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Short Communication

High-level production of calcium malate from glucose by *Penicillium sclerotiorum* K302

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HIGHLIGHTS

- The high-level calcium malate-producing *Penicillium sclerotiorum* K302 was obtained.
- The titer of calcium malate reached 92.0 g/l.
- The main product was calcium malate in the culture.
- The fermentation period was 3 days.

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ABSTRACT

In this study, after screening of 9 fungal strains for their ability to produce calcium malate, it was found that *Penicillium sclerotiorum* K302 among them could produce high-level of calcium malate. Under the optimal conditions, the titer of calcium malate in the supernatant was 88.6 g/l at flask level. During 10-l fermentation, the titer of 92.0 g/l, the yield of 0.88 g/g of glucose and the productivity of 1.23 g/l/h were reached within 72 h of the fermentation, demonstrating that the titer, yield and productivity of calcium malate by this strain were very high and the fermentation period was very short. After analysis of the partially purified product with HPLC, it was found that the main product was calcium malate. The results showed that *P. sclerotiorum* K302 obtained in this study was suitable for developing a novel one-step fermentation process for calcium malate production from glucose on large scale.

1. Introduction

Malic acid is a four-carbon dicarboxylic acid and an intermediate in the tricarboxylic acid (TCA) cycle. It has many applications in the beverage and food industry as an acidulant and flavor enhancer, in metal cleaning, textile finishing and pharmaceuticals and it is also used as a feedstock for chemical synthesis of polymalic acid (PMA) (Liu and Jarboe, 2012). It can be synthesized by chemical ways through the hydration of fumaric acid under high temperature and pressure, yielding a racemic mixture of D- and L-malate (Goldberg et al., 2006) or by enzymatic ways that transform fumaric acid to L-malate using the fumarase and the microbial cells containing fumarase (Presecki and Vasic-Racki, 2005). However, these processes have many disadvantages. In recent years, L-malic acid production from glucose by one step fermentation has received more and more attention and many microorganisms were found be able to produce L-malate. Unfortunately, the direct malic acid production by microbial fermentation is limited by low product yield, titer, and productivity due to end-product inhibition (Zou et al., 2013). Therefore, it is very important to screen the microbial strains which can actively transform glucose to high level of L-malic acid and can highly secrete it into medium.

In this study, several strains of *Penicillium* spp isolated from different marine environments were screened for their ability to produce calcium malate. It was found that the strain K302 could produce higher level of calcium malate than any other strains tested in this study. Therefore, it was used for calcium malate production in this study. The calcium malate produced can be easily transformed into L-malic acid and $CaSO_4$ (undissolved) using H_2SO_4.

2. Methods

2.1. Fungal strains and cultivation media

Nine strains (strains 99, 69, 83, 159, 72, 1120, 1062, 1075 and K302) of *Penicillium* spp isolated from different marine environments were used in this study and stored at −80 °C in this laboratory. The medium for growth of the seed culture contained 60.0 g/l

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of glucose, 3.0 g/l of yeast extract, 3.0 g/l of NH_4NO_3, 10.0 g/l of $CaCO_3$. The cultivation time and temperature were 48 h and 28 °C, respectively. The medium for calcium malate production consisted of 140.0 g/l of glucose, 2.0 g/l of NH_4NO_3, 0.1 g/l of KH_2PO_4, 0.1 g/l of $MgSO_4{\cdot}7H_2O$, 0.5 g/l of KCl, and 50.0 g/l of $CaCO_3$. The Potato Dextrose Agar was 100 ml of potato extract containing 2.0 g glucose and 2.0 g agar.

2.2. Morphological analysis

All the fungal strains were inoculated at Potato Dextrose Agar (PDA, for microscopic analysis and colony characters). The plates were incubated in the dark at 28 °C for 6 days. Microscopic observations employed Olympus U-LH100HG fluorescent microscope with 40 × objective under blue light. Images were recorded using the cellSens Standard software.

2.3. DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Total genomic DNA from the strain K302 was extracted. To estimate phylogenetic relationships among strain K302 and the typical strains reported on internet, amplification and sequencing of ITS (Internal transcribed spacer) from the strain K302 were performed using the primers IT5: 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′ (Pedersen et al., 1997). The sequence obtained above was aligned using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed 2007.10.18). The sequences which shared over 98% similarity with currently available sequences were considered to be the same species and multiple alignments were performed using Clustal X 1.83 and phylogenetic tree was constructed using MEGA 4.0 (Tamura et al., 2007).

2.4. Optimization of the medium for calcium malate production

The strain K302 was grown in the seed culture medium at 28 °C and 180 rpm for 48 h. A total of 2.5 ml of the cultures was inoculated into the flask containing 50.0 ml of the calcium malate production medium supplemented with different concentrations of glucose and $CaCO_3$. The flasks were aerobically grown at 28 °C and 180 rpm for 4 days. The culture obtained was centrifuged at 5000 g and 4 °C for 5 min. Calcium malate in the supernatant was obtained and quantitative determination of calcium malate was performed as described below.

2.5. Batch fermentation

Calcium malate production by the strain K302 was also carried out in the 10-l fermenter [BIOQ-6005-6010B, Huihetang Bio-Engineering Equipment (Shanghai) CO-LTD]. The seed cultures were prepared as described above. The fermentation was carried out in the fermenter equipped with baffles, a stirrer, heating element, oxygen sensor, and temperature sensor. Three hundreds of milliliters of the seed culture were transferred into 6 l of the calcium malate production medium. The fermentation was performed under the conditions of the agitation speed of 300 rpm, aeration rate of 8 l/min, the temperature of 28 °C and the fermentation period of 96 h. Only 10.0 ml of the culture was collected in the interval of 24 h and was centrifuged at 5000 g and 4 °C for 5 min and calcium malate, cell mass and reducing sugar in the supernatant obtained were determined as described below. The cell dry weight in 20.0 ml of the culture during the 10-l fermentation was also determined as described below

2.6. Purification of calcium malate

The culture obtained during the 10-l fermentation was centrifuged at 5000 g and 4 °C for 5 min. Calcium malate in 100 ml of the supernatant was purified with the repeated methanol precipitation. Briefly, the first addition of 50 ml of methanol was to selectively remove exopolysaccharide (EPS) as precipitates. After the EPS was removed by centrifugation, 300 ml of methanol was added into the supernatant and the mixture was incubated at 4 °C for overnight. The resulting calcium malate precipitates were collected and dried by evaporating at 60 °C. After the precipitates were dissolved in 50 ml of distilled water, 100 ml of methanol was added into the solution and the mixture was incubated at 4 °C for overnight, the above procedures were repeated several times until the pure calcium malate was obtained. Finally, the amount of calcium malate in 100 ml of the supernatant was calculated.

2.7. Determination of reducing sugar

Reducing sugar in the fermented media was determined by the Nelson-Somogyi method (Spiro, 1966).

2.8. Measurement of cell dry weight

Cell dry weight was measured according to the methods described by Chi et al. (2001).

2.9. Analysis of the partially purified calcium malate by HPLC

The partially purified calcium malate obtained above was dissolved in distilled water. The solution was analyzed by HPLC (Agilent 1200 LC, USA) for determination of the purity of calcium malate. First, calcium malate was separated on ZORBAXSB-C18 column (5.0 μm, 4.6 mm × 150 mm). The HPLC conditions were that mobile phase was 0.01 M $(NH_4)_2HPO_4$ in 10.0% methanol solution which was adjusted to pH 2.7 using 1.0 M phosphoric acid and degassed by microwave; flow rate was 1.0 ml/min; column temperature was 30 °C; the sample volume was 20.0 μl; detector was waters 996 Diode-Array Detector; detection wavelength was 210 nm; sensitivity was 0.02 AUFS. The pure calcium malate bought from Sigma (USA) was used as the standard.

3. Results and discussion

3.1. Screening of the strains of Penicillium spp for their ability to produce high-level of calcium malate

After the ability to yield calcium malate by 9 strains of *Penicillium* spp isolated from different marine environments was examined, the results in Table 1 showed that the strain K302 among

Table 1
The titers of calcium malate produced by different strains of *Penicillium* spp.

Strain	Titer (g/l)	Sources
99	60.0 ± 2.3	Seawater at China Eastern S Seawater at Yellow sea
69	0	Sea sediments at Yellow sea
83	50 ± 1.3	Marine fish at Yellow sea
159	0	Marine algae
72	40 ± 1.1	Sea water at Yellow sea
1120	30 ± 2.1	Intertidal zones of Qingdao
1062	50 ± 2.0	Intertidal zones of Qingdao
1075	0	Intertidal zones of Qingdao
K302	64.7 ± 2.3	Sediment at Yellow sea

Data are given as means ± SD, $n = 3$.

them could produce the highest level of calcium malate (64.7 g/l). After ANOVA analysis, there were significant differences ($P = 0.05$) between the titers of calcium malate of the different mold strains. Therefore, it was used for the subsequent investigation.

3.2. Identification of the strain K302

The colonies of the strain K032 grown on PDA plate were of characteristics of *Penicillium* spp (data not shown). The mycelium of the strain K302 typically consisted of a highly branched network of multinucleate, septate and colorless hyphae and the chains of its conidia resembled a broom (data not shown). ITS sequence of the strain K302 was determined and aligned and phylogenetic tree was constructed as described in Section 2. The search for similarities between ITS of the isolate and those in the NCBI database showed that many phylogenetically related *Penicillium* species were similar to the strain K302 obtained in this study (data not shown). The topology of the phylograms confirmed that the strain K302 was assigned to *Penicillium sclerotiorum*. It has been evidenced that *Aspergillus flavus*, *Aspergillus niger*, *Zygosaccharomyces rouxii*, *Schizophyllum commue*, the metabolically engineered *E. coli*, *Saccharomyces cerevisiae*, and *Bacillius subtilis* 168 can synthesize malate from glucose and secrete it into medium (Battat et al., 1991; West, 2011; Taing and Taing, 2007; Kawagoe et al., 1997; Zelle et al., 2008; Moon et al., 2008; Mu and Wen, 2013). Therefore, this is the first time to report that *P. sclerotiorum* isolated from the sediment at Yellow sea can be a candidate for calcium malate production.

3.3. Optimization of the medium for calcium malate production

It has been well known that the high C/N ratio in the medium is required for microbial malate biosynthesis like microbial fatty acid biosynthesis (Wang et al., 2012). It also has been confirmed that $CaCO_3$ is required for calcium malate production in the fermentation medium in which $CaCO_3$ plays an important role in malate biosynthesis by keeping pH constant of around 6.5 and providing CO_2 as a substrate for efficient production of malate (Goldberg et al., 2006). Therefore, it is important to optimize glucose and $CaCO_3$ concentrations in the medium in order to enhance calcium malate production by *P. sclerotiorum* K302 used in this study. It can be seen from our data that 140 g/l of glucose was the most suitable for calcium malate production by *P. sclerotiorum* K302. Under this condition, its cells grew also well (data not shown). The results also indicated that 50 g/l of $CaCO_3$ was the most suitable for calcium malate production by *P. sclerotiorum* K302 (data not shown). Under this condition, its cells grew also well. The results showed that the titer of calcium malate was 88.6 g/l ± 2.1, the yield was 0.81 g/g ± 0.2 of glucose and productivity was 1.23 g/l/h ± 0.1 at the flask level (data not shown).

3.4. Calcium malate production by 10-l fermentation

To scale up for calcium malate production from glucose, 10-l fermentation was carried out. During the fermentation, calcium malate yield, cell growth and changes in reducing sugar concentration were monitored. The results in Fig. 1 showed that during the 10-l fermentation, 92.0 g/l ± 4.5 of calcium malate in the fermented medium were attained from glucose of 140.0 g/l and dried cell mass in the culture was 9.7 g/l within 72 h. However, the amount of calcium malate was decreased after 72 h due to consumption of it by the mold. It also can be observed from the data in Fig. 1 that the yield of 0.88 g/g ± 0.04 of the consumed glucose and the productivity of 1.23 g/l/h ± 0.1 were reached within 72 h of the fermentation. However, a considerable amount of glucose (35.8 g/l ± 8.3) was still left in the fermented medium, suggesting that only

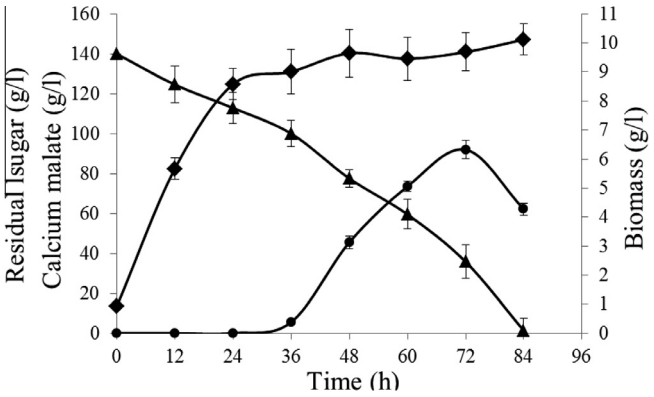

Fig. 1. The time course of calcium malate production (●), cell growth (♦) and residual sugar concentration change (▲) during the batch fermentation in a 10-l fermentor. Data are given as means ± SD, $n = 3$.

74.4% of the sugar was used for calcium malate production and cell growth by the strain K302. Although the titer (113 g/l) of L-malate produced by *A. flavus* was higher than that (92.0 g/l) produced by the strain K302 used in this study (Battat et al., 1991), *A. flavus* produced L-malate (0.59 g/l/h, the fermentation period 8 days) more slowly than the strain K302 used in this study (Battat et al., 1991). However, the titers, yield and productivity of L-malate produced by *A. niger*, *Z. rouxii*, *S. commue*, the metabolically engineered *E. coli*, *S. cerevisiae*, and *B. subtilis* 168 were much lower than those of L-malate produced by the strain K302 used in this study (West, 2011; Taing and Taing, 2007; Kawagoe et al., 1997; Zelle et al., 2008; Moon et al., 2008; Mu and Wen, 2013). This demonstrates that *P. sclerotiorum* K302 used in this study may be the most suitable for calcium L-malate production from glucose on large scale in industry. However, it is still a problem how to enhance the utilization of glucose in the medium by this fungus.

3.5. Analysis of the fermentation products by HPLC

After analysis of the partially purified precipitate and the pure calcium malate with HPLC, the results confirmed that the final product was indeed calcium malate (data not shown). It also can be seen from the results that the partially purified precipitate was mainly composed of calcium malate, indicating that the main product produced by *P. sclerotiorum* K302 was calcium malate (data not shown). However, in addition to malic acid, aflatoxin and other acids such as succinic acid and fumaric acid were also produced by *A. flavus* so that the L-malic acid yield from glucose was reduced (Battat et al., 1991). *Z. rouxii* V19 could produce both 74.9 g/l of malic acid and 7.7 g/l of succinic acid in the culture (Taing and Taing, 2007). *A. niger* can produce both malic acid and citric acid (West, 2011). *E. coli*, *B. subtilis* and *S. cerevisiae* produce L-malic acid from glucose only after they are metabolically engineered (Kawagoe et al., 1997; Zelle et al., 2008; Moon et al., 2008; Mu and Wen, 2013) and some of the engineered microorganisms still produce other organic acids, such as acetate (Mu and Wen, 2013). This means that as a calcium malate producer, *P. sclerotiorum* K302 is better than any other microorganisms.

4. Conclusions

In this study, it was found that *P. sclerotiorum* K302 used in this study may be the most suitable for calcium L-malate production from glucose on large scale in industry as the titer, yield and productivity of calcium malate are very high, fermentation period is short and calcium malate obtained is pure. During 10-l

fermentation, the titer of 92.0 g/l of calcium malate, the yield of 0.88 g/g of glucose and the productivity of 1.23 g/l/h were reached within 72 h of the fermentation. However, it is still a problem how to enhance the utilization of glucose in the medium.

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