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Full Length Article

Effect of Rare Earth Elements on Vitamin C Fermentation by Mixed Cultures

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Abstract

In recent years, the research on rare earth metals has expanded to the field of microbiology. In China, the production of Vitamin C is through mixed culture fermentation, which transforms L-sorbose to 2-keto-L-gulonic acid (2-KGA), the precursor of Vitamin C. In this study we compared the yields of 2-KGA by an acid-producing strain of *Ketogulonigenium vulgare* that was co-cultured with different companion bacterial strains in the presence of four light rare earth elements, lanthanum, cerium, neodymium, and samarium at selected concentrations. We found that all of the tested rare earth elements had a promoting effect on 2-KGA production at concentration up to 5 mM. At or above 10 mM, the yield of 2-KGA was reduced. When cultured separately with 5 mM of given rare earth elements, the growth of two companion bacterial strains (*Bacillus megaterium* 25-B and *B. subtilis* A9) were both significantly reduced, while that of the *K. vulgare* strain was enhanced. © 2014 Friends Science Publishers

Keywords: 2-keto-L-gulonic acid; *Ketogulonigenium vulgare*; Companion strain; Mixed cultures; Rare earth elements

Introduction

The mixed culture fermentation of L-sorbose for producing 2-keto-L-gulonic acid (2-KGA), the precursor of Vitamin C (Vc), is the main method of industrial Vc production in China (Zhang *et al.*, 2008, 2010; Ai *et al.,* 2013). The mixed culture fermentation is realized by the cooperation of two microorganisms: *Ketogulonigenium vulgare* and *Bacillus megaterium*. As a Gram-negative bacterium, the strain of *K. vulgare* is usually referred to as the acid-producing strain, which grows poorly and its production of 2-KGA is low under *K. vulgare* mono-culture system; As a Gram-positive bacterium the strain of *B. megaterium* is the companion strain (or co-culture helper) which promotes the growth and 2-KGA production of *K. vulgare* in mixed culture fermentation of vitamin C (Feng *at al*., 1998).

Rare earth elements (REEs), also known as lanthanides, are a group of 15 metals with similar properties, which are used for a broad spectrum of applications in industry, agriculture and medicine. REEs also affect the growth of microorganisms. Generally low concentrations of REEs promote and high concentrations of REEs inhibit, the growth of microorganisms (Tang *et al.*, 2001; Wang *et al*., 2005; Jiang *et al.*, 2008).

In this study, an acid-producing strain of *K. vulgare* was cultured with two different companion bacterial strains, *B. megaterium* 25-B and *B. subtilis* A9. Four light REEs, lanthanum, cerium, neodymium and samarium, were added at selected concentrations to the mixed cultures. Our results demonstrated that these light REEs could increase the yield of 2-KGA significantly. These results of the experiments could provide critical information that can be referenced for large-scale production of vitamin C.

Materials and Methods

Materials

The acid producing strain of *K. vulgare* and the companion strain of *B. megaterium* 25-B used in this study for vitamin C fermentation were provided by Northeast Pharmaceutical General Factory of Vitamin C Company (Shenyang, China). The *B. subtilis* A9 strain was from Shenyang Agriculture University collection. The slant agar in g/L consisted of: L-sorbose, 5; peptone, 10; corn steep liquor, 5; yeast extract, 3; beef extract, 3; $MgSO_4$, 0.2; agar, 25. The isolation medium in g/L consisted of: L-sorbose, 20; peptone, 10; corn steep liquor, 3; yeast extract, 3; beef extract, 3; urea, 1; $MgSO_4$, 0.2; KH_2PO_4, 1; $CaCO_3$, 1; agar, 25. The seed medium in g/L consisted of: L-sorbose, 20; glucose, 2; corn steep liquor, 5; urea, 1; $CaCO_3$, 1. The companion strain medium in g/L consisted of: glucose, 2; corn steep liquor, 5; urea, 1. The fermentation medium in g/L consisted of: L-sorbose, 90; corn steep liquor, 10; urea, 12; KH_2PO_4, 1; $MgSO_4$, 0.2; $CaCO_3$, 1.

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Methods

Fermentation conditions used in this study consisted of 5 mL of fermentation medium mixed with 10% (v/v) of seed culture in a 50 mL flask containing different concentrations of the four rare earth elements. The inoculated mixed culture was incubated at 29°C for 24~72 h, with orbital shaking aeration at 180 rpm. The initial culture acidity was adjusted to pH 6.8~7.0 (Gao *et al*., 2012). The 2-KGA concentration was determined using the iodometric method as previously described (Chen and Yan, 1981), with three independent repetitions. The conversion rate of 2-KGA was calculated using the formula:

Conversion rate = [2-KGA Yield (g/L)/L-sorbose Input (g/L)]× 1.0776 × 100%

Where: 1.0776 is the relative molecular mass ratio of 2-KGA and L-sorbose.

For determining the growth characteristics of the strains, any strain (including *K. vulgare*, *B. subtilis* A9 and *B. megaterium* 25-B) in this study was pure cultured in the seed medium at 29°C for 0~60 h with the initial culture acidity adjusting to pH 6.8~7.0, measured every 3 hours by using the spectrophotometer at 650 nm, with five independent repetitions.

Statistical Analysis

Design of the experiments was completely randomized with three to five replications. Statistical analyses for all experiments were performed using the Proc Mixed model (general linear model, GLM) of SAS 9.3 (SAS Inst. Inc., Cary, NC.). When effect(s) were statistically significant, mean comparisons were performed with Sidak adjusted P values to maintain experiment-wise error (α) at 0.05.

Results

Effect of REEs on the Yield of 2-KGA with *B. megaterium* 25-B as Companion Strain

For each of the light REEs, concentrations of 1, 5, 10, and 15 mM were tested for the effect on the yields of 2-KGA by *K. vulgare* cultured with companion strain of *B. megaterium* 25-B for three independent repetitions. Similar effects on the yields of 2-KGA were observed for all of the four tested REEs (Fig. 1). At concentrations up to 5 mM, the yields of 2-KGA increased comparably for all four REEs. The average increases of 2-KGA yields were 13.6 and 25.6% in the presence of 1 and 5 mM of REEs, respectively. The highest yield of 2-KGA was obtained with the addition of 5 mM of La^{3+}, which reached 73.4 g/L. In comparison to the control (54.4 g/L), this represented a 34.9% increase of 2-KGA yield. The calculated conversion rate (from L-sorbose to 2-KGA) increased from 63.1 to 87.8%. At or above concentrations of 10 mM, each of the four REEs resulted in a sharp decrease in 2-KGA yields. Addition of each of the light REEs at 10 mM resulted in a drop of 2-KGA yields to approximately 25% of that of the control.

Yield of 2-KGA by *K. vulgare* stimulated by 5 mM of La^{3+} with *B. subtilis* A9

With the concentration of each of the REEs from 0~5 mM, the curves of the yield of 2-KGA are similar to Fig. 1. With three independent repetitions, the yield of 2-KGA increased with the addition of up to 5 mM of each of the REEs. When the concentration of REEs was 5 mM, the yield of 2-KGA was maximum. When REE was added at concentration at or above 10 mM, the amount of 2-KGA decreased significantly (Fig. 2). The yield of 2-KGA with the addition 5 mM of La^{3+} reached 83.5 g/L, in contrast to the control which yielded 72.1 g/L and resulted in the conversion rate (from L-sorbose to 2-KGA) increasing from 86.3 to 99.98%. Of interest, the yield of 2-KGA by *K. vulgare* companion with *B. subtilis* A9 increased 13.8% than that of *K. vulgare* cultured with companion strain of *B. megaterium* 25-B. This suggested that the companion strain *B. subtilis* A9 plays more important role in stimulating *K. vulgare* to produce 2-KGA than that of *B. megaterium* 25-B.

Effect of Rare Earth Elements on the Growth of *K. vulgare* and Companion Bacterial Strains

The effect of the REEs was further examined by cultivating *K. vulgare* and the companion strains of *B. megaterium* 25-B and *B. subtilis* A9, individually in the presence or absence of 5 mM of the REEs respectively. In five independent repetitions, the growth of the *K. vulgare* strain was all significantly stimulated by the addition of REEs in the seed medium (Fig. 3), but that of both *B. megaterium* 25-B and *B. subtilis* A9 were strongly inhibited (Fig. 4 and 5). In the absence of a companion strain, 2-KGA production by *K. vulgare* in the fermentation medium is less than 10% of that in the presence of *B. megaterium* 25-B or *B. subtilis* A9. Although adding 5 mM of La^{3+} to *K. vulgare* culture stimulated 2-KGA production, the yield of 2-KGA was much higher in the mixed cultures of *K. vulgare* and companion strain of 25-B (or A 9) under the same condition (Fig. 6).

Discussion

Three bacterial strains used in this study, *K. vulgare*, *B. megaterium* 25-B, and *B. subtilis* A9, appeared to have different capacities of accumulating REEs (Tsuruta, 2006). With the concentrations of La^{3+} at 5 mM, the growth of *K. vulgare* was promoted, while that of *B. megaterium* 25-B and *B. subtilis* A9 strongly inhibited. However, it was previously shown that the growth of *B. megaterium* 25-B and *B. subtilis* A9 was promoted at much lower concentrations of La^{3+} (Yang *et al*., 2008). Therefore, it can be concluded that 5 mM of La^{3+} exceeded the tolerance limits of these strains.

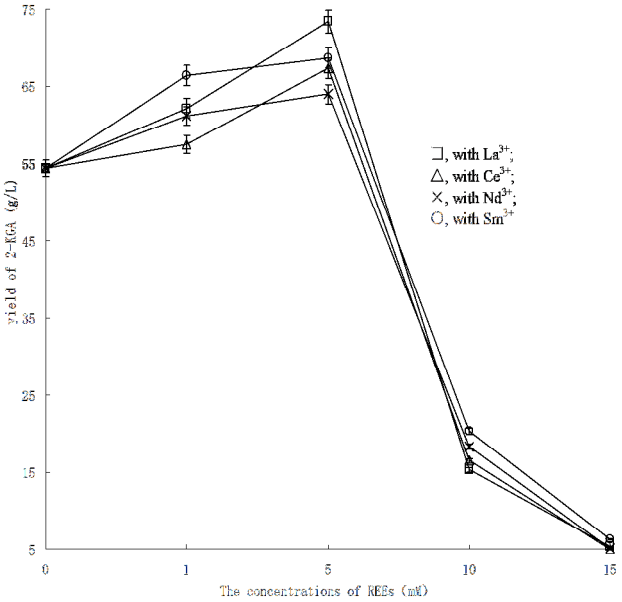

Fig. 1: The yield of 2-KGA produced by *K. vulgare* with companion *B. megaterium* 25B at different concentrations of the four REEs. The error bars represent the standard deviation for three replicates

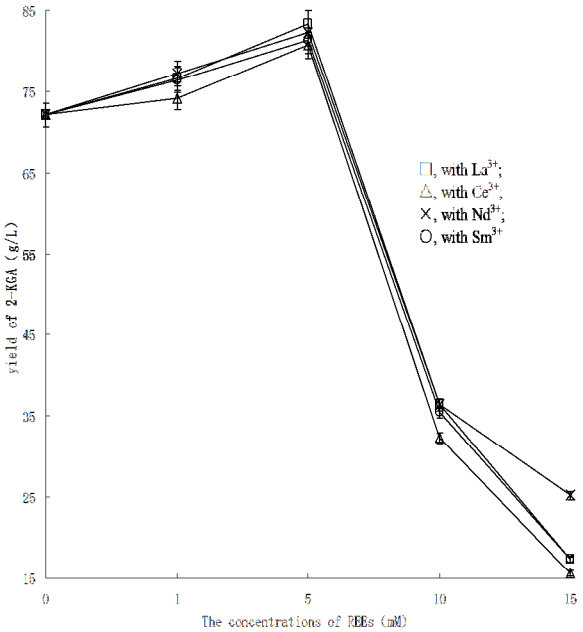

Fig. 2: The yield of 2-KGA produced by *K. vulgare* with companion *B. subtilis* A9 at different concentrations of the four light REEs. The error bars represent the standard deviation for three replicates

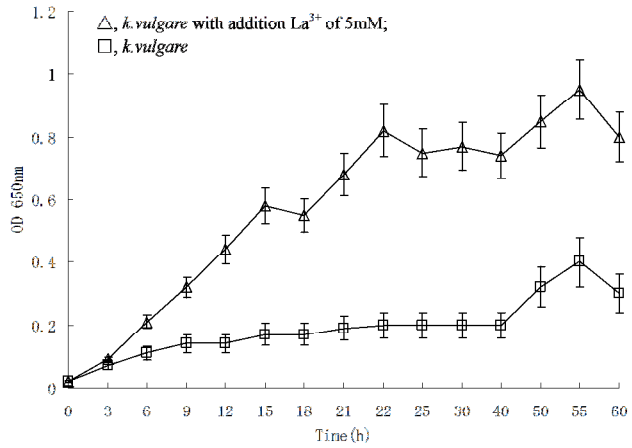

Fig. 3: The growth of *K. vulgare* with addition La^{3+} of 5 mM. The error bars represent the standard deviation for five replicates

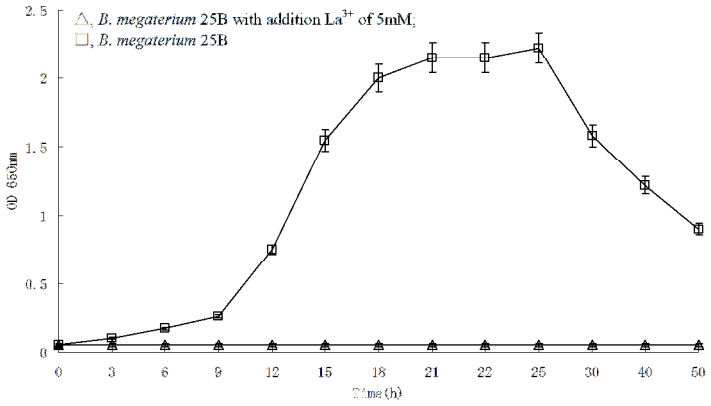

Fig. 4: The growth of *B. megaterium* 25B with addition of 5 mM of La^{3+} (△, *B. megaterium* 25B with addition La^{3+} of 5 mM;□, *B. megaterium* 25B). The error bars represent the standard deviation for five replicates

In general, Gram-positive bacteria accumulate more REEs than Gram-negative bacteria (Tsuruta, 2006). Therefore, with the same concentration of 5 mM REEs, the Gram-negative bacterium of *K. vulgare* could be expected to accumulate low concentration of REEs that is less than the amount needed to trigger a toxic reaction, resulting in a stimulation of growth. In contrast, the Gram-positive bacteria of *B. megaterium* 25-B and *B. subtilis* A9 would be expected to accumulate much higher concentrations of the REEs that are sufficient to trigger a toxic reaction, resulting in the inhibition of growth.

In Gram-positive bacteria, the teichoic acid polymers rich in ionized phosphate groups confer a strong negative charge on the surface of the cell wall. In contrast, little teichoic acid is found in Gram-negative bacteria. The teichoic acids play a significant role in binding metal ions to the cell walls of the strains of *B. subtilis* (Tsuruta, 2006). Therefore, it is believed that the accumulation of La^{3+} on the cell surface in Gram-positive bacteria is stronger than that in Gram-negative bacteria (Yang *et al*., 2005). Consequently, it is reasonable that Gram-positive bacteria accumulate higher REEs than Gram-negative bacteria.

The synthesis of RNAs, proteins in bacterial cell could be accelerated at low concentration of REEs, and so are cell

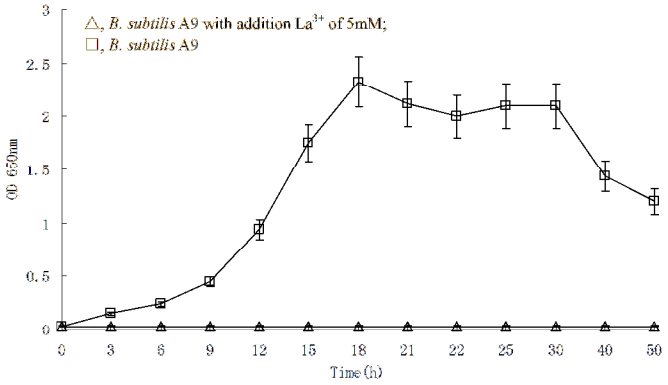

Fig. 5: The growth of *B. subtilis* A9 with addition La^{3+} of 5 mM. The error bars represent the standard deviation for five replicates

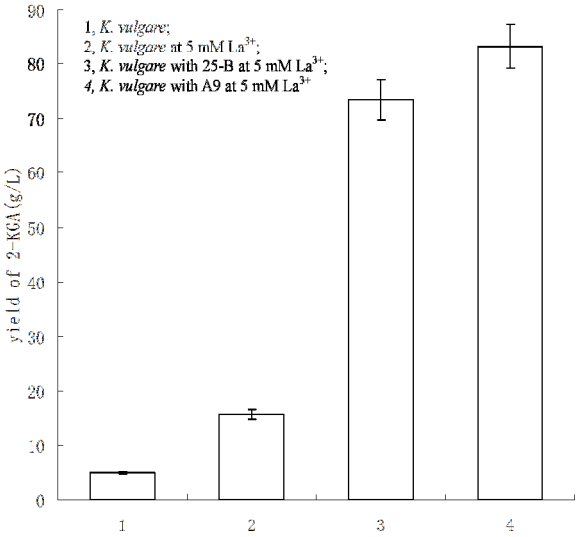

Fig. 6: The effect of different culture conditions to the yield of 2-KGA. The error bars represent the standard deviation for five replicates

growth and division (Xu *et al*., 1992). However, at high concentrations of REEs, the bacteriostatic mechanism of REEs is triggered (Huo *et al.*, 2002). Ca^{2+}, which is crucial for multiple biological functions in cells, may be replaced by REEs. Liu (2004) proposed that due to La^{3+} replacement of Ca^{2+} at low La^{3+} concentrations, increased cell permeability in *Escherichia coli* led to a more efficient nutrient uptake, while at higher La^{3+} concentrations, *E. coli* accumulates toxic levels of La^{3+}, resulting in inhibition of growth (Zhao *et al*., 2000; Luigi *et al*., 2009).

When separately cultured, the growth of *K. vulgare* was significantly enhanced, and that of the companion bacteria (25-B and A9) were significantly inhibited by the individual REEs at 5 mM. But without the companion strain

of *B. megaterium* 25-B or *B. subtilis* A9 in fermentation medium, there was only a slight increase in the yield of 2-KGA by *K. vulgare* cultured alone when 5 mM of La^{3+} were added. We propose that a symbiotic relationship exists between *K. vulgare* and *B. megaterium* 25-B (or *B. subtilis* A9), which results in a two-staged growth in the mixed culture fermentation when an effective concentration of REE is added. In the first stage, the more REE tolerant *K. vulgare* would grow rapidly and sequesters REEs through surface accumulation. The decreased level of REE in the medium would allow the more REE sensitive companion bacteria to grow in the second stage. Then in turn the growth of the companion bacteria may generate more extracellular protein (Lu *et al*., 2001) to accelerate the growth of *K. vulgare*, resulting in significantly increase in the yield of 2-KGA.

In conclusion, four light REEs at concentration of 5 mM significantly increased the yields of 2-KGA in Vc fermentation by mixed cultures. The REE of La^{3+} at 5 mM significantly stimulated the growth of *K. vulgare*, but inhibited that of companion strains of *B. megaterium* 25-B and *B. subtilis* A9 in pure cultured respectively.

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