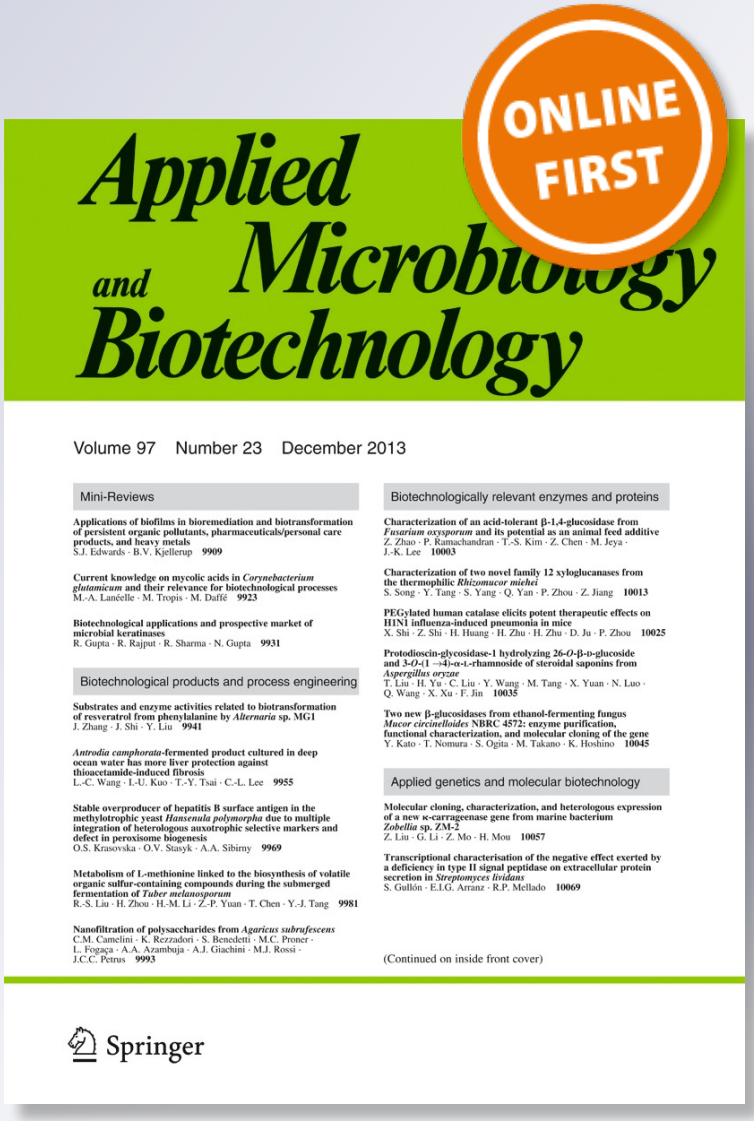
Calcium malate overproduction by Penicillium viticola *152 using the medium containing corn steep liquor*

Ibrar Khan, Kiran Nazir, Zhi-Peng Wang, Guang-Lei Liu & Zhen-Ming Chi

Applied Microbiology and Biotechnology

ISSN 0175-7598

Appl Microbiol Biotechnol
DOI 10.1007/s00253-013-5326-7



Springer

Appl Microbiol Biotechnol
DOI 10.1007/s00253-013-5326-7

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Calcium malate overproduction by *Penicillium viticola* 152 using the medium containing corn steep liquor

Ibrar Khan · Kiran Nazir · Zhi-Peng Wang · Guang-Lei Liu · Zhen-Ming Chi

Received: 29 September 2013 / Revised: 6 October 2013 / Accepted: 9 October 2013

Abstract In this study, after screening of eight fungal strains for their ability to produce calcium malate, it was found that *Penicillium viticola* 152 isolated from marine algae among them could produce the highest titer of calcium malate. At the same time, it was found that corn steep liquor (CSL) could stimulate calcium malate production and 0.5 % (v/v) CSL was the most suitable for calcium malate production. Under the optimal conditions, a titer of calcium malate in the supernatant was 132 g/l at flask level. During a 10-l fermentation, a titer of 168 g/l, a yield of 1.28 g/g of glucose, and a productivity of 1.75 g/l/h were reached within 96 h of the fermentation, and 93.4 % of the sugar was used for calcium malate production and cell growth, demonstrating that the titer, yield, and productivity of calcium malate by this fungal strain were very high and the fermentation period was very short. After analysis of the partially purified product with high-performance liquid chromatography, it was found that the main product was calcium malate. The results demonstrated that *P. viticola* 152 obtained in this study was the most suitable for developing a novel one-step fermentation process for calcium malate production from glucose on a large scale.

Keywords L-Malic acid · Calcium malate · *P. viticola* · One-step fermentation · Corn steep liquor

Introduction

L-Malic acid, a naturally occurring four-carbon dicarboxylic acid, is an intermediate of cell metabolisms, which is involved in two respiratory metabolic cycles: the tricarboxylic acid and glyoxylic acid cycles. L-Malic acid is commonly used directly, or as an alternative to citric acid because of it having greater acid taste and better taste retention, as a food and beverage acidulant and, to a lesser extent, in amino acid infusions to treat hyperamonemia and liver disease. Malic acid is also mediated in aluminum phosphate solubilization in soil (Gadagi et al. 2007). It is also used as a feedstock for chemical synthesis of polymalic acid (Liu and Jarboe 2012). Calcium malate formed during fermentation can be used as a medicine to efficiently provide human and animals with calcium. So, L-malic acid and calcium malate have many applications in the beverage, food, cosmetic, agricultural, chemical, and pharmaceutical industries. It can be synthesized by chemical ways through 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 fumarase and 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 increasing 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). Since current production costs are too high to allow a more widespread use of L-malic acid, in which production is assumed that could increase from the current 100,000 to over 200,000 ton/year, a competitive production process is needed (Stojkovic and Znidarsic-Plazl 2012). 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 our previous study (Wang et al. 2013), *Penicillium sclerotiorum* K302 was found to be able to produce 92.0 g/l of calcium malate within 3 days.

I. Khan · K. Nazir · Z.-P. Wang · G.-L. Liu · Z.-M. Chi (✉)
UNESCO Chinese Center of Marine Biotechnology, Ocean University of China, Yushan Road, No. 5, Qingdao 266003, China
e-mail: zhenming@sdu.edu.cn

Published online: 15 November 2013

According to the non-oxidative pathway of L-malic acid biosynthesis in microorganisms (Fig. 1), pyruvate is carboxylated by fixation of CO_2, to oxaloacetate, followed by reduction of oxaloacetate to malate. Therefore, pyruvate carboxylase can play an important role in malic acid biosynthesis. Pyruvate carboxylase is a biotin-dependent tetrameric enzyme, suggesting that biotin is required during biosynthesis of malic acid (Goldberg et al. 2006). It has been reported that corn steep liquor (CSL) is rich in biotin (Sharma et al. 2013). So, CSL may play an important role in biosynthesis of malic acid.

In this study, several strains of *Penicillium* spp. isolated from different marine environments were further screened for their ability to produce calcium malate. It was found that the strain 152 could produce higher level of calcium malate than any other strains tested in this study and 0.5 % (v/v) CSL could promote calcium malate production.

Materials and methods

Fungal strains and cultivation media

Eight strains [strains 152 (collection number 2F00182 at the Marine Microorganisms Culture Collection of China-MCCC), 99 (2F00183), 83 (2F00184), 102 (2F00185), 72 (2F00186), 1120 (2F00187), 1080 (2F00188), and K302 (2E00189)] 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 of glucose, 3.0 g/l of yeast extract, 3.0 g/l of NH_4NO_3, and 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, 0.5 % (v/v) corn steep liquor, 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 40.0 g/l of $CaCO_3$. Potato dextrose agar (PDA), malt extract agar (MEA), Czaoek yeast extract agar (CYA), and yeast extract, polypeptone, and dextrose (YPD) media were used for the fungal identification (Nonaka et al. 2011; Kurtzman and Fell 2000).

Isolation of fungal strains

Two grams of seawater, sea sediment, marine algae, and marine fish from different places at Yellow Sea were immediately suspended in 50.0 ml of sterile PDA medium supplemented with 0.5 g/l chloramphenicol in 250-ml shaking flasks after the sampling and cultivated at 28 °C for 5 days. After suitable dilution of the cell cultures, the dilute was plated on the PDA plates with 0.5 g/l chloramphenicol, and the plates

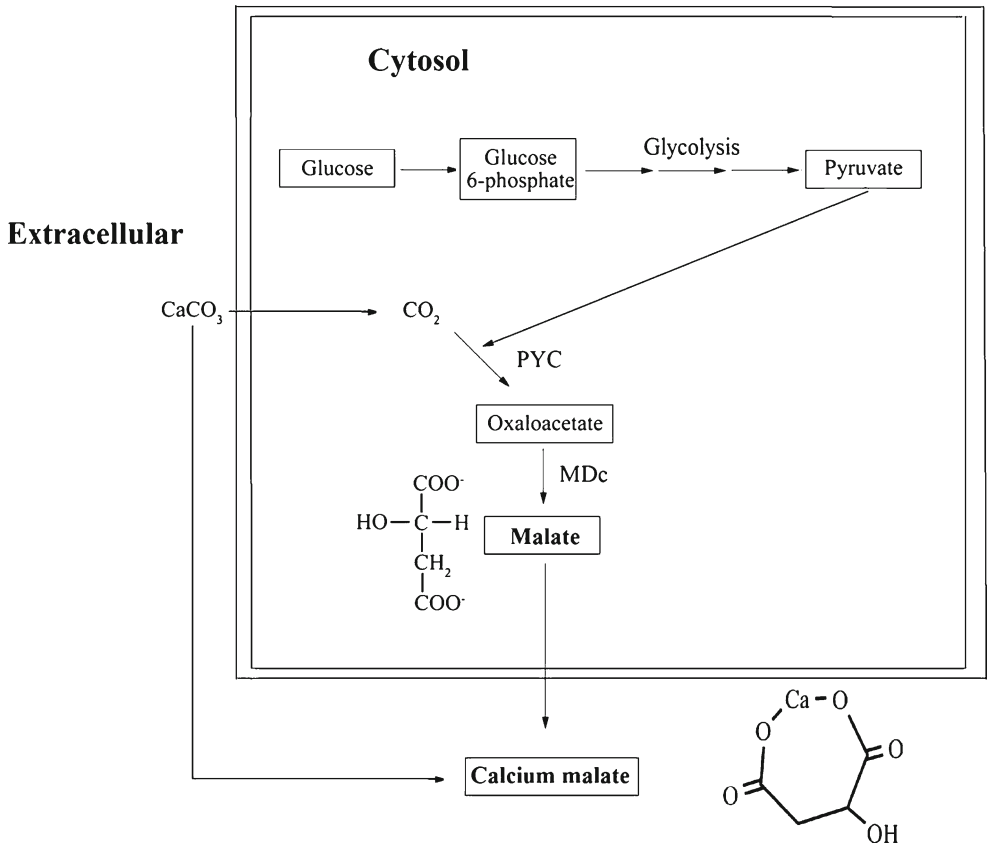


Fig. 1 The proposed non-oxidative pathway of L-malic acid biosynthesis in microorganisms. *PYC* pyruvate carboxylase, *MDc* cytosolic malate dehydrogenase

were incubated at 28 °C for 5 days. Different mold colonies from the plates were transferred to the YPD slants.

Morphological analysis

All the fungal strains were inoculated at potato dextrose agar (PDA, for microscopic analysis and colony characterization). The plates were incubated in the dark at 28 °C for 6 days. Microscopic observations were performed as described by Wang et al. (2013).

DNA extraction, polymerase chain reaction amplification, sequencing, and phylogenetic analysis

Total genomic DNA from the strain 152 was extracted. To estimate phylogenetic relationships among strain 152 and the typical strains reported at the National Center for Biotechnology Information (NCBI), amplification and sequencing of internal transcribed spacer (ITS) from the strain 152 were performed using the primers IT5: 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′ (Pederson et al. 1997). The sequence obtained above was aligned using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences which shared over 98 % similarity with currently available sequences were considered to be the same species. Multiple alignments were performed using Clustal X 1.83, and a phylogenetic tree was constructed using MEGA 4.0 (Tamura et al. 2007). The accession number of the ITS sequence is JX003129.1.

Corn steep liquor

Corn steep liquor was kindly supplied by Dr. Dawei Liu working at Genyuan Biotech Company in Qingdao.

Optimization of the medium for calcium malate production

The strain 152 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, $CaCO_3$, and CSL. The flasks were shaken at 28 °C and 180 rpm for 4 days. The culture obtained was centrifuged at 5,000×*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.

Ten-liter fermentation

Calcium malate production by the strain 152 was also carried out in a 10-l fermentor [BIOQ-6005-6010B, Huihetang Bioengineering Equipment (Shanghai) Co., Ltd] as described by Wang et al. (2013).

Purification of calcium malate

Purification of calcium malate was performed according to the methods described by Wang et al. (2013).

Determination of reducing sugar

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

Measurement of cell dry weight

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

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 high-performance liquid chromatography (HPLC; Agilent 1200 LC, USA) for determination of the purity of calcium malate as described by Wang et al. (2013).

Results

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

After the ability to produce calcium malate by eight strains of *Penicillium* spp. isolated from different marine environments was examined, our results showed that the strain 152 among them could produce the highest level of calcium malate (80.0±2.4 g/l). This fungal strain was isolated from marine algae at Yellow Sea in China. After analysis of variance, there were significant differences ($P=0.05$) between the titers of calcium malate of the different mold strains. However, *Penicillium sclerotiorum* K302 (the positive control) used in our previous study only produced 64.7 g/l calcium malate after primary screening (Wang et al. 2013). Therefore, the fungal strain 152 was used for the subsequent investigation.

Identification of the strain 152

The colonies of the strain 152 grown on CYA and MEA plates were of characteristic of *Penicillium viticola* (data not shown) (Nonaka et al. 2011). The front colonies on the PDA plate were 34–35 mm in diameter after 7 days at 25 °C (Fig. 2a),

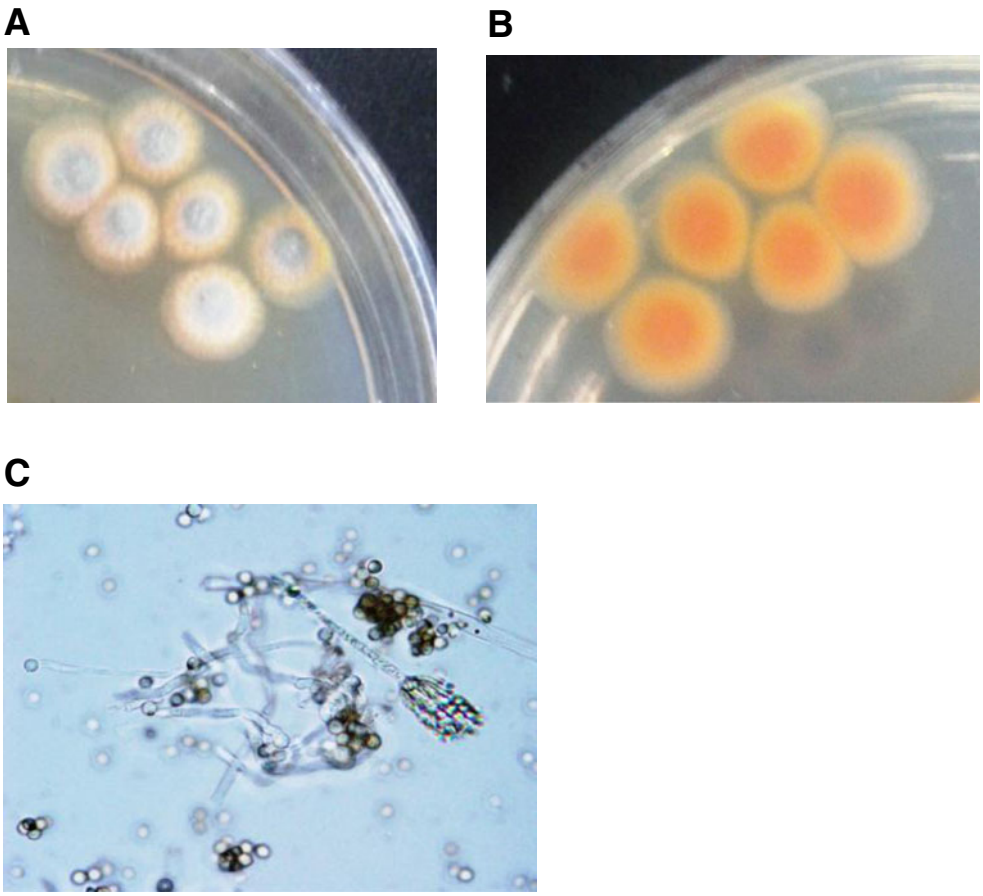


Fig. 2 The morphologies of the colony (**a** front and **b** back), mycelium (**c**), and the chains of its conidia (**c**) of the strain 152

adially sulcate and velutinous. Their mycelia were at the pink margins and the colonies were covered with olive gray conidia in their center. Their margins were entire and soluble pigment was not produced. The back colonies were red (Fig. 2b). The mycelium of the strain 152 grown on PDA typically consisted of a highly branched network of multinucleate, septate, and colorless hyphae, and the chains of its conidia resembled a broom (Fig. 2c).

ITS sequence of the strain 152 was determined and aligned, and a phylogenetic tree was constructed as described in "Materials and methods." The search for similarities between ITS of the isolate and those in the NCBI database showed that many phylogenetically related *Penicillium* spp. were similar to the strain 152 obtained in this study (Fig. 3). The topology of the phylograms confirmed that the strain 152 was assigned to *Penicillium viticola*.

Optimization of the medium for calcium malate production

As $CaCO_3$ can play an important role in malate biosynthesis (Zhang et al. 2011), it is important to optimize $CaCO_3$ concentrations in the medium in order to enhance calcium malate production by *P. viticola* 152 used in this study. Our results indicated that 40 g/l of $CaCO_3$ was the most suitable for calcium malate production by *P. viticola* 152 and 99±2.3 g/l of calcium malate was produced under this condition (data not shown). Our results also showed that 140 g/l of glucose was the most suitable for calcium malate production by *P. viticola* 152 and 110±3.4 g/l of calcium malate was produced under this condition (data not shown).

In order to develop a cost-effective process for production of calcium malate by using agri-industrial residues, corn steep liquor (CSL) was tried to be used to replace nitrogen source in the malate production medium. First, effects of different concentrations of CSL on calcium malate production by the strain 152 grown in the medium only with 140 g/l glucose and 40 g/l $CaCO_3$ were tested. The results in Fig. 4 showed that when the medium contained 0.5 % (v/v) CSL, the highest amount of calcium malate (122.0±3.9 g/l) was gained. However, when the strain 152 was grown in the medium with 0.5 % (v/v) CSL, 140.0 g/l glucose, 40.0 g/l $CaCO_3$, and various mineral salts shown in "Materials and methods," it could produce 132±3.5 g/l calcium malate (Fig. 4). The results in Fig. 4 indicated that as the CSL concentrations were increased, dry cell weight was also increased. The results in Fig. 4 demonstrated that in the presence of CSL, calcium malate production was enhanced.

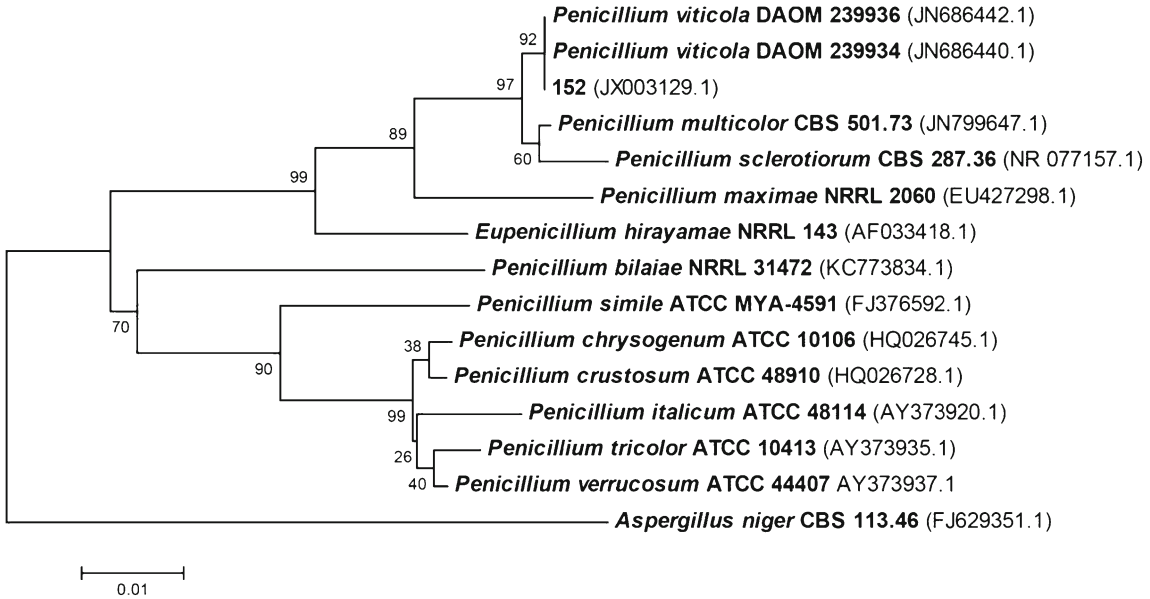


Fig. 3 The phylogenetic tree of the strain 152 and 11 *Penicillium* species relatives based on a neighbor-joining analysis of ITS sequences. Bootstrap values (1,000 pseudoreplications) were ≥38 %, and *Aspergillus niger* CBS 113.46 was used as the outgroup

Calcium malate production by 10-l fermentation

In order to scale up for calcium malate production from glucose, a 10-l fermentation was carried out. During the fermentation, calcium malate yield, cell growth, and changes in reducing sugar concentration were measured. The results in Fig. 5 showed that during the 10-l fermentation, 168.0±4.5 g/l of calcium malate in the fermented medium was attained from 140.0 g/l glucose and dried cell mass in the culture was 14.0 g/l within 96 h. It also can be observed from the data in Fig. 5 that a yield of 1.28 g/g of glucose and a productivity of 1.75 g/l/h were reached within 96 h of the fermentation, demonstrating

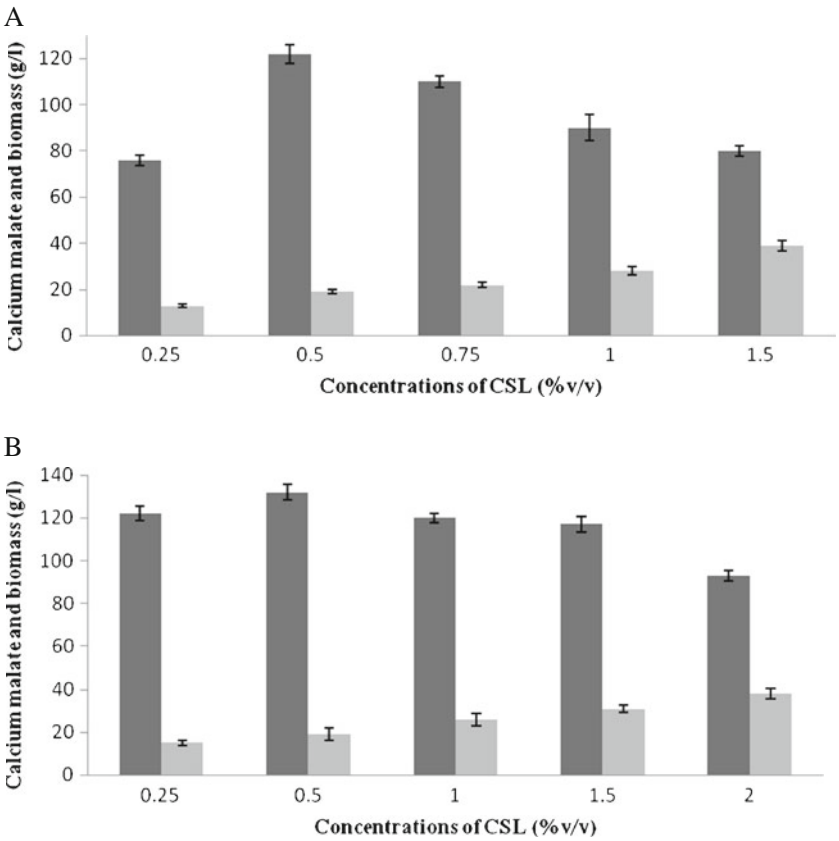


Fig. 4 Effects of different concentrations of CSL on calcium malate production (*black*) and dry cell weight (*gray*). **a** The medium with only 140 g/l glucose and 40 g/l $CaCO_3$; **b** the medium with 140 g/l glucose, 40 g/l $CaCO_3$, and mineral salts. Data are given as means±SD, $n=3$

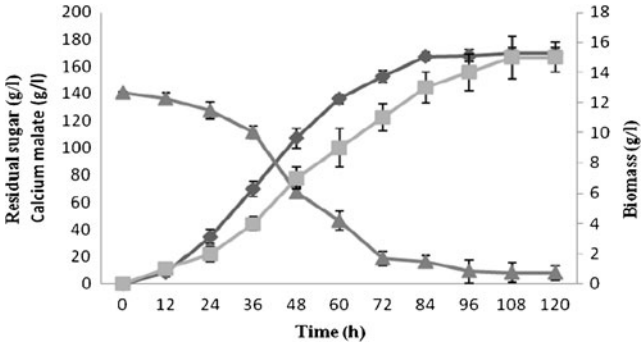


Fig. 5 The time course of calcium malate production (■), dry cell weight (■), and residual sugar concentration change (▲) during the batch fermentation in a 10-l fermentor. Data are given as means±SD, $n=3$

that the titer, yield, and productivity of calcium malate by this fungal strain were very high and the fermentation period was very short. Furthermore, a small amount of glucose (9.19± 8.3 g/l) was left in the fermented medium, suggesting that 93.4 % of the sugar was used for calcium malate production and cell growth by the strain 152. It is interesting to note that after the supernatant of the culture was left for 24 h at room temperature, the calcium malate formed was precipitated automatically (data not shown).

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 (Fig. 6). 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. viticola* 152 was calcium malate.

Discussion

Penicillium viticola 152 isolated from marine algae at Yellow Sea was found to be able to produce higher yield of calcium malate than any other fungal strains including *P. sclerotiorum*

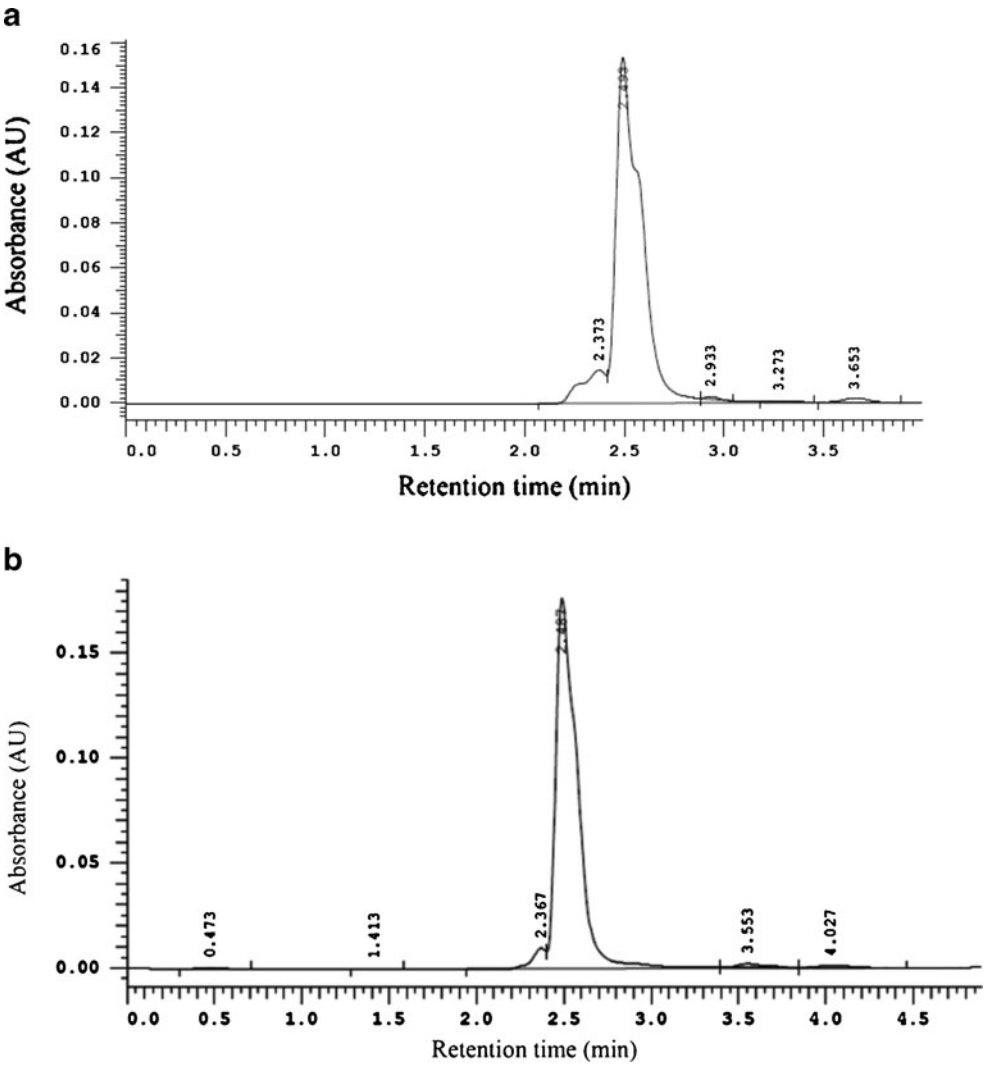


Fig. 6 HPLC chromatogram of standard calcium malate (**b**) and fermentation product (**a**)

K302 (Wang et al. 2013). All the characteristics of *P. viticola* 152 used in this study were consistent with those of *P. viticola* isolated from a grape cultivated in Yamanashi Prefecture, Japan (Nonaka et al. 2011) (Figs. 2 and 3). It has been reported that *Aspergillus flavus*, *Aspergillus niger*, *Zygosaccharomyces rouxii*, *Schizophyllum commune*, the metabolically engineered *Escherichia 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; Zhang et al. 2011; Zelle et al. 2008; Moon et al. 2008; Mu and Wen 2013). In our previous study (Wang et al. 2013), it has been reported that *P. sclerotiorum* isolated from the sediment at Yellow Sea can also produce high level of calcium malate.

According to the non-oxidative pathway of L-malic acid biosynthesis (Fig. 1), $CaCO_3$ 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). It is also thought that Ca^{2+} may be helpful in the secretion of malic acid by changing the permeability of the plasma membrane of the producer (Cassio and Leao 1993). After optimization of $CaCO_3$ and glucose concentrations, it was found that 40.0 g/l of $CaCO_3$ and 140.0 g/l of glucose were the most suitable for calcium malate production by *P. viticola* 152 (data not shown). Corn steep liquor is an inexpensive by-product obtained from corn starch production industries and produced in large quantity. It has been reported that CSL contains 40 % protein, 21 % lactic acid, and 16 % nitrogen-free extract and is also very rich in amino acids and vitamins, especially biotin, which make it an excellent but inexpensive source of essential nutrients (Sharma et al. 2013). The results in Fig. 4 indeed showed that calcium malate production in the presence of 0.5 % (v/v) CSL was stimulated. The pullulan production by *Aureobasidium pullulans* RBF 4A3 was also promoted by use of CSL as nutrient (Sharma et al. 2013). This may be related to the key enzyme pyruvate carboxylase that is a biotin-dependent tetrameric enzyme in L-malic acid biosynthesis (Fig. 1).

P. viticola 152 grown in the optimized medium produced 168.0±4.5 g/l of calcium malate. The yield was 1.28 g/g of glucose, and the productivity was 1.75 g/l/h (Fig. 5). However, in our previous study (Wang et al., 2013), a titer of 92.0 g/l, a yield of 0.88 g/g of glucose, and a productivity of 1.23 g/l/h were reached by *P. sclerotiorum* K302 within 72 h of the 10-l fermentation, and only 74.4 % of the sugar was used for calcium malate production and cell growth by the strain K302. Although a titer (113 g/l) of L-malate was produced by *A. flavus* (Battat et al. 1991), *A. flavus* produced L-malate (0.59 g/l/h, the fermentation period 8 days) much more slowly than the strain 152 used in this study. However, the titers, yield, and productivity of L-malate produced by *A. niger*, *Z. rouxii*, *S. commune*, the metabolically engineered *E. coli*, *S. cerevisiae*, and *B. subtilis* 168 were much lower than those of L-malate produced by the strain 152 used in this study (West 2011; Taing and Taing 2007; Kawagoe et al. 1997; Zhang et al. 2011; Zelle et al. 2008; Moon et al. 2008; Mu and Wen 2013). This demonstrates that *P. viticola* 152 used in this study may be the most suitable for calcium L-malate production from glucose on large scale in industry.

The calcium malate in the supernatant of the culture could be precipitated automatically (data not shown). *A. flavus* also produced unusual crystals during its L-malic acid production phase (Peleg et al. 1988). They thought that it was possible that in *A. flavus*, malic, succinic, and fumaric acids were excreted to the broth from the hyphae. Immediately upon secretion, these acids reacted with $CaCO_3$ present in the medium to form insoluble calcium salts of the acids which crystallized on the hyphae. However, during the fermentation, calcium malate produced by *P. viticola* 152 was soluble (data not shown). Therefore, it is completely unknown how calcium malate precipitates are formed during storage at room temperature.

The main product produced by *P. viticola* 152 was calcium malate (Fig. 6). In our previous study (Wang et al. 2013), the main fermentation product produced by *P. sclerotiorum* K302 was also calcium malate. However, in addition to malic acid, 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; Zhang et al. 2011; 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 an excellent calcium malate producer, *P. viticola* 152 is much better than any other microorganisms.

Acknowledgments This research was supported by Hi-Tech Research and Development Program of China (Grant No. 2013BAB01B05).

References

Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I (1991) Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. Biotechnol Bioeng 37:1108–1116

Cassio F, Leao C (1993) A comparative study on the transport of L(−)-malic acid and other short-chain carboxylic acids in the yeast

Candida utilis: evidence for a general organic acid permease. Yeast 9:743–752

Chi ZM, Liu J, Zhang W (2001) Trehalose accumulation from soluble starch by *Saccharomycopsis fibuligera* sdu. Enz Microb Technol 38:240–246

Gadagi RS, Shin WS, Sa TM (2007) Malic acid mediated aluminum phosphate solubilization by *Penicillium oxalicum* CBPS-3F-Tsa isolated from Korean paddy rhizosphere soil. In: E. Vela′ zquez and C. Rodrı′guez-Barrueco (eds.), First International Meeting on Microbial Phosphate Solubilization, Springer, pp: 285–290

Goldberg I, Rokem JS, Pines O (2006) Organic acids: old metabolites, new themes. J Chem Technol Biotechnol 81:1601–1611

Kawagoe M, Hyakumura K, Suye SI, Miki K, Naoe K (1997) Application of bubble column fermentors to submerged culture of *Schizophyllum commune* for production of L-malic acid. J Ferment Bioeng 84:333–336

Kurtzman CP, Fell JW (2000) The yeasts. A taxonomic study, fourth revised and enlarged ed. Elsevier, Amsterdam, Lausanne, New York, Oxford, Shannon, Singapore, Tokyo, pp. 222–360

Liu P, Jarboe LR (2012) Metabolic engineering of biocatalysts for carboxylic acids production. Comput Struct Biotechnol J 3:1–9

Moon SY, Hong SH, Kim TY, Lee SY (2008) Metabolic engineering of *Escherichia coli* for the production of malic acid. Biochem Eng J 40:312–320

Mu L, Wen J (2013) Engineered *Bacillus subtilis* 168 produces L-malate by heterologous biosynthesis pathway construction and lactate dehydrogenase deletion. World J Microbiol Biotechnol 29:33–41

Nonaka K, Masuma R, Iwatsuki M, Shiomi K, Otoguro K, Omura S (2011) *Penicillium viticola*, a new species isolated from a grape in Japan. Mycosci 52:338–343

Pedersen LH, Skouboe P, Boysen M, Souleb J, Rossen L (1997) Detection of *Penicillium* species in complex food samples using the polymerase chain reaction. Intern J Food Microbiol 35:169–177

Peleg Y, Rahamin E, Kessel M, Goldberg I (1988) Malic acid accumulation by *Aspergillus flavus*. II, Crystals and hair-like processes formed by *A. flavus* in a L-malic acid production medium. Appl Microbiol Biotechnol 28:26–29

Presecki AV, Vasic-Racki D (2005) Production of L-malate by permeabilized cells of commercial *Saccharomyces* sp. strains. Biotechnol Lett 27:1835–1839

Sharma N, Prasad GS, Choudhury AR (2013) Utilization of corn steep liquor for biosynthesis of pullulan, an important exopolysaccharide. Carboh Polym 93:95–101

Spiro RG (1966) Analysis of sugars found in glycoproteins. Meth Enzymol 8:3–26

Stojkovic G, Znidarsic-Plazl P (2012) Continuous synthesis of L-malic acid using whole-cell microreactor. Proc Biochem 47: 1102–1107

Taing O, Taing K (2007) Production of malic and succinic acids by sugar-tolerant yeast *Zygosaccharomyces rouxii*. Eur Food Res Technol 224:343–347

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Wang ZP, Wang GY, Khan I, Chi ZM (2013) High-level production of calcium malate from glucose by *Penicillium sclerotiorum* K302. Biores Technol 143:674–677

West TP (2011) Malic acid production from thin stillage by *Aspergillus* species. Biotechnol Lett 33:2463–2467

Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, Winkler AA, Geertman JMA, van Dijken JP, Pronk JT, van Maris AJA (2008) Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. Appl Environ Microbiol 74:2766–2777

Zhang H, Cai J, Dong J, Zhang D, Huang L, Xu Z, Cen P (2011) High level production of poly (β-L-malic acid) with a new isolated *Aureobasidium pullulans* strain. Appl Microbiol Biotechnol 92:295–303

Zou X, Zhou Y, Yang ST (2013) Production of polymalic acid and malic acid by *Aureobasidium pullulans* fermentation and acid hydrolysis. Biotechnol Bioeng 11:2105–2113