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# Medium optimization for palmarumycin $C_{13}$ production in liquid culture of endophytic fungus *Berkleasmium* sp. Dzf12 using response surface methodology

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## Abstract

**Background:** *Berkleasmium* sp. Dzf12, an endophytic fungus from *Dioscorea zingiberensis*, was a high producer of palmarumycin $C_{13}$ with various bioactivities. In the present study, the experimental designs based on statistics were employed to evaluate and optimize the medium for palmarumycin $C_{13}$ production in mycelia liquid culture of *Berkleasmium* sp. Dzf12.
**Results:** Among various carbon and nitrogen sources, glucose, peptone and yeast extract were found to be the most favourable for palmarumycin $C_{13}$ production based on the one-factor-at-a-time experiments. After Plackett-Burman test on the medium, glucose, peptone and yeast extract were further verified to be the most significant factors to stimulate palmarumycin $C_{13}$ accumulation. These three factors (*i.e.*, glucose, peptone and yeast extract) were then optimized through the experiments of central composite design (CCD) and analysis of response surface methodology (RSM). The optimized medium compositions for palmarumycin $C_{13}$ production were determined as 42.5 g/l of glucose, 6.5 g/l of peptone, 11.0 g/l of yeast extract, 1.0 g/l of $KH_2PO_4$, 0.5 g/l of $MgSO_4$ x $7H_2O$, 0.05 g/l of $FeSO_4$ x $7H_2O$, and pH 6.5. Under the optimal culture conditions, the maximum palmarumycin $C_{13}$ yield of *Berkleasmium* sp. Dzf12 was increased to 318.63 mg/l, which was about 2.5-fold in comparison with that (130.44 mg/l) in the basal medium.
**Conclusions:** The results indicate that the optimum production of palmarumycin $C_{13}$ in *Berkleasmium* sp. Dzf12 liquid culture can be achieved by addition of glucose, peptone and yeast extract with their appropriate concentrations in the modified Sabouraud medium.

**Keywords:** *Berkleasmium* sp. Dzf12; central composite design; endophytic fungus; mycelia liquid culture; palmarumycin C13; Plackett-Burman test; response surface methodology.

## INTRODUCTION

Plant endophytic fungi are the fungal microorganisms which spend the whole or part of their lifecycle colonizing inter- and/or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease (Rodríguez et al. 2009; Aly et al. 2011). They have been regarded as an important and novel resource of natural bioactive products with their potential applications in agriculture, medicine and food industry, and attracted many researchers' attentions (Gunatilaka, 2006; Zhao et al. 2011a; Zhi-Lin et al. 2012). In the past two decades, many valuable bioactive secondary metabolites with antimicrobial, insecticidal, cytotoxic, and anticancer activities have been successfully obtained from the endophytic fungi. These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinones, lignans, phenols, and lactones (Zhang et al. 2006; Kharwar et al. 2011).

Spirobisnaphthalenes are a group of naphthoquinone derivatives with notable antibacterial, antifungal, allelochemical, antileishmanial, cytotoxic, antitumor, and enzyme inhibitory activities (Cai et al. 2010; Zhou et al. 2010). Palmarumycin $C_{13}$ (also named Sch 53514, diepoxin ζ or cladospirone bisepoxide), a notable spirobisnaphthalene with various bioactivities, was first isolated from the endophytic fungus LL-07F725 associated with a tree growing in Panama by Schlingmann et al. (1993). Later, it was also successfully obtained from other fungal species such as *Nattrassia mangiferae* (Chu et al. 1994), *Coniothyrium* sp. (Krohn et al. 1994), *Cladosporium* sp. (Petersen et al. 1994), and endophytic fungus *Berkleasmium* sp. Dzf12 (Cai et al. 2009). In the previous investigations, palmarumycin $C_{13}$ exhibited strong antitumor activity with the $IC_{50}$ values as 0.2 μM in PLD assay (Schlingmann et al. 1993) and 0.37 μM in HT 1080 human fibro-sarcoma invasion assay (Chu et al. 1994), as well as strong antibacterial activity with the $IC_{50}$ values ranging from 5.0 to 12.5 μg/ml (Cai et al. 2009). These discoveries indicated that palmarumycin $C_{13}$ could be developed as the potent antibiotic and anticancer agents, and showed great potential applications in modern agriculture and medicinal industry.

Endophytic fungus *Berkleasmium* sp. Dzf12 was isolated from the healthy rhizomes of a Chinese medicinal plant *Dioscorea zingiberensis* C.H. Wright (Cai et al. 2009). In our previous studies, six spirobisnaphthalenes were successfully obtained from this fungus by bioassay-guided fractionation, and palmarumycin $C_{13}$ was found to be the most abundant and active antimicrobial compound in *Berkleasmium* sp. Dzf12 (Cai et al. 2009; Li et al. 2012c). In order to speed up application of palmarumycin $C_{13}$, one of the most important approaches is to increase palmarumycin $C_{13}$ yield in fermentation culture of *Berkleasmium* sp. Dzf12. Various strategies have been developed to increase metabolite yield in microorganism or plant cultures, which include optimization of medium, utilization of two-phase culture systems, addition of precursors and metal ions, as well as application of elicitation by using polysaccharides and oligosaccharides (Zhou and Wu, 2006; Zhou et al. 2007; Xu et al. 2009; Zhang et al. 2009; Zhao et al. 2010; Li et al. 2011).

It is well-known that the medium optimization is an early and essential step, and plays an important role in enhancement of metabolite production in the development of any microbial fermentation process. The classical "one-factor-at-a-time approach" is an operation frequently used in medium optimization to obtain high yields of the desired products in a microbial system. However, this method disregards the complex interactions among various factors, and is unsuitable for multifactor optimization (Wang and Liu 2008). The limitation of such method can be avoided by using statistical experimental design, a powerful and useful tool, which can allow explaining interactions between the different variables, decreasing the process variability, as well as reducing the overall cost (Mu et al. 2009). The most popular choices are the Plackett-Burman design (PBD), the Box-Behnken design (BBD), and the central composite design (CCD), along with the response surface analysis (Liu et al. 2005; Djekrif-Dakhmouche et al. 2006; Xu et al. 2008). The response surface methodology (RSM) can be used to study the effects of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments (Li et al. 2012a; Li et al. 2012b). There are some examples on successful optimization of medium for microbial metabolite production by statistical optimization techniques (Pio and Macedo, 2007; Turlo et al. 2008; Xu et al. 2010; Mou et al. 2013).

To the best of our knowledge, there are no reports available on the medium optimization for palmarumycin $C_{13}$ accumulation. The aim of this study was to examine and optimize the nutrient medium for palmarumycin $C_{13}$ production in mycelia liquid culture of *Berkleasmium* sp. Dzf12. In this study, the one-factor-at-a-time experiments were first used to select the optimal carbon and nitrogen sources. And then, the statistical screening test based on Plackett-Burman design was conducted on numerous nutrient components to determine the significant factors affecting the palmarumycin $C_{13}$ accumulation. Moreover, the significant variables were further optimized by the experiment of central composite design (CCD) and the analysis of response surface methodology (RSM).

## MATERIALS AND METHODS

### Endophytic fungus and culture conditions

The endophytic fungus *Berkleasmium* sp. Dzf12 was isolated from the healthy rhizomes of a medicinal plant *Dioscorea zingiberensis* C.H. Wright (Dioscoreaceae), and identified through its morphological characteristics and internal transcribed spacer (ITS) rRNA gene sequence analysis (GenBank accession number EU543255) (Cai et al. 2009). The living culture has been deposited at the China

General Microbiological Culture Collection Center (CGMCC) under the number of CGMCC 2476. The stock culture of *Berkleasmium* sp. Dzf12 mycelia was maintained on solid potato-dextrose-agar (PDA) medium at 25ºC, and in 40% glycerol at -70ºC at the Herbarium of the College of Agronomy and Biotechnology, China Agricultural University. Liquid culture experiments were carried out in 150-ml Erlenmeyer flasks, each filled with 30 ml of liquid medium, maintained on a rotary shaker at 150 rpm and 25ºC for 13 days or a specified culture period. The basal medium (before optimization) used in this study was the modified Sabouraud broth medium (consisted of 40.0 g/l of glucose, 10.0 g/l of peptone, 1.0 g/l of $KH_2PO_4$, 0.5 g/l of $MgSO_4$ x $7H_2O$, 0.05 g/l of $FeSO_4$ x $7H_2O$, pH 6.5), which was favourable for the mycelia growth and palmarumycin $C_{13}$ production in *Berkleasmium* sp. Dzf12 culture in our preliminary investigation (Zhao et al. 2011b). The inoculum for the shake-flask culture was prepared by shaking incubation of the mycelia from the solid stock culture in potato dextrose broth for 4 days, and 0.9 ml of mycelia broth was inoculated to each flask (3.0%, v/v).

**Measurement of mycelia biomass and palmarumycin $C_{13}$ content**

Measurement of mycelia biomass and palmarumycin $C_{13}$ content were carried out as previously described (Zhao et al. 2011b). Briefly, the mycelia of *Berkleasmium* sp. Dzf12 were separated from the fermentation broth by filtration under vacuum and rinsed thoroughly with distilled water, and then dried at 50-55ºC in an oven to a constant dry weight (dw). Palmarumycin $C_{13}$ was extracted from the dry mycelia powder (10 mg/ml) with methanol-chloroform (9:1, v/v) in an ultrasonic bath (three times, 60 min each). After removal of the solid by filtration, the liquid extract was evaporated to dryness and re-dissolved in 1 ml of methanol. For analysis of palmarumycin $C_{13}$ content in medium, 5 ml of the culture broth was evaporated to dryness and extracted with 5 ml of methanol-chloroform (9:1, v/v), and the liquid extraction was then evaporated to dryness and re-dissolved in 1 ml of methanol. The palmarumycin $C_{13}$ content was then analyzed by the high performance liquid chromatography (HPLC) system (Shimadzu, Japan), which consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, an SPD-M20A photodiode array detector, and CBM-20Alite system controller. The reversed-phase Agilent TC-$C_{18}$ column (250 mm x 4.6 mm *i.d*., particle size 5 µm) was used for separation by using methanol-$H_2O$ (50:50, v/v) as the mobile phase at a flow rate of 1 ml/min. The temperature was maintained at 40ºC, and UV detection at 226 nm. The sample injection volume was 10 µl. The LCsolution multi-PDA workstation was employed to acquire and process chromatographic data. The palmarumycin $C_{13}$ was detected and quantified with the standard obtained from our previous study, which was identified according to its physicochemical and spectrometric data (Cai et al. 2009; Li et al. 2012c).

**Evaluation of carbon and nitrogen sources**

Before the statistical optimization experiments, various carbon and nitrogen sources beneficial to *Berkleasmium* sp. Dzf12 mycelia growth and palmarumycin $C_{13}$ production were preliminarily investigated. Six carbon sources including glucose (Glu), sucrose (Suc), fructose (Fru), xylose (Xyl), lactose (Lac), and maltose (Mal), were tested at a fixed concentration of 40.0 g/l. Three organic nitrogen sources including peptone (PEP), corn steep liquor (CSL), and yeast extract (YE), as well as three inorganic nitrogen sources including $KNO_3$, $NH_4Cl$, and urea were tested both separately and in various combinations, all at a fixed total nitrogen source of 10.0 g/l (each at 5.0 g/l for two in combination).

**Statistical experimental design and data analysis**

A screening test was constructed by the Plackett-Burman (P-B) design (Cheilas et al. 2000) on all six nutrients of the basal medium along with the medium pH, to evaluate their effects and to determine the significant factors on palmarumycin $C_{13}$ production. P-B design allows for the screening of $n$ variables with $n$ + 1 runs, each variable set at two levels, high (H) and low (L) shown in Table 1. Therefore, 12 runs in all were performed for the seven test variables plus four dummy variables.

The significant variables screened out by the above P-B test were further optimized by the experiment of central composite design (CCD) and analysis of response surface methodology (RSM). According to the experimental design, a $2^3$ factorial design was required which contained six star points ($\alpha = \pm 1.68$) and six replicates at the center points with a total of 20 runs. The coded and actual values of these three significant factors in the CCD design are shown in Table 2, and the other medium components of the basal medium were fixed as 1.0 g/l of $KH_2PO_4$, 0.5 g/l of $MgSO_4$ x $7H_2O$, 0.05 g/l of $FeSO_4$ x $7H_2O$,

and pH 6.5. Based on the experimental results, a predictive quadratic polynomial equation for the correlation of response to the test variables was determined, and it could be expressed by the following equation (Equation 1):

**Table 1. Levels and values of variables tested in Plackett-Burman design.**

| Level | Component (g/l) | | | | | | pH |
|---|---|---|---|---|---|---|---|
| | Glucose | Peptone | Yeast extract | $KH_2PO_4$ | $MgSO_4$ x $7H_2O$ | $FeSO_4$ x $7H_2O$ | |
| High | 50 | 10 | 10 | 1.5 | 0.75 | 0.075 | 7.0 |
| Low | 25 | 5 | 5 | 0.5 | 0.25 | 0.025 | 5.5 |

$$Y = \beta_0 + \sum_{i=1}^{i=3} \beta_i x_i + \sum_{i=1}^{i=3} \beta_{ii} x_i^{\,2} + \sum_{i<j}^{j=3} \beta_{ij} x_i x_j$$

**[Equation 1]**

Where $Y$ is the predicted response, $\beta_0$ is a constant, $\beta_i$ is the linear coefficient, $\beta_{ii}$ is the quadratic coefficient, and $\beta_{ij}$ isthe cross product coefficient. The response considered in the optimization study is volumetric palmarumycin $C_{13}$ yield. The equation uses coded ones of the actual variables as given by $x_i = (X_i - X^*_i)/\Delta X_i$, where $x_i$ is the coded value of the $i$th test variable, $X_i$ is the actual value of the $i$th test variable, $X^*_i$ is the value of $X_i$ at the central point of the investigated area, and $\Delta X_i$ the step size.

**Table 2. Coded values ($x$) and actual values ($X$) of variables in CCD test.**

| $x_i$ | $X_1$ (Glucose, g/l) | $X_2$ (Peptone, g/l) | $X_3$ (Yeast extract, g/l) |
|---|---|---|---|
| +1.68 | 56.82 | 11.05 | 14.73 |
| +1 | 50 | 9 | 12 |
| 0 | 40 | 6 | 8 |
| -1 | 30 | 3 | 4 |
| -1.68 | 23.18 | 0.95 | 1.27 |

The "Design-Expert" software (version 7.0, Stat-Ease, Inc., Minneapolis, USA) was used for the experimental design and data analysis. All experiments were carried out in triplicate, and the results were represented by their mean values and the standard deviations (SD). The data were submitted to analysis of variance (one-way ANOVA) to detect significant differences by PROC ANOVA of SAS version 8.2. The term significant has been used to denote the differences for which $p \leq 0.05$. The test of significance for the regression coefficients were determined by Fischer's test. The fitness of the second-order polynomial model equation was evaluated by the coefficient of determination, $R^2$. In order to visualize the relationship between the response and test variables, the fitted polynomial equation was separately expressed as 3D-response surfaces curves and 2D-contour plots.

## RESULTS AND DISCUSSION

### Kinetics of *Berkleasmium* sp. Dzf12 in normal batch culture

The time courses of mycelia growth and palmarumycin $C_{13}$ accumulation in liquid cultureof *Berkleasmium* sp. Dzf12 with the modified Sabouraud broth as the basal medium are shown in Figure 1. The mycelia culture exhibited a lag phase in the first three days, and a rapid growth (or exponential) period between day 3 and day 9. A slow growth was observed between day 9 and day 12, and a stationary period in the rest 2 to 3 days. The maximum mycelia biomass of 7.11 g/l was obtained

around day 12. The palmarumycin $C_{13}$ accumulation of *Berkleasmium* sp.Dzf12 remained at a very low level from day 0 to day 5, and then increased steadily from day 5 to day 13, achieving the highest content of 4.07 mg/g dw around day 13. Correspondingly, the maximum palmarumycin $C_{13}$ yield (intracellular palmarumycin $C_{13}$ in mycelia plus extracellular palmarumycin $C_{13}$ in medium) of 130.44 mg/l was obtained on day 13. From day 13 to day 15, palmarumycin $C_{13}$ yield remained stable. The results indicated that day 13 to day 15 was a suitable time for harvesting palmarumycin $C_{13}$ in liquid culture of *Berkleasmium* sp. Dzf12 in the basal medium which should be a reference for the following medium optimization experiments for palmarumycin $C_{13}$ production.

**Comparison of carbon and nitrogen sources**

Table 3 shows the mycelia biomass, palmarumycin $C_{13}$ content in mycelia, palmarumycin $C_{13}$ yield in broth, and total palmarumycin $C_{13}$ yield in *Berkleasmium* sp. Dzf12 mycelia liquid cultures supplemented with different carbon and nitrogen sources. Of the sugars tested, glucose was the most favourable for mycelia growth, and achieved the maximum biomass (6.86 g/l), which was much higher than that (4.01 to 6.06 g/l).

For the palmarumycin $C_{13}$ production in mycelia liquid cultureof *Berkleasmium* sp. Dzf12, the palmarumycin $C_{13}$ contents of mycelia (3.81 mg/g dw with glucose as carbon source, and 3.67 mg/g dw with maltose as carbon source) were higher than with the other four sugars (2.56 to 3.43 mg/g dw). The palmarumycin $C_{13}$ yield (98.64 mg/l with glucose as carbon source, and 85.27 mg/l with maltose as carbon source) in medium were also higher than those (36.88 to 78.25 mg/l) with the other four sugars. Correspondingly, the highest total palmarumycin $C_{13}$ yield (intracellular palmarumycin $C_{13}$ in mycelia plus extracellular palmarumycin $C_{13}$ in medium) of 124.80 mg/l wasobtained with the glucose, which was notably higher than with other sugars (47.16 to 106.34 mg/l) ($p < 0.05$).

For the nitrogen source screening test (Table 3), the *Berkleasmium* sp. Dzf12 mycelia liquid cultures supplemented with an organic nitrogen nutrient (*i.e*., peptone, corn steep liquor, and yeast extract) all achieved fairly high biomass (5.94 to 6.93 g dw/l), while the cultures with only inorganic nitrogen (*i.e*., $KNO_3$, $NH_4Cl$, and urea) showed poor growth with the very low biomass (0.97 to 3.08 g dw/l). Among all nitrogen sources, the combined nitrogen source of peptone with yeast extract (PEP+YE) was the most favorable for the mycelia growth, and yielded the maximum mycelia biomass (8.46 g dw/l), which was notably higher ($p < 0.05$) than with any other nitrogen sources (0.97 to 6.93 g dw/l). For the palmarumycin $C_{13}$ accumulation in *Berkleasmium* sp. Dzf12, the palmarumycin $C_{13}$ contents (7.32 mg/g dw with YE, 6.35 mg/g dw with PEP+YE, and 5.37 mg/g dw with YE+CSL) of mycelia were statistically higher than those with other nitrogen sources. The palmarumycin $C_{13}$ yield in medium (161.35 mg/l with PEP+YE, and 154.58 mg/l with YE) were also statistically different than those with other nitrogen sources (5.13 to 161.35 mg/l). Correspondingly, the maximum total palmarumycin $C_{13}$ yield (215.11 mg/l) was obtained with PEP+YE, which was statistically different than those with the rest of nitrogen sources (6.31 to 202.21 mg/l).

By virtue of the experimental results above, glucose was selected as the preferable carbon source, peptone and yeast extract as the nitrogen sources for mycelia growth and palmarumycin $C_{13}$ production in mycelia liquid culture of *Berkleasmium* sp. Dzf12 in the following optimization experiments.

**Screening of the significant factors using Placket-Burman design**

The ANOVA data for the experimental results from the 12-run Placket-Burman (P-B) screening test are listed in Table 4. Of the seven factors tested, six had the positive effects (glucose, peptone, yeast extract, $KH_2PO_4$ and $FeSO_4 \times 7H_2O$, along with medium pH). On the contrary, $MgSO_4 \times 7H_2O$ had a negative effect on palmarumycin $C_{13}$ accumulation. Three factors including glucose, peptone and yeast extract, had a remarkable effect on palmarumycin $C_{13}$ production at a confidence level $p < 0.05$. Therefore, these three components were selected as the significant variables in the following optimization experiments.

**Table 3. Effects of carbon and nitrogen sources on mycelia growth and palmarumycin $C_{13}$ production in liquid culture of *Berkleasmium* sp. Dzf12.**

| Nutrient | Biomass (g dw/l) | C13 content in mycelia (mg/g dw) | C13 yield in medium (mg/l) | Total C13 yield (mg/l) |
|---|---|---|---|---|
| **Carbon source (all with 10 g/l peptone)** | | | | |
| Glucose | 6.86 ± 0.42$^{a}$ | 3.81 ± 0.27$^{a}$ | 98.64 ± 7.16$^{a}$ | 124.80 ± 9.03$^{a}$ |
| Sucrose | 5.82 ± 0.31$^{b}$ | 3.35 ± 0.25$^{b}$ | 73.12 ± 6.48$^{c}$ | 92.63 ± 7.92$^{c}$ |
| Fructose | 6.06 ± 0.37$^{b}$ | 3.43 ± 0.26$^{b}$ | 78.25 ± 8.12$^{bc}$ | 99.01 ± 9.67$^{c}$ |
| Xylose | 4.94 ± 0.24$^{c}$ | 2.85 ± 0.21$^{c}$ | 45.25 ± 5.36$^{d}$ | 59.30 ± 6.42$^{d}$ |
| Lactose | 4.01 ± 0.40$^{d}$ | 2.56 ± 0.24$^{d}$ | 36.88 ± 5.68$^{e}$ | 47.16 ± 6.62$^{e}$ |
| Maltose | 5.75 ± 0.29$^{b}$ | 3.67 ± 0.26$^{a}$ | 85.27 ± 6.53$^{b}$ | 106.34 ± 8.04$^{b}$ |
| **Nitrogen source (all with 40 g/l glucose)** | | | | |
| Pep | 6.93 ± 0.27$^{b}$ | 3.82 ± 0.32$^{d}$ | 102.35 ± 7.86$^{c}$ | 128.80 ± 10.04$^{d}$ |
| YE | 6.50 ± 0.37$^{b}$ | 7.32 ± 0.35$^{a}$ | 154.58 ± 8.12$^{a}$ | 202.21 ± 10.40$^{b}$ |
| CSL | 5.94 ± 0.45$^{c}$ | 3.61 ± 0.36$^{e}$ | 66.23 ± 6.48$^{e}$ | 87.69 ± 8.63$^{e}$ |
| $KNO_3$ | 2.26 ± 0.36$^{g}$ | 1.83 ± 0.22$^{h}$ | 14.16 ± 1.35$^{h}$ | 18.29 ± 1.84$^{i}$ |
| $NH_4Cl$ | 3.08 ± 0.15$^{f}$ | 2.10 ± 0.19$^{g}$ | 21.25 ± 1.56$^{gh}$ | 27.73 ± 2.13$^{h}$ |
| Urea | 0.97 ± 0.08$^{h}$ | 1.22 ± 0.08$^{i}$ | 5.13 ± 0.56$^{i}$ | 6.31 ± 0.64$^{j}$ |
| Pep + YE | 8.46 ± 0.35$^{a}$ | 6.35 ± 0.32$^{b}$ | 161.35 ± 7.56$^{a}$ | 215.11 ± 10.25$^{a}$ |
| Pep + CSL | 6.71 ± 0.24$^{b}$ | 3.53 ± 0.26$^{e}$ | 93.65 ± 8.64$^{d}$ | 117.35 ± 10.40$^{d}$ |
| Pep + $NH_4Cl$ | 4.76 ± 0.18$^{d}$ | 2.72 ± 0.22$^{f}$ | 46.34 ± 4.28$^{f}$ | 59.26 ± 5.30$^{f}$ |
| YE + CSL | 5.80 ± 0.32$^{c}$ | 5.37 ± 0.31$^{c}$ | 116.39 ± 4.37$^{b}$ | 147.50 ± 6.14$^{c}$ |
| YE + $NH_4Cl$ | 3.89 ± 0.41$^{e}$ | 3.64 ± 0.18$^{e}$ | 51.35 ± 2.46$^{f}$ | 65.52 ± 3.18$^{f}$ |
| CSL + $NH_4Cl$ | 3.64 ± 0.32$^{e}$ | 2.54 ± 0.16$^{f}$ | 28.63 ± 1.88$^{g}$ | 37.85 ± 2.45$^{g}$ |

Note: All values represent means ± standard deviations; Values marked with different letters (*i.e.*, a-j) indicated significant differences among the treatments in each column at $p$ = 0.05. Nutrient symbols: Pep, peptone; YE, yeast extract; CSL, corn steep liquor. Palmarumycin $C_{13}$ was abbreviated as C13.

**Table 4. Main effects of medium components on palmarumycin $C_{13}$ accumulation of *Berkleasmium* sp. Dzf12 by P-B screening test ($R^2$ = 0.9810).**

| Factor | Palmarumycin $C_{13}$ production | |
|---|---|---|
| | Effect | *p*-Value |
| Glucose | 22.72 | 0.0025* |
| Peptone | 15.40 | 0.0077* |
| Yeast extract | 49.36 | 0.0003* |
| $KH_2PO_4$ | 0.20 | 0.9379 |
| $MgSO_4 x 7H_2O$ | -4.73 | 0.1434 |
| $FeSO_4 x 7H_2O$ | 4.47 | 0.1597 |
| pH | 0.38 | 0.8844 |

Note: * means significance at p < 0.05.

**CCD experiments, model building and statistical analysis**

According to the screening of factors by the P-B test, a central composite design (CCD) was developed for variables significantly affecting palmarumycin $C_{13}$ production in *Berkleasmium* sp. Dzf12 liquid cultures. The central composite design of three selected factors (Table 4) in coded along with the palmarumycin $C_{13}$ yield as responses is presented in Table 5. The obtained CCD experimental results of palmarumycin $C_{13}$ yield ($Y$, mg/l) at various conditions ranged from 166.23 to 313.82 mg/l. In order to determine the maximum palmarumycin $C_{13}$ production in *Berkleasmium* sp. Dzf12 mycelia liquid cultures corresponding to the optimum levels of glucose, peptone, and yeast extract, a quadratic polynomial model was proposed to calculate the optimum levels of these three test variables. By employing multiple regression analysis on the experimental data, the response variable ($Y$) and the tested variables were related by the following second-order polynomial equation (Equation 2):

$$Y = -173.30 + 12.41\ X_1 + 14.67\ X_2 + 33.95\ X_3 - 0.07\ X_1X_2 + 0.14\ X_1X_3 - 0.07\ X_2X_3 - 0.16\ X_1^2 - 0.83\ X_2^2 - 1.80\ X_3^2$$

**[Equation 2]**

Where $Y$ is the predicted palmarumycin $C_{13}$ yield, and $X_1$, $X_2$, and $X_3$ are the actual values of glucose, peptone, and yeast extract, respectively. Table 6 showed the results of the quadratic polynomial model in the form of ANOVA. The Fisher's $F$-test with a high model $F$-value of 107.93 but a very low probability value [($p > F$) < 0.0001] indicated the model was highly significant. The fitness of the model can be checked by the determination coefficient ($R^2$ = 0.9898), which suggested that the sample variation of 98.98% was attributed to the independent variables and only about 1.02% of the total variance could not be explained by the model. The closer the value of $R$ is to 1, the better the fitness of the model is (Pujari and Chandra, 2000; Xiao et al. 2007; Mishra et al. 2012). The adjusted determination coefficient (Adj-$R^2$ = 0.9806) and predicted determination coefficient ($R^2$ = 0.9377) were also satisfactory to confirm the significance of the model. The lack-of-fit measures the failure of the model to represent the data in the experimental domain at points which were not included in the regression. The model showed statistically insignificant lack of fit [($p > F$) = 0.1127], so the model was supposed to be adequate for prediction within the range of variables employed.

**Response surface curve and contour plot analyses**

The two-dimensional (2D) contour plot and three-dimensional (3D) response surface curve are generally the graphical representation of the regression equation. The contour plots are helpful in identification of the types of interactions between two test variables, and the response surface curve could provide a convenient method to locate the optimum ranges of the variables, as well as to predict the response (palmarumycin $C_{13}$ yield) efficiently. In the present study, each figure presented the effect of two test variables on palmarumycin $C_{13}$ production, while the third variable was fixed at the zero level.

The 3D response surface curves and 2D contour plots in Figure 2 showed the effects of glucose, peptone, yeast extract, and their interactions on palmarumycin $C_{13}$ accumulation in mycelia liquid cultures of *Berkleasmium* sp. Dzf12.

It can be clearly obtained from Figures $2A_1$, $2B_1$ and $2C_1$ thatthe 3D response surface curves were convex in nature implying that there were well-defined concentrations for glucose, peptone and yeast extract. In addition, as the 2D contour lines were nearly horizontal shown in Figures $2A_2$ and $2C_2$, the interactions between glucose and peptone, or between peptone and yeast extract were not significant. That can also be checked by the cross-product coefficient of glucose and peptone with their $p$ value of 0.3730, as well as that of 0.7217 for peptone and yeast extract (Table 6). As shown in Figure $2B_2$, the contour line plot on glucose and yeast extract was oriented diagonally, which implied a significant interaction between the two test variables on palmarumycin $C_{13}$ production. Moreover, the 2D contour line plots shown in Figure $2B_2$ (on glucose and yeast extract) and Figure $2C_2$ (on peptone and yeast extract) were much denser than those in Figure $2A_2$ (on glucose and peptone), indicating that the effect of yeast extract on palmarumycin $C_{13}$ accumulation was more notable than that of glucose or peptone. This was in accordance with previous observations (Yu et al. 2010; Zhao et al. 2011b).

**Table 5. Central composition design (CCD) of three variables in coded along with palmarumycin $C_{13}$ yield as response value.**

| Run | Glucose ($x_1$) | Peptone ($x_2$) | Yeast extract ($x_3$) | Palmarumycin $C_{13}$ (mg/l) |
|---|---|---|---|---|
| **1** | -1 | 1 | -1 | 218.52 |
| **2** | 0 | 0 | 0 | 305.80 |
| **3** | 0 | 0 | -1.68 | 166.23 |
| **4** | 0 | 0 | 0 | 312.64 |
| **5** | 1 | -1 | 1 | 305.61 |
| **6** | 0 | 0 | 0 | 310.82 |
| **7** | 0 | -1.68 | 0 | 284.55 |
| **8** | 0 | 0 | 0 | 304.82 |
| **9** | -1 | -1 | 1 | 283.71 |
| **10** | -1 | 1 | 1 | 293.30 |
| **11** | 1 | -1 | -1 | 204.54 |
| **12** | 1 | 1 | 1 | 306.92 |
| **13** | 0 | 0 | 1.68 | 297.65 |
| **14** | 0 | 0 | 0 | 313.82 |
| **15** | -1.68 | 0 | 0 | 259.25 |
| **16** | 0 | 0 | 0 | 303.52 |
| **17** | 1 | 1 | -1 | 209.03 |
| **18** | 0 | 1.68 | 0 | 299.81 |
| **19** | 1.68 | 0 | 0 | 277.52 |
| **20** | -1 | -1 | -1 | 205.56 |

**Table 6. ANOVA for the fitted quadratic polynomial model for optimization of palmarumycin $C_{13}$ yield.**

| Source | Sum of squares | Degree of freedom | Mean square | *F*-value | *p*>F |
|---|---|---|---|---|---|
| Model | 39398.76 | 9 | 4377.64 | 107.93 | <0.0001 |
| $X_1$ | 227.80 | 1 | 227.80 | 5.62 | 0.0393 |
| $X_2$ | 214.56 | 1 | 214.56 | 5.29 | 0.0443 |
| $X_3$ | 24040.29 | 1 | 24040.29 | 592.70 | <0.0001 |
| $X_1X_1$ | 3649.99 | 1 | 3649.99 | 89.99 | <0.0001 |
| $X_2X_2$ | 810.63 | 1 | 810.63 | 19.99 | 0.0012 |
| $X_3X_3$ | 11954.60 | 1 | 11954.60 | 294.74 | <0.0001 |
| $X_1X_2$ | 35.28 | 1 | 35.28 | 0.87 | 0.3730 |
| $X_1X_3$ | 264.50 | 1 | 264.50 | 6.52 | 0.0287 |
| $X_2X_3$ | 5.44 | 1 | 5.44 | 0.13 | 0.7217 |
| Residual | 405.60 | 10 | 40.56 | | |
| Lack of fit | 309.45 | 5 | 61.89 | 3.22 | 0.1127 |
| Pure error | 96.16 | 5 | 19.23 | | |
| Corrected total | 39804.37 | 19 | | | |

$R^2$ = 0.9898; Adj-$R^2$ = 0.9806; Predicted $R^2$ = 0.9377.

### Validation of the experimental design

Based on the statistical analysis of the experimental results obtained, the optimal values of the test variables were calculated as follows: 42.5 g/l of glucose ($X_1$), 6.5 g/l of peptone ($X_2$), and 11.0 g/l of yeast extract ($X_3$), with which the maximum palmarumycin $C_{13}$ yield could be predicted as much as 325.4 mg/l. Verification experiments were accomplished by using the above optimized culture conditions, and attained a mean value of palmarumycin $C_{13}$ yield as 318.63 mg/l ($n$ = 5), which was in close agreement with the model-predicted response of 325.45 mg/l. Therefore, the repeat experiments corroborated the predicted values and the accuracy of the model equation. Moreover, under the optimized culture medium, the total palmarumycin $C_{13}$ yield was most effectively enhanced about 2.5-fold (318.63 mg/l versus 130.44 mg/l in the basal media), implying that the optimized medium favors the palmarumycin $C_{13}$ production in mycelia liquid culture of *Berkleasmium* sp. Dzf12.

## CONCLUDING REMARKS

In this work, the experimental designs based on statistics were proved to be valuable and efficient tools in optimizing medium for palmarumycin $C_{13}$ production in liquid culture of *Berkleasmium* sp. Dzf12. Through the one-factor-at-a-time experiments, glucose was selected as the most favourable carbon source, and peptone and yeast extract were found to be the most favourable nitrogen sources for palmarumycin $C_{13}$ accumulation. Then the method of Plackett-Burman test was employed to screen glucose, peptone and yeast extract to be the significant factors to stimulate palmarumycin $C_{13}$ production. Moreover, these three factors were further optimized through the experiments of central composite design (CCD) and the analysis of response surface methodology (RSM), and the optimized medium for palmarumycin $C_{13}$ production was determined as 42.5 g/l of glucose, 6.5 g/l of peptone, and 11.0 g/l of yeast extract. Under the optimal culture conditions, the maximum palmarumycin $C_{13}$ production of *Berkleasmium* sp. Dzf12 was increased to 318.63 mg/l, which was about 2.5-fold in comparison with that (130.44 mg/l) in the basal medium. The present study demonstrates the feasibility and promising potential of *Berkleasmium* sp. Dzf12 mycelia fermentation for efficient production of palmarumycin $C_{13}$. After a series of optimizations for palmarumycin $C_{13}$ biosynthesis conditions, we could achieve the final optimized media for maximum production of palmarumycin $C_{13}$ by natural fermentation of the endophytic fungus *Berkleasmium* sp. Dzf12.

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## REFERENCES

ALY, A.H.; DEBBAB, A. and PROKSCH, P. (2011). Fungal endophytes: Unique plant inhabitants with great promises. *Applied Microbiology and Biotechnology*, vol. 90, no. 6, p. 1829-1845. **[CrossRef]**

CAI, X.; SHAN, T.; LI, P.; HUANG, Y.; XU, L.; ZHOU, L.; WANG, M. and JIANG, W. (2009). Spirobisnaphthalenes from *Berkleasmium* sp. Dzf12 of *Dioscorea zingiberensis* and their antimicrobial activities. *Natural Product Communications*, vol. 4, no. 11, p. 1469-1472.

CAI, Y.S.; GUO, Y.W. and KROHN, K. (2010). Structure, bioactivities, biosynthetic relationships and chemical synthesis of the spirodioxynaphthalenes. *Natutal Product Reports*, vol. 27, no. 12, p. 1840-1870. **[CrossRef]**

CHEILAS, T.; STOUPIS, T.; CHRISTAKOPOULOS, P.; KATAPODIS, P.; MAMMA, D.; HATZINIKOLAOU, D.G.; KEKOS, D. and MACRIS, B.J. (2000). Hemicellulolytic activity of *Fusarium oxysporum* grown on sugar beet pulp. Production of extracellular arabinanase. *Process Biochemistry*, vol. 35, no. 6, p. 557-561. **[CrossRef]**

CHU, M.; TRUUMEES, I.; PATEL, M.G.; GULLO, V.P.; BLOOD, C.; KING, I.; PAI, J.K. and PUAR, M.S. (1994). A novel class of antitumor metabolites from the fungus *Nattrassia mangiferae*. *Tetrahedron Letters*, vol. 35, no. 9, p. 1343-1346. **[CrossRef]**

DJEKRIF-DAKHMOUCHE, S.; GHERIBI-AOULMI, Z.; MERAIHI, Z. and BENNAMOUN, L. (2006). Application of a statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. *Journal of Food Engineering*, vol. 73, no. 2, p. 190-197. **[CrossRef]**

GUNATILAKA, A.A.L. (2006). Natural products from plant-associated microorganisms: Distribution, structural diversity, bioactivity, and implications of their occurrence. *Journal of Natural Products*, vol. 69, no. 3, p. 509-526. **[CrossRef]**

KHARWAR, R.N.; MISHRA, A.; GOND, S.K.; STIERLE, A. and STIERLE, D. (2011). Anticancer compounds derived from fungal endophytes: Their importance and future challenges. *Natural Product Reports*, vol. 28, no. 7, p. 1208-1228. **[CrossRef]**

KROHN, K.; MICHEL, A.; FLORKE, U.; AUST, H.J.; DRAEGER, S. and SCHULZ, B. (1994). Biologically active metabolites from fungi. 5. Palmarumycins $C_1$-$C_{16}$ from *Coniothyrium*sp.: Isolation, structure elucidation, and biological activity. *Liebigs Annalen der Chemie*, vol. 1994, no. 11, p. 1099-1108. **[CrossRef]**.

LI, P.; MAO, Z.; LOU, J.; LI, Y.; MOU, Y.; LU, S.; PENG, Y. and ZHOU, L. (2011). Enhancement of diosgenin production in *Dioscorea zingiberensis* cell cultures by oligosaccharides from its endophytic fungus *Fusarium oxysporum* Dzf17. *Molecules*, vol. 16, no. 12, p. 10631-10644. **[CrossRef]**

LI, P.; LU, S.; SHAN, T.; MOU, Y.; LI, Y.; SUN, W. and ZHOU, L. (2012a). Extraction optimization of water-extracted mycelial polysaccharide from endophytic fungus *Fusarium oxysporum* Dzf17 by response surface methodology. *International Journal of Molecular Sciences*, vol. 13, no. 5, p. 5441-5453. **[CrossRef]**

LI, P.; XU, L.; MOU, Y.; SHAN, T.; MAO, Z.; LU, S.; PENG, Y. and ZHOU, L. (2012b). Medium optimization for exopolysaccharide production in liquid culture of endophytic fungus *Berkleasmium* sp. Dzf12. *International Journal of Molecular Sciences*, vol. 13, no. 9, p. 11411-11426. **[CrossRef]**

LI, Y.; SHAN, T.; MOU, Y.; LI, P.; ZHAO, J.; ZHAO, W.; PENG, Y.; ZHOU, L. and DING, C. (2012c). Enhancement of palmarumycin $C_{12}$ and $C_{13}$ production in liquid culture of the endophytic fungus *Berkleasmium* sp. Dzf12 by oligosaccharides from its host plant *Dioscorea zingiberensis*. *Molecules*, vol. 17, no. 4, p. 3761-3773. **[CrossRef]**

LIU, J.G.; XING, J.M.; CHANG, T.S.; MA, Z.Y. and LIU, H.Z. (2005). Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. *Process Biochemistry*, vol. 40, no. 8, p. 2757-2762. **[CrossRef]**

MISHRA, P.; KSHIRSAGAR, P.R.; NILEGAONKAR, S.S. and SINGH, S.K. (2012). Statistical optimization of medium components for production of extracellular chitinase by *Basidiobolus ranarum*: A novel biocontrol agent against plant pathogenic fungi. *Journal of Basic Microbiology*, vol. 52, no. 5, p. 539-548. **[CrossRef]**

MOU, Y.; LUO, H.; MAO, Z.; SHAN, T.; SUN, W.; ZHOU, K. and ZHOU, L. (2013). Enhancement of palmarumycins $C_{12}$ and $C_{13}$ production in liquid culture of endophytic fungus *Berkleasmium* sp. Dzf12 after treatment with metal ions. *International Journal of Molecular Sciences*, vol. 14, no. 1, p. 979-998. **[CrossRef]**

MU, W.M.; CHEN, C.; LI, X.F.; ZHANG, T. and JIANG, B. (2009). Optimization of culture medium for the production of phenyllactic acid by *Lactobacillus* sp. SK007. *Bioresource Technology*, vol. 100, no. 3, p. 1366-1370. **[CrossRef]**

PETERSEN, F.; MOERKER, T.; VANZANELLA, F. and PETER, H.H. (1994). Production of cladospirone bisepoxide, a new fungal metabolite. *The Journal of Antibiotics*, vol. 47, no. 10, p. 1098-1103. **[CrossRef]**

PIO, T.F. and MACEDO, G.A. (2007). Optimizing the production of cutinase by *Fusarium oxysporum* using response surface methodology. *Enzyme and Microbial Technology*, vol. 41, no. 5, p. 613-619. **[CrossRef]**

PUJARI, V. and CHANDRA, T.S. (2000). Statistical optimization of medium components for enhanced riboflavin production by a UV-mutant of *Eremothecium ashbyii*. *Process Biochemistry*, vol. 36, no. 1-2, p. 31-37. **[CrossRef]**

RODRÍGUEZ, R.J.; WHITE, J.F.; ARNOLD, A.E. and REDMAN, R.S. (2009). Fungal endophytes: Diversity and functional roles. *New Phytologist*, vol. 182, no. 2, p. 314-330. **[CrossRef]**

SCHLINGMANN, G.; WEST, R.R.; MILNE, L.; PEARCE, C.J. and CARTER, G.T. (1993). Diepoxins, novel fungal metabolites with antibiotic activity. *Tetrahedron Letters*, vol. 34, no. 45, p. 7225-7228. **[CrossRef]**

TURLO, J.; GUTKOWSKA, B.; HEROLD, F.; KRZYCZKOWSKI, W.; BLAŻEWICZ, A. and KOCJAN, R. (2008). Optimizing vitamin $B_{12}$ biosynthesis by mycelial cultures of *Lentinula edodes* (Berk.) Pegl. *Enzyme and Microbial Technology*, vol. 43, no. 4-5, p. 369-374. **[CrossRef]**

WANG, Z.W. and LIU, X.L. (2008). Medium optimization for antifungal active substances production from a newly isolated *Paenibacillus* sp. using response surface methodology. *Bioresource Technology*, vol. 99, no. 17, p. 8245-8251. **[CrossRef]**

XIAO, Z.J.; LIU, P.H.; QIN, J.Y. and XU, P. (2007). Statistical optimization of medium components for enhanced acetoin production from molasses and soybean meal hydrolysate. *Applied Microbiology and Biotechnology*, vol. 74, no. 1, p. 61-68. **[CrossRef]**

XU, H.; SUN, L.P.; SHI, Y.Z.; WU, Y.H.; ZHANG, B. and ZHAO, D.Q. (2008). Optimization of cultivation conditions for extracellular polysaccharide and mycelium biomass by *Morchella esculenta* As51620. *Biochemical Engineering Journal*, vol. 39, no. 1, p. 66-73. **[CrossRef]**

XU, L.J.; LIU, Y.S.; ZHOU, L.G. and WU, J.Y. (2009). Enhanced beauvericin production with in situ adsorption in mycelial liquid culture of *Fusarium redolens* Dzf2. *Process Biochemistry*, vol. 44, no. 10, p. 1063-1067. **[CrossRef]**

XU, L.J.; LIU, Y.S.; ZHOU, L.G. and WU, J.Y. (2010). Optimization of a liquid medium for beauvericin production in *Fusarium redolens* Dzf2 mycelial culture. *Biotechnology and Bioprocess Engineering*, vol. 15, no. 3, p. 460-466. **[CrossRef]**

YU, J.; LI, Z.; YE, Q.; YANG, Y. and CHEN, S. (2010). Development of succinic acid production from corncob hydrolysate by *Actinobacillus succinogenes*. *Journal of Industrial Microbiology & Biotechnology*, vol. 37, no. 10, p. 1033-1040. **[CrossRef]**

ZHI-LIN, Y.; YI-CUN, C.; BAI-GE, X. and CHU-LONG, Z. (2012). Current perspectives on the volatile-producing fungal endophytes. *Critical Reviews in Biotechnology*, vol. 32, no. 4, p. 363-373. **[CrossRef]**

ZHANG, H.W.; SONG, Y.C. and TAN, R.X. (2006). Biology and chemistry of endophytes. *Natural Product Reports*, vol. 23, no. 5, p. 753-771. **[CrossRef]**

ZHANG, R.; LI, P.; XU, L.; CHEN, Y.; SUI, P.; ZHOU, L. and LI, J. (2009). Enhancement of diosgenin production in *Dioscorea zingiberensis* cell culture by oligosaccharide elicitor from its endophytic fungus *Fusarium oxysporum* Dzf17. *Natural Product Communications*, vol. 4, no. 11, p. 1459-1462.

ZHAO, J.L.; ZHOU, L.G. and WU, J.Y. (2010). Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in *Salvia miltiorrhiza* cell cultures. *Applied Microbiology and Biotechnology*, vol. 87, no. 1, p. 137-144. **[CrossRef]**

ZHAO, J.; SHAN, T.; MOU, Y. and ZHOU, L. (2011a). Plant-derived bioactive compounds produced by endophytic fungi. *Mini-Reviews in Medicinal Chemistry*, vol. 11, no. 2, p. 159-168. **[CrossRef]**

ZHAO, J.; ZHENG, B.; LI, Y.; SHAN, T.; MOU, Y.; LU, S.; LI, P. and ZHOU, L. (2011b). Enhancement of diepoxin ζ production by yeast extract and its fractions in liquid culture of *Berkleasmium*-like endophytic fungus Dzf12 from *Dioscorea zingiberensis*. *Molecules*, vol. 16, no. 1, p. 847-856. **[CrossRef]**

ZHOU, L.G. and WU, J.Y. (2006). Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. *Natural Product Reports*, vol. 23, no. 5, p. 789-810. **[CrossRef]**

ZHOU, L.; CAO, X.; ZHANG, R.; PENG, Y.; ZHAO, S. and WU, J. (2007). Stimulation of saponin production in *Panax ginseng* hairy roots by two oligosaccharides from *Paris polyphylla* var. *yunnanensis*. *Biotechnology Letters*, vol. 29, no. 4, p. 631-634. **[CrossRef]**

ZHOU, L.; ZHAO, J.; SHAN, T.; CAI, X. and PENG, Y. (2010). Spirobisnaphthalenes from fungi and their biological activities. *Mini-Reviews in Medicinal Chemistry*, vol. 10, no. 10, p. 977-989. **[CrossRef]**

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# Figures

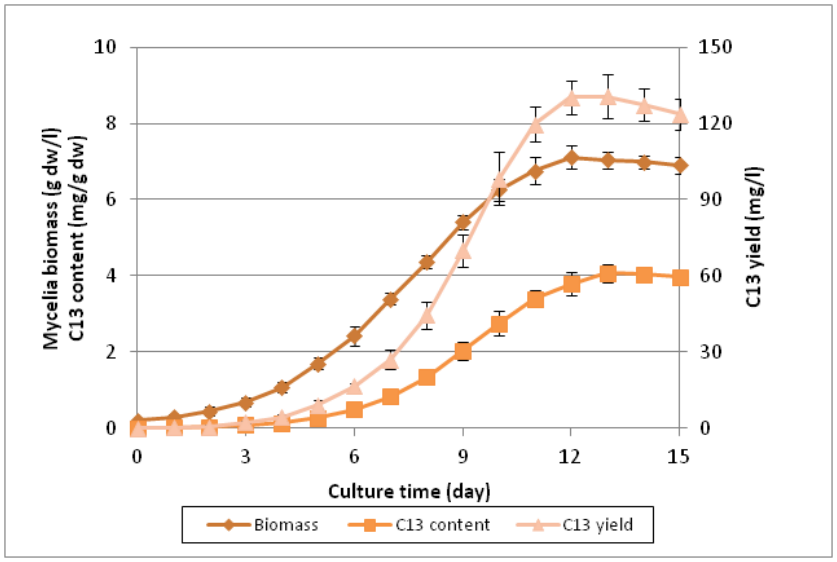


**Fig. 1 Time courses of mycelia growth and palmarumycin $C_{13}$ production in liquid culture of *Berkleasmium* sp. Dzf12 (error bars for standard deviations, n = 3).** Palmarumycin $C_{13}$ was abbreviated as C13.

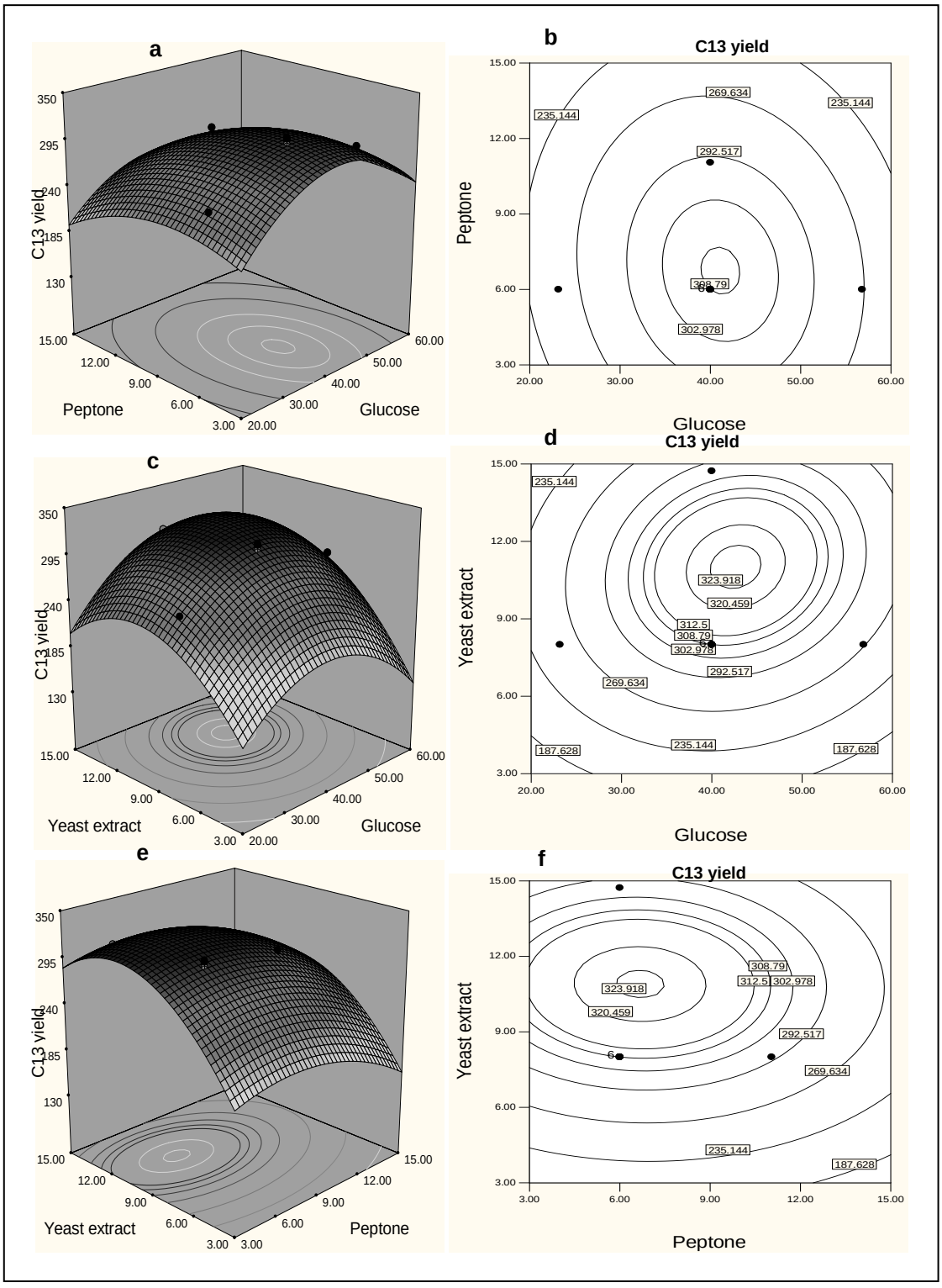


**Fig. 2. 3D-response surface curves (a, c and d) and 2D-contour plots (b, d and f) of palmarumycin $C_{13}$ yield (mg/l) versus the test variables (glucose, peptone and yeast extract at g/l in medium) and their reciprocal interactions.**