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SHORT COMMUNICATION

Isolation and characterization of *Paenibacillus polymyxa* LY214, a camptothecin-producing endophytic bacterium from *Camptotheca acuminata*

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Abstract Camptothecin (CPT) is mainly produced and extracted from *Camptotheca acuminata* and *Nothapodytes foetida* for pharmaceutical use, i.e., the starting material for chemical conversion to the clinical CPT-type drugs. As the third largest plant anticancer drug, the heavy demand on CPT from global market leads to many research efforts to identify new sources for CPT production. Herein we report the isolation and characterization of a CPT-producing endophytic bacterium *Paenibacillus polymyxa* LY214 from *Camptotheca acuminata*. A 10.7 μg l^{-1} of CPT was presented in the fermentation broth of *P. polymyxa* LY214. Its CPT production decreased sharply when the strain of the 2nd generation of *P. polymyxa* LY214 was cultured and fermented. However, the CPT production remained relatively constant from 2.8 μg l^{-1} of the 2nd generation to 0.8 μg l^{-1} of the 8th generation of *P. polymyxa* LY214 under optimized fermentation conditions. A 15- to 30-fold increase of CPT yield was observed when the optimized fermentation conditions, together with the addition of putative biosynthetic precursors of CPT and adsorbent resin XAD16, were applied to ferment the strains of the 7th and 8th generation of *P. polymyxa* LY214. Bioinformatics analysis of the relative species of *P. polymyxa* LY214 indicates its potential to produce CPT, which will be helpful to decipher the mysteries of CPT biosynthesis.

Keywords Camptothecin · Endophytic bacterium · *Paenibacillus polymyxa* LY214 · Adsorbent resin

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Camptothecin (CPT, **1**, Fig. 1), a complex pentacyclic pyrroloquinoline alkaloid, was first identified from a China native tree *Camptotheca acuminata* [23]. Since CPT was proven to be an effective and specific inhibitor of DNA topoisomerase I [3], it was chemically converted to the clinical CPT-type drugs, including 10-hydroxycamptothecin (**2**), topotecan (**3**), irinotecan (**4**), and SN-38 (**5**) (Fig. 1) that were used as anticancer drugs against a broad band of tumor types such as small lung cancer and refractory ovarian cancer [1, 20]. CPT-type drugs were recognized as the third largest anticancer drug from plant sources [1]. Most CPT was extracted and purified from two primary CPT-producing plants, *C. acuminata* in China and *Nothapodytes foetida* in India [10, 13]. However, the plant-derived CPT cannot support the heavy demand in global market [10, 13]. Thus research efforts are needed to identify new sources of CPT from both new plant sources and endophytic microorganisms from CPT-producing plants [13, 14, 17, 18].

In the course of screening CPT-producing endophytic microorganisms from *C. acuminata*, a fungus *Trichoderma atroviride* LY357 was proven to produce CPT in the fermentation broth [13]. A substantial decrease of CPT production with subsequent successive subculturing under axenic monoculture conditions was evidenced. To the best of our knowledge, the reported CPT-producing microorganisms were fungi whose CPT-producing capacity was attenuated with repeat subculturing [2, 4–6, 13, 16]. However, the CPT-production of *T. atroviride* LY357 could be

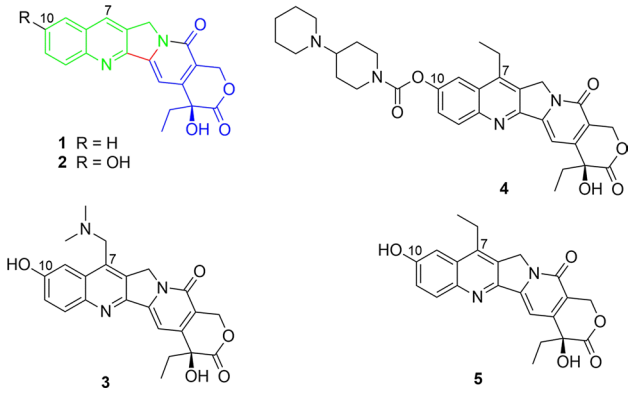

Fig. 1 Chemical structures of camptothecin (CPT, **1**) and its clinically used derivatives 10-hydroxycamptothecin (**2**), topotecan (**3**), irinotecan (**4**) and SN-38 (**5**). The monoterpenoid moiety of CPT from mevalonate and/or non-mevalonate pathway was highlighted in *blue*; whereas the indole moiety from shikimate pathway was in *green*. The chemical bonds formed in the Pictet-Spengler condensation reaction was in *red*

increased by the addition of elicitor and adsorbent resin to the optimized culture media. Recently endophytic bacteria from *Miquelia dentata*, a CPT-producing plant identified in 2013 [14], were reported to produce CPT and its analogues in the fermentation broth for the first time [18]. In the continuing efforts to find CPT-producing microorganisms, an endophytic bacterium *Paenibacillus polymyxa* LY214 from *C. acuminata* was proven to produce CPT in the fermentation broth. Herein we report the isolation and characterization of *P. polymyxa* LY214.

Following the same experimental procedure as that for the isolation of CPT-producing *T. atroviride* LY357 [13], the different plant parts of *C. acuminata* were placed on the Petri dishes containing Gause medium G-1 agar (GA1A), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), and water agar (WA), respectively. Total 125 endophytes, including 40 from roots, 43 from stem barks, 12 from leaves, and 30 from twigs, were isolated and maintained on the isolation medium. Each isolate was grown and fermented in the medium from which it was isolated. The fermentation broth and the cell pellets were extracted with organic solvents, respectively, to afford the crude extracts that were subjected to HPLC–DAD analyses. The results showed that a peak with identical retention time (panel II, Fig. 2a) and parallel UV–Vis profile (panel II, Fig. 2b) to that of the authentic CPT (panel I, Fig. 2a, b) was detected in the crude extract of the fermentation broth of the isolate LY214. HPLC–DAD-HRMS analyses showed the molecular ion at *m/z* 349.1233 ($[M + H]^+$) of the bacterial CPT produced by LY214 (panel II, Fig. 2c) is consistent with the molecular ion at *m/z* 349.1251 ($[M + H]^+$) of the standard CPT (panel I, Fig. 2c). UPLC-DAD-ESI–MS/MS analysis (Fig. 2d–f) of the crude extract of LY214 showed the pattern of the fragmentation ion (*m/z* 305, 249, 219, 206, 181, and 168, Fig. 2f) of the bacterial CPT was characteristic and identical to that of the reported bacterial CPT [18] and the standard CPT [11], which confirmed that the isolate LY214 can produce CPT in the fermentation broth.

The optical photography (Fig. 3a) and the morphologic characteristics of LY214 by scanning electron microscope (Fig. 3b) indicated that the isolate LY214 should be a *Paenibacillus* sp. The universal primers 27F (5′- AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′- TAC GGY TAC CTT GTT ACG ACT T -3′) [7] were synthesized to amplify the 16S rRNA gene of the isolate LY214 using its genomic DNA as template. The PCR amplification products were gel-purified and sequenced. The nucleotide sequence of the 16S rRNA gene of the isolate LY214 (GenBank accession number KR058350) was set as a query to perform a sequence similarity search in the NCBI database using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nih.gov/BLAST). The result showed that the isolate LY214 had an identity value of >99 % with other *P. polymyxa* strains (Fig. 3c). A phylogenetic tree of the strain LY214 and its relatively proximate species was constructed on the basis of the 16S rRNA gene sequence alignment using the neighbor-joining method in the MEGA 4.0 software. The typical strain of *P. polymyxa* LY214 was deposited at China General Microbiological Culture Collection Center under the accession number CGMCC 6841.

Attenuation of CPT production with subsequent successive subculturing is a common event in the CPT-producing fungi and bacteria. *P. polymyxa* LY214 showed similar attenuation trend towards CPT production as that of CPT-producing bacteria from *Miquelai dentata* [18] and CPT-producing fungi [2, 4–6, 13, 16]. A substantial decrease of CPT production (2.8 $\mu g\ l^{-1}$) was witnessed in the fermentation broth of the 2nd generation of *P. polymyxa* LY214 when the strain was subcultured in the same medium under the same fermentation conditions. However, the fermentation conditions, including culture media composition (Fig. 4a), fermentation time (Fig. 4b), pH of the media (Fig. 4c), fermentation temperature (Fig. 4d), and agitation rate of flasks (Fig. 4e) were optimized for CPT production using the strains of the 2nd generation of *P. polymyxa* LY214. It should be noted that as an endophytic bacterium, *P. polymyxa* LY214 can produce 1.5 and 2.5 $\mu g\ l^{-1}$ of CPT when it was grown and fermented in LB and M9 media, respectively (Fig. 4a). However, a higher CPT production was observed when *P. polymyxa* LY214 was grown and fermented in SDB medium for fungi (Fig. 4a). Thus SDB medium was set as a basic medium for *P. polymyxa* LY214 to optimize other fermentation conditions. Under the optimal fermentation

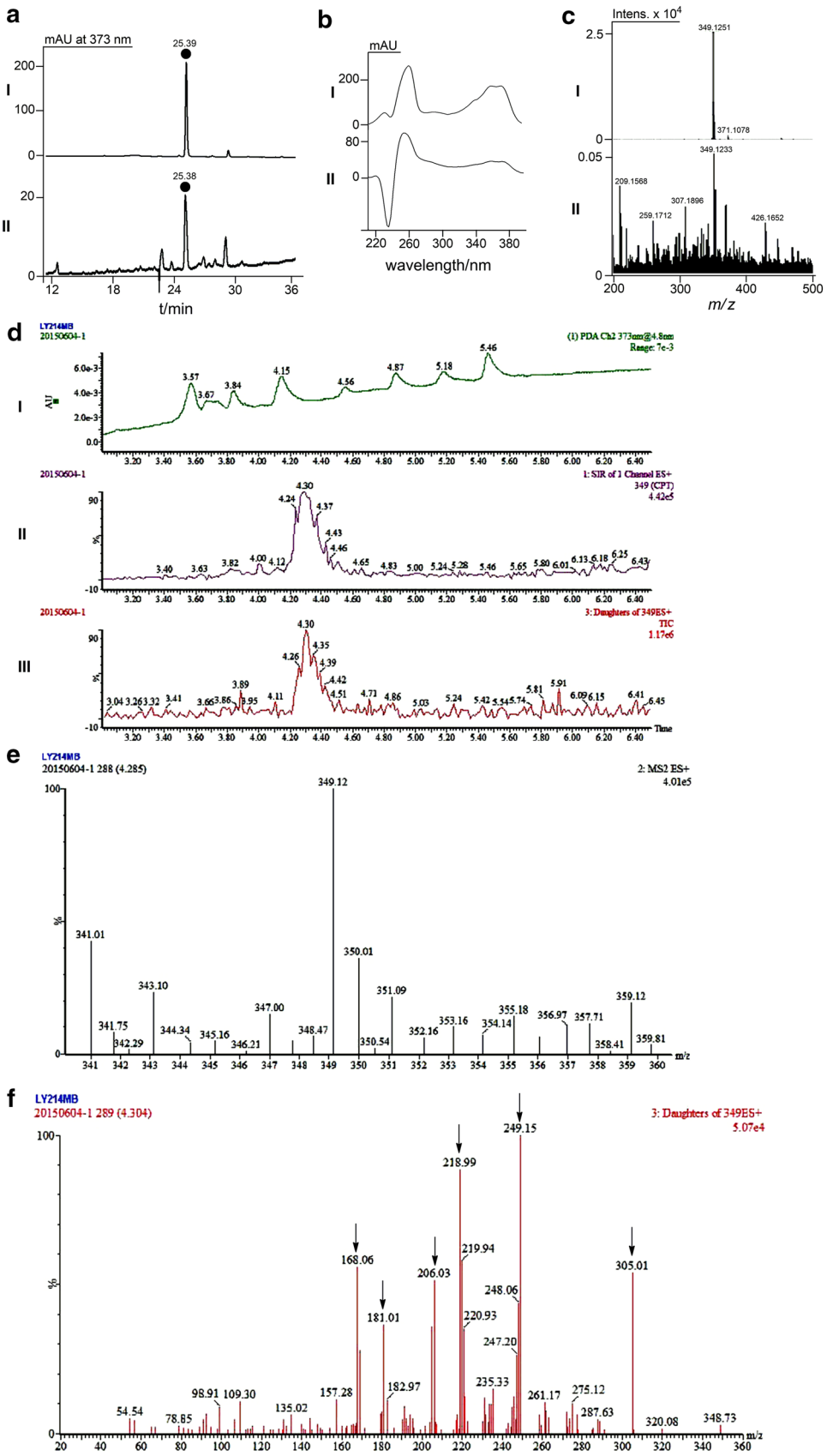

Fig. 2 Analyses and confirmation of CPT production in the fermentation broth of *Paenibacillus polymyxa* LY214. HPLC–DAD analyses showed that the CPT produced by *P. polymyxa* LY214 has identical retention time (panel *II*, **a**) and parallel UV profile (panel *II*, **b**) to that of the authentic CPT (panel *I*, **a** and **b**). HPLC–DAD-HRMS analysis indicated that the exact molecular weight of the bacterial CPT (panel *II*, **c**) is consistent with that of the standard CPT (panel *I*, **c**). UPLC-DAD-MS analysis (**d**, **e**, **f**) showed that the pattern of the fragmentation ion highlighted with arrow of bacterial CPT (**f**) was identical to the reported fragmentation pattern of standard CPT [11] and that of reported bacterial CPT [18]

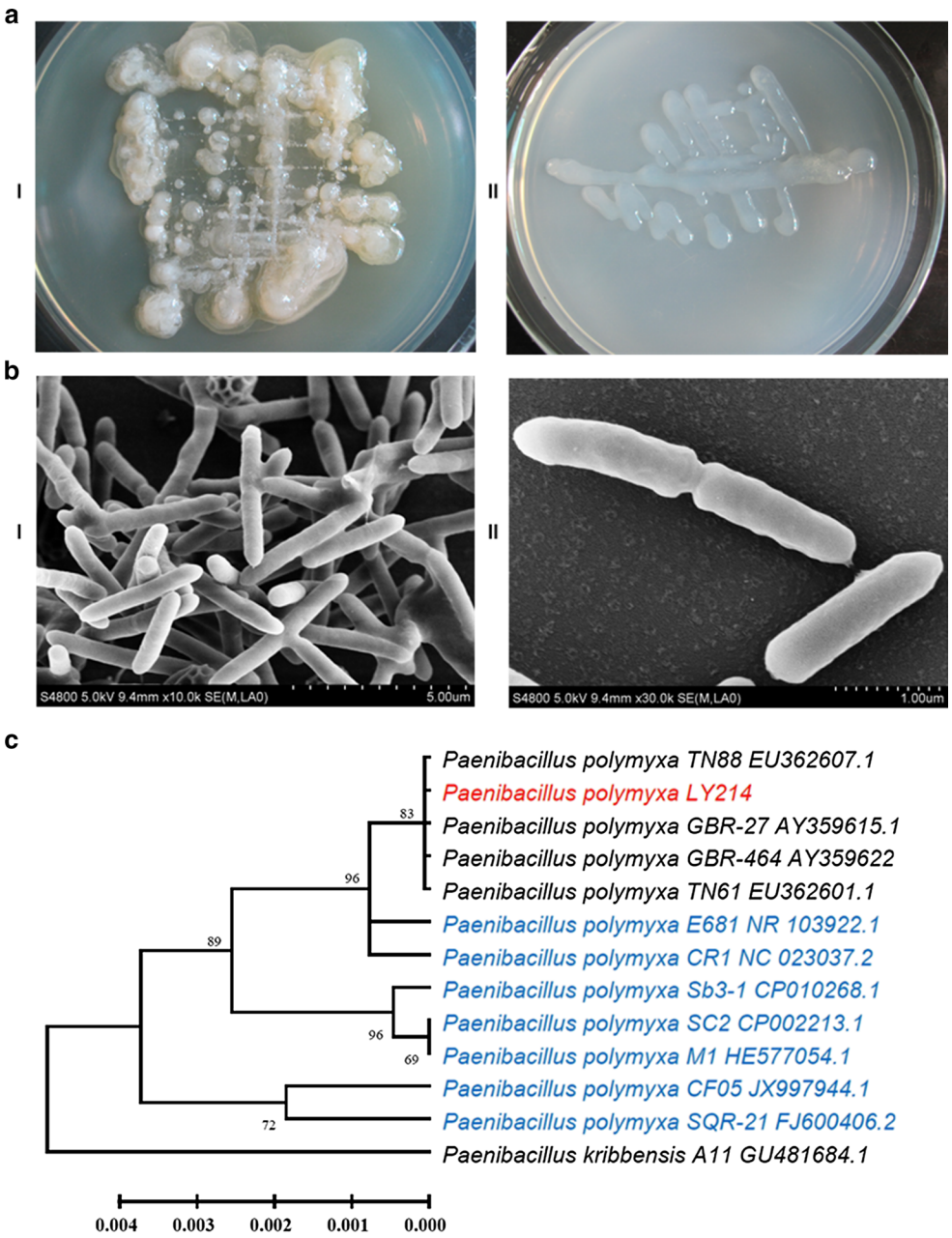

Fig. 3 Endophytic bacterium strain identification. Morphological analyses of LY214 grown on SDA (*panel I*, **a**) and CZA (*panel II*, **a**) media by optical photography and scanning electron microscope with different *scale bar* (*panels I* and *II*, **b**); and (**c**) neighbor-joining tree based on 16S rRNA gene sequence of *P. polymyxa* LY214 and its closest 16S rRNA gene matches in the GeneBank. The strain LY214 was highlighted in *red*. The strains whose genome were sequenced and annotated were in *blue* and their GenBank assembly accession numbers were GCA_000164985.2 for *P. polymyxa* SC2, GCA_000146875.2 for *P. polymyxa* E681, GCA_000237325.1 for *P. polymyxa* M1, GCA_000507205.2 for *P. polymyxa* CR1, GCA_000597985.1 for *P. polymyxa* SQR-21, GCA_000819665.1 for *P. polymyxa* Sb3-1, GCA_000785455.1 for *P. polymyxa* CF05

conditions, CPT production remained relatively constant, i.e., 2.8 μg l^{-1} of the 2nd generation, 2.4 μg l^{-1} of the 3rd generation, 1.2 μg l^{-1} of the 4th generation, 1.6 μg l^{-1} of the 5th generation, 1.2 μg l^{-1} of the 6th generation, 0.9 μg l^{-1} of the 7th generation, and 0.8 μg l^{-1} of the 8th generation of *P. polymyxa* LY214 was observed.

According to previous reports that the metal ions, secondary metabolism activators, and extractive resins had effects on the production of natural products [8, 9, 12, 13, 19]. In this work, HPLC–DAD quantification [13] analysis indicated that the metal ions Ca^{2+}, Cu^{2+}, Li^{+}, and Mn^{2+} and the two known activators salicylic acid (SA) and methyl jasmonate (MeJA) showed slight or adverse effects on CPT production (Fig. 4f). The addition of secologanin and tryptamine, the two putative biosynthetic precursors of CPT, were proven to increase 5- and 4-fold CPT production, respectively (Fig. 4f). Regarding the extractive adsorbent resins HP20 and XAD16, CPT yield increased sixfold when XAD16 (10 %, w/v) was added to the 5-day-fermentation broth of the 2nd generation of LY214 (Fig. 4f). The optimized fermentation conditions and the addition of the biosynthetic precursors and adsorbent resin XAD16 were combined and applied to ferment the strains of the 7th and 8th generation of LY214. A 15- to 30-fold increase of CPT yield was observed and the CPT yield was increased to 12.0–23.0 μg l^{-1}.

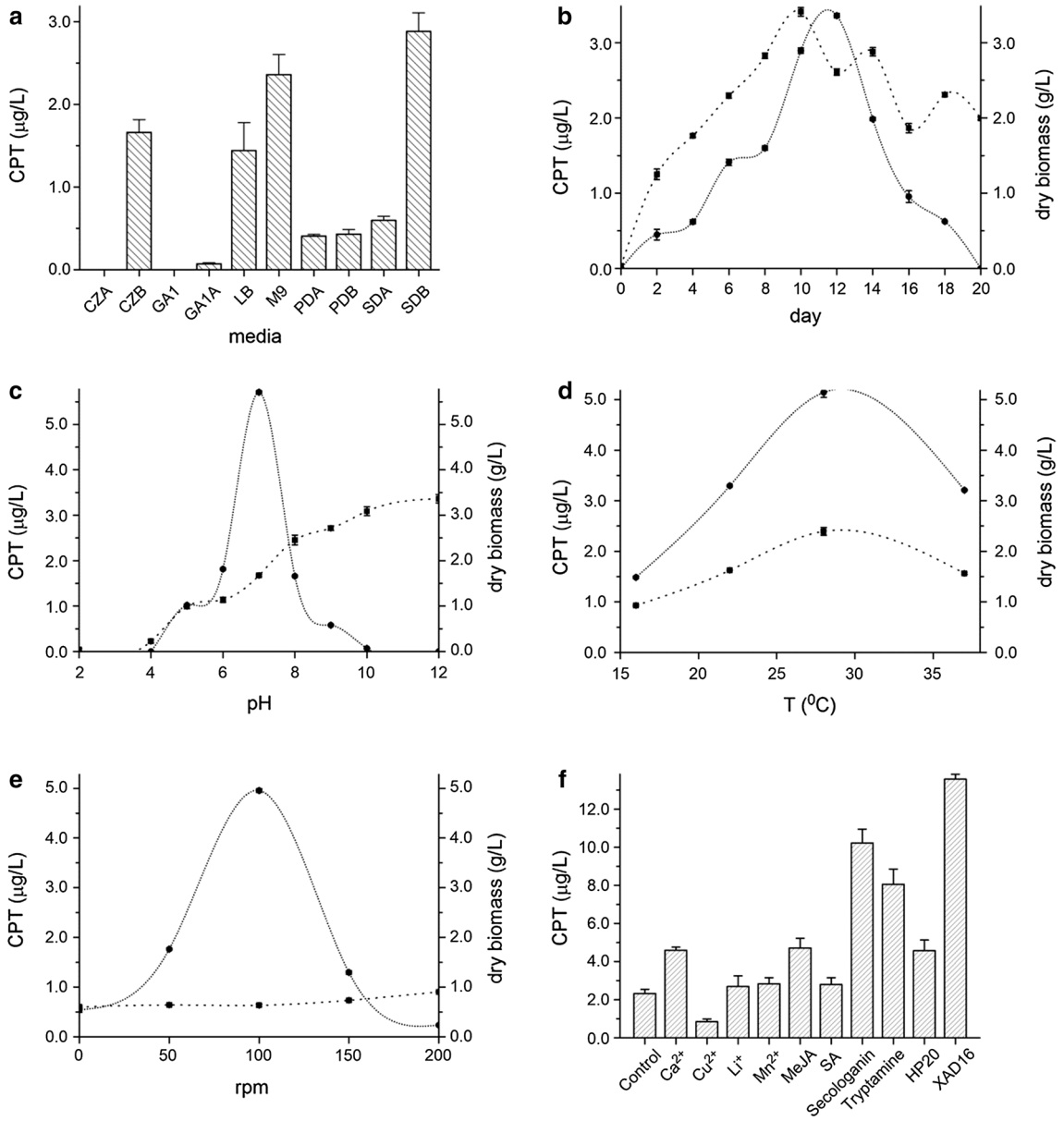

Fig. 4 Effects of medium composition (**a**), fermentation days (**b**), pH of medium (**c**), fermentation temperature (**d**), agitation rate of flasks (**e**), and the addition of metal ions, elicitors, biosynthetic precursors, and adsorbent resins (**f**) on CPT production of *P. polymyxa* LY214. CZA, Czapek yeast extract agar; CZB, Czapek yeast extract broth; GA1, Gause medium G-1; GA1A, Gause medium G-1 agar; LB, Lauria-Bertani medium; M9, M9 minimal medium; PDA, potato dextrose agar; PDB, potato dextrose broth; SDA, Sabouraud dextrose agar; SDB, Sabouraud dextrose broth; *filled circle*, CPT; *filled square*, biomass. The experiments were performed in triplicate. The mean values were used to plot and the *error bar* indicated the variation

The strains of the genus *Paenibacillus* have been isolated from a variety of sources, including soil, water, food, plant, and clinical patients [15]. Some species of the genus *Paenibacillus* are versatile and productive, which is a basic requirement for industrial use. Genome sequencing and bioinformatics annotation of 17 strains of *P. polymyxa* indicated that these strains may produce terpenoids and/or indole derivatives in view of that the encoding genes involved in the biosynthesis of terpenoid and indole moieties were annotated and deposited in the NCBI database. CPT was proposed to be a hybrid of a monoterpenoid (highlighted in blue, Fig. 1) from mevalonate and/or non-mevalonate pathway and an indole moiety (highlighted in green, Fig. 1) from shikimate pathway [21]. Strictosidine synthase, a member of gluconolactonase super family [21], is a key enzyme catalyzes the crucial Pictet-Spengler condensation reaction between the monoterpenoid and the indole moieties to form strictosidine—the common

intermediate of most, if not all, monoterpenoid indole alkaloids including CPT, vinblastine, and vincristine [22]. Although many CPT-producing microorganisms were reported, there is not any gene for strictosidine synthase or strictosidine synthase-like was reported [6, 16]. However, at least one gluconolactonase encoding gene was annotated in some *P. polymyxa* strains (highlighted in blue, Fig. 3c) that are relatively close to *P. polymyxa* LY214, which implied that LY214 may contain the encoding genes for CPT biosynthesis and can produce CPT in the fermentation broth.

In summary, *P. polymyxa* LY214 is an endophytic bacterium that can produce CPT in the fermentation broth although substantial decrease of CPT production was observed with subsequent successive subcluturing. A 15- to 30-fold increase of CPT yield was observed when the optimized fermentation conditions and the addition of the biosynthetic precursors and adsorbent resin XAD16 were combined and applied to ferment the strains of the 7th and 8th generation of LY214. A phylogenic tree analysis based on 16S rRNA gene sequences showed *P. polymyxa* LY214 has the potential to synthesize CPT and more investigations are needed to decipher the biosynthesis of CPT.

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