Research Square

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Enhanced Cercosporin Production by Co-culturing *Cercospora* Sp. JNU001 With Leaf-Spot-Disease-Related Endophytic Bacteria

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Abstract

Background: Owing to the excellent properties of photosensitization, cercosporin, one of naturally occurring perylenequinonoid pigments, has been widely used in photodynamic therapy, or as an antimicrobial agent and an organophotocatalyst. However, because of low efficiency of total chemical synthesis and low yield of current microbial fermentation, the limited production restricts its broad applications. Thus, the strategies to improve the production of cercosporin were highly desired. Besides traditional optimization methods, here we screened leaf-spot-disease-related endophytic bacteria to co-culture with our previous identified *Cercospora* sp. JNU001 to increase cercosporin production.

Results: *Bacillus velezensis* B04 and *Lysinibacillus* sp. B15 isolated from leaves with leaf spot diseases were found to facilitate cercosporin secretion into the broth and then enhance the production of cercosporin. After 4 days of co-culture, *Bacillus velezensis* B04 allowed to increase the production of cercosporin from 128.2 mg/L to 984.4 mg/L, which was 7.68-fold of the previously reported one. *Lysinibacillus* sp. B15 could also enhance the production of cercosporin with a yield of 626.3 mg/L, which was 4.89-fold higher than the starting condition. More importantly, we found that bacteria B04 and B15 employed two different mechanisms to improve the production of cercosporin, in which B04 facilitated cercosporin secretion into the broth by loosening and damaging the hyphae surface of *Cercospora sp.* JNU001 while B15 could absorb cercosporin to improve its secretion.

Conclusions: We here established a novel and effective co-culture method to improve the production of cercosporin by increasing its secretion ability from *Cercospora sp.* JNU001, allowing to develop more potential applications of cercosporin.

Background

Cercosporin, one of naturally occurring perylenequinonoid pigments (PQPs) with a characterized 3,10-dihydroxy-4,9-perylenequinone chromophore core structure (Fig. 1A), was first isolated in the mycelium of *Cercospora kikuchii* in 1957 and then was widely found in many pathogenic fungus *Cercospora* [1–3], which is a causal agent of leaf spot diseases in a wide range of crops [4], including agriculturally important crops such as soybean [5], maize [6], and olive [7]. Owing to its excellent properties of photosensitization, it is widely investigated in the aspects of photophysics, photochemistry and photobiology [8–13], and has been used in photodynamic therapy and photophysical diagnosis, or as antimicrobial agents [14–16]. For instance, it has broad applications in the treatment of refractory skin diseases caused by certain fungi and cancer [16, 17]. Cercosporin is also a potent inhibitor of protein kinase C (PKC) [18], which regulates numerous intracellular signal transduction, including cell differentiation, cell proliferation and inflammatory response, by controlling the function of other proteins through the phosphorylation. More recently, we have developed cercosporin as a new class of metal-free photocatalyst to catalyze a series of chemical transformations, including selective photxidation, C-H activation, C-N coupling and C-S coupling [19–23]. Meanwhile, it has also been utilized to fabricate a novel HARCP/HAp photocatalyst (HexaAcetyl-Reductive Cercosporin/Hydroxyapatite) after a simple

structural modification, allowing to efficiently photoremove tetracycline in water pollution under natural sunlight [24]. Based on these applications of cercosporin and potential industrial demands, a large quantity of cercosporin is highly desired.

Currently, cercosporin is mainly produced by total chemical synthesis and fermentation engineering. However, owing to its structural complexity (Fig. 1A), the total chemical synthesis of perylenequinonoid pigments, including cercosporin, needs more than twenty steps [18], which limits its practical application. Thus, the production of cercosporin is mainly depended on fermentation engineering by culturing fungus *Cercospora* sp. [22, 25–27]. So far, many previous studies have focused on increasing the productivity of cercosporin by optimizing numerous fermentation factors, including medium, salts, buffers and ions, and delivered the maximum production of 75.59 mg/L after 31 days culture of a *Cercospora* strain from symptomatic leaves of waterhyacinth [26]. However, both of solid-state and liquid fermentation are currently not able to produce stable and high-yield of cercosporin in a large scale within a reasonable culture time. Additionally, its productivity highly varies from different sources of *Cercospora* sp. [2]. Thus, the improvement of cercosporin production through different strategies by a promising *Cercospora* strain is still awaiting to be explored.

Recently, we identified a new cercosporin-producing strain *Cercospora* sp. JNU001, which was isolated from bark of *Taxus chinensis*, with the ability to produce cercosporin at the yield of 128.2 mg/L when it was cultivated under continuous light illumination with S-7 medium after 11 days [22], which is substantially higher than previous studies, even within a shortened culture time [26]. Therefore, herein we attempted to improve the production of cercosporin using *Cercospora* sp. JNU001 strain. We initiated the improvement of cercosporin production of *Cercospora* sp. JNU001 by typical medium optimization and culture condition optimization, and then employed the co-cultivation method, a powerful ecologically driven approach to increase the production of specific metabolites or to produce some new substances by mimicking natural situations [28–33]. To our delight, the production of cercosporin was still remarkably increased with the yield of 984.4 mg/L and 626.3 mg/L by co-culturing with two new identified leaf-spot-disease-related endophytic bacteria *Bacillus velezensis* B04 and *Lysinibacillus* sp. B15 after traditional medium and culture condition optimizations, respectively. They were 7.68-fold and 4.89-fold higher than the starting condition, respectively. Furthermore, we found that these two bacteria applied different mechanisms to improve the production of cercosporin.

Results And Discussion

Determination and optimization of liquid fermentation conditions

Considering the limited production of cercosporin (CP) on PDA plate [22], S-7 culture medium was firstly chosen as the basic medium to optimize the liquid fermentation conditions [19, 22, 34], including culture time, medium pH, temperature, carbon source and nitrogen source (Fig. 1B-F). It was found that the production of cercosporin was dramatically increased from 128.2 mg/L to 467.8 mg/L when *Cercospora*

sp. JNU001 was cultured at 25℃ with the optimized S-7 medium (initial pH = 8.5) (Fig. 1B-D), in which glucose was used as carbon source and soy peptone as nitrogen source (Fig. 1E, F), for 11 days under continuous light illumination. The total amount of cercosporin was 6.19-fold and 3.65-fold higher than the previously reported condition and the original condition, respectively. For the *Cercospora* sp. JNU001 strain, its production ability reached the maximum at 11 days and then part of cercosporin was degraded when the culture time was increased (Fig. 1B), which is consistent with previous studies [35]. Surprisingly, the production of cercosporin was almost inhibited when the culture temperature was set at more than 27℃ (Fig. 1D). Moreover, no cercosporin was produced when inorganic ammonia was used as nitrogen source (Fig. 1F). Thus, we obtained the highest productivity of cercosporin after typical optimizations through liquid fermentation, allowing us to further improve cercosporin production by co-culture strategy.

Screening of leaf-spot-disease-related bacteria

As co-cultivation often enhances the production of metabolites by mimicking natural situations [29, 36], we began with screening the endophytic bacteria community related to leaf spot diseases to mimic the phenomenon caused by pathogen *Cercospora* sp. and then co-cultured each of them with *Cercospora* sp. JNU001 to further increase cercosporin production. After extensive purification, a total of 16 pure bacteria were isolated from the leaf-spot-disease-related leaves (Additional file 1: Table S1). Next, each of them was co-cultured with *Cercospora* sp. JNU001 using the above optimized conditions to investigate which of them would enhance the production of cercosporin. It was found that most of them had a negative effect on its production (Additional file 1: Table S1). The B10 strain even caused the death of *Cercospora* sp. JNU001 (Additional file 1: Table S1). To our delight, B04 and B15 strains had a positive effect to increase the production of cercosporin (Fig. 2 and Additional file 1: Table S1). Furthermore, ^{1}H NMR analysis showed that the product purified from co-cultivation was still cercosporin (Additional file 1: Fig. S1). Thus, these results allowed us to further characterize B04 and B15 strains and then investigated how they improved the production of cercosporin.

Identification and characterization of B04 and B15 strains

It showed that the B04 colony appeared round, rough and white in color (Fig. 3A), while the B15 colony appeared round, smooth, small and white in color (Fig. 3B), suggesting that different molecular mechanisms would be applied by B04 and B15 to increase the production of cercosporin. Based on the analysis of 16S rDNA nucleotide sequences (GenBank accession number MW418038.1 for B04, MW418069.1 for B15, respectively), the phylogenetic trees for B04 and B15 strains were established through the alignment and cladistics analysis of homologous nucleotide sequence (Fig. 3C, D). B04 strain and B15 strain belonged to *Bacillus velezensis* and *Lysinibacillus* sp., respectively. Compared to *Bacillus velezensis* CBMB205 (GenBank accession number, NR_116240.1), B04 strain had a similarity of 99.57%, which was then designated as *Bacillus velezensis* B04 (Fig. 3B). B15 strain showed a similarity of 99.43% with *Lysinibacillus macroides* LMG18474 (GenBank accession number, NR_114920.1) (Fig. 3D), and then was named as *Lysinibacillus* sp. B15.

Optimization of co-culture conditions to enhance cercosporin production

Next, we optimized the co-culture conditions by adding different amounts of B04 or B15 strains to culture medium with *Cercospora* sp. JNU001, which initially grew overnight and then was diluted to customized concentrations with the optimized S-7 medium (Fig. 4A, B). It showed that B04 could obviously enhance the production of cercosporin at different concentrations, and the cercosporin production reached a maximum of 984.4 mg/L when B04 was added at the final concentration of 0.20 OD_{600} (Fig. 4A), which was 2.67-fold and 7.68-fold higher than the one in optimized S-7 medium and the original condition, respectively. However, its production was decreased when more B04 was added (Fig. 4A). Interestingly, the production of cercosporin was only increased when the B15 strain was added around 0.20 OD_{600}, in which the highest production of cercosporin was achieved at 626.3 mg/L, which was 1.33-fold higher than that of the control (Fig. 4B). No significant increase was observed when less or more B15 strain was used (Fig. 4B).

To better understand the effect of co-culturing *Cercospora* sp. JNU001 with B04 or B15 strain, the time-course of the growth of *Cercospora* sp. JNU001 with or without bacterium strain was analyzed. It showed that the amount of cercosporin was very low at the beginning phase (Fig. 4C), and then started to secrete more cercosporin after day 7. Under the control strain, the production of cercosporin reached the maximum at day 11 (Fig. 4C), similar with the unmodified S-7 medium (Fig. 1B). These results suggested that the appropriate time to add B04 or B15 was around day 7. To verify the hypothesis, the effect of the adding time of B04 at day 5, 7, 8 and 9 was analyzed. Surprisingly, no cercosporin was detected when B04 was added at day 5 (Fig. 4D). Interestingly, although the amount of cercosporin was also enhanced when the B04 strain was added at day 8 and 9, the production of cercosporin was significantly impaired when compared with the condition at day 7 (Fig. 4D), illustrating that the optimal time to add B04 was day 7. Moreover, the maximum of cercosporin production also happened at day 11 (Fig. 4C). After day 11, *Cercospora* sp. JNU001 appeared to autolyze and had a negative effect on cercosporin production (Additional file 1: Fig. S2). Similarly, the same phenomenon was observed for the strain B15 (Fig. 4C).

To further support the aforementioned conclusions, we also investigated the glucose utilization by measuring the remaining glucose concentration during the time-course of fermentation (Fig. 4E). It clearly showed that the glucose utilization was greatly increased after day 7 no matter with or without co-culturing with B04 or B15 (Fig. 4E), which was well correlated with the production of cercosporin (Fig. 4C). Interestingly, the glucose utilization was similar under control condition and B15 co-culture condition, but more glucose was consumed under B04 co-culture condition after day 7, probably owing to the requirement of more energy to synthesize cercosporin as it delivered much more cercosporin than the other two conditions. Moreover, the remaining glucose was very limited after day 12 under B04 co-culture condition, which could explain the autolysis of *Cercospora* sp. JNU001 (Additional file 1: Fig. S2).

Effect of live B04 and B15 on fungal growth and cercosporin secretion

To understand molecular mechanisms that improved the production of cercosporin by B04 or B15 strain, we next performed *in vitro* fungal-bacterial confrontation bioassays (Fig. 5A, B) [37, 38]. It showed that B04 and B15 strains resulted in different phenomena (Fig. 5C). Surprisingly, *Cercospora* sp. JNU001 was unable to cross the boundary of the B04 strain (Fig. 5Ca-d)), but it clearly induced the secretion of cercosporin as it was well distributed outside of the boundary of *Cercospora* sp. JNU001 with the disappearance of the red ring of cercosporin (Fig. 5Cd), in which the growth of both of B04 was somehow inhibited (Fig. 5Cc, d). On the contrary, *Cercospora* sp. JNU001 obviously crossed over the boundary of B15 (Fig. 5Ce-h). Moreover, the red ring of cercosporin still existed and B15 bacteria were also became red once they got contacted with *Cercospora* sp. JNU001 (Fig. 5Ch), suggesting that B15 had the ability to absorb cercosporin to stimulate its secretion and then enhance its production. To verify this hypothesis, we then investigated whether B15 could emit the red fluorescence from cercosporin after co-culturing with *Cercospora* sp. JNU001. As expected, the B15 strain alone did not show any fluorescence, but became red after co-culturing with *Cercospora* sp. JNU001 (Additional file 1: Fig. S3), confirming that cercosporin could be absorbed by B15 strain to facilitate its production.

Next, we investigated whether the above phenomena would also happen in the liquid fermentation condition. It was found that there was no obvious difference of dry biomass between the control strain and B04 co-culture condition (Fig. 6A), suggesting that there was no influence on fungal growth under B04 co-culture conditions. However, the dry biomass of *Cercospora* sp. JNU001 was slightly decreased when it was co-cultured with B15. Interestingly, the amount of cercosporin extracted from the dry biomass of B15 co-culture was similar with the control strain, but slightly decreased in the B04 co-culture system (Fig. 6B), probably due to the excellent secretion ability of cercosporin induced by B04 (Fig. 5Cd, 6C). However, the amount of cercosporin secreted into the culture broth was dramatically increased in both B04 and B15 co-culture situations (Fig. 6C), which mainly contributed to the production of cercosporin (Fig. 6D). Moreover, the secretion ability of cercosporin induced by the B04 strain was much better than the B15 strain (Fig. 6C), resulting in a higher production of cercosporin (Fig. 6D), which was consistent with the results of *in vitro* fungal-bacterial confrontation assays (Fig. 5C). Together, it suggests that B04 and B15 employed two different mechanisms to improve the production of cercosporin.

Morphological observation of co-culture samples

To further support the above conclusion that two different mechanisms were applied by B04 and B15 to increase the production of cercosporin through enhancing its secretion ability, field emission scanning electron microscope (FESEM) was employed to investigate the morphology of co-culture samples (Fig. 7), which was derived from the optimized liquid fermentation. It showed that the bacteria B04 were attached on the fungal hyphae surface and seemed to have the capacity to loosen it (Fig. 7D-F), even to damage the fungal hyphae (Fig. 7E), which was very tight in the original *Cercospora* sp. JNU001 strain (Fig. 7B, C). The clear puncta on the fungal hyphae surface were observed (Fig. 7F). To further support these phenomena, the Congo red differential medium with glucan (Additional file 1: Fig. S4), which is the main component of fungal cytoderm and can be degraded by glucanase [39–41], was employed to determine whether it would be degraded by B04. Indeed, the glucan around the B04 strain was able to be degraded

(Additional file 1: Fig. S4), indicating that B04 probably have an ability to secrete glucanase to loosen and damage the fungal hyphae, which could facilitate cercosporin secretion and then resulted in the improvement of cercosporin production. Furthermore, it showed that bacteria B04 were somehow shrunk and became unhealthy when compared with the untreated ones (Fig. 7A, D-F).

However, as for the B15 co-culture condition, no obvious appearance change of *Cercospora* sp. JNU001 was observed, and only a few bacteria B15 had physical attachment on the surface of hyphae (Additional file 1: Fig. S5), in which the shape of B15 bacteria was also deformed like the bacteria B04.

Together with the result that cercosporin was absorbed and inserted into B15 bacteria (Additional file 1: Fig. S3), we further confirmed that bacteria B04 and B15 employed different mechanisms to enhance the production of cercosporin, in which B04 could loosen and damage the hyphae of *Cercospora* sp. JNU001 to facilitate cercosporin secretion while B15 had an ability to absorb cercosporin to improve its secretion.

Conclusions

To improve the production of cercosporin, besides traditional optimization methods, including optimization of culture medium and culture conditions, the co-culture method was also employed in this study. After extensive screening and purification, two new identified bacteria *Bacillus velezensis* B04 and *Lysinibacillus* sp. B15 further enhanced the production of cercosporin by separately co-culturing with fungus *Cercospora* sp. JNU001 after traditional optimization, with the yield of 984.4 mg/L and 626.3 mg/L, which were 7.68-fold and 4.89-fold higher than the starting condition, respectively. Moreover, two different mechanisms were found to increase the production of cercosporin by these two bacteria. *Bacillus velezensis* B04 had the ability to loosen or damage the surface of hyphae and then to improve the secretion ability of cercosporin, while *Lysinibacillus* sp. B15 could absorb and accumulate cercosporin to increase cercosporin production. Thus, we here provided a novel effective co-culture method to enhance cercosporin production of *Cercospora* sp., which allows to develop more applications of cercosporin.

Materials And Methods

Cercosporin-producing fungal strain and culture conditions

Cercosporin was produced by an endophytic fungus *Cercospora* sp. JNU001, which was isolated from the tree bark of *Taxus chinensis* in Lishui, Zhejiang, China and reserved in China Center for Type Culture Collection (CCTCC 2017842). The strain was stored on modified S-7 solid medium (glucose: 20 g/L, sodium acetate: 1 g/L, soy peptone: 2 g/L, phenylalanine: 5 mg/L; sodium benzoate: 100 mg/L, 1M KH_2PO_4 buffer: 1 mL, biotin: 1 mg/L, $Ca(NO_3)_2$: 6.5 mg/L, pyridoxal: 1 mg/L, calcium pantothenate: 1 mg/L, thiamine: 1 mg/L, $MnCl_2$: 5 mg/L, $FeCl_3$: 2 mg/L, $Cu(NO_3)_2$: 1 mg/L, $MgSO_4$: 3.6 mg/L, $ZnSO_4$: 2.5 mg/L, agar powder: 15 g/L) at 4°C, or in cryotubes with glycerol (20%) at -80°C.

The traditional S-7 medium before optimization was shown below: Glucose: 1 g/L, fructose: 2 g/L, saccharose: 6 g/L sodium acetate: 1 g/L, soy peptone: 2 g/L, phenylalanine: 5 mg/L; sodium benzoate: 100 mg/L, 1M KH_2PO_4 buffer: 1 mL, biotin: 1 mg/L, $Ca(NO_3)_2$: 6.5 mg/L, pyridoxal:1 mg/L, calcium pantothenate: 1 mg/L, thiamine: 1 mg/L, $MnCl_2$: 5 mg/L, $FeCl_3$: 2 mg/L, $Cu(NO_3)_2$: 1 mg/L, $MgSO_4$: 3.6 mg/L, $ZnSO_4$: 2.5 mg/L, agar powder: 15 g/L.

Cercospora sp. JNU001 was inoculated into 500 mL flasks with 100 mL modified S-7 liquid medium and then cultured at 25°C in a shaker (ZQZY-AF8, Zhichu, China) with 135 rpm upon continuous light illumination.

Separation and purification of cercosporin

50 mL dichloromethane (DCM) was added to the fermentation broth after 11 days and then flasks were put back to the shaker at 135 rpm for 36 h to ensure the complete extraction of cercosporin. This procedure was repeated twice. The organic phase containing cercosporin was collected and DCM was evaporated (RV8, IKA, Germany) to obtain the raw cercosporin, which was then dissolved in methanol and purified by a Sephadex column LH-20.

Quantitative determination of cercosporin production and glucose consumption

To rapidly determine the content of cercosporin, the raw cercosporin was analyzed by HPLC (2695, Waters, America) at 472 nm, which was the characteristic absorption wavelength of cercosporin, with purified cercosporin as a reference. Then, the concentration of cercosporin of each sample could be calculated. To study the time-course of cercosporin production, 2 mL of culture broth was taken out at each day and extracted three times by 2 mL DCM. The extracted fraction was collected and DCM was evaporated. The raw materials with cercosporin was dissolved in 200 µL methanol and loaded into HPLC. The amount of cercosporin was detected by HPLC.

To study the glucose consumption, 100 µL culture broth was taken out from the flask each day and diluted with double distilled water (dd H_2O). The diluted broth was centrifuged at 12000 rpm to remove bacterium and fungal mycelia, and then injected to SBA-40E Biosensor Analyzer, which was made by Institute of Biology, Shandong Academy of Science, to measure the glucose concentration.

Isolation and identification of leaf-spot-disease-related endophytic bacteria

Leaves with leaf spot diseases was collected from August to October in Wuxi, Jiangsu, China. All leaf samples were immediately stored in sealed bags on ice and then sterilized as the following procedures [42]. The fresh leaves were washed by tap water for 1–2 hours and cut into small pieces (2–3 cm long and 0.5 cm wide), which were then rinsed by sterilized water for 3 times, dipped in 75% ethyl alcohol for 1 min and again rinsed by sterilized water for 3 times. Next, some new wounds of leaf pieces were made by the sterilized scalpel to contact LB agar plate (tryptone: 10 g/L, yeast extra: 5 g/L, sodium chloride: 10

g/L, agar powder: 15 g/L, pH: 7.0-7.2) after drying with sterilized filter tissue paper and then cultured at 37℃ without light for 48 h. The single colony was obtained by streak plate method, and then inoculated into LB medium to cultivate without light at 37℃ in a shaker at 200 rpm for 24h. Each of purified bacteria was stored in cryovial tubes with glycerol (25%) at -80℃.

To characterize each of purified bacteria, the bacterial general primers, 27F (5’-AGAGTTTGATCATGGCTCAG-3’) and 1492R (5’-TACGGCTACCTTG TTACGACTT-3’) were used to amplify the 16S rDNA of each of them. The PCR reaction was performed in a final volume of 50 μL: 1 μL bacteria culture medium containing DNA, 25 μL of 2×Premix, 1 μL of 27F (5 nmol/L) and 1 μL of 1492R (5 nmol/L), 22 μL double distilled water (ddH_2O). The amplified PCR product was purified and sequenced by GENEWIZ Inc. (Suzhou, China). The obtained sequences were uploaded to GenBank in NCBI database and the cladogram was constructed by neighbor-joining method in MEGA-X (version 10.1.8) after homologous comparisons with the existed bacterial sequences in NCBI.

Establishment of co-culture conditions

To improve the production of cercosporin, two small pieces (5 mm×5 mm) of *Cercospora* sp. JNU001 from the modified S-7 agar plate were firstly inoculated into 100 mL modified S-7 liquid medium at 25℃ on a rotating shaker at 135 rpm for 7 days. At day 6, the single colony of each of two isolated bacteria B04 and B15 were inoculated into 50 mL LB medium and cultured at 37℃ on a rotating shaker at 200 rpm overnight. At day 7, the customized number of bacteria cells was harvested, centrifuged and resuspended by modified S-7 liquid medium, and then added into pre-culture *Cercospora* sp. JNU001. The co-culture samples grew for another 3−5 days at 25℃ on a rotating shaker at 135 rpm with continuous light illumination. After that, 50 mL DCM was added into culture broth to extract cercosporin using the aforementioned method. The dry biomass was measured immediately after treatment in vacuum freeze dryer (FreeZone 6 Plus, LABCONCO, America) for 3 days. Similarly, 50 mL DCM was also used to extract cercosporin from dry biomass of *Cercospora* sp. JNU001. The content of cercosporin was detected by HPLC.

In vitro **fungal-bacterial confrontation bioassay**

Based on the method reported by Wang et. al [38], different kinds of confrontation bioassays (*in vitro*) were conducted between *Cercospora* sp. JNU001 and each of two isolated bacteria. Firstly, a small piece (5 mm×5 mm) of marginal mycelium of *Cercospora* sp. JNU001 with agar was dug out and reset in the center of a 10 cm modified S-7 agar plate and let it grow for 5 days. Next, the single colony of different bacteria was inoculated in LB medium at 37°C on a rotating shaker at 200 rpm for 24 h on day 5, respectively. As it is shown in Fig. 5, bacterial suspension (10 μL) was streaked in four parallel rectangular areas (approximately 3 cm×0.5 cm) and cultured for another 10 days. The morphology was observed.

Morphological observation

The physical attachment between *Cercospora* sp. JNU001 and B04 or B15 was observed by FESEM (SU-8220, Hitachi, Japan). The samples were collected at day 11 (3 days after co-culturing). Meanwhile, pure bacteria and fungus were also observed as a control. To study whether B15 had an ability to absorb cercosporin, B15 samples were collected from *in vitro* fungal-bacterial confrontation assays when *Cercospora* sp. JNU001 partially reached the edge of the rectangular area of B15, and then dissolved by double distilled water. 10 μL was dropping onto a glass slide and measured by a fluorescence microscope (CKX53, OLYMPUS, Japan) as cercosporin has the capacity to emit the fluorescence. Meanwhile, B15 samples far from *Cercospora* sp. JNU001 on the *in vitro* fungal-bacterial confrontation assays were also collected and used as a control.

Congo red stain

To study whether B04 had an ability to secret glucanase to damage the fungal hyphae, Congo red agar plate (0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.05% NaCl, 0.2% $(NH_4)_2SO_4$, 0.5% glucan, 0.2% Congo red and 1.5% agar) was prepared. Next, B04 on LB plate was dug out, transferred onto the center of glucan Congo red agar plate, and let it grow for another 2–3 days. The transparent zone was scanned.

Declarations

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional file.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

TZ, SY, YH, YS and JC performed the experiments. TZ, YZ and CL analyzed the data. YR and TZ wrote the manuscript. YR designed and supervised this project. All authors read and approved the final manuscript.

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Figures

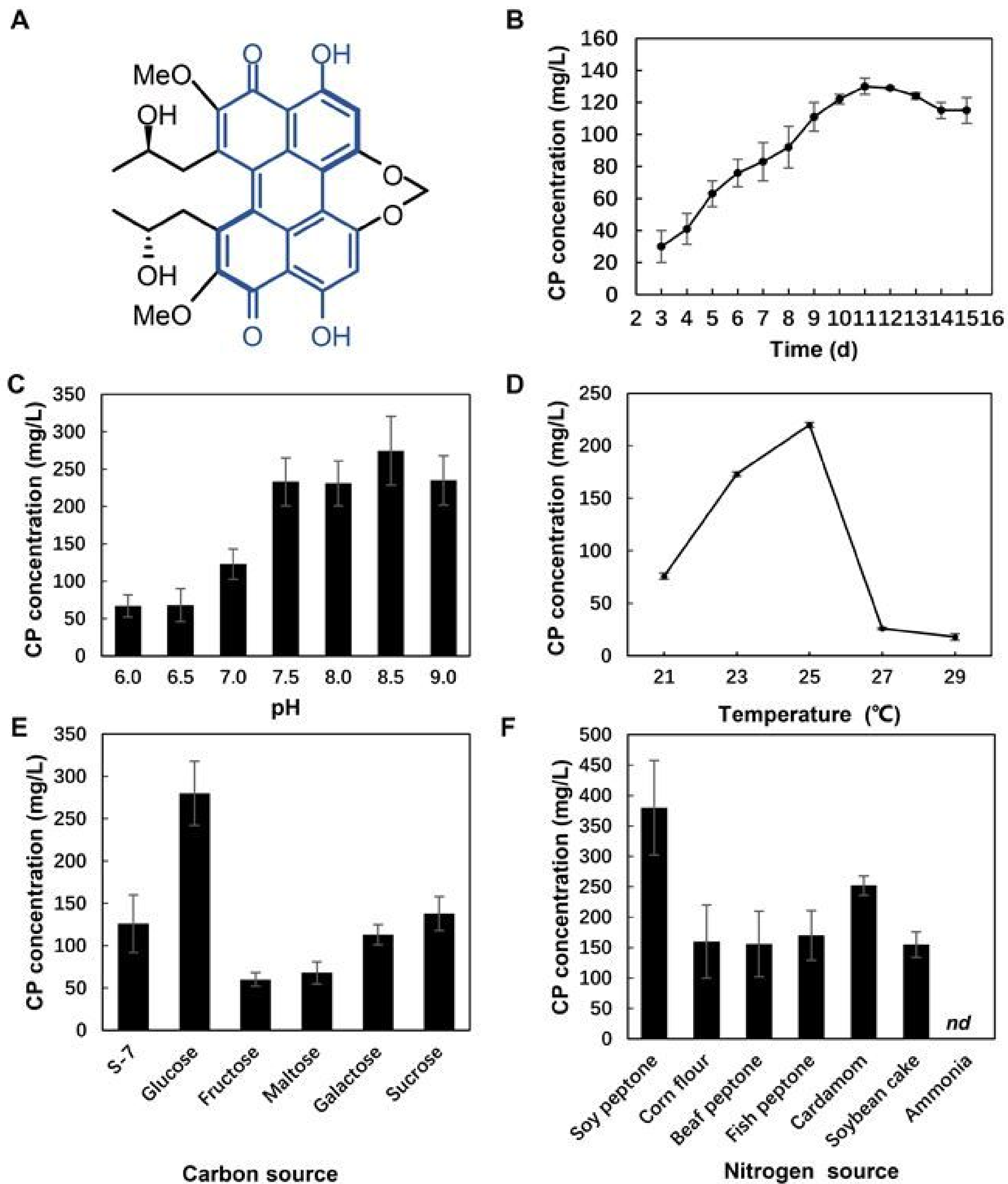

Figure 1

Improvement of cercosporin production by optimizing culture medium and culture conditions. A. Molecular structure of cercosporin. The characterized 3,10-dihydroxy-4,9-perylenequinone chromophore core structure was labelled in blue. B. Influence of culture time on CP production. d=day. C. pH optimization of S-7 medium. D. Optimization of culture temperature. E. Selection of carbon source in S-7

medium. F. Screening of nitrogen source in S-7 medium (cardamom: cardamom powder, ammonia: ammonia sulfate, nd: no detected).

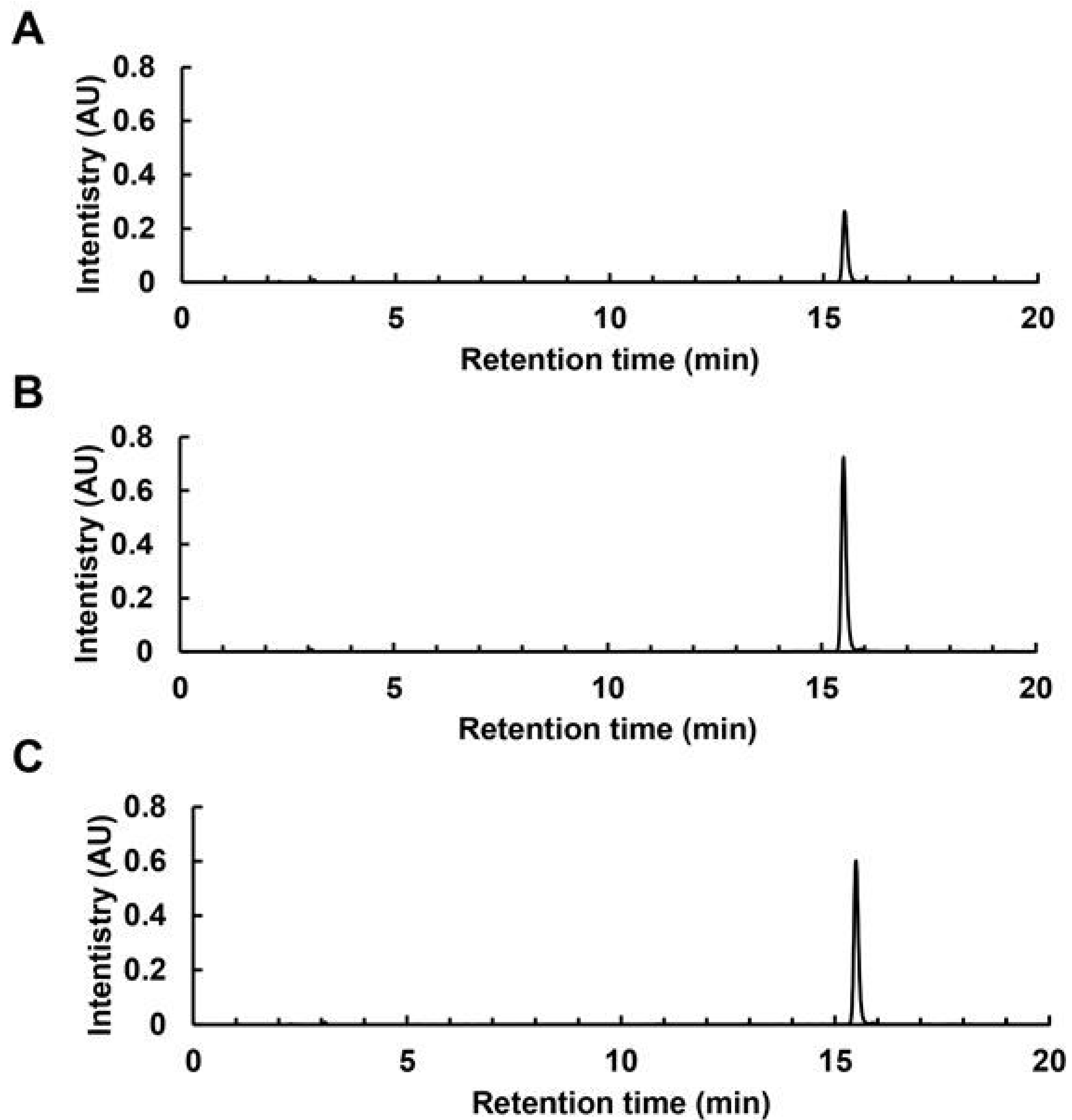

Figure 2

Enhanced cercosporin production by co-culturing with bacteria B04 and B15. A. The chromatogram of CP production of Cercospora sp. JNU001 alone. B. The chromatogram of enhanced CP production by co-culturing Cercospora sp. JNU001 with B04. C. The chromatogram of enhanced CP production by co-culturing Cercospora sp. JNU001 with B15.

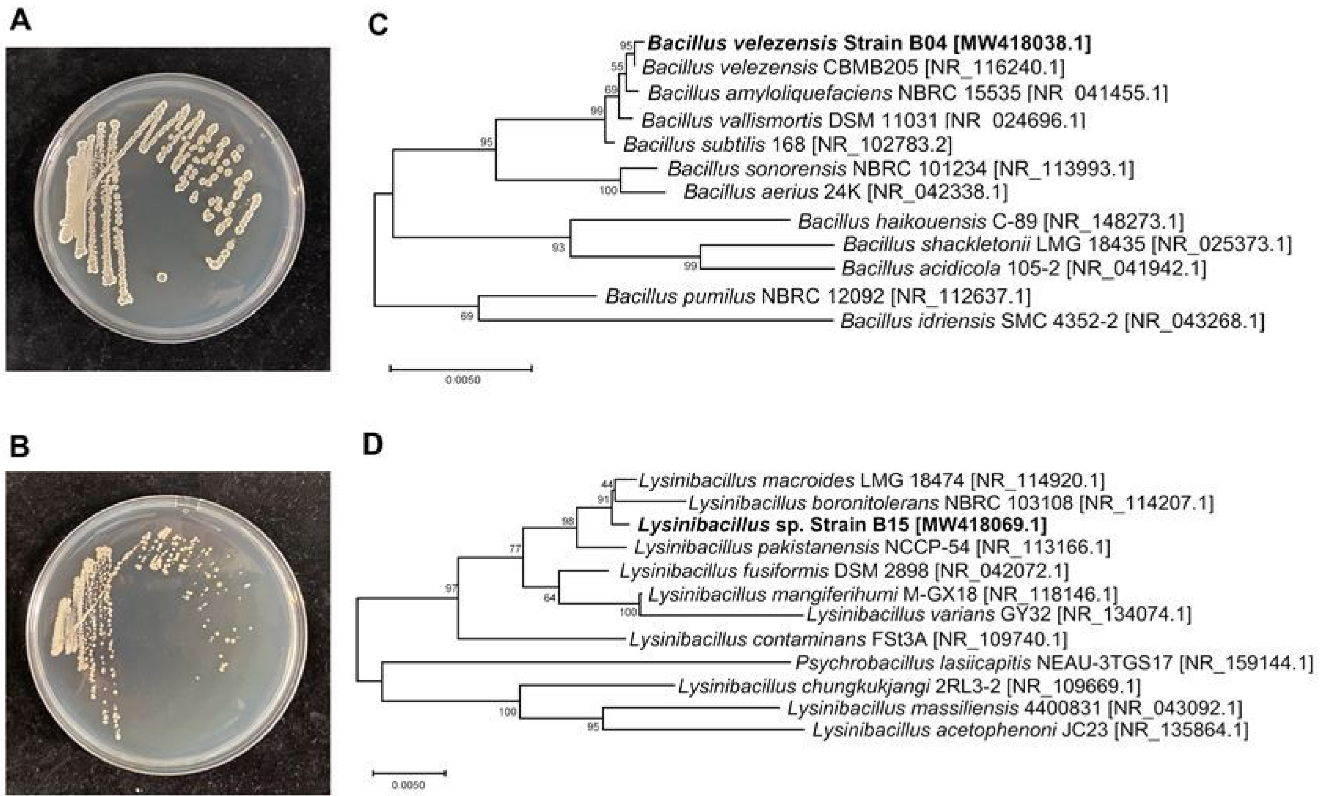

Figure 3

Identification and characterization of two isolated bacteria B04 and B15. A. Microscopic appearance of culture colony of B04 strain on LB plate after 24 h. B. Microscopic appearance of culture colony of B15 strain on LB plate after 24 h. C. The phylogenetic tree of B04 strain and its relationship with other Bacillus species. Confidence values above 55% obtained from 1000-replicate bootstrap are indicated at the branch nodes. The scale bar indicates the number of base substitutions per site. D. The phylogenetic tree of B15 and its relationship with other Lysinibacillus species. Confidence values above 44% obtained from 1000-replicate bootstrap are indicated at the branch nodes. The scale bar indicates the number of base substitutions per site.

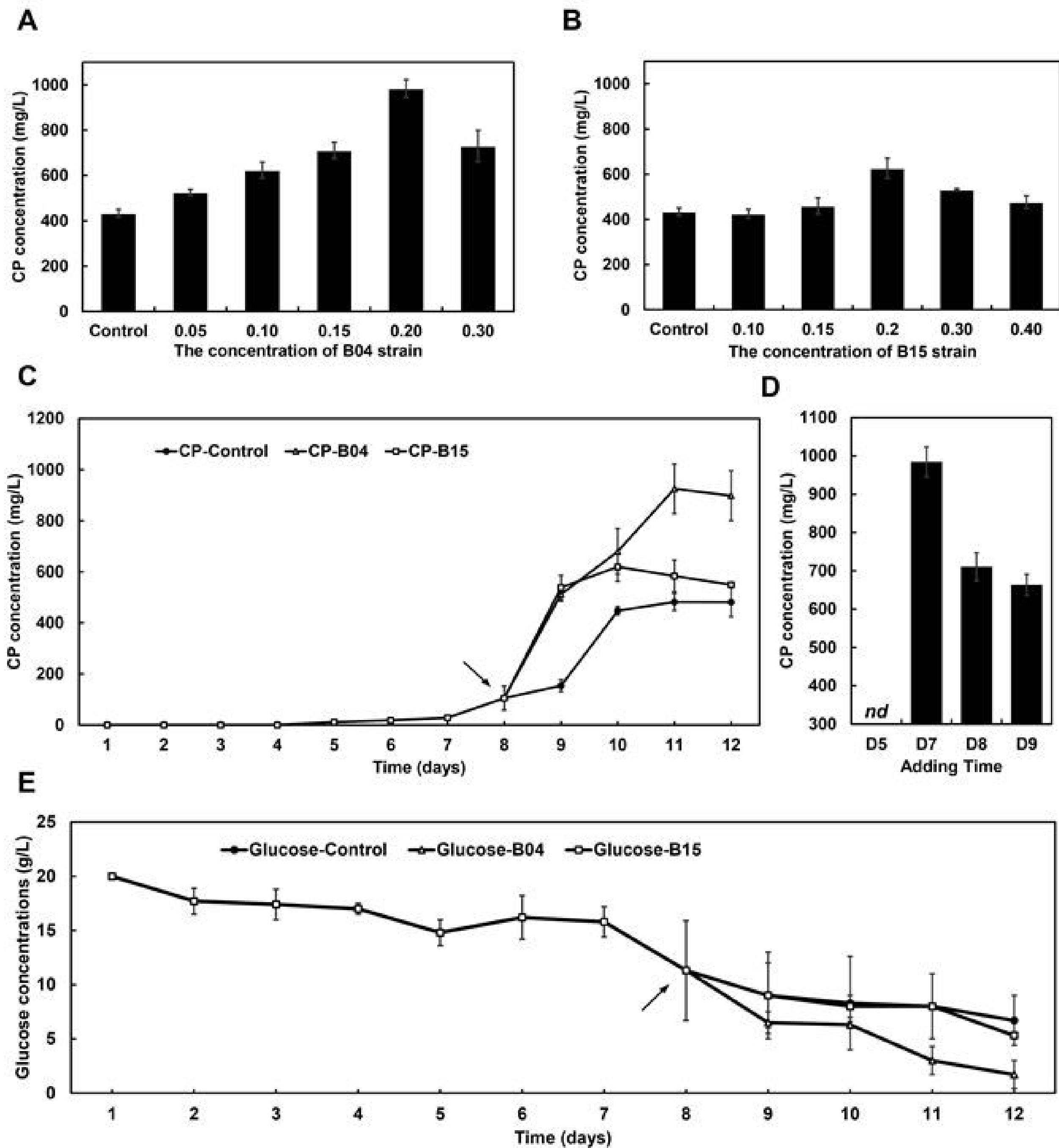

Figure 4

Effect of cercosporin production by co-culturing B04 or B15 strain with Cercospora sp. JNU001. A. Effect of the concentration of B04 strain on cercosporin production. B. Effect of the concentrations of B15 on cercosporin production. C. Effect of culture time on cercosporin production when B04 with OD600=0.20 or B15 with OD600=0.20 was added to Cercospora sp. JNU001 at day 7. D. Effect of the adding time of B04 on cercosporin production. E. The glucose consumption of Cercospora sp. JNU001 grew in the optimized S-7 medium and under co-culture with B04 or B15 strain condition. The arrows indicate the beginning of significant differences caused by co-culture.

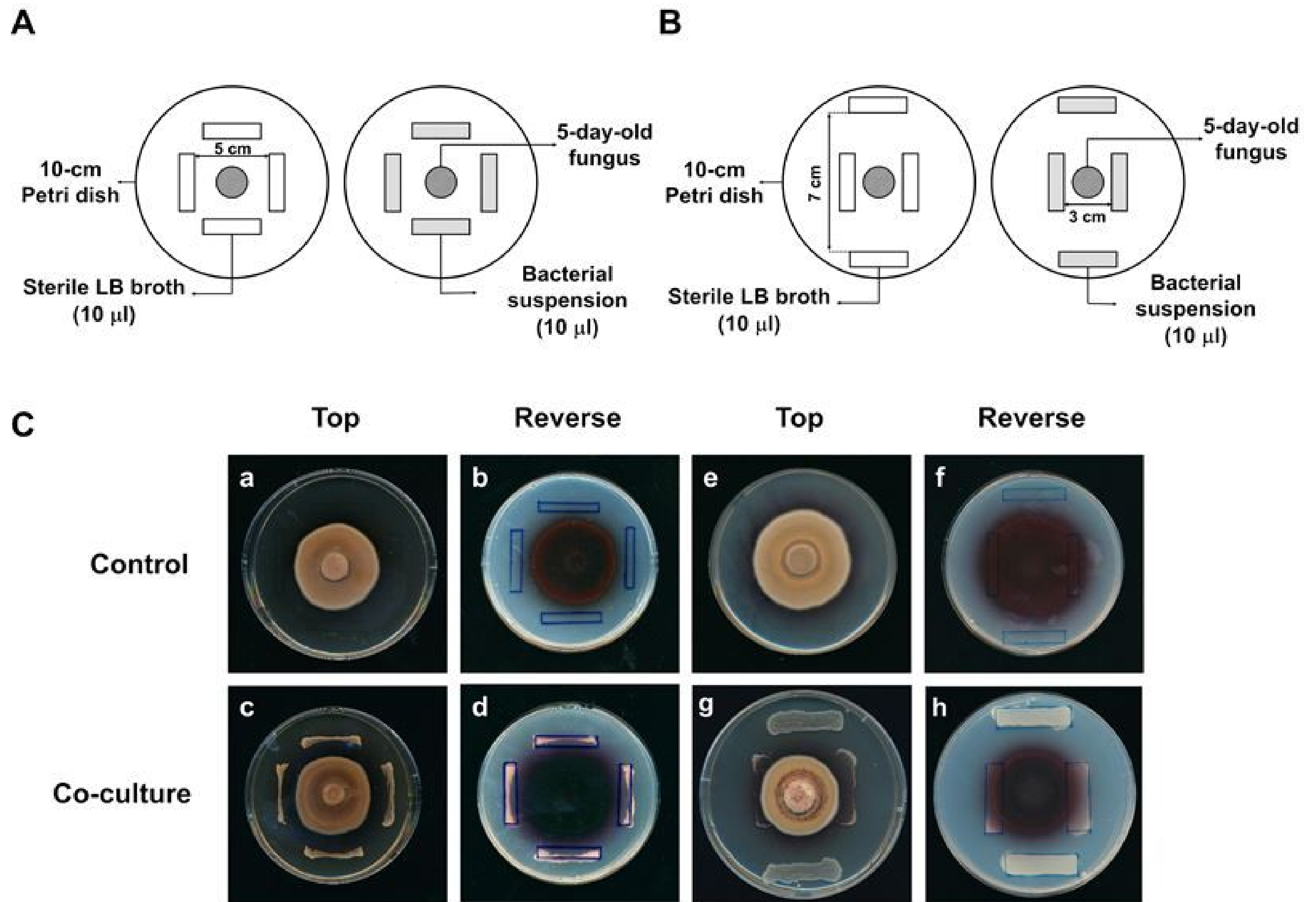

Figure 5

In vitro confrontation bioassays between bacteria and Cercospora sp. JNU001. A. Schematic diagram of in vitro confrontation bioassay between B04 and Cercospora sp. JNU001. B. Schematic diagram of in vitro confrontation bioassay between B15 and Cercospora sp. JNU001. C. Effects of B04 (a-d) or B15 (e-h) on Cercospora sp. JNU001.

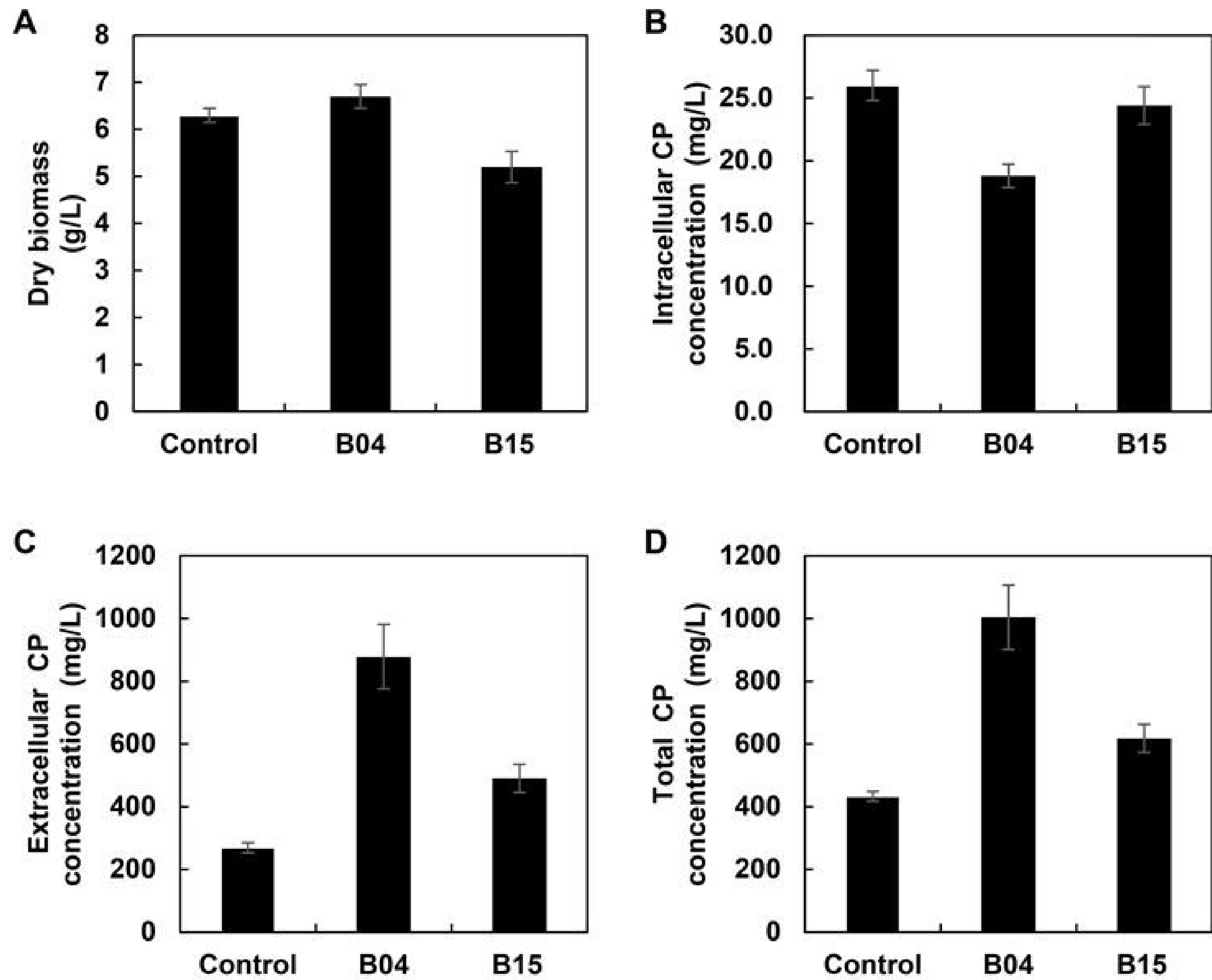

Figure 6

Effects of B04 and B15 on fungal growth and cercosporin secretion. A. Dry fungal biomass of Cercospora sp. JNU001 without or with bacteria. B Intracellular cercosporin production of Cercospora sp. JNU001 extracted from A. C Extracellular cercosporin production purified from culture broth after co-culture without or with bacteria. D Total cercosporin production, which was calculated by intracellular cercosporin production (B) and extracellular cercosporin production (C).

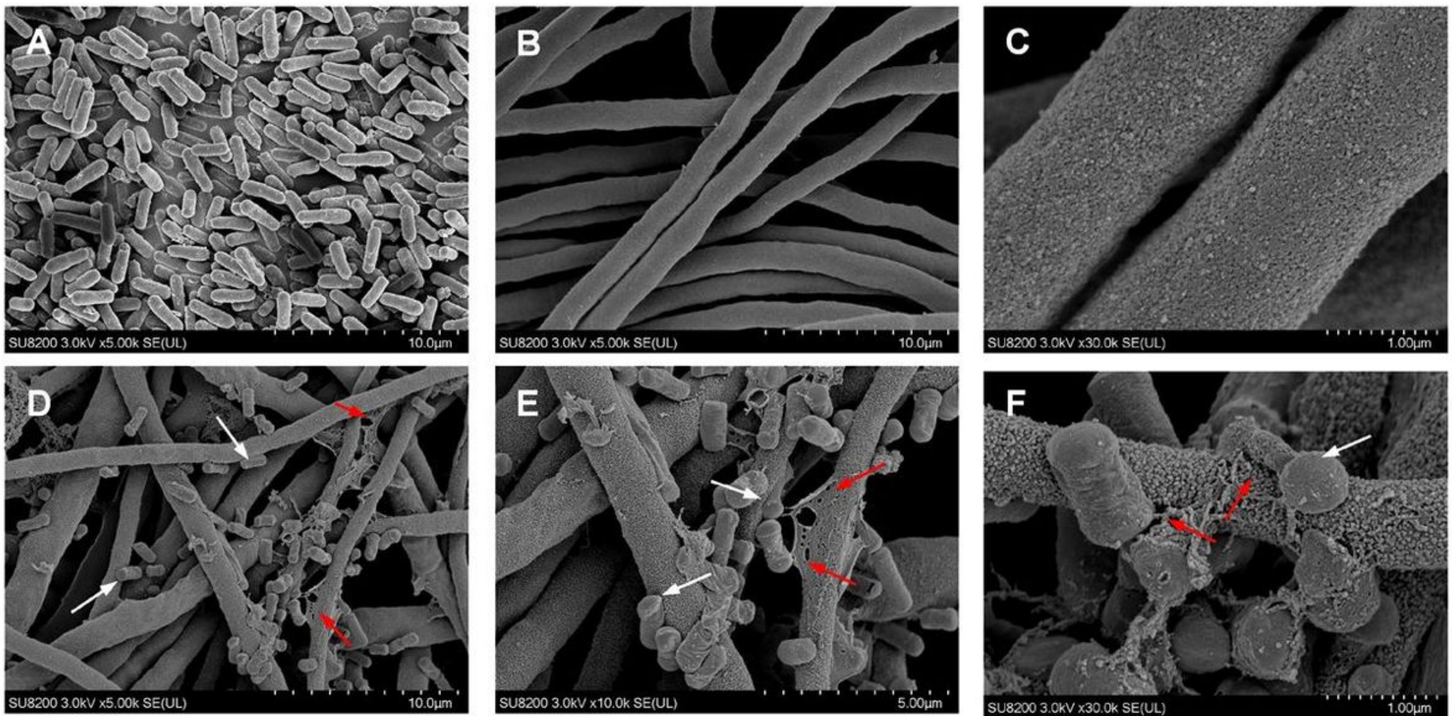

Figure 7

FESEM observation of co-culture Cercospora sp. JNU001 with B04. A. B04 samples. B, C. Cercospora sp. JNU001 samples. D-F. Co-culture samples of B04 and Cercospora sp. JNU001. White arrows indicate bacteria B04, and red arrows indicate the damage of fungal hyphae. Scale bar was indicated.

Supplementary Files

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