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Short communication

Manipulation of *B. megaterium* growth for efficient 2-KLG production by *K. vulgare*

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ABSTRACT

In the two-step Vitamin C fermentative production, its precursor 2-keto-L-gulonic acid (2-KLG) was synthesized by *Ketogulonicigenium vulgare* through co-culture with *Bacillus megaterium*. The rates of *K. vulgare* cell growth and 2-KLG production were closely related with *B. megaterium* concentration in the co-culture system. To enhance the 2-KLG production efficiency, a strategy of manipulating *B. megaterium* growth in the co-culture system and properly releasing its intracellular components was introduced. Lysozyme was used specifically to damage *B. megaterium* cell wall structure and subsequently inhibit its cell growth. When 10,000 U mL^{-1} lysozyme was fed to the co-culture system at 12 h, the growth rate of *K. vulgare*, sorbose consumption rate, and 2-KLG productivity could increase 27.4%, 37.1%, and 28.2%, respectively.

1. Introduction

Vitamin C or L-ascorbic acid, an essential and exogenous nutrient for humans and other primates, plays crucial roles in biochemical and regulatory reactions, such as scavenging reactive oxygen [1] and acting as a cofactor for metalloenzymes of living organisms [2,3]. Numerous biotechnological methods have been developed and exploited to increase its yield and productivity [4–8]. The synthesized vitamin C production world-wide (approximately 110,000 tons annually) mostly originates from glucose via the Reichstein process or the two-step fermentation process [9,10]. The modern two-step fermentation process, originally developed in China during 1960s [11], uses *Ketogulonicigenium vulgare* [12] and *Bacillus megaterium* [13] as the industrial strains to synthesize 2-keto-L-gulonic acid (2-KLG), the precursor of vitamin C. Among the two strains, *B. megaterium* acts as a companion that secretes some metabolites to stimulate the growth of *K. vulgare* and thus enhances 2-KLG production [14]. In the past few decades, much effort has been devoted to identify those internal and external metabolites from *B. megaterium* through biochemical and molecular methods [14–16], and certain proteins or amino acids were suggested to play a role in this process. Two different proteins isolated from *B. megaterium* capable of improving 2-KLG productivity by *K. vulgare* have been identified. Recently, a novel 58-kDa protein excreted from *B. megaterium* BM302 with sorbose dehydrogenase activity has been identified [15]. However, the name and specific function of this protein, particularly its role in promoting the metabolites secretion remains unclear. As a result, it is difficult to achieve the target of 2-KLG production enhancement in the co-culture system because the rational or targeted operational way is not available.

Disrupting the cell wall of *B. megaterium* could be considered as one of the effective ways to stimulate the secretion. Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase (EC 3.2.1.17), is one of the candidates for the cell wall disruption. Although the function and structure of lysozyme have been extensively explored [17–19], application of lysozyme as the cell walls disrupting agent for industrial strains in order to improve titer, yield, and productivity of fermentation products has not been previously reported. In this study, lysozyme was employed to disrupt the *B. megaterium* cell wall, and the consequent effect on the co-culture system performance, namely, the *K. vulgare* growth and 2-KLG production were carefully investigated.

2. Materials and methods

2.1. Microorganisms

The strains of *K. vulgare* and *B. megaterium* used in this study were kindly provided by Jiangsu Jiangshan Pharmaceutical Co., Ltd., and stored in the Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University.

2.2. Culture conditions

Medium composition for seed culture (medium A) and fermentation (medium B) were same as that reported by Yan et al. [20]. The seed culture inoculated from a

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slant was cultivated at 30 °C, 200 rpm, in a 750-mL flask containing 75 mL medium A on a reciprocal shaker for 32 h (*K. vulgare*), 9 h (*B. megaterium*), respectively. The seeds were mixed and cultured for another 18 h, and then the mixture of *K. vulgare* and *B. megaterium* was transferred into the fermentation medium. The fermentations were carried out in 750-mL flasks containing 75 mL medium B, buffered by 5 g L^{-1} $CaCO_3$, or in a 7-L jar fermentor (KF-7 L, Korea Fermentor Co., Inchon, Korea) with 4 L medium B. The inoculums amount was 10% (v/v). In the fermentation with jar fermentor, pH was automatically controlled at 7.0 using 8 M NaOH solution, and the agitation speed was controlled at 400 rpm. The air flow rate was 1.5 L min^{-1}. All experiments were done in biological triplicate. All cultivations were carried out at 30 °C.

2.3. Preparation of culture supernatant and cytosolic matrix of *B. megaterium*

10 mL *B. megaterium* cell samples were collected at 40 h, and then centrifuged at 4 °C, 7000 × *g* for 10 min to obtain culture supernatants. The supernatants were sterilized with 0.22 μm filters. To obtain cytosolic matrix of *B. megaterium*, 75 mL culture medium were collected at 12 h and then centrifuged to separate the cell pellets from the culture. The cell pellets were washed twice with sterile water and disrupted by ultrasonication at 0 °C for 20 min with 30 s on–off intervals. Cell debris was removed by centrifugation at 4 °C, 13,000 × *g* for 30 min. The cytosolic matrix was also sterilized with 0.22 μm filters. At last, the content of proteins in the supernatants and cytosolic matrix of *B. megaterium* was examined by Bradford assay [21].

2.4. Experimental design

2.4.1. Enhancement of *K. vulgare* growth and 2-KLG production by *B. megaterium*

In order to determine the *K. vulgare* growth stimulating components, the following five experiments were designed: (1) *K. vulgare* were grown alone; (2) *K. vulgare* were grown with *B. megaterium* supernatants (0.05 mg mL^{-1} protein); (3) *K. vulgare* were grown with *B. megaterium* cytosolic matrix (0.05 mg mL^{-1} protein); (4) *K. vulgare* were grown with *B. megaterium* cytosolic matrix and supernatants (0.05 mg mL^{-1} protein); (5) *B. megaterium* (10% v/v) and *K. vulgare* were in mixed and cultured.

2.4.2. Effect of lysozyme concentration on *K. vulgare* and *B. megaterium* cell viability and 2-KLG production

Resting cells were prepared as follows: *B. megaterium* (12 h) and *K. vulgare* (30 h) were collected separately and washed twice with water, then re-suspended and mixed in 750-mL flask with medium B. Lysozyme dissolved in sterile water was added to *B. megaterium* and *K. vulgare* resting cells in amount of 1000, 5000 and 10,000 U mL^{-1}, respectively.

2.5. Assay methods

2-KLG in the fermentation broth was determined by HPLC using an Aminex HPX-87H column (Bio-Rad) at 35 °C with a flow rate of 0.5 mL min^{-1} and 0.4 mM H_2SO_4 as the eluent [22]. Sorbose concentration was determined via the sulfuric anthrone reaction and detected at 620 nm [23]. *K. vulgare* and *B. megaterium* were enumerated by hemacytometer count and confirmed by standard plate count.

2.6. Statistical analysis

Student's *t*-test was employed to investigate statistical differences of *K. vulgare* growth and 2-KLG production with or without *B. megaterium* addition. Samples with *P* values of <0.05 were considered statistically different.

3. Results and discussion

3.1. Enhancement of *K. vulgare* growth by *B. megaterium*

Although *K. vulgare* can self-survive in the fermentation medium, but the growth is very poor (cfu 7.85×10^8, 36 h) (Fig. 1A). Interestingly, the growth of *K. vulgare* can be promoted by the presence of *B. megaterium* in the *K. vulgare* culture broth, and a positive relationship between *K. vulgare* growth and the number of *B. megaterium* bacteria in the mixed culture could be observed as shown in Fig. 1A. When 5% and 10% (v/v) *B. megaterium* broth were added to the culture broth, the *K. vulgare* amount increased to 1.23×10^9 and 1.25×10^9 (cfu), respectively, which was 56.6% and 59.2% higher than that of the control. More importantly, the presence of *B. megaterium* can efficiently reduce the lag time (6 h, red line in Fig. 1A) and the time to achieve the highest cfu (9 h, blue line in Fig. 1A). The effect of the addition time of *B. megaterium* on

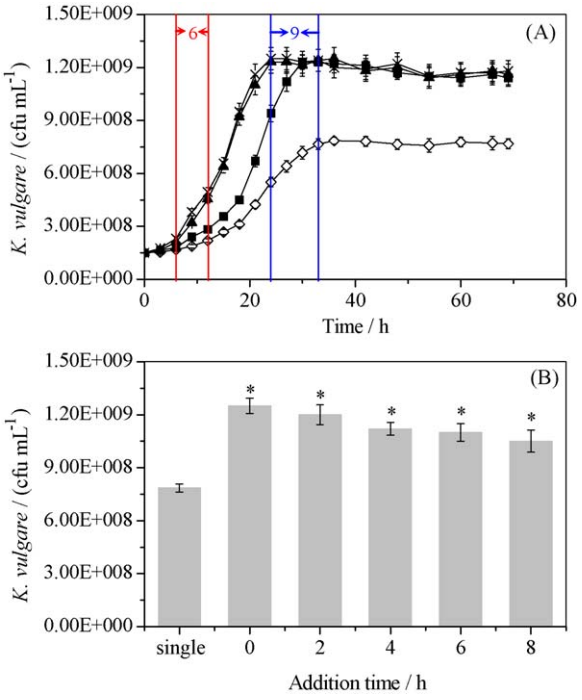

Fig. 1. Effect of *B. megaterium* addition volume (A) and time (B) on K. *vulgare* growth. 5%, 10% and 15% (v/v) *B. megaterium* broth were added to the culture broth at the beginning of the fermentation (A), and *B. megaterium* (10%, v/v) added at 0, 2, 4, 6, 8 h and harvested at 68 h (B). Initial sorbose concentration was 80 g L^{-1}. Error bars indicate standard deviations ($n = 3$). Statistically significant differences ($P < 0.05$) were determined by Student's *t*-test and are indicated with an asterisk. ◇, Without *B. megaterium*; ■, 5% (v/v) *B. megaterium*; ▲, 10% (v/v) *B. megaterium*; ×, 15% (v/v) *B. megaterium*.

K. vulgare growth was also investigated in detail (Fig. 1B). It was found that adding *B. megaterium* at different time could all significantly enhance *K. vulgare* growth. The highest final cell density of *K. vulgare* was achieved when *B. megaterium* was added at the beginning of the fermentation. Delaying addition time, even though the promotion effect of *K. vulgare* growth is continuously decreased, the final amount of *K. vulgare* in presence of *B. megaterium* is still larger than that of the control (without *B. megaterium* addition). This result indicated that *B. megaterium* may secrete certain bioactive component to promote *K. vulgare* growth. This result also strongly suggested that *B. megaterium* itself or the metabolites produced is vital for obtaining high level or amount of *K. vulgare*.

A question remaining unanswered is that, whether the intracellular or extracellular metabolites, or both of them stimulated the growth of *K. vulgare*. To answer the question, five experiments in the 7-L jar fermentor were designed and conducted (see Section 2.4.1) and the results were summarized in Table 1. No enhanced *K. vulgare* growth was detected only when the centrifugal supernatant of *B. megaterium* was supplemented to the culture. However, the concentration of *K. vulgare* reached 1.19×10^9 cfu when adding the sonicated extracts of *B. megaterium*, and similar cell levels could also be achieved for the cases #4 and #5 (refer to Table 1). This result strongly suggested that both the cytosolic matrix of *B. megaterium* and *B. megaterium* cells stimulated the growth of *K. vulgare*, and the highest 2-KLG production were achieved in mixed culture. Among the different culture strategies, the cytosolic matrix of *B. megaterium* played a central role in achieving high growth of *K. vulgare*. The major results could be summarized and interpreted as follows: (1) the highest *K. vulgare* concentration was detected when adding *B. megaterium* at beginning of the fermentation; (2) *K. vulgare* growth