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Induced mutation breeding of *Brevibacillus brevis* FJAT-0809-GLX for improving ethylparaben production and its application in the biocontrol of *Lasiodiplodia theobromae*

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ABSTRACT

Ethylparaben is the main antimicrobial substance in the culture supernatant of *Brevibacillus brevis* FJAT-0809-GLX. The present study aimed to obtain and partially characterize mutant strains with higher ethylparaben production through mutagenesis of *B. brevis* FJAT-0809-GLX by UV irradiation and LiCl treatment. The results showed that the positive mutant rates of the UV irradiation, LiCl treatment and combination of UV irradiation and LiCl treatment were 7.8%, 0% and 12%, respectively. There were no morphological and growth rate differences between the mutants and the wild type strain. However, the mutant strains Bb-M1, Bb-M2 and Bb-M3 were more stable than the wild type strain FJAT-0809-GLX against temperature and pH changes. Mutant strains Bb-M1, Bb-M2 and Bb-M3 produced 5 times, 3 times and one time as much ethylparaben, respectively, when compared with the wild type strain FJAT-0809-GLX. The inhibition zones of the mutant strains to different pathogens, such as *Fusarium oxysporum*, *Colletotrichum orbiculare*, *Lasiodiplodia theobromae*, *Escherichia coli* and *Salmonella typhimurium*, were significantly larger than those of the wild type strain. The mutant strains and wild type strain showed antibacterial activities, with an inhibition zone of 13.2 mm, 13.3 mm, 13.8 mm and 11.1 mm against *E. coli* K88, respectively. When the storage time was 4 d, the percentages of fruit decay in the Bb-M1-, Bb-M2- and Bb-M3-treated wax apple fruits were 8.3%, 5.0%, and 5.0%, respectively; These values were significantly lower than the control treatment, which was 25.0%. These results indicated that *B. brevis* FJAT-0809-GLX mutant strains with higher ethylparaben yields may be more suitable as a candidate biocontrol agent for the control of *L. theobromae*.

1. Introduction

Brevibacillus brevis has a broad-spectrum antimicrobial activity (Seddon et al., 2000; Wafaa et al., 2013; Panda et al., 2014; Che et al., 2015a) and can inhibit the growth of many bacteria and fungi, such as *Botrytis cinerea* Pers. (Edwards and Seddon, 2001), *Fusarium oxysporum* (Sunita et al., 2010; Bouqellah et al., 2011), *Lasiodiplodia theobromae* (Che et al., 2015a,b) and *Ralstonia solanacearum* (Che et al., 2012; Hou et al., 2015), suggesting that it is a candidate biocontrol agent for many diseases. *B. brevis* produces different antibiotics, such as gramicidin S (Murray et al., 1986; Schmitt et al., 1999), lipopeptides (Wang et al., 2010), chitinase (Li et al., 2002), bacteriocin (Ghadbane et al., 2013) and ethylparaben (Che et al., 2015a,b). Among these, ethylparaben has been reported to control postharvest pathogens on different fruits (Valencia-Chamorro et al., 2009; Moscose-Ramírez et al., 2013; Che et al., 2015a,b). The antimicrobial activity of *B. brevis* FJAT-0809-GLX has been shown to be positively correlated with the concentration of ethylparaben in its culture supernatant (Che et al., 2015a,b). Therefore, improving the ethylparaben yield from *B. brevis* might increase its overall antimicrobial activity.

Many efforts have been made to increase the yields of antimicrobial compounds produced by *Bacillus* spp. (Lin et al., 2007; Rathakrishnan et al., 2011), some are based on modifying culture conditions that influence the production of antimicrobial compounds of *Bacillus* spp. (Akpa et al., 2001; Ray et al., 2012). The medium of *Bacillus subtilis* was optimized by response surface methodology and central composite rotary design, resulting in a 2-fold increased level of nattokinase production compared to initial levels (Deepak et al., 2008). The optimization of submerged culture conditions of *B. licheniformis* KBR6 resulted in a tannase yield that was enhanced by 2.18-fold from its unoptimized condition (Mohapatra et al., 2009). However, the modification of microbial strains has a greater influence on the production of

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antimicrobial compounds (Kachan and Evtushenkov, 2013; Sangkharak and Prasertsan, 2013). *B. amyloliquefaciens* mutant strain M3-7 produced 7-fold more antifungal metabolites than the wild type strain in MC medium. However, the optimized medium achieved a 1.62-fold enhancement in antifungal compound production, compared to that achieved in production medium MC (containing 25 g/L sucrose, 20 g/L peptone, 4.5 g/L yeast extract, 2 g/L KH_2PO_4, 0.6 g/L $MgSO_4$ and 6 mg/L $MnSO_4$) (Masmoudi et al., 2017). Modification of microbial strains could be achieved by physical and chemical mutagenesis (Diep et al., 2016). In recent years, there are some achievements in radiation-induced mutagenesis technique, which have been applied in practice. *B. subtilis* mutant colonies, which had protease activity higher than their parent strain, were obtained by gamma ray irradiation (Diep et al., 2016). Use of different mutagenic agents in combination for microbial strain improvement was employed for higher yields of antimicrobial compounds. Induced mutation by UV light and acridine orange enhanced the ability of *Bacillus* strains to produce up to 3-fold more polygalacturonase enzyme as compared to the wild type strains (Muzzamal and Latif, 2016). *B. subtilis* mutant M2 was obtained using a combination of UV irradiation and nitrous acid treatment, which produced twice the amount of biosurfactant compared to the wild type strain (Bouassida et al., 2017). Ethylparaben is the main antimicrobial substance in the culture supernatant of *B. brevis* FJAT-0809-GLX. It was found to inhibit different types of microbes to different extents (Che et al., 2015a,b). Modification of *B. brevis* FJAT-0809-GLX by physical and chemical mutagenesis to improve the yield of ethylparaben might improve its antimicrobial activities against different types of microbes. However, the induced mutation breeding of *B. brevis* for improving ethylparaben production has not yet been reported.

Lasiodiplodia theobromae is one of the dominant fungi and is capable of causing black spot on fruits and leaves and shortening the postharvest storage time of wax apple (*Syzygium samarangense* Merr. et Perry) (Yang et al., 2009). *B. brevis* strain FJAT-0809-GLX could reduce the black spot decay of wax apple fruit caused by *L. theobromae* (Che et al., 2015a,b). In the present study, we sought to improve the yield of ethylparaben in *B. brevis* strain FJAT-0809-GLX by induced mutation breeding with UV irradiation and LiCl. High ethylparaben mutant strains were selected and their partial characterizations were observed. Furthermore, their efficacy in inhibiting the growth of *L. theobromae* was investigated in *in vitro* and *in vivo* conditions.

2. Materials and methods

2.1. Microbial strains and culture conditions

Ethylparaben-producing *B. brevis* strain FJAT-0809-GLX was obtained from soil in Yongtai, Fujian Province, PR China, isolated in our laboratory (Che et al., 2013, 2015a,b), and used for all of the experiments. The liquid Luria-Bertani (LB) medium was used to culture this strain.

All fungal and bacterial strains used in this study are shown in Table 1. *L. theobromae* FJAT-9860, *F. oxysporum* FJAT-282 and *Colletotrichum orbiculare* FJAT-30256 were isolated from wax apple fruits, tomato plant and loquat fruits, respectively, in our lab (Zhan et al., 2013; Che et al., 2015a,b). The fungi were maintained on potato dextrose agar (PDA) medium at 4 °C and cultured on the same medium at 28 °C for 48 h before they were used in this study. *Escherichia coli* K88 and *Salmonella typhimurium* ATCC14028 were provided by China Institute of Veterinary Drug Control and Fujian Institute of Microbiology, respectively. Liquid LB medium was used for the culture of bacterial strains.

Table 1
Fungal and bacterial strains used in this study.

Strains	No.	Host plants/Resources
Lasiodiplodia theobromae	FJAT-9860	Wax apple
Fusarium oxysporum	FJAT-282	Tomato
Escherichia coli K88	FJAT-301	China Institute of Veterinary Drug Control
Salmonella typhimurium ATCC14028	FJAT-8720	Fujian Institute of Microbiology
Colletotrichum orbiculare	FJAT-30256	Loquat

Note: FJAT is an abbreviation for Fujian Agricultural Technology.

2.2. Induction of mutagenesis of Brevibacillus brevis FJAT-0809-GLX

2.2.1. Treatment of cells with UV rays

A single colony of the *B. brevis* strain FJAT-0809-GLX was cultured in 20 mL of LB medium and incubated for 12 h at 170 r min^{-1} and 30 °C. One milliliter of the broth culture was transferred to 100 mL of LB medium and incubated for 10 h at 170 r min^{-1} and 30 °C. A 10-mL sample was taken and centrifuged at 10,000 r min^{-1} for 2 min. The cells were collected and washed twice with sterilized saline water. Subsequently, the cells were diluted with sterilized saline water to 10^6 CFU mL^{-1}. A 10-mL cell suspension (10^6 CFU mL^{-1}) was put in petri plates with a diameter 9 cm and irradiated with UV radiation using an X-30 G UV lamp (Spectronics Corporation, Westbury, NY, USA) with 254 nm light (30 W) for 0 s, 20 s, 40 s, 50 s, 60 s, 70 s and 80 s. The distance between the petri plates and the UV lamp was adjusted to 20 cm. Three replicates were carried out for each treatment. The treated and non-treated cells were incubated in the dark at 30 °C for 48 h. The colony forming units (CFU) were counted, and the mortality percentage was calculated according to the following formula: 100 × [1-(CFU of treated suspension/CFU of non-treated suspension)].

2.2.2. Treatment of cells with LiCl

A cell suspension (10^6 CFU mL^{-1}) was spread on LB plates containing LiCl at the following concentrations: 0%, 0.1%, 0.3%, 0.5% and 0.7% (g g^{-1}). After incubation at 30 °C for 48 h, the percentage of lethality was calculated as described above.

2.2.3. Treatment of cell suspension with combined UV rays and LiCl treatment

A cell suspension (10^6 CFU mL^{-1}) was exposed to an X-30 G UV lamp with 254 nm light (30 W) at a distance of 20 cm for 30 s and 50 s. Serial dilutions of treated cells were incubated on LB plates containing LiCl with final concentrations of 0%, 0.1%, 0.3%, 0.5% and 0.7% (g g^{-1}) and incubated in the dark at 30 °C for 48 h.

2.2.4. Preliminary screening of potential mutant strains

All the colonies were selected and incubated in 20 mL of liquid LB medium on a shaker at 30 °C at 170 r min^{-1} for 48 h. A 1-mL sample was taken and centrifuged at 13,000 r min^{-1} for 5 min, and the culture supernatant was collected for the antifungal assay. The antifungal activity of the culture supernatant against the *L. theobromae* FJAT-9860 was tested using an agar well diffusion method (Che et al., 2015a,b). Strains showing different antifungal activities from wild type strain *B. brevis* FJAT-0809-GLX were selected as potential mutant strains. The experiment was conducted three times.

2.3. Molecular characterization of selected mutant strains

The identification of selected mutant strains was conducted by using specific detection primers BREV174 F (5′-AGACCGGGATAACATAGGG AAACTTAT-3′) and 1377R (5′-GGCATGCTGATCCGCGATTACTAGC-3′) (Shida et al., 1996), which were synthesized by Boshang Biotechnology Co., Ltd. (Shanghai, China). The total genomic DNA was isolated following the procedure of Takagi et al. (1993). The PCR reaction mixture (25 μL) contained 2.5 μL of 10 × buffer (Sangong, Shanghai, China), 200 μM of each dNTP (Sangong, Shanghai, China), 1 U of TaKaRa Ex