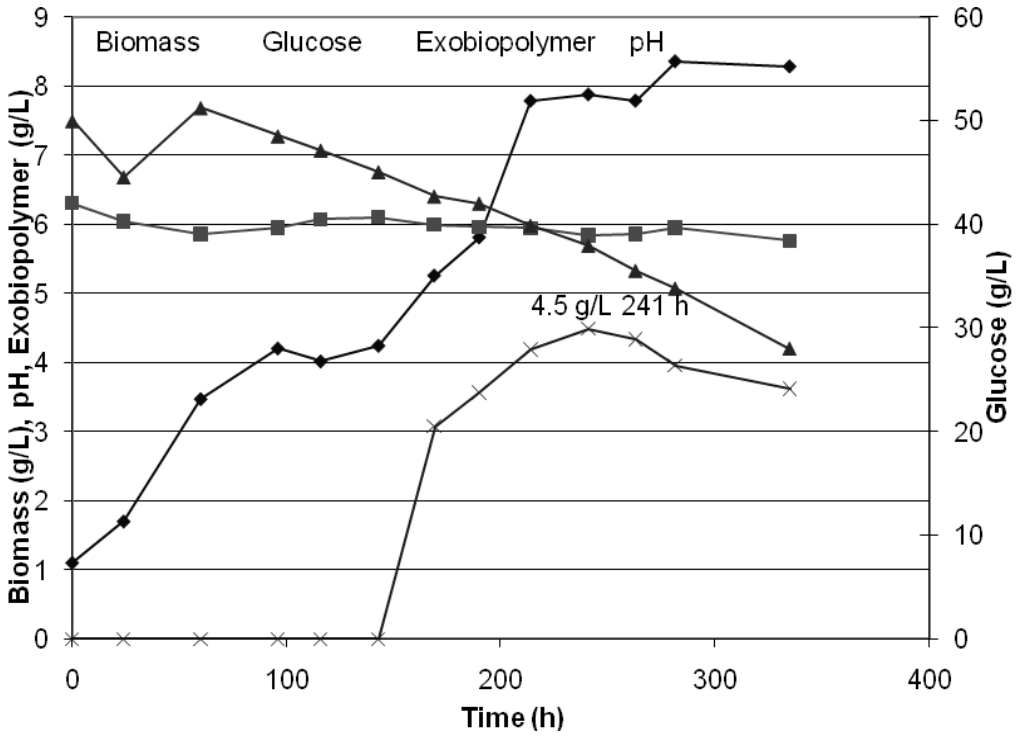

Figure 2. Time profile of growth and exobiopolymer production of *A. pistillariiformis* BCC 2694 on 60 g/L glucose and 20 g/L meat extract in a 20-L bioreactor.

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