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Optimization of submerged culture conditions for roridin E production from the poisonous mushroom *Podostroma cornu-damae*

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ABSTRACT: Roridin E, produced by *Podostroma cornu-damae*, is a mycotoxin with anticancer activity. To increase the content of roridin E, submerged culture conditions were optimized using response surface methodology. Three factors, namely, medium initial pH, incubation time and agitation speed were optimized using a Box–Behnken design. The optimum submerged culture conditions to increase the content of roridin E included a medium with an initial pH of 4.0, an incubation time of 12.90 days, and an agitation speed of 63.03 rpm. The roridin E content in the submerged culture, under the aforementioned conditions, was 40.26 mg/L. The findings of this study can help lower the current price of roridin E and promote its related research.

KEYWORDS: *Podostroma cornu-damae*, Poisonous mushroom, Response surface methodology, Roridin E, Submerged culture conditions

INTRODUCTION

Mushrooms have long been consumed in oriental countries because of their unique aroma, texture, and various pharmacological properties (Wasser, 2010). More than 14000 mushroom species are known worldwide, and, at present, approximately 7000 species can be consumed and about 10 species are cultivated on an industrial scale. It has been reported that approximately 2000 mushroom species have medicinal properties. Of these, poisonous mushrooms are less than 1%; however, their presence cannot be ignored as they can be dangerous and lethal (Miles and Chang, 2004). Therefore, studies on the biological activity, structure, biosynthetic pathway, and action mechanism of the toxic compounds present in poisonous mushrooms have been conducted (Sharma *et al*., 2018; Jo *et al*., 2014; Zhu *et al*., 2020).

Podostroma cornu-damae belongs to the Hypocreaceae family and produces a deadly poison called trichothecene. Since this mushroom is indistinguishable from the young fruiting bodies of reishi mushrooms or cordyceps, which are known as healthy foods, accidental poisoning by ingestion have been frequently reported (Park *et al*., 2016; Yang *et al*., 2013; Yu *et al*., 2013). In *P. cornu-damae*, substances such as roridin E, satratoxin H, 5 12-episatratoxin H, roridin L-2, and trichoverritone are present.

Recently, it was reported that roridin E has a more potent activity in breast cancer cells than doxorubicin, an existing therapeutic substance (Lee *et al*., 2018). It also has a potential as an antimalarial agent (Zhang *et al*., 2002). However, roridin E is very expensive substance (selling for $245 for 1 mg, Biovision, Inc.), and therefore, an optimization study is required to increase the concentration of roridin E extracted from *P. cornu-damae* mycelia.

The response surface methodology (RSM) was used to optimize the liquid culture conditions. It is one of the most

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popular optimization techniques, which greatly reduces the number of experiments required and provides a set of mathematical equations for process optimization (Cui and Yuan., 2011; Wu *et al*., 2017).

The Box–Behnken design (BBD), consisting of central and middle points on the edges of the cube circumscribed on the sphere, has been widely used to optimize the RSM. The BBD produces a statistical model with fewer design points than that of a central composite design (Ferreira *et al*., 2007). Muthukumar *et al*. (2003) utilized BBD for optimization 6 variables.

In this study, we aimed to optimize the submerged culture conditions (medium, incubation time, medium initial pH, and agitation speed) by using the RSM, to increase the content of roridin E extracted from *P. cornu-damae*.

MATERIALS AND METHODS

Reagents and standards

The Roridin E standard was purchased from BioVision, Inc. (California, USA). Potato dextrose agar (PDA), potato dextrose broth (PDB), and yeast extract were purchased from BD (New Jersey, USA). Glucose was purchased from Sigma-Aldrich Co. (Darmstadt, Germany). Mushroom complete medium (MCM) broth was purchased from MB cells (Seoul, South Korea). KH_2PO_4 was purchased from Junsei Chemical Co. (Tokyo, Japan). $MgSO_4 \cdot 7H_2O$ was purchased from Samchun (Seoul, South Korea). KCl, $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, HCl, and NaOH were purchased from Daejung Chemicals & Metals Co. (Gyeonggi-do, South Korea).

Microorganism

P. cornu-damae was obtained from the BCRC (FU31130, Bioresource Collection and Research Center, Hsinchu, Taiwan). The stock cultures were maintained on PDA plates, and the plates were incubated at 25°C for 30 days and stored at 4°C for use as subcultures every two months. *P. cornu-damae* was initially grown on PDA medium at 25°C, and the mycelia were harvested after 30 days for further experiments.

Culture media

Czapek mineral salts broth (CZB) medium (Jackson *et al*., 1991) (glucose 30 g/L; yeast extract 2.8 g/L; KH_2PO_4 1 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; KCl 0.5 g/L; $FeSO_4 \cdot 7H_2O$ 0.01 g/L; $ZnSO_4 \cdot 7H_2O$ 0.01 g/L; $CuSO_4 \cdot 5H_2O$ 0.005 g/L). MCM broth (glucose 20 g/L; peptone 2 g/L; yeast extract 2 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; KH_2PO_4 0.46 g/L; K_2HPO_4 1 g/L). PDB medium (glucose 20 g/L; potato extract 4 g/L). Yeast extract $MgSO_4 \cdot 7H_2O$ KH_2PO_4 (YMK) medium (glucose 20 g/L; yeast extract 5 g/L; $MgSO_4 \cdot 7H_2O$ 1 g/L; KH_2PO_4 2 g/L).

Submerged Culture

Submerged culture using liquid medium has the advantage of not only being able to control the seed amount to some extent but also greatly shortens the cultivation period of the mycelia (Stamets, 1993). Three mycelial agar disks (5 mm) were obtained using a sterilized cork borer and transferred to 100 mL Erlenmeyer flasks containing 50 mL of media. The incubation time was 15 days with intervals of 3 days in between (3, 6, 9, 12, and 15 days). The initial pH of the medium was adjusted using 1N HCl and 1N NaOH (pH 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0). The mycelia were cultivated at 24°C in a shaking incubator (SI-900R, JEIO TECH, Daejeon, South Korea), and the agitation speeds were set at 0, 50, 100, and 150 rpm.

Determination of mycelial dry weight

The samples collected from the shake flasks were centrifuged at 6000×g for 15 min, and the supernatant was filtered through a pre-weighed No. 2 Whatman filter paper (Whatman plc, Maidstone, UK). The centrifuged mycelia were washed with distilled water and collected by filtration through the Whatman filter paper. The dry weights of the mycelia were measured after oven drying at 60°C for 48 h to a constant dry weight.

Determination of roridin E

Mycelia (whole cultured) were extracted overnight with 90% MeOH (30 mL). The MeOH phase was filtered and mixed with distilled water (30 mL). And aqueous phase was evaporated in vacuo to obtain the water layer. The water layer was extracted with ethyl acetate (30 mL ×3). A culture broth (30 mL) of *P. cornu-damae* was extracted with ethyl acetate (30 mL ×3), and the ethyl acetate layers were filtered using a 0.2 μm syringe filter (16534k, Sartorius, Göttingen, Germany).

To measure roridin E, an HPLC system (YL9100 plus, YOUNG IN Chromass, Gyeonggi-do, South Korea) was equipped with an Eclipse plus C18 column (5 μm, 4.6 × 250 mm, Agilent Technologies, Inc., California, USA). Samples were eluted with gradients of 25% to 100%

Table 1. Code of three factor, Box–Behnken experimental design

Independent variables	Levels		
	-1	0	1
X_1: Initial pH	4.0	6.0	8.0
X_2: Incubation time, day	9	12	15
X_3: Agitation speed, rpm	50	100	150

acetonitrile in water for 30 min as the mobile phase. The injection volume was 10 μL and the flow rate was 2 mL/min. The eluent was detected using a UV/visible detector at 260 nm and quantified using the external calibration based on the UV signal response by 0.0625-1 μg/mL of roridin E.

Experimental design by the RSM

A three-level BBD in the RSM was employed in the present study, and the optimal conditions were determined using minimal set of experiments, compared with other designs (Dong *et al*., 2009). The BBD has been applied to optimize culture conditions for submerged cultivation of mushrooms (Jing *et al*., 2018); in this study also, BBD was used to optimize the roridin E (Y) of *P. cornu-damae*. The three factors chosen for this study (Table 1) were initial pH (pH, X_1), incubation time (days, X_2), and agitation speed (rpm, X_3) with three levels of each factor, namely high (coded as 1), middle (coded as 0), and low (coded as -1). For a three-factor, three-level design, 17 experimental trials were conducted. The BBD results were fitted to a second-order polynomial equation (Eq. (1)) using a multiple regression technique,

$$Y = \beta_0 + \Sigma_{i=1}^{4}\beta_i x_i + \Sigma_{i=1}^{4}\beta_{ii} x_i^2 + \Sigma_{i=1}^{3}\Sigma_{i<j}^{4}\beta_{ij} x_i x_{ij} \quad (1)$$

where Y is the predicted response (roridin E yield in this study, mg/L), β0, βi, βii, and βij are constant coefficients, and x_i and x_{ij} are the coded independent variables or factors. Design-Expert software (Version 13, Stat-Ease, Inc., Minnesota, USA) was used for regression analysis of the experimental data and responses.

Statistical analysis

Data are presented as mean ± standard deviation (n = 3). Statistical analyses of the results were performed at a 5% significance level using Statistical Analysis System (SAS) software (SAS Institute, Inc., North Carolina, USA). Differences between the means of individual groups were assessed using SAS with Duncan's multiple-range test.

RESULTS AND DISCUSSION

Effect of various media on mycelial dry weight and roridin E production

It has been reported that an acidic pH and appropriate media components are favorable for the growth of mycelia and for the production of metabolites in many of the ascomycetes and basidiomycetes members (Osińska-Jaroszuk *et al*., 2015). The effects of liquid media on the mycelium dry weight and roridin E content of *P. cornu-damae* are depicted in Fig. 1. When cultured for 15 days, the dry weight of mycelium in each medium was 6.55 ± 0.64 g/L, 5.89 ± 0.39 g/L, 6.02 ± 1.15 g/L, 6.39 ± 0.46 g/L, and for CZB, MCM, PDB and YMK media, respectively.

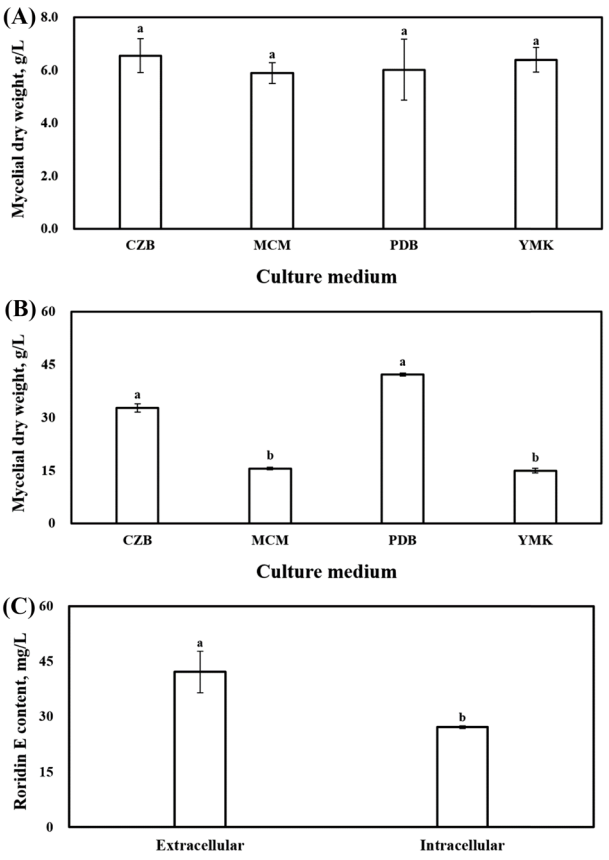

Fig. 1. The effects of various media on the mycelial dry weight and roridin E content in the submerged cultures of *P. cornu-damae*. A: mycelial dry weight; B: roridin E content; and C: roridin E presence in PDB medium. Each value is expressed as mean±SE (n = 3). Different letters on the top of the line represent statistically significant at 5% probability level. CZB, Czapek mineral salts broth medium; MCM, mushroom complete medium; PDB, potato dextrose broth; YMK, yeast extract $MgSO_4 \cdot 7H_2O$ KH_2PO_4.

There was no significant difference in the mycelial dry weight between the groups, although the content of roridin E was different. After 15 days, the maximum roridin E content was observed in PDB media (42.14 ± 5.63 mg/L) followed by CZB (32.75±6.11 mg/L), MCM (15.59±5.24 mg/L), and YMK (14.98±1.99 mg/L). The roridin E content in the extracellular and intracellular form in *P. cornu-damae* cultivated in PDB medium was also determined. It was observed that the roridin E content in the extracellular form (42.14±5.63 mg/L) was 1.6 times higher than that in the intracellular form (27.16±0.37 mg/L). Therefore, the PDB medium displaying the highest roridin E content of *P. cornu-damae* was selected for further experiments.

One-factor-at-a-time experiment

Effect of incubation time on roridin E production

To evaluate the production of roridin E we optimized the culture condition using PDB medium. The culture was performed for 15 days at intervals of 3 days at 100 rpm and incubated at 24°C. It was found that the content of roridin E increased as the incubation time increased. The highest content was observed on day 15 (40.33±6.29 mg/L), although there was no significant difference as compared to the content on day 12 (37.45±5.61 mg/L). This tendency was similar to the result of maintaining a constant after increasing the content of metabolites as the incubation time increased (Adnan *et al*., 2017). Therefore, for efficiency, it was determined that a culture time of 12 days was most suitable for the culture of *P. cornu-damae*.

Effect of initial pH on roridin E production

Fig. 3 depicts the concentration of roridin E in *P. cornu-damae* at different initial pH values. The initial pH of the medium was set to pH 2.0–pH 12.0. Culturing was performed at 100 rpm and 24 °C using PDB medium for 12 days. Firstly, it was observed that roridin E content was very low under the extreme conditions of pH 2.0 (3.08± 0.99 mg/L) and pH 12.0 (7.12±0.3 mg/L). The highest content of roridin E was observed in the mildly acidic conditions, including pH 4.0 (24.48±8.70 mg/L) and pH 6.0 (28.07±0.29 mg/L). Among these, the content of roridin E was the highest at pH 6.0, although there was no significant difference between the two. These results were consistent with reports of increased metabolite production in several mushrooms when the initial pH was mildly acidic (Meng *et al*., 2016; Osińska-Jaroszuk *et al*., 2015)

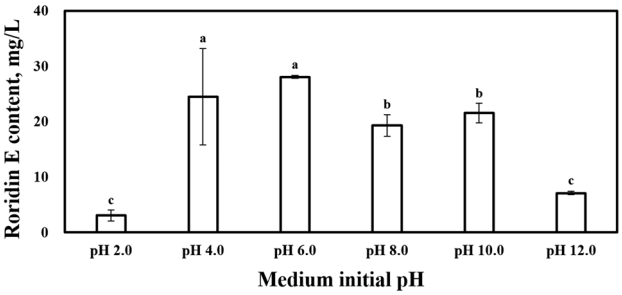

Fig. 3. The effects of medium initial pH on roridin E content by *P. cornu-damae* in submerged culture. Each value is expressed as mean ± SE (n = 3). Different letters on the top of the line represent statistically significant at 5% probability level.

Effect of agitation speed on roridin E production

To compare the content of roridin E based on the agitation speed during the submerged culture, culturing was performed at 0, 50, 100, and 150 rpm (Fig. 4). It was observed that an agitation speed of 100 rpm (38.45±2.15 mg/L) was most suitable for producing

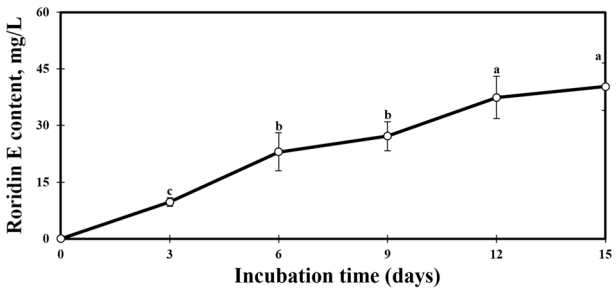

Fig. 2. The effects of incubation time on roridin E content by *P. cornu-damae* in submerged culture. Each value is expressed as mean±SE (n = 3). Different letters on the top of the line represent statistically significant at 5% probability level.

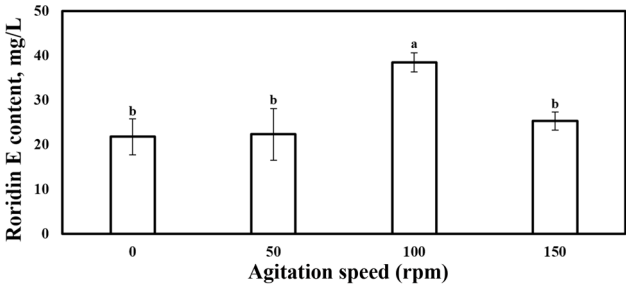

Fig. 4. The effects of agitation speed on roridin E content by *P. cornu-damae* in submerged culture. Each value is expressed as mean±SE (n = 3). Different letters on the top of the line represent statistically significant at 5% probability level.

Table 2. Result of three factor, Box–Behnken experimental design

Run	Independent variables (coded)			Independent variables (actual)			Roridin E content, mg/L
	X_1	X_2	X_3	X_1	X_2	X_3	Y
1	-1	-1	0	4.0	9	100	13.58±9.05
2	1	-1	0	8.0	9	100	7.06±1.36
3	-1	1	0	4.0	15	100	29.90±4.23
4	1	1	0	8.0	15	100	5.84±1.79
5	-1	0	-1	4.0	12	50	31.55±12.63
6	1	0	-1	8.0	12	50	36.65±9.17
7	-1	0	1	4.0	12	150	6.32±1.58
8	1	0	1	8.0	12	150	11.88±3.50
9	0	-1	-1	6.0	9	50	11.51±3.61
10	0	1	-1	6.0	15	50	19.99±8.58
11	0	-1	1	6.0	9	150	3.74±3.25
12	0	1	1	6.0	15	150	16.19±18.31
13	0	0	0	6.0	12	100	41.82±6.28
14	0	0	0	6.0	12	100	47.09±5.41
15	0	0	0	6.0	12	100	17.07±7.07
16	0	0	0	6.0	12	100	51.02±8.29
17	0	0	0	6.0	12	100	12.54±8.97

X_1: Initial pH; X_2: Incubation time, day; X_3: Agitation speed, rpm.

roridin E from *P. cornu-damae*. This could be due to the difference in air saturation caused by agitation in the submerged culture of mushrooms (Asadi *et al*., 2021).

Optimization of roridin E production by the RSM

The conventional variation of the one-factor-at-a-time approach of optimization is not only time-consuming but also costly. Therefore, an experimental design using the RSM is much more efficient. To achieve the desired target (i.e., highest roridin E content), optimization was performed by applying the BBD to predict the optimal levels of the independent variables (initial pH, incubation time, and agitation speed). The response surface plots for the BBD are depicted in Fig. 5 together with the observed experimental data (Table 2). A second-order polynomial model (Eq. (2)):

$$\text{Roridin E content, mg/L} = -345.31204 + 15.26432\ X_1 + 53.84132\ X_2 + 0.306343\ X_3 - 0.972611 X_1X_2 + 0.039587\ X_2X_3 - 0.002831\ X_1X_3 - 0.797459\ X_1^2 - 1.92938\ X_2^2 - 0.003396 X_3^2$$

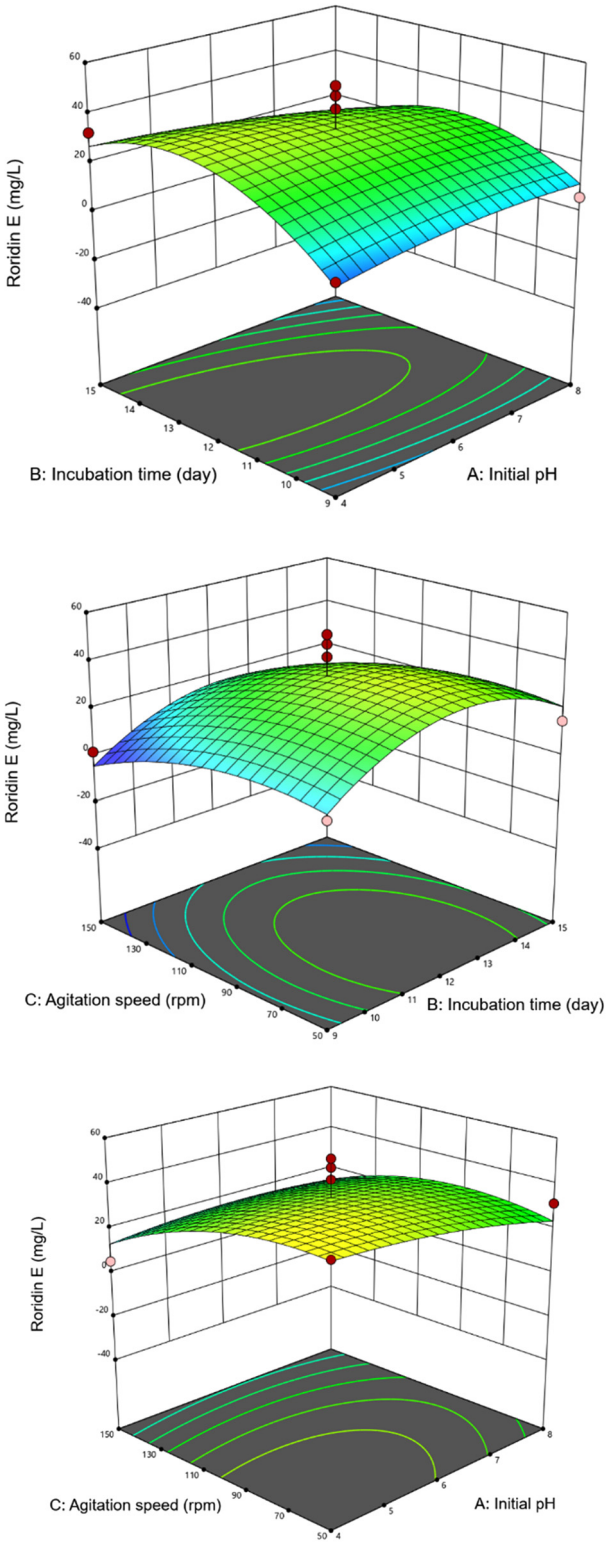

Fig. 5. Regression analysis of the Box–Behnken design experiments.

Statistical analysis of the BBD data revealed that the order of the three factors affecting roridin E production

Table 3. Results of the verified experiment

	Initial pH	Incubation time, day	Agitation speed, rpm	Roridin E, mg/L
Optimal culture condition	4.0	12.90	63.03	40.26

in the submerged cultures of *P. cornu-damae* was agitation speed > incubation time > initial pH. The optimal conditions, as per Eq. (2), included an initial pH of 4.0, an incubation time of 12.90 days, and an agitation speed of 63.03 rpm (Fig. 5). This model predicted a maximum roridin E yield of 40.26 mg/L.

Roridin E is a trichothecene belonging to the macrocyclic sesquiterpenes. Trichothecenes are found in *Myrothecium roridum*, *M. verrucaria*, *Stachybotrys chartarum*, *Cylindrocarpon* sp., *Verticimonosporium diffractum*, *Cryptomola acutispora*, *Phomopsis leptostromiformis*, and *Cercophora* (Jarvis and Wang, 1999). Trichothecene is a highly toxic substance (Rocha *et al*., 2005); therefore, limited studies are performed on increasing its content. However, due to the strong anticancer activity of roridin E, further research is required to increase its content. To our knowledge, no studies have been conducted to control the increase the roridin E content of *P. cornu-damae*. In this study, in the predicted roridin E content (40.26 mg/L) of *P. cornu-damae* was higher than that reported in *P. cornu-damae* cultured by stationary culture by Saikawa *et al*. (2001) (22.1 mg/L). They were cultured for 3 weeks by stationary culture. In addition, Bean *et al*. (1984) reported a higher content than that reported for *M. roridum* (less than 5 mg/L). The findings of this study will help lower the price of roridin E and promote related research.

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