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Efficient calcium fumarate overproduction from xylose and corncob-derived xylose by engineered strains of *Aureobasidium pullulans* var. *Aubasidani* DH177

Peng Wang[1], Hao Chen[1], Xin Wei[1], Guang-Lei Liu[1], Zhe Chi[1,3], Bo Jiang[2*] and Zhen-Ming Chi[1,3*]

Abstract

Background Xylose from lignocellulose is one of the most abundant and important renewable and green raw materials. It is very important how to efficiently transform xylose into useful bioproducts such as fumaric acid and so on.

Results In this study, it was found that the GC1 strain (***Δgox***, in which the ***GOX*** gene encoding glucose oxidase which could transform glucose into gluconic acid was removed) of ***A. pullulans*** var. ***aubasidani*** DH177 had the high ability to utilize xylose and corncob-derived xylose with CO_2 fixation derived from $CaCO_3$ to produce calcium fumarate. Overexpression of the ***XI*** gene encoding xylose isomerase, the ***XK*** gene encoding xylose kinase and the ***TKL*** gene coding for transketolase made the strain TKL-4 produce 73.1 g/L of calcium fumarate from xylose. At the same time, the transcriptional levels of the key ***ASS*** gene coding for argininosuccinate synthase and the ***ASL*** gene coding for argininosuccinate lyase in the ornithine-urea cycle (OUC) were also obviously enhanced. The results also demonstrated that the TKL-4 strain could produce more calcium fumarate from xylose and corncob-derived xylose than from glucose. During 10-liter fermentation, the TKL-4 strain could produce 88.5 g/L of calcium fumarate from xylose, the productivity was 0.52 g/h/L. Meanwhile, it could yield 85.6 g/L of calcium fumarate from corncob-derived xylose and the productivity was 0.51 g/h/L. During the same fermentation, the TKL-4 strain could transform the mixture containing 75.0 g/L glucose and 45.0 g/L xylose to produce 78.7 ± 1.1 g/L calcium fumarate.

Conclusions This indicated that the TKL-4 strain constructed in this study indeed could actively transform xylose and corncob-derived xylose into calcium fumarate through the green ways.

Keywords *A. Pullulans* var. ***Aubasidani*** DH177, Calcium fumarate, Xylose, Corncob-derived xylose, Green calcium fumarate overproduction

*Correspondence:
Bo Jiang
jiangboqdy@126.com
Zhen-Ming Chi
chi@ouc.edu.cn
[1]College of Marine Life Sciences, Ocean University of China, Yushan Road, No. 5, Qingdao, China
[2]Department of Urology, Qingdao Hospital, University of Health and Rehabilitation Sciences (Qingdao Municipal Hospital), Qingdao, Shandong 266000, China
[3]Laboratory for Marine Biology and Biotechnology, Qingdao Marine Science and Technology Center, Qingdao, China

Introduction

Plant biomass (lignocellulose) produced by agricultural crops and forestry, is one of the most abundant and important renewable and green raw materials and the output each year can reach about 170 billion tons in this world. Lignocellulose consists of cellulose, hemicellulose and lignin [1]. It has been well known that cellulose can be enzymatically hydrolyzed into glucose which is the most commonly used carbon source for microbial cell growth and various bioproduct production. Hemicelluloses consist of pentoses (D-xylose and D-arabinose) and/or hexoses (D-mannose, D-glucose, and D-galactose) with xylose as the major monosaccharide. Hemicelluloses mainly from corncobs, straws of corn, wheat and rice straws and sugarcane bagasse also can be enzymatically hydrolyzed to produce major xylose which also can be utilized by some microorganisms for cell growth and various green bioproduct production. Recently, it has been found that the waste xylose mother liquor (globally, over 100,000 tons), the lignocellulosic hydrolysate from corncob, is a xylose-rich hydrolysate [2] and is also suitable for various green bioproduct production [3].

So far, glucose catabolism and regulation in most of microbial cells have been very clear. However, it is still not very clear how xylose is efficiently catabolized and transformed into cell mass and various bioproducts [4]. Furthermore, lignocellulose is regarded as a sustainable, eco-friendly and renewable raw material and can replace unsustainable, eco-unfriendly and un-renewable fossil resources for the synthesis of cell mass, biochemicals, biomaterials and biofuels. However, so far, no microorganisms have been found to efficiently secrete all kinds of lignocellulolytic enzymes to decompose the natural plant cell mass to rapidly release glucose, xylose and other sugars [4]. Therefore, it is very important to screen and develop fungal strains that secrete different kinds of lignocellulolytic enzymes and efficiently transform xylose into cell mass and useful bioproducts.

Fumaric acid is a four-carbon unsaturated dicarboxylic acid. It has many applications in food, beverage, chemical, pharmaceutical, animal feeds, paper sizing and detergent industries [5]. At this moment, it is commercially produced in petrochemical industries using petrochemical maleic anhydride or maleic acid [6]. However, the chemical fumaric acid production process has severe environmental concerns and conflicts with the sustainable and green development goals in this world, such as formation of carbon monoxide and carbon dioxide, release of greenhouse gas, the high price and continuous depletion of crude oil, intense reaction conditions such as high temperature and high pressure [7]. Therefore, many researchers and commercial manufacturers are trying very hard to develop bio-production of fumarate by using renewable and green raw materials, such as xylose from natural xylan, a sustainable and green alternative to the petroleum-based production [7]. It has been found that bioengineered strains of *E. coli* [8], *Rhizopus oryzae* [9, 10], *Aspergillus oryzae* [11], *Saccharomyces cerevisiae* [12], *Torulopsis glabrata* and *Scheffersomyces stipitis* [5] could produce a considerable amount of fumaric acid from glucose. But only some strains of *R. oryzae* could produce less than 78.0 g/L. calcium fumarate from glucose [5, 13, 14]. However, *R. oryzae* as fumaric acid producer has many drawbacks, such as low fungal growth, low yield of fumarate, mycelial clumps and pellets formation, simultaneous production of ethanol and lactate, the complexities of this fungal strain mediated with fumaric acid production, difficulties in genetic engineering, cell growth control, the control of the pellet size and oxygen transfer [14]. In our recent studies [15]. the engineered strain e-PYC of *A. pullulans* var. *aubasidani* DH177 has been found to be the best fungal strain for calcium fumarate production from glucose. Much research also shows that xylose is the second most abundant sugar after glucose and it could be alternative source for fumaric acid production. But only less than 49.0 g/L of fumaric acid could be obtained from xylose or corncob hydrolytes by *R. oryzae*, *Rhizopus arrhizus*, *S. stipitis* and *E. coli* [6, 16–20]. In our previous studies [15], it was the first time to have found that the ornithine-urea cycle (OUC) is involved in high level calcium fumarate biosynthesis of *A. pullulans* var. *aubasidani* DH177 which is controlled by the Ca^{2+} signaling pathway. Deletion of both the *GOX* gene encoding glucose oxidase for gluconic acid production and the *PKS1* gene encoding the polyketide synthase for 3,5-dihydroxydecanoic acid biosynthesis and overexpression of the *PYC* gene encoding pyruvate carboxylase enable the strain e-PYC to produce 93.9±0.8 g/L of calcium fumarate from glucose during the fed-batch fermentation. It has been strongly confirmed that *A. pullulans* var. *aubasidani* as a new fumaric acid producer and a yeast-like fungus have many advantages over *Rhizopus* spp., such as easy cultivation in various sizes of bioreactor, easy whole genomic DNA editing and metabolic engineering for high fumarate production, fixation of CO_2 and high titer of fumarate. In addition, different strains of *A. pullulans* have been widely applied to fermentation, food and pharmaceutical industries to produce pullulan and polymalic acid [3, 21]. Therefore, *A. pullulans* var. *aubasidani* DH177 used in this study is very safe. Meanwhile, it was also found that abolishment of the *GOX* gene encoding glucose oxidase made the disruptant *Δgox* produce only 60.0 g/L of calcium fumarate [15]. In another study [4, 21], we also found that *Aureobasidium* spp. had a high xylan-degrading and xylose-utilizing ability and are suitable for bioproduct production from xylan and its hydrolysate xylose. Therefore, in this study, the xylose metabolic pathways in the disruptant

Δgox (GC1 strain in which the *GOX* gene encoding glucose oxidase for gluconic acid biosynthesis was deleted) were engineered to efficiently produce high level of calcium fumarate from xylose, a mixture of glucose and xylose and corncob-derived xylose by a new green way (Fig. 1).

Methods

The yeast-like fungal strains, *E. Coli* strain, plasmids, media and raw materials

The yeast-like fungal mutant, a glucose oxidase deficient *Δgox* mutant (here called a GC1 strain) of *A. pullulans* var. *aubasidani* DH177 was obtained in our previous study [15]. This strain and other engineered strains obtained in this study are listed in Table S1. The competent cells of *E. coli* DH5α were prepared in this laboratory.

The plasmid pMD19-T used to amplify target genes in *E. coli* was bought from the TaKaRa Company in Dalian, China. The plasmid pLB-simple was bought from the TIANGEN company in Beijing, China. The disruption vector pFL4A-NAT-loxp carrying the nourseothricin resistance gene (*NAT* gene) was used for abolishment of the target genes. The expression vector pNATX13-loxp carrying the same *NAT* gene was used for overexpression of the target genes. In order to remove the antibiotics resistance gene (*NAT* gene) in the transformants and express or delete another gene, the plasmid pAMCRE-1 carrying the autonomously replicating DNA sequence, Cre recombinase gene and hygromycin B resistance gene (*HPT* gene) constructed in this laboratory was used to clean the *NAT* gene in all the transformants [22–24].

The YPD (Yeast extract, peptone and Dextrose) medium for cultivation of the yeast-like fungal strains contained 10.0 g/L yeast extract, 20.0 g/L peptone, 20.0 g/L glucose. The YPD media with 1.0 M sorbitol or with 100.0 μg/mL of nourseothricin or hygromycin B were used to cultivate the yeast-like fungal transformants. The medium for calcium fumarate production contained 113.0 g/L glucose or xylose or corncob-derived xylose or a mixture of 75.0 g/L glucose and 45.0 g/L xylose, 2.0 g/L $(NH_4)_2SO_4$, 0.3 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.3 g/L $ZnSO_4$, 2.0 g/L corn steep liquid (CSL) and 80.0 g/L $CaCO_3$. The CSLs were purchased from Angel Bio-company, Yichang, Hubei, China, Houman Biotechnology Company in Shanghai, Qingyuyuan Materials Company in Jinan, Shandong Weiduofeng Biotechnology Company in Jinan and Yihao Agricultural Biotechnology Company in Hangzhou, respectively. The pure xylose and the corncob-derived xylose were brought from Xie-Li Biotechnology Company, Jinan, China. A LB (Luria-Bertani) medium for cultivation of *E. coli* contained 5.0 g/L yeast extract, 10.0 g/L NaCl, 10.0 g/L tryptone. LA medium was the LB medium with 100.0 μg/mL of ampicillin.

Cloning and characterization of the relevant genes

The genomic DNAs of *A. pullulans* var. *aubasidani* DH177 or its mutant GC1 were isolated and purified based on the methods described by Chi et al. [23]. The *XR* gene encoding xylose reductase, the *XI* gene encoding xylose isomerase, the *XK* gene encoding xylulose kinase, the *TKL* gene encoding transketolase and the *XPKA* gene encoding xylulose-5-phosphate phosphoketolase (Fig. 1 and Table S2) were PCR amplified using

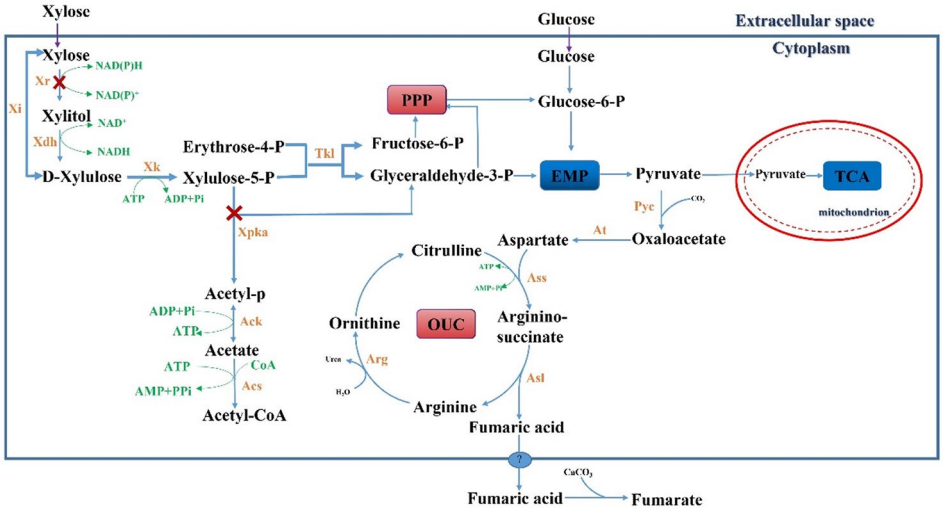

Fig. 1 The pathway of xylose conversion to fumarate in *Aureobasidium* spp. Xr: Xylose reductase; Xdh: Xylose Dehydrogenase; Xi: Xylose isomerase; Xk: Xylulose kinase; Tkl: Transketolase; Xpka: Xylulose-5-phosphate phosphoketolase; Pyc: Pyruvate carboxylase; Acs: acetyl-CoA synthetase; Ack: Acetate kinase; At: Aspatrate aminotransferase; Ass: Argininosuccinate synthase; Asl: Argininosuccinate lyase; Arg: Arginase; PPP: Phosphate pentose pathway; OUC: Ornithine-Urea Cycle; ×: deletion. Thick Blue Arrow means enhanced reactions by overexpression of the relevant genes

the primers (SXR-F/SXR-R; XI-F/XI-R; XK-F/XK-R, SXPKA-F/S*XPKA-R* (Table S3) designed according to the genomic DNA sequence of *A. pullulans* var. *aubasidani* DH177 (the GenBank accession number was JADDKP000000000) and the genomic DNAs or cDNAs of the DH177 strain as templates. The conserved DNA and amino acid sequences of the cloned genes were analyzed and characterized using the software in Table S4.

Construction of the disruption and expression vectors

In order to completely remove the *XR* gene (Fig. 1 and Table S2) from the genomic DNA of the GC1 strain, 5'-arm and 3'-arm of the *XR* gene were PCR amplified from the genomic DNA of the GC1 strain using the primers (XR-5 F/XR-5R and XR-3 F/XR-3R, Table S3) and the amplified PCR products were digested with the enzymes *Sph*I/*Pst*I and *Bam*HI/*Eco*RI (Table S3). The digested and purified DNA fragments were ligated into the disruption plasmid pFL4A-NAT-loxp as mentioned above, generating pFL4A-NAT-loxp-*ΔXR* (Fig. S1A). Similarly, in order to delete the *XPKA* gene, 5'-arm and 3'-arm of the *XPKA* gene were PCR amplified from the genomic DNA of the GC1 strain using the primers (XPKA-5 F/XPKA-5R XPKA-3 F/XPKA-3R, Table S3) and the amplified PCR products were digested with the enzymes *Sph*I/*Sal*I and *Bam*HI/*Eco*RI (Table S3). The digested and purified DNA fragments were ligated into pFL4A-NAT-loxp as mentioned above, forming pFL4A-NAT-loxp-*ΔXPKA* (Fig. S1B).

To overexpress the *XI*, *XK* and *TKL* genes (Table S2) in the corresponding strains, they were PCR amplified using the primers XI-F/XI-R, XK-F/XK-R and TKL-F/TKL-R, respectively (Table S3) and cDNAs of the DH177 strain as templates. The PCR products were hydrolyzed with the enzymes *Pst*I/*Sal*I, *Sph*I/*Pst*I, *Bam*HI/*Eco*RI, respectively. The hydrolyzed PCR products were linked into the expression plasmid pNATX13-loxp hydrolyzed with the same enzymes, resulting in pNATX13-loxp-*XI* (Fig. S1C), pNATX13-loxp-*XK* (Fig. S1D) and pNATX13-loxp-*TKL* (Fig. S1E).

Transformation and isolation of deletants and expressing strains

The linear DNA fragments 5'-arm-loxp-polyA-NAT-PGK-loxp-3'-arm were PCR amplified from the knock-out plasmid pFL4A-NAT-loxp-*ΔXR* (Fig. S1A) constructed above using the primers XR-5 F/XR-3R (Table S3). The obtained linear DNA fragment 5'-arm-loxp-polyA-NAT-PGK-loxp-3'-arm was transformed into the competent cells of the GC1 strain as mentioned above and the pure *Δxr-6*, *Δxr-16* and *xr-20* mutants were isolated. Then, the pAMCRE-1 plasmid was introduced into the *Δxr-6* mutant to remove the *NAT* and *HPT* genes and the *Δxr-6-cre* mutant was gained (Table S1). The expression plasmid pNATX13-loxp-*XI* (Fig. S1C) was linearized with the enzyme *Sma*I. The obtained linear 18SrDNA-TEF-XI-loxp-polyA-NAT-PGK-loxp-26SrDNA was transformed and expressed in the competent cells of the GC1 strain. The overexpressing strains XI-2, XI-6, XI-7, XI-10, XI-17, XI-19 were obtained. In addition, the same linear 18SrDNA-TEF-XI-loxp-polyA-NAT-PGK-loxp-26SrDNA was also transformed and expressed in the competent cells of the *Δxr-6-cre* mutant and the overexpressing strains xi-36 and xi-39 were acquired.

The linear DNA fragments 5'-arm-loxp-polyA-NAT-PGK-loxp-3'-arm were PCR amplified from the disruption plasmid pFL4A-NAT-loxp-*ΔXPKA* (Fig. S1B) constructed above using the primers XPKA-5 F/XPKA-3R (Table S3). The obtained linear DNA fragment 5'-arm-loxp-polyA-NAT-PGK-loxp-3'-arm from the pFL4A-NAT-loxp-*ΔXPKA* was introduced into the competent cells of the GC1 strain as mentioned above and the disruptants *Δxpka-4*, *Δxpka-23* and *Δxpka-28* were isolated. Similarly, the *NAT* and *HPT* genes in the overexpressing XI-6 were removed by introducing the pAMCRE-1 plasmid into it and the XI-6-cre strain was acquired. The expression plasmid pNATX13-loxp-*XK* (Fig. S1D) was digested with the enzyme *Sma*I. The linear 18SrDNA-TEF-XK-loxp-polyA-NAT-PGK-loxp-26SrDNA was introduced and expressed in the competent cells of the XI-6-cre strain and the overexpressing strains XK-5, XK-6 and XK-28 were gotten. In the same principle, the *NAT* and *HPT* genes in the overexpressing XK-6 were removed by introducing the pAMCRE-1 plasmid into the overexpressing XK-6 and the new XK-6-cre strain was isolated. Finally, the expression plasmid pNATX13-loxp-*TKL* (Fig. S1E) was linearized with the enzyme *Sma*I. The obtained linear 18SrDNA-TEF-TKL-loxp-polyA-NAT-PGK-loxp-26SrDNA was further transformed and expressed in the competent cells of the XK-6-cre strain and the overexpressing strains TKL-2, TKL-4, TKL-12, TKL-30 and TKL-43 were isolated and purified.

Calcium fumarate production from glucose or xylose or corncob-derived xylose and identification of the produced fumaric acid from xylose

In order to examine effects of glucose, xylose and corncob-derived xylose on calcium fumarate production, first, the GC1 strain was aerobically grown in 50.0 mL of the YPD medium for 24 h at 28 °C and at shaking speed of 300 rpm. The seed culture (5.0 mL, 10^7 cells/mL) was inoculated into 45.0 mL of the calcium fumarate production media containing xylose or corncob-derived xylose or glucose at 28 ℃ and 180 rpm for 7 d, respectively. During the cultivation, calcium fumarate titer, cell growth, residual reducing sugars were measured as described

below. The calcium fumarate produced by the GC1 strain (the *Δgox* mutant mentioned above) grown in the calcium fumarate production media containing glucose, xylose and corncob-derived xylose (Table S1) was further purified as described by Wei et al. [15]. Briefly, after removal of all the yeast-like fungal cells and the unused $CaCO_3$ in the culture by centrifugation, all the calcium fumarate in the supernatants was precipitated by adding cold methanol, all the precipitated calcium fumarate was purified by using the same precipitation and centrifugation for three times and the purified calcium fumarate was dried at 80 °C until its dried weight was constant. At the same time, the residual xylose in the supernatants was determined as described below. Finally, g of calcium fumarate/g of the consumed xylose was calculated. The calcium in the purified calcium fumarate was totally removed by addition of 0.1 N H_2SO_4 and the formed calcium sulphate was totally removed by centrifugation at 10,000 × g for 10 min and the formed supernatant was filtrated using 0.22 μm membrane. The filtrate obtained was incubated in the refrigerator (4 °C) for one night and fumaric acid was crystalized. The isolated crystals were dissolved in pure water at 80 °C and the formed solution was incubated in the refrigerator again and fumaric acid was re-crystalized at 4 °C. The same procedures were repeated several times until fumaric acid was completely pure. The pure fumaric acid solution was filtered again using 0.22 μm membrane, the filtrate was analyzed by using HPLC. The sample of 1.0 μL was injected into an Agilent 7890 A/5975 C machine with the column Agilent HP-INNOWax Polyethylene Glyco (30 m × 50 μm × 0.25 μm). The conditions for HPLC were 100 ℃ of the initial temperature (then the temperature was increased to 240 ℃ at the rate of 15 ℃/min within 20 min) and 20 min of the running time.

At the same time, the GC1 strain, all the deletants and overexpressing strains (Table S1) obtained above were aerobically cultivated in 50.0 mL of the liquid YPD medium in the 300-mL flask at 28 ℃ and 180 rpm for 24 h. The seed culture (5.0 mL) was inoculated into 45.0 mL of the calcium fumarate production medium and the new culture in the 300-mL flask was continued to be aerobically grown at 28 ℃ and 180 rpm for 7 d. Each culture (10.0 mL) obtained was centrifuged at 8000 × g for 10 min. All the calcium fumarate in the supernatant was precipitated using methanol cooled in the refrigerator and dissolved in pure water based on the methods described by Wei et al. [15] and the same procedures were repeated three times. The purified calcium fumarate was dried and weighed and the amount of calcium fumarate per liter of culture was calculated. At the same time, cell dry weight in the culture was assayed and calculated according to the methods described by Chi et al. [25].

Measurements of expression of the relevant genes in the GC1 strain, the deletants and overexpressing strains

The GC1 strain and its various deletants and transformants obtained above (Table S1) were cultivated in 50.0 mL of the liquid YPD medium at 28 ℃ and 180 rpm for 24 h as described above. The seed culture (5.0 mL) was inoculated into 45.0 mL of the calcium fumarate production medium as described above and the new culture was continued to be cultivated at 28 ℃ and 180 rpm for 3 d. The yeast-like fungal cells in the cultures were harvested and washed with sterile distilled water by centrifugation at 11,000 × *g* for 3 min. The total RNAs in the washed cells were extracted using a Fungal RNA prep pure kit (OMEGA, USA) and the bands of the RNAs were checked by agarose electrophoresis. The reverse transcription was conducted using a PrimeScript RT reagent Kit (TaKaRa, Japan) according to the manufacturer's protocol. The fluorescent real-time RT-PCR assay was carried out using a Rotor-Gene Q RT-PCR analyzer (QIAGEN Hilden, Germany) in triplicate based on the methods described by Liu et al. [26]. All the primers for the fluorescent real-time PCR are shown in Table S5. The relative transcriptional levels were calculated using the formula $RATE=2^{-\Delta\Delta Ct}$ and Rotor-Gene Q 2.0.2 Real-time Date Acquisition and Analysis Software. The sample data obtained from the real-time PCR analysis were subjected to One-way Analysis of Variance (ANOVA) [27]. P values were calculated by Student's t-test ($n=3$). P values less than 0.05 were considered statistically significant. Statistical analysis was performed using a SPSS 11.5 for Windows (SPSS Inc., Chicago, IL). The expression of the house-keeping β-actin gene was used as a control.

10-Liter fermentation using the TKL-4 strain

First, effects of different concentrations of xylose or corncob derived xylose (6.0%, 8.0%, 10.0%, 10.5%, 11.0%, 11.5%, 12.0%, 14.0% and 16.0% w/v) and CSL (0.05%, 0.1%, 0.15%, 0.2%, 0.25% w/v) in the calcium fumarate production medium on calcium fumarate production, cell growth and changes in residual xylose by the TKL-4 strain obtained above were tested. During the cultivation, the calcium fumarate titer, cell dry weight and residual xylose concentration of the culture were measured every 24 h as described above and below. In order to test the effects of glucose, corncob derived xylose and xylose on calcium fumarate production and cell growth, the TKL-4 strain was also cultivated in the calcium fumarate production media containing 113.0 g/L of glucose or xylose or corncob derived xylose for 168 h and the calcium fumarate titer and cell dry weight of the cultures were assayed as described above.

A 10-Litter fermenter (bioq-6005-6010b, Shanghai huihetang Bioengineering Equipment Co., Ltd. Shanghai, China) was utilized for the fermentation tests using

the optimized calcium fumarate production medium as mentioned above. The seed culture of the TKL-4 strain was prepared as described above. Then, 700.0 mL of the seed culture (the cell concentration 8.0×10^7 cells/mL) was inoculated into 6.3 L of the optimized calcium fumarate production medium containing 113.0 g/L xylose or corncob derived xylose or glucose and 0.2% (w/v) CSL in the 10-Liter fermenter and the cultivation was conducted at temperature of 28 ℃, agitation of 280 rpm, aeration of 300.0 L/h for 168 h. 30.0 mL of the culture was sampled every 12 h to determine the cell dry weight and the amount of calcium fumarate as described above. The residual reducing sugar concentration in the culture was quantitatively measured based on the Anthrone method [28]. A 500 µL sample was thoroughly mixed with an equal volume of anthrone solution, following which the mixture was heated in boiling water for 15 min. After cooling, the optical density of the total reducing sugar in the sample was measured at 620 nm.

Because the hydrolysate of lignocellulose from plants contained a mixture of glucose and xylose, effects of the different mixtures of 0% xylose (100.0% glucose), 100.0% xylose (0% glucose), 20.0% xylose (80.0% glucose), 40.0% xylose (60.0% glucose), 50.0% xylose (50.0% glucose) and 60.0% xylose (40.0% glucose) in the calcium fumarate production media on calcium fumarate production by the TKL-4 strain at the 300-mL flask level were examined. Then, the mixture containing 75.0 g/L glucose and 45.0 g/L xylose (63.0% glucose and 37.0% xylose) in the calcium fumarate production medium in the 10-Litter fermenter was used for calcium fumarate production as described above. During the fermentation, xylose and glucose concentrations in the cultures were determined using HPLC 1260 Infinity with the Refractive Index Detector (Agilent Technologies, Palo Alto, CA, USA).

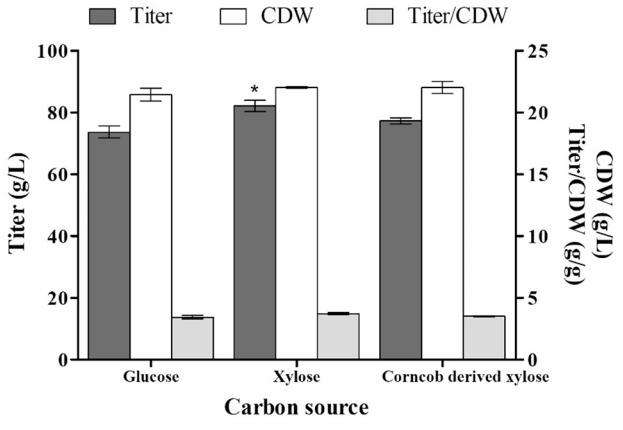

Fig. 2 Comparison of fumarate production and cell growth between xylose (from pure sources) and corn cob-derived xylose using the GC1 strain, in a fumarate production medium, against glucose. The data represented the standard deviation of three independent experiments, $n=3$. Compared with those of glucose, $^*P<0.05$ means difference

The column was an Aminex HPX-87 H (300×7.8 mm (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 5 mM H_2SO_4. The flow rate was 0.5 mL/min. The column temperature was 50 ℃.

Statistical analysis

All the experiments were conducted in triplicate ($n=3$) as required, and the results were expressed as mean ± standard deviation. Comparative studies of means were performed using one-way ANOVA. $^*p<0.05$ was considered statistically difference and $^{**}p<0.01$ was considered statistically significant difference.

Results

Effects of different sugars on calcium fumarate production by GC1 strain and identification of fumaric acid

In our previous study [15], we had shown that the GC1 strain (*Δgox* mutant as mentioned above) only produced calcium fumarate from glucose. So, conversion of glucose, xylose, and corncob-derived xylose to calcium fumarate by the GC1 strain were examined in this study. The results in Fig. 2 showed that when the GC1 strain was grown in the calcium fumarate production media containing glucose or xylose, the produced calcium fumarate titer (60.6 g/L) from glucose was little higher than that (57.8 g/L) from xylose. The cell growth (23.3 g/L) from glucose was also little higher than that (22.0 g/L) from xylose. Therefore, the values of the amount of the produced calcium fumarate/g of cell dry weight were almost the same. This meant that the GC1 strain grew a little poorer on xylose than on glucose. However, the results in Fig. 2 revealed that when the GC1 strain was grown in the calcium fumarate production medium containing corncob-derived xylose, calcium fumarate titer (55.7 g/L) from corncob-derived xylose was less than that (60.6 g/L) from glucose and that (57.8 g/L) from xylose and the cell growth (21.2 g/L) from corncob-derived xylose was also little lower than that (22.0 g/L) from xylose and that (23.3 g/L) from glucose. Therefore, the values of the amount of the produced calcium fumarate/g of cell dry weight were also almost the same (Fig. 2). This meant that the GC1 strain grew a little poorer on xylose and corncob-derived xylose than on glucose, but calcium fumarate titers produced from different sugars were not affected.

So, when the GC1 strain was grown on xylose and glucose, the time courses of calcium fumarate production, cell growth and changes in residual reducing sugars during cultivation at the flask level were compared. It can be seen from the data in Fig. S2 that glucose was utilized more rapidly than xylose and calcium fumarate production from xylose was more slowly than that from glucose. For example, within 120 h, only 3.4 g/L of glucose was left in the culture and 53.2 g/L of calcium fumarate

was produced from glucose, but 34.9 g/L of xylose was still maintained in the culture and 38.5 g/L of calcium fumarate was yielded from xylose under the same conditions. However, within 168 h of cultivation, the amounts of residual xylose and glucose were 8.6 g/L and 0.9 g/L, respectively while calcium fumarate titers from glucose and xylose were 53.5 g/L and 57.6 g/L, respectively. At the same time, during the cultivation, cell growth on xylose and glucose was also almost the same. This again indicated that the GC1 strain grew on xylose as well as on glucose. All the results demonstrated that GC1 strain indeed was suitable for calcium fumarate bioproduction from xylose. However, GC1 strain was needed to be further genetically modified to enhance xylose utilization for promotion of calcium fumarate biosynthesis as described below.

After recrystallization and purification of the produced products and identification of the purified products by using HPLC, the results in Fig. S3 indicated that when the GC1 strain was grown on xylose and corncob derived xylose, they indeed could produce a large amount of calcium fumarate and a minor amount of malate. This meant that the major product produced from xylose and corncob-derived xylose by the GC1 strain was still calcium fumarate. Wei et al. [15] also reported that the same GC1 strain (the *Δgox* mutant as described above) only produced calcium fumarate from glucose.

Enhanced calcium fumarate production from xylose by genetic engineering

Wei et al. [15] reported that the engineered strain e-PYC of the GC1 strain could produce 88.1±4.3 g/L of calcium fumarate from glucose at a flask level and 93.9±0.8 g/L of fumarate from glucose during the fed-batch fermentation. So, in this study, the xylose metabolism pathway of the GC1 strain was genetically engineered to stimulate calcium fumarate production from xylose.

As shown in Fig. 1, xylose reductase (XR) uses NAD(P)H as cofactor, while xylitol dehydrogenase is strictly NAD^+-dependent. This NAD(P)H dependence causes redox imbalance and leads to xylitol accumulation [4]. Therefore, the XR gene encoding xylose reductase was removed first to investigate its role in the GC1 strain in calcium fumarate production. Interestingly, the calcium fumarate titer and cell growth of all the disruptants (*Δxr-6*, *Δxr-16*, *Δxr-20*) were greatly reduced compared to those of their parent GC strain (Fig. 3A). For example, the disruptant *Δxr-6* only produced 38.2 g/L of calcium fumarate, 38.2% less than that (56.3 g/L) produced by the GC1 strain and its cell mass was only 19.5 g/L, 32.1% less than that (21.1 g/L) of the GC1 strain. This meant that the *XR* gene, and other functional aldehyde reductase genes, such as *GRE3* gene in the xylose reduction pathway of the GC1 strain (Fig. 1) were also important for calcium fumarate production and cell growth by the GC1 strain. In order to further indicate if the xylose isomerase pathway (Fig. 1) took part in the conversion of xylose to calcium fumarate, the *XI* gene, the key gene in the xylose isomerase pathway (Fig. 1) was overexpressed in the genomic DNAs of both the GC1 strain and the disruptant *Δxr-6*, respectively. It can be observed from the data in Fig. 3C that all the strains overexpressing the *XI* gene (XI-2, XI-6; XI-7, XI-10, XI-17 and XI-18) could produce more calcium fumarate from xylose than their parent GC1 strain. For example, the XI-6 strain could produce 62.8 g/L of calcium fumarate, 9.6% more than that (57.3 g/L) produced by the GC1 strain and its cell mass was 22.3 g/L. However, when the *XI* gene was overexpressed in the disruptant *Δxr-6* obtained above, all the overexpressing strains (xi-36 and xi-39) still produced more calcium fumarate than the GC1 strain, but produced less calcium fumarate than that produced by XI-6 strain (Fig. 3D). For example, the xi-36 strain produced only 61.5 g/L of calcium fumarate, a little less than that produced by the XI-6 strain and its cell mass was 22.9 g/L. This suggested that it was necessary to keep the *XR* gene in the genome in order to promote calcium fumarate production from xylose.

Moreover, the *XK* gene encoding xylulose kinase (Fig. 1) was further expressed in the XI-6 strain in order to further enhance calcium fumarate production as described in Methods. It can be seen from the data in Fig. 3E that all the expressing strains including XK-5, XK-6 and XK-28 could yield more calcium fumarate than the XI-6 strain, but cell growth was not affected. For instance, the expressing strain XK-6 yielded 68.5 g/L of calcium fumarate, 8.6% more than that (63.1 g/L) produced by the XI-6 strain (Fig. 3D). Furthermore, to test if the formed 5-P-xylulose can be catalyzed into acetyl-P and glyceralde-3-P under catalysis of xylulose-5-phosphate phosphoketolase (Xpka) (Fig. 1), the gene encoding Xpka was deleted from the genomic DNA of the GC1 strain. The results in Fig. 3B showed that calcium fumarate production and cell growth of all the obtained disruptants (*Δxpka-4*, *Δxpka-25* and *Δxpka-28*) were not affected compared to those of their parent strain GC1 strain. This suggested that the phosphoketolase (Pk) pathway was not involved in calcium fumarate biosynthesis in the parent strain GC1 strain (Fig. 1). So, according to xylose metabolisms shown in Fig. 1, the *TKL* gene encoding transketolase was also introduced and overexpressed in the expressing strain XK-6 as described in Methods. The results in Fig. 3F revealed that all the expressing strains (TKL-2, TKL-4, TKL-12, TKL-30 and TKl-43) yielded more calcium fumarate than the expressing strain XK-6. But their cell growth was almost the same (Fig. 3F). Especially, fumarate titer produced by the expressing strain TKL-4 reached to 73.1 g/L, 7.5%

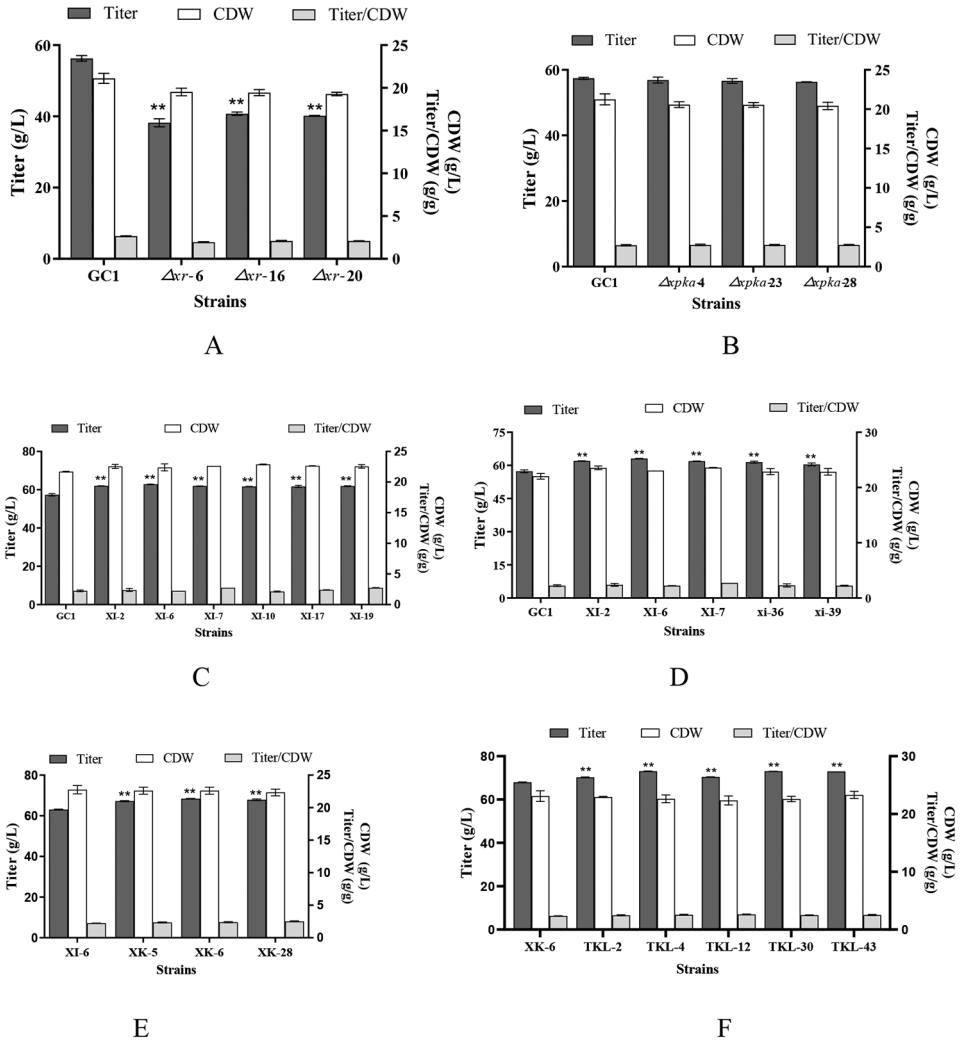

Fig. 3 Fumarate production and cell growth by the GC1 strain and its various mutants. (**A**): Deletants of the *XR* gene and their parent GC1 strain; (**B**): Disruptants of the *XPKA* gene and their parent GC1 strain, (**C**): Overexpressing strains of the *XI* gene and their parent GC1 strain; (**D**). Overexpressing strains of the *XI* gene in the disruptant *Δxr-6* and some of overexpressing strains of the *XI* gene in the GC1 strain; (**E**): Overexpressing strains of both the *XI* gene and the *XK* gene and the XI-6 strain; (**F**): Overexpressing strains of all the *XI* gene, the *XK* gene and the *TKL* gene and the XK-6 strain. The data represented the standard deviation of three independent experiments, $n = 3$. Compared with those of their parent strains, $^{*}P < 0.05$ means difference, $^{**}P < 0.01$ means significant difference

higher than that (68.0 g/L) produced by the XK-6 strain. The results in Fig. 3B and F suggested that the formed xylulose-5-phosphate would enter the non-oxidative pentose phosphate pathway (PPP) whose end-product is fructose-6-phosphate for intermediates of glycolysis for calcium fumarate formation based on Fig. 1 and the calcium fumarate biosynthesis pathway in the GC-1 strain confirmed by Wei et al. [15]. All the results indicated that expression of the *XI* gene, the *XK* gene and the *TKL* gene indeed could obviously enhance calcium fumarate production from xylose by the TKL-4 strain.

Determination of transcriptional levels of the genes related to xylose metabolism and calcium fumarate biosynthesis in Ornithine-Urea Cycle (OUC) showed that after overexpression of the *XI* gene, the *XK* gene and the *TKL* gene closely related to xylose metabolisms (Fig. 1) of the TKL-4 strain, the transcriptional levels of the *XI* gene, the *XK* gene and the *TKL* gene in the TKL-4 strain were greatly enhanced compared to those of the *XI* gene, the *XK* gene and the *TKL* gene in the parent GC1 strain (Table 1). At the same time, the transcriptional levels of the *ASS* gene and the *ASL* gene in the OUC (Fig. 1) of the TKL-4 strain were also greatly promoted compared to those of the *ASS* gene and the *ASL* gene in the OUC of the GC1 strain (Table 1). However, the transcriptional levels of the *XR* gene, the *XPKA* gene and the *ARG* gene (encoding arginase) were not affected (Table 1). This meant that the *XI* gene, the *XK* gene and the *TKL* gene in the TKL-4 strain were indeed overexpressed so that the

Table 1 The relative transcription levels (%) of the genes responsible for xylose metabolism and fumaric acid biosynthesis in the GC1 strain and the TKL-4 strain

Genes	GC1	TKL-4
XR	100	99.5 ± 0.7
XPKA	100	98.3 ± 2.7
XI	100	199.3 ± 1.9**
XK	100	248.4 ± 1.2**
TKL	100	207.9 ± 2.6**
PYC	100	95.9 ± 3.9
ASS	100	160.7 ± 1.9 **
ASL	100	148.24 ± 0.58**
ARG	100	93.1 ± 2.8

Data are given as mean ± SD, $n=3$. ** meant to have significant difference ($P<0.01$) compared with the strain GC1. The transcriptional levels of all the genes in the GC1 strain were regarded as 100%. All the genes were mentioned in Table S2 and Fig. 1. Compared with those of the GC1 strain, *P < 0.05, **P < 0.01. The functions of all the genes are mentioned in Table 52

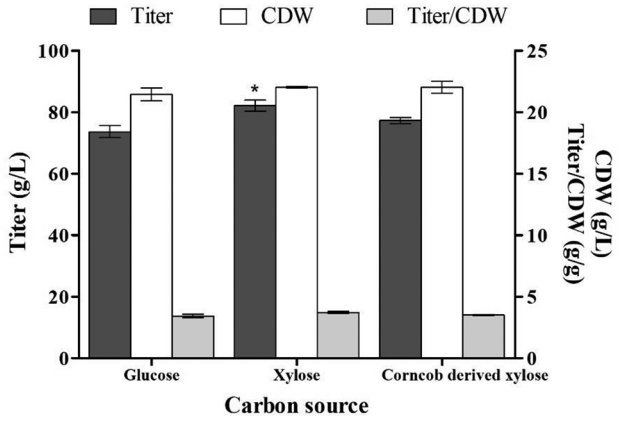

Fig. 4 Effects of different sugars on fumarate production and cell growth of the TKL-4 strain. Compared with those of the TKL-4 strain grown in the fumarate production medium containing glucose. The data represented the standard deviation of three independent experiments, $n=3$. Compared with those of glucose, *$P<0.05$ means difference

TKL-4 strain could produce more calcium fumarate from xylose than any other strains used in this study (Fig. 3F).

The maximum calcium fumarate production from xylose and corncob-derived xylose by the TKL-4 strain at the flask level

After optimization of the calcium fumarate production medium containing different concentrations (100.0 g/L, 105.0 g/L, 115.0 g/L and 120.0 g/L) of added xylose, it was found that at 115.0 g/L of xylose, the TKL-4 strain could produce the highest amount of calcium fumarate (76.3 g/L) (data not shown). In contrast, optimization of the calcium fumarate production medium containing different concentrations (100.0 g/L, 105.0 g/L, 115.0 g/L and 120 g/L) of the added corncob-derived xylose) indicated that at 115.0 g/L of corncob-derived xylose, the TKL-4 strain only produced 70.8 g/L of calcium fumarate (data not shown). This meant that the TKL-4 strain grown on xylose produced little more calcium fumarate than that cultivated on corncob-derived xylose, suggesting that corncob-derived xylose might contain some compounds toxic to the TKL-4 strain, such as furfural and hydroxymethyl furfural [2]. After influences of different kinds of CSLs on calcium fumarate production by the TKL-4 strain were examined, it was found that at 0.2% (w/v) of CSL from the Angel Bio-company was the most suitable for calcium fumarate production by the TKL-4 strain. Under these conditions, the TKL-4 strain could produce 83.7 g/L calcium fumarate (data not shown). Finally, the TKL-4 strain constructed in this study was found to be able to produce 73.6 g/L of calcium fumarate from glucose and its cell mass was 21.4 g/L and to yield 82.1 g/L of calcium fumarate from xylose and its cell dry weight was 22.0 g/L while it could accumulate 77.3 g/L of calcium fumarate from corncob-derived xylose in the culture and its cell growth was 22.0 g/L (Fig. 4). These results (Fig. 4) strongly demonstrated that the TKL-4 strain overexpressing the *XI* gene, the *XK* gene and the *TKL* gene could produce more calcium fumarate from xylose and corncob-derived xylose than that from glucose, but cell growth was not influenced. This meant that enhancement of xylose isomerase pathway and pentose phosphate pathway indeed could greatly improve calcium fumarate production from xylose and corncob-derived xylose (Fig. 1) compared to the calcium fumarate titers produced by their parent strain GC1 strain (Fig. 2). Even the TKL-4 strain could produce more calcium fumarate (73.6 g/L) from glucose than that (60.6 g/L) produced by its parent strain GC1. At the same time, it was confirmed that the major products from xylose and corncob-derived xylose by the TKL-4 strain were also calcium fumarate (Fig. S3E and F).

Calcium fumarate production from xylose and corncob-derived xylose during the 10-liter fermentation

After optimization of the calcium fumarate production medium, the TKL-4 strain was grown in the 10-liter fermenter with the optimized medium containing 113.2 g/L xylose as described in Methods. It can be observed from the data in Fig. 5A that within 168 h of the cultivation, the TKL-4 strain could produce 88.5 g/L of calcium fumarate and 22.6 g/L of cell mass, leaving 4.3 g/L of residual xylose in the fermented medium. Therefore, the productivity was 0.5 g/h/L (Fig. 5A). In addition, when it was cultivated in the optimized calcium fumarate production medium containing 112.5 g/L corncob-derived xylose within the same fermentation period, 85.6 g/L of calcium fumarate and 22.6 g/L of cell dry weight were accumulated in the fermented medium, leaving 4.5 g/L of residual xylose in the culture (Fig. 5B). This again revealed that the TKL-4 strain grown in the optimized calcium

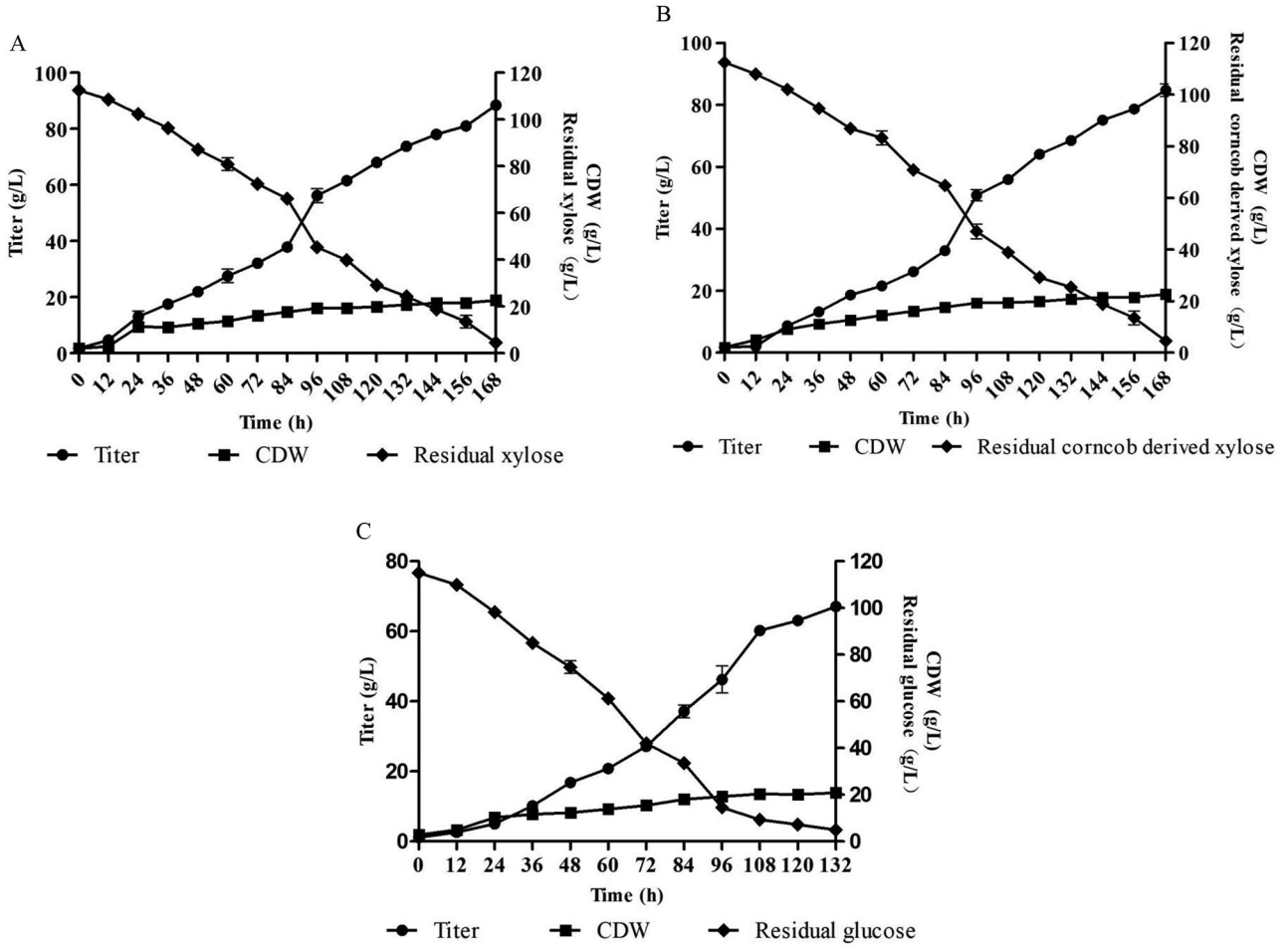

Fig. 5 The time courses of fumarate production, cell growth and changes in reducing sugar in the cultures containing 115 g/L xylose (**A**), corn-cob derived xylose (**B**) and glucose (**C**) in the 10-liter fermenters. Data are given as means ± SD, $n = 3$

fumarate production medium containing xylose could produce little more calcium fumarate than that cultivated in the optimized fumarate production medium containing corncob-derived xylose. In contrast, when the TKL-4 strain was grown in the optimized calcium fumarate production medium containing 115.1 g/L glucose, only 67.1 g/L of calcium fumarate was produced from glucose and cell mass was 20.8 g/L within 132 h of the cultivation, leaving 4.9 g/ L glucose in the culture (Fig. 5C). This demonstrated that the yield (0.6 g/g of glucose) was much less than those (0.81 g/g of xylose and 0.79 g/g of corncob-derived xylose), indicating that the calcium fumarate yield and titer from xylose in the engineered strain TKL-4 was much higher than those from glucose. Compared to the results in Figs. 2 and 5, it also can be clearly seen that calcium fumarate yield and titers produced from xylose and corncob-derived xylose by the genetically modified TKL-4 strain were indeed greatly improved compared to those produced by their parent strain GC1. This demonstrated that after the genetic engineering in this study, the xylose conversion to calcium fumarate by the engineered strain TKL-4 was greatly improved.

Calcium fumarate production from the mixture of xylose and glucose during the 10-liter fermentation

It has been known that the hydrolysate from lignocellulose in the hardwood contained 33.4 g/L glucose and 19.9 g/L xylose [1]. At the flask levels, the mixture with 40.0% xylose and 60.0% glucose was found to be the most suitable for the TKL-4 to produce calcium fumarate (75.5 g/L calcium fumarate) (data not shown). Therefore, the mixture containing 75.0 g/L glucose and 45.0 g/L xylose (63.0% glucose and 37.0% xylose) in the calcium fumarate production medium in the 10-liter fermenter was used to produce calcium fumarate by the genetically modified TKL-4 strain. It was found that within 168 h, the genetically modified TKL-4 strain could accumulate 78.7±1.1 g/L calcium fumarate and cell mass was 24.7±0.5 g/L, leading to the residual glucose of 0.5±0.1 g/L, residual xylose of 0.3±0.03 g/L and the productivity of 0.5 g/L/h (Fig. 6). At the same time, the

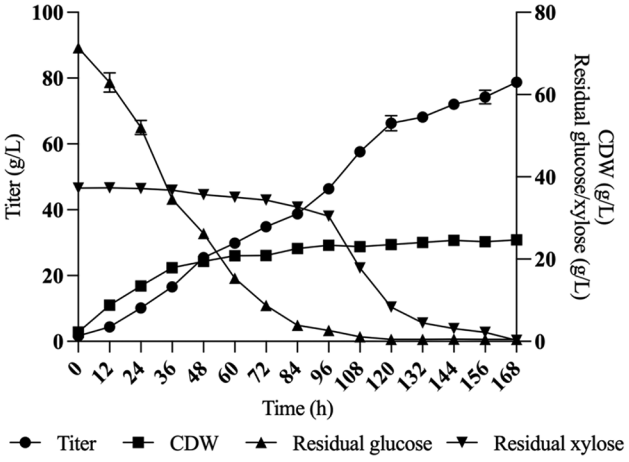

Fig. 6 The time course of fumarate production, cell growth, changes of the residual glucose and residual xylose in the mixture of glucose and xylose of the culture of the TKL-4 strain during the 10-liter fermentation. The data represented the standard deviation of three independent experiments, $n=3$

results in Fig. 6 also revealed that within 0–84 h of the fermentation, glucose (residual glucose was 3.9±0.1 g/L) was utilized quickly while xylose utilization (the residual xylose was 40.8±1.3 g/L) was slow, resulting in calcium fumarate production of 38.8±1.0 g/L. In contrast, after 84 h of the fermentation, xylose utilization was fast while glucose was almost used up, leading to quick calcium fumarate production from xylose. This meant that in the presence of the high concentration of glucose, xylose utilization was repressed while in the absence of glucose, xylose utilization was derepressed and calcium fumarate production was greatly promoted. This again demonstrated that the genetically modified TKL-4 strain constructed in this study was indeed suitable for calcium fumarate production from xylose.

Discussion

We have confirmed that the GC1 strain had high ability to use xylose and corncob-derived xylose as sole carbon sources to produce calcium fumarate. This may be related to its parent strain *A. pullulans* var. *aubasidani* DH177 which was isolated from leaves of the plant [29]. Similarly, Zou et al. [30] also reported that *A. pullulans* YJ 6–11 grew better on xylose than on glucose and had a better potential to produce polymalate from xylose than from glucose when xylose and $CaCO_3$ were utilized in this study as well as in the other studies. This meant that many strains of *Aureobasidium* spp. indeed have high ability to use xylose which is an important renewable and green raw material. However, *Scheffersomyces stipitis* is generally regarded as the yeast with excellent xylose-utilizing ability [15]. On contrast, this study demonstrated that many strains of *Aureobasidium* spp. had different characteristics in xylose utilization as mentioned above. It has been well evidenced that xylose is the second most abundant sugar after glucose on the earth [31]. However, it is generally regarded that xylose utilization rates are much slower than glucose utilization rates [32]. After the GC1 strain was genetically modified to enhance xylose utilization, the data showed that overexpression of the *XR* gene, the *XK* gene and the *TKL* gene could greatly stimulate calcium fumarate production from xylose by the TKL-4 strain. It was found that deletion of the *XI* gene also reduced calcium fumarate production (Fig. 3A). This meant that xylose metabolism in the GC1 strain was different from that in any other fungal strains based on the Fig. 1 [2, 4]. However, the detailed xylose metabolism in the GC1 strain is still awaited to be elucidated at molecular level. This indicated that promotion of xylose isomerase pathway and pentose phosphate pathway indeed could greatly improve calcium fumarate production from xylose and corncob-derived xylose (Fig. 1). Meanwhile, abolishment of an oxidoreductase pathway and overexpression of an isomerase pathway in *A. melanogenum* P10 rendered the XI-15 strain to accumulate intracellular 53.7% (w/w) of lipids from xylose [4]. In contrast, the removal of the *XPKA* gene (encoding xylulose-5-phosphate phosphoketolase) and *ACK* gene (encoding acetate kinase) in the phosphoketolase (Pk) pathway could lead to the reduced lipid production from xylose in the mutants of *A. melanogenum* P10 strain, indicating the PK pathway also plays some roles in conversion of xylose into lipid in this strain. However, overexpression of the *XPKA* gene and *ACK* gene could not further enhance lipid biosynthesis [4]. This meant that xylose metabolism in different strains of *Aureobasidium* spp. was very similar, but also varied according to different strains. Indeed, when D-xylose or arabinose are used as carbon source, they are metabolized into xylulose-5-phosphate to enter the glycolysis [33]. Overexpression of both *STB5* gene (encoding a transcription factor involved in response to oxidative stress) and *TKL1* gene make the expressing strain increase D-xylose consumption and shinorine (mycosporine, or mycosporine-like amino acid) production [34]. Ledesma-Amaro and Nicaud [35] overexpressed endogenous *XK* gene in addition to *S. stipitis XYL1* and *XYL2* encoding xylose dehydrogenase in the PO1d strain of *Yarrowia lipolytica*, achieving a growth rate on xylose as fast as on glucose. This suggested that the formed xylulose-5-phosphate in many yeasts will enter the non-oxidative pentose phosphate pathway (PPP) for further metabolisms.

At the same time, there was coordinated regulation of the expression of the genes in OUC of the yeast-like fungal cells after the expression of the key genes for xylose metabolism (Fig. 1; Table 1). However, the real mechanism of the coordinated regulation is still awaited to be understood [36]. In fact, such a coordinated regulation

also happens in lipid metabolism in yeasts and melanin biosynthesis in *Aureobasidium* spp [37, 38].

The TKL-4 strain obtained through overexpression of the *XI* gene, the *XK* gene and the *TKL* gene could make 73.6 g/L, 82.1 g/L and 77.3 g/g L of calcium fumarate from glucose, xylose and corncob-derived xylose, respectively, at the flask level (Fig. 4). It has been reported that the highest concentration of 41.3 g/L fumaric acid was reached from 20% (w/v) of the pretreated corncob by *Rhizopus oryzae* CICC 40,351 strain under the conditions of the combined hydrolysis and fermentation and fed-batch simultaneous saccharification and fermentation as well as at 38 °C [19]. In contrast, only 4.7 g/L of fumaric acid was obtained from 50.0 g/L of xylose by *S. stipitis* PSYPMFfS after the introduction and expression of xylose metabolic pathway in it [17]. Through immobilization of *R. arrhizus* cells and fermentation of the immobilized fungal cells in the culture containing xylose, 45.3 g/L fumaric acid was obtained from xylose, and the xylose conversion attained 0.7 g/g [5]. After genetic modification, the fungal strain *Rhizopus arrhizus* only yielded 28.5 g/L of fumaric acid from 80.0 g/L of xylose [16]. This has demonstrated that the TKL-4 strain constructed in this study indeed could produce much more calcium fumarate from xylose and corncob-derived xylose than any other fungal strains obtained so far.

During the 10-liter fermentation, the TKL-4 strain could produce the highest titers (88.5 g/L and 85.6 g/L) of calcium fumarate from xylose and corncob-derived xylose, respectively. It has been reported that the fumaric acid titer was only 49.1 g/L from corncob hydrolytes by growing *R. oryzae* WHT5 in a 7-Liter bioreactor [20]. This again demonstrated that the genetically engineered TKL-4 strain could produce the highest amount of calcium fumarate from xylose during the fermentation. In our previous studies [15], it was found that the engineered e-PYC strain of the same parent strain GC1 could produce 93.9±0.8 g/L of calcium fumarate from glucose during the fed-batch fermentation within 168 h. In this study, the conversion efficiencies of xylose and corncob-derived xylose to calcium fumarate were 29% and 25% higher, respectively, than the efficiency obtained from glucose, which was reported previously [15]. It has been reported that the wild type strain *R. oryzae* produced 56.5 g/L of calcium fumarate from glucose [5, 6] while the engineered *E. coli* EF02 (pSCppc) [19], *R. oryzae* ppc [39], *S. cerevisiae* FMME 006 *FUM1* (encoding fumarase)+*RoPYC* (encoding pyruvate carboxylase)+*RoMDH* (encoding malate dehydrogenase)+*RoFUM1*, *T. glabrata* (*Δade12* for denylosuccinate synthetase)−PMS−P160A and *S. stipites* [12, 40] only yield 41.5 g/L, 25.0 g g/L, 5.64 g/L, 9.2 g/L and 4.7 g/L of fumarate from glucose, respectively. Even, *C. glabrata* expressing *pyc*, *fumR* and *mdhR* from *R. oryzae*, *dct* from *S. pombe*, *fdh* (encoding formate dehydrogenase) from *S. cerevisiae* produced only 21.6 g/L fumarate and the productivity was 0.3 g/h/L [41] and *R. oryzae* expressing endogenous *pyc* and *ppc* genes produced 25.0 g/L of fumarate and the productivity was 0.26 g/L/h [10]. This again strongly demonstrated that the productivity (0.5 g/h/L and 0.51 g/h/L) by the genetically engineered TKL-4 strain obtained in this study were also the highest. It was also found that the genetically modified TKL-4 strain could accumulate 78.7±1.1 g/L calcium fumarate from the mixture of 75.0 g/L glucose and 45.0 g/L xylose (Fig. 6). Therefore, the genetically engineered TKL-4 strain will be the most suitable for calcium fumarate production from xylose and corncob-derived xylose in the future. Ko et al. [42, 43] also showed that the mixture with 70.0 g/L glucose and 40.9 g/L xylose in the lignocellulosic hydrolysates was the most suitable for ethanol production by the engineered *S. cerevisiae* harboring xylose isomerase-based pathway. In order to relieve glucose repression and improve utilization of xylose in lignocellulose, *R. oryzae* was first grown in hydrolysate containing 30.0 g/L of xylose. Then, fumaric acid fermentation was initiated in the hydrolysate containing 80.0 g/L of glucose. In this case, only 27.8 g/L of fumaric acid could be reached [44].

Conclusions

The TKL-4 strain overexpressing the *XI* gene, the *XK* gene and the *TKL* gene could produce the highest titers (88.5 g/L, 85.6 g/L and 78.7±1.1 g/L) of calcium fumarate from xylose, corncob-derived xylose and the mixture of 75.0 g/L glucose and 45.0 g/L xylose. All the results demonstrated that *A. pullulans* var. *aubasidani* DH177 and its genetically modified strains had the high ability to utilize xylose and corncob-derived xylose with CO_2 fixation derived from $CaCO_3$ to produce calcium fumarate. This would be helpful to keep the global sustainable development and carbon neutrality. Therefore, the genetically engineered TKL-4 strain obtained in this study was the most suitable for calcium fumarate production from xylose, corncob-derived xylose and the mixture of xylose and glucose. In the future, the fermentation period (168 h) should be shortened by overexpression of the genes for high affinity xylose transporter and addition of $CaCO_3$ during the fermentation must be avoided to reduce pollution of the produced $CaSO_4$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02608-6.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

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Author contributions
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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors have read and checked the revised manuscript and agree that the manuscript is submitted to Microbial Cell Factories and the corresponding authors are Dr. Bo Jiang and Dr and Professor Zhen-Ming Chi.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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