J Ind Microbiol Biotechnol (2014) 41:1169–1173
DOI 10.1007/s10295-014-1445-0

SHORT COMMUNICATION

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pH-Dependent accumulation of anticancer compound on mycelia in fermentation of marine fungus

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Received: 27 January 2014 / Accepted: 2 April 2014 / Published online: 24 April 2014

Abstract The real-time distribution of anticancer 1403C in fermentation broth of marine fungus *Halorosellinia* sp. was investigated. It was closely related with pH variations, which was, 1403C in the supernatant decreased while that in the mycelia increased with pH rising. There was only 0.5 % of the total 1403C left in the supernatant when pH reached 7.0. The scanning electron microscope then provided information that compounds precipitated on the mycelia when pH rose. Then, the pH-regulation experiments proved that 1403C mainly secreted extracellular and easily dissolved in acidic condition but precipitated and absorbed on the mycelia with the increase of broth pH. Thereby, a pH-regulation strategy was proposed and applied to accumulate 1403C on the mycelia before draw-off of fermentation broth. It significantly simplified purification process and is critical for 1403C preparation of industrial scale.

Keywords pH-Dependent · Marine *Halorosellinia* sp. · Anticancer 1403C · Product distribution

Introduction

Recently, a new medicinal resource of marine fungi has attracted wide attention all around the world [2–4]. Thereinto, a large number of endophytic fungi have been isolated from a mangrove ecosystem. They usually synthesize abundant bioactive compounds and drug candidates [8, 9].

Halorosellinia sp. (No. 1403) was isolated from the leaves of mangrove in the South China Sea [6]. This fungus produces a novel compound, 1403C (also called SZ-685C), which could induce apoptosis of breast cancer cell by suppression of the Akt/FOXO pathway and showed great potential to be a new antitumor drug [6, 12]. However, the lack of 1403C pure product severely inhibited its preclinical study. As previously reported, 1403C production has been improved to 1.09 g/l in 500-l bioreactor fermentation via systematical nutrition and bioprocess optimizations [13]. However, the follow-up treatment of large-scale fermentation was inhibited by the multi-step and time-consuming extraction process of the fermentation broth.

For microbial fermentation, broth pH is a critical parameter reflecting both cell metabolism and external culture environment. The functional enzyme activity and cell metabolism are often determined by broth pH, which in turn leads to different kinds and amount of products (compounds and enzymes) [1, 10, 11]. Also, pH could greatly affect variation of product distribution intracellular and extracellular [7]. For marine *Halorosellinia* sp. (No. 1403), pH also significantly affected its fermentation process and the biosynthesis of 1403C, and its production was enhanced dramatically by pH-regulation strategy [13].

Electronic supplementary material The online version of this article (doi:10.1007/s10295-014-1445-0) contains supplementary material, which is available to authorized users.

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In this study, the pH-dependent 1403C distribution was investigated so as to provide applicable information for downstream process purification of this anticancer compound. With this effort, we aim to simplify the product extraction procedure and reduce the industrial cost of 1403C purification by controlling the fermentation process.

Materials and methods

Strain and culture conditions

Halorosellinia sp. (No. 1403) (CCTCCM 201018) was kindly provided by Sun Yat-sen University. The seed medium and culture condition were reported previously [13]. The second stage seed was then inoculated with 5 ml of the first stage seed and incubated at 28 °C and 170 rpm on a rotary platform shaker for 36 h. For fermentation medium, the basic components were 12.36 g/l glucose, 1.05 g/l tryptone, 6.08 g/l beef extract, 0.246 g/l $MnSO_4{\cdot}H_2O$, dissolved in 40 % (v/v) modified artificial seawater (MASW). As to the MASW, 19.624 g NaCl, 4.908 g Na_2SO_4, 1.392 g $CaCl_2$, 6.240 g $MgCl_2{\cdot}6H_2O$, 0.032 g H_3BO_3, 0.081 g KBr and 0.161 g $NaHCO_3$ were dissolved in 1 l of distilled water. Each medium was autoclaved at 121 °C for 20 min. All cultures were performed in triplicates, and the samples were analyzed every 12 h.

Analytical methods

The off-line pH of the broth was measured by a pH meter (FE20, Mettler-Toledo). The total biomass was determined by dry cell weight (DCW) [13]. Residual glucose was analyzed as previously reported [13]. The extraction and quantification of 1403C were the same as before [13].

Mycelial ultrastructure was photographed by a scanning electron microscopy (S-4800, 10 kV, Hitachi). Before scanning, a 10-ml sample was filtrated and washed with distilled water, and some hypha was freeze-dried under high vacuum on a lyophilizer (Alpha 1–2, Christ) for 12 h after prefreezing at −20 °C. Then the freeze-dried hypha was fixed onto doubled-sided tape, which was mounted directly on an SEM stub and coated with gold–palladium using an ion sputter-coater (E-1010, Hitachi) [5].

Results and discussion

Variations of 1403C distribution in submerged culture

Time profiles of *Halorosellinia* sp. in submerged culture in a 250-ml shake flask and the real-time 1403C distribution were shown in Fig. 1. The residual glucose declined sharply during 12–48 h and it dropped to lower than 0.3 g/l after 48 h. The broth pH underwent a fast decline in the first 24 h and kept at a stable level fluctuated around 3.8 in the following 36 h. However, when glucose nearly exhausted, the pH level rose rapidly and increased about two units in 12 h. In addition, the cell growth increased quickly during the early phase (0–48 h) until it reached a stationary phase (48–96 h) when the DCW was about 5.5 g/l.

As for 1403C production, there was almost no 1403C in the mycelia before 70 h. However, the distribution of 1403C showed a great turning point when the pH level began to rise at 60 h. The 1403C in the filtrate decreased but that in the mycelia accumulated dramatically. It seemed that the pH level was the regulatory signal for 1403C distribution. The highest total production of 1403C reached 1.86 g/l but that in the filtrate was only 20 mg/l, which indicated that 99.5 % of the 1403C located in the mycelia

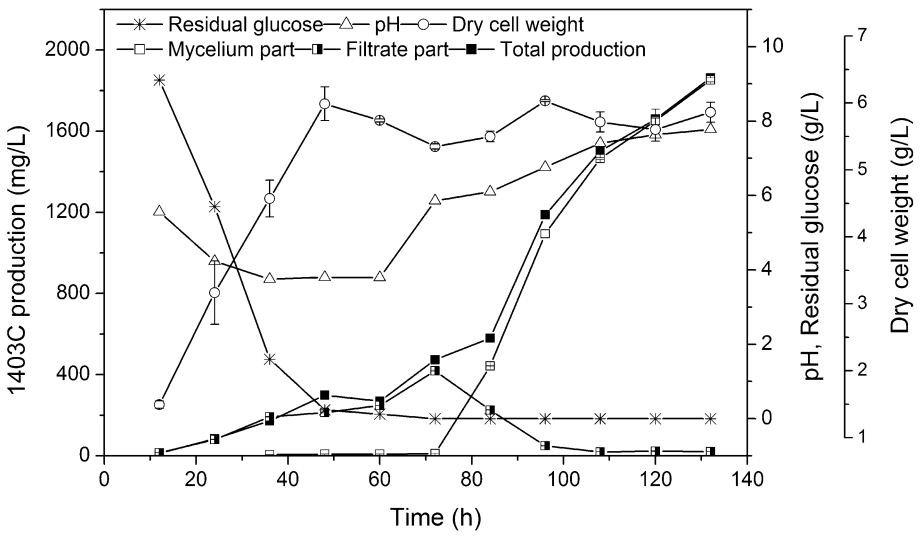

Fig. 1 Time profiles of pH, DCW, residual glucose, and 1403C production (intracellular and extracellular) in submerged culture of *Halorosellinia* sp. (No. 1403)

finally. Product diversity was also analyzed. The HPLC spectra of the extracted sample were presented in Fig. S1. Obviously, 1403C production increased rapidly during 48–84 h, whereas 1403R production firstly increased (48–60 h), then decreased (60–72 h), and even disappeared eventually (84 h). As 1403R shows a reduction chemical structure of 1403C, there should be a close conversion between 1403C and 1403R during fermentation. Two small chromatographic peaks in the front and back of 1403C were also decreased with pH increasing. They were identified as the hydrogenation and dehydrogenation of 1403C by mass spectrometry (data not shown). Interestingly, the production of griseofulvin, a major by-product in 1403C purification, only slightly increased with pH rising. Therefore, regulating pH environment to concentrate 1403C onto the mycelia could be a good choice for simplifying the purification process.

Effects of pH on mycelial morphology

The mycelial morphology and broth color of the 5-ml samples were photographed by a digital camera (Fig. S2). The mycelial morphologies of *Halorosellinia* sp. exhibited as large pellets and some dispersed hyphae. After the middle of fermentation period, the center of the pellet turned to dark red and more robust. Moreover, the color of the supernatant changed to brown, which indicated that the content of red compound (including 1403C) dissolved in the supernatant decreased. Thus, it was deduced that 1403C could secrete extracellularly but its dissolubility was markedly influenced by pH levels. With broth pH increasing, 1403C continued precipitating and absorbing onto the hyphal surface, which finally led to the 1403C accumulation on the mycelia (Fig. 1).

Effects of pH regulation on product distributions

In order to confirm the fact that extracellular 1403C was accumulated on the mycelia when pH rose to 7.0, the mycelial ultrastructure was observed by scanning electron microscopy (SEM). A well-mixed broth sample of 108 h was divided into three parts equally, and the corresponding pH levels were then adjusted to 3.0, 7.2, (natural broth pH) and 11.0, respectively. As shown in Fig. 2a, some substance adhered to the superficial ultrastructure of the mycelia and the substance content was easily distinguished among three samples with different pH levels. The results indicated that the accumulation of substance enhanced with the increase of pH level.

The content of 1403R, 1403C, 1403B, and griseofulvin in the corresponding filtrates with different pH-regulation treatments (3.0, 7.2, and 11.0) were then analyzed respectively. As shown in Fig. 2b, the original color of untreated filtrate (pH 7.2) was ruby red, whereas it turned dark brown

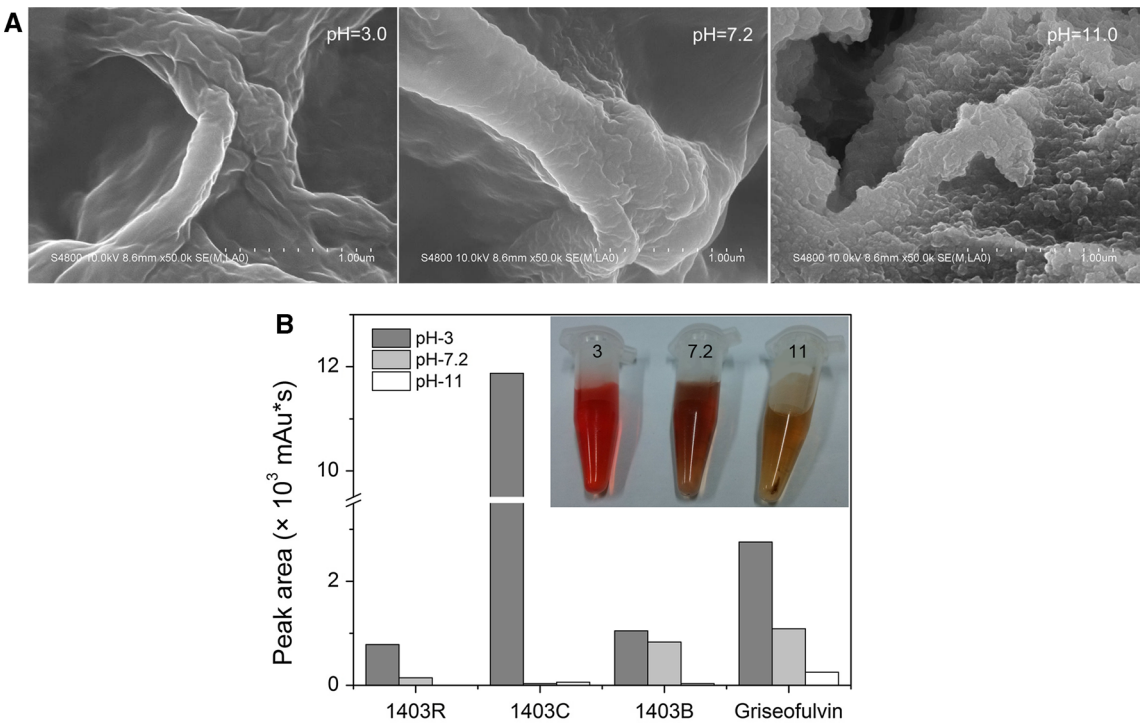

Fig. 2 **a** High magnification view (×50,000) image of mycelial surface by scanning electron microscopy under different pH levels. **b** Productions of compounds in the supernatant from the same fermentation broth (120 h) but adjusted to different pH values (3.0, 7.2, and 11.0)

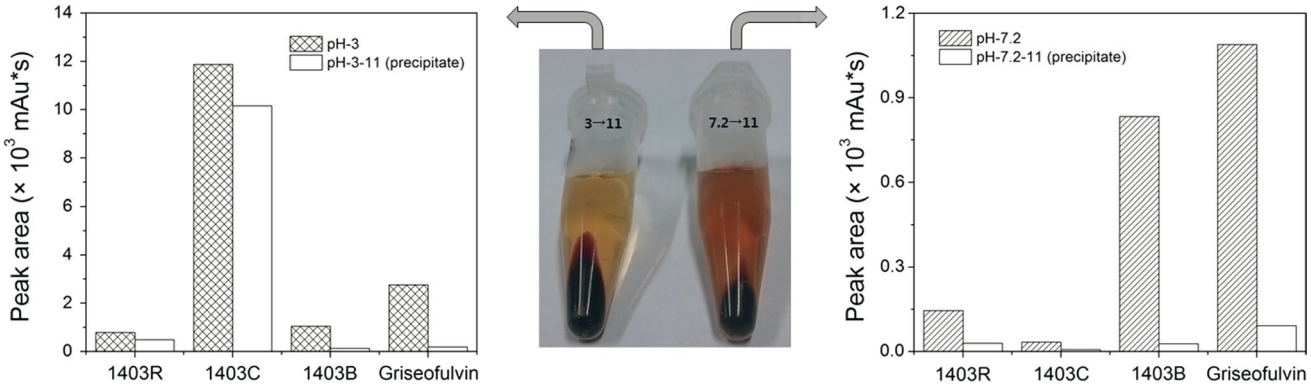

Fig. 3 Production of compounds in the supernatant and precipitation of fermentation broth sample (120 h). The same fermentation broth was adjusted to different pH values (3.0 and 7.2) and then centrifugated to harvest the supernatant. Then, the obtained supernatant was subsequently adjusted to pH 11.0 and then centrifugated to harvest the precipitation

(pH 3.0) and light brown (pH 11.0), respectively. The results indicated that the solubility of 1403C was most sensitive to pH change. The content in the supernatant of pH 7.2 even decreased to zero as compared to the HPLC peak area of 1.19×10^4 mAu s (0.54 g/l) in the supernatant of pH 3.0. However, the by-products precipitated less sensitively to pH changes (Fig. 2b). To further analyze the pH-dependent product distribution pattern, we then adjusted the pH level of the pH-regulated supernatant to determine the production variations (Fig. 3). It seemed that 1403C in the supernatant effectively transferred into the precipitation when pH was adjusted from 3.0 to 11.0, whereas 1403B and griseofulvin were not effectively accumulated into the precipitant. Comparing to Fig. 2b, it could be induced that they might decompose or transform to other compounds.

This work provided useful information for downstream processing for 1403C preparation. For industrial fermentation, a pH-regulation strategy (pH up to 11.0) could be applied to accumulate 1403C on the mycelia before draw-off of fermentation broth. With this strategy, we have successfully simplified the extraction procedure during 1403C purification process by concentrating the product on the mycelia in 500-l bioreactor fermentation (1403C of 1.09 g/l). Based on this strategy, a total amount of 102 g of uniform 1403C (purity of 99.2 %) was finally purified from 300 l of broth and greatly facilitated its preclinical study (data not published). This work offers enlightenment for production and purification of bioactive compounds from marine microorganisms and it is valuable for industrial applications.

Conclusions

A real-time pH-dependent 1403C distribution was clarified during submerged culture of marine fungus *Halorosellinia* sp. (No. 1403). The compound could secrete extracellularly and precipitate on the mycelia with the increase of broth pH. On the basis of this advance, a time- and labor-saving and cost-effective postprocessing in large-scale fermentation was determined. The purification process was then significantly simplified by concentrating 1403C onto the mycelia using a simple pH-increase regulation.

Acknowledgments This work was financially supported by the Chinese National High Technology Research and Development Program (2011AA090702, 2012AA092103, 2012AA092105). We thank Prof. Zhigang She, Sun Yat-Sen University for supplying the strain.

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