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Activation of glycerol metabolism in *Xanthomonas campestris* by adaptive evolution to produce a high-transparency and low-viscosity xanthan gum from glycerol

Zichao Wang [a], Jianrong Wu [a], Li Zhu [b], Xiaobei Zhan [a,*]

[a] Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu 214122, China
[b] Wuxi Galaxy Biotech Co. Ltd., Wuxi, Jiangsu 214125, China

HIGHLIGHTS

- Adaptive evolution makes mutant strain can use glycerol to produce xanthan.
- Xanthan gum produced by glycerol was high-transparency and low-viscosity.
- Impurities in crude glycerol have no effect on the activity of mutant strain.
- Starter substrate decreased cell growth time by 12 h.
- RT levels of related genes were compared between mutant and parent strains.

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ABSTRACT

Many studies have focused on using crude glycerol from biodiesel to obtain valuable products, but few of these studies have focused on obtaining polysaccharides. A mutant strain of *Xanthomonas campestris* CCTCC M2015714 that could use glycerol to produce high-transparency and low-viscosity xanthan gum was obtained by adaptive evolution, and the yield of xanthan gum reached 11.0 g/L. We found that transcriptional levels of genes related to glycerol metabolism (*glpF*, *glpK*, *glpD*, and *fbp*) in the mutant strain were all higher than those from the parent strain. Using 5 g/L sucrose or glucose as starter substrate, cell growth time decreased from 36 h to 24 h and xanthan gum yield increased. Moreover, the mutant strain can tolerate high titer glycerol, and its activity was not affected by the impurities in crude glycerol. All these results proved that crude glycerol from biodiesel industries can be used for xanthan gum production.

1. Introduction

Biodiesel is the focus of new clean energy in the 21st century. In a common biodiesel process, plant or animal oils are treated by trans-esterification with methanol or ethanol to produce long-chain fatty acid monoesters (methyl or ethyl ester), which has lower pollution but the similar combustibility to fossil fuel (Anastacio et al., 2014). However, glycerol is generated as a by-product in the process of trans-esterification and accounts for 10% of biodiesel's total product (Sarma et al., 2014). Global biodiesel production will reach 37 trillion gallons in 2016, which means that 400 million gallons of crude glycerol will also be produced (Yang et al., 2012; Gallardo et al., 2014).

The massive volume of crude glycerol has become a burden to biodiesel industry; thus, a practical way to utilize crude glycerol is the key to sustainable development of the biodiesel industry. Current utilization of crude glycerol varies in different industries. For one, crude glycerol enters into traditional glycerol market through refining process. For another, crude glycerol goes to the low value-added markets, such as feed or bio-fuel markets. Finally, crude glycerol is used as raw material in developing high value-added products. Bioreaction conditions of microorganisms converting glycerol to high value-added products are mild and are more vital in long-term development and environment protection. For instance, bulk chemicals, such as 1,3-propanediol, 1,2-propanediol, ethanol, butanol, docosahexaenic acid, succinic acid, propionic acid, lactic acid, kojic acid, and citric acid, are produced by microbial fermentation using glycerol as substrate (Almeida et al., 2012; Mattam et al., 2013).

* Corresponding author.
E-mail address: xbzhan@yahoo.com (X. Zhan).

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Xanthan gum is an extracellular polysaccharide discovered in the late 1950s and is produced by the bacterium *Xanthomonas campestris* (Jeanes et al., 1961). Xanthan gum has desirable properties, such as water solubility, emulsibility, suspensibility, and thickening. Meanwhile, xanthan gum's aqueous solution is highly viscous even at lower gum concentration and is shear-thinned; it is also stable in salt, temperature, and pH. Xanthan gum is widely used in many industries, such as food, oil recovery, pharmaceuticals, cosmetics, and textiles because of these properties (Rosalam and England, 2006; Palaniraj and Jayaraman, 2011). In general, the carbon source used to produce xanthan gum is corn starch; glucose and sucrose are also commonly used substrates for xanthan gum production in some cases. Other renewable resources, such as xylose, whey, bagasse, and syrup, are used to produce xanthan gum in bench scale (Faria et al., 2011; Rosalam and England, 2006). In the fermentation production of xanthan gum, the capital cost of substrate is an important factor for commercial production. Due to its low cost, glycerol can be a potential alternative substrate in the production of xanthan gum.

Microorganisms can survive in adverse conditions and adapt rapidly to new environmental conditions, especially through the artificial evolution. Therefore, adaptive evolution has become a useful tool for biotechnological applications in industrial processes, such as activating latent pathways, increasing tolerance to rugged environment, utilizing nonnative substrates, and improving productivity (Portnoy et al., 2011; Wang et al., 2016). For instance, Huang et al. (2015) reported the glycerol metabolism pathway in *Rhizopus oryzae* was activated by the process of evolutionary engineering and, led to high fumaric acid yield. According to Nwachukwu et al. (2012), an evolved mutant strain of *Enterobacter aerogenes* can tolerate 200 g/L glycerol and 30 g/L ethanol through adaptive evolution. To date, Kurosawa et al. (2015) improved triacylglycerols yield in *Rhodococcus opacus* from glycerol through adaptive evolution.

In the initial test and preliminary work, a mutant strain, named *X. campestris* CCTCC M2015714, was screened to grow well on glycerol through adaptive evolution. In this work, the capacity of the mutant strain on xanthan gum production was further evaluated and enhanced. The mutant strain can tolerate the impurities in crude glycerol and shows excellent commercial potential. Transcriptional analysis indicated that genes related to glycerol metabolism in the mutant strain were up regulated. This study provides another way of utilizing crude glycerol from biodiesel.

2. Materials and methods

2.1. Microorganisms and chemicals

X. campestris NRRL B-1459 was bought from Agricultural Research Service Culture Collection (USA). We obtained *X. campestris* CCTCC M2015714 through adaptive evolution from *X. campestris* NRRL B-1459, which was collected in China Center for Type Culture Collection (China).

Chemicals (excluding crude glycerol) used in this work were of analytical grade and were bought from Sinopharm Chemical Reagent Co., Ltd. (China). The crude glycerol used, which contains the following (w/w): glycerol 80%, sodium salts 2.0%, methanol 1.5%, glycerides 4.5%, soap and other organic materials 4.0%, and water 8.0%, was bought from Nanjing Yangtze Jiang Yu Oil Co., Ltd. (China).

2.2. Medium and cultivation conditions

2.2.1. Seed medium

The seed medium used for *X. campestris* NRRL B-1459 contains (g/L): sucrose, 20; peptone from fish, 5; beef extract, 3; yeast extract, 1; pH 7.0 ± 0.02. The seed medium was cultured in 250 mL flask with 50 mL working volume and was shaken in a rotary shaker at 200 rpm (28 °C). The composition of the seed medium used for *X. campestris* CCTCC M2015714 is similar to that of *X. campestris* NRRL B-1459; the only difference is glycerol was used instead of sucrose but the culture conditions are the same.

2.2.2. Adaptive medium

The initial adaptive medium used was the seed medium for *X. campestris* NRRL B-1459. Along with sequential adaptive evolution process, the concentration of sucrose was reduced gradually and glycerol's concentration was increased stepwise; other compositions in the medium were unchanged. The screening plate medium contained 20 g/L agar based on the adaptive medium.

2.2.3. Batch fermentation

Fermentation medium used for *X. campestris* NRRL B-1459 contained the following (g/L): sucrose (glycerol), 40; peptone from fish, 3; yeast extract, 1; $NaNO_3$, 0.8; $FeSO_4$, 0.01; $MgSO_4$, 2.5; KH_2PO_4, 2; K_2HPO_4, 3.5; and antifoam (Foam-free powder, Sangon, Shanghai, China), 0.5. The culture temperature was 30 °C and the inoculation volume of the seed medium was 10% (v/v). In the process of fermentation, pH was maintained at 7.0 ± 0.02 with 1 M NaOH and 1 M HCl, while the agitation speed and aeration volume were set at 600 rpm and 0.4 vvm, respectively. The experiments were conducted in 7.0 L fermenters (BioFlo 115, New Brunswick, USA) with 4 L working volume.

Compositions of fermentation medium used for *X. campestris* CCTCC M2015714 are similar to *X. campestris* NRRL B-1459; however, the carbon source was replaced by sucrose, glycerol, crude glycerol, sucrose and glycerol, and glucose and glycerol.

2.2.4. Fed-batch fermentation

In order to study the effect of glycerol concentration on the growth of mutant strain *X. campestris* CCTCC M2015714, the glycerol concentrations for batch fermentation were set at 10, 20, 30, 40, 50, 60, 70, and 80 g/L. To study glycerol feeding strategies on *X. campestris* CCTCC M2015714 growth and xanthan production, the initial glycerol concentration used for the fermentation medium was 10 g/L before glycerol feeding. Then, the first strategy was to pulse 40 g glycerol to the batch at 36, 57, and 78 h, respectively. Another feeding strategy is to feed glycerol at a constant rate of 2 g/h during the culture time of 36–96 h.

2.3. Adaptive evolution

X. campestris NRRL B-1459 was initially cultured in a seed medium with 20 g/L sucrose. When the bacterium entered into mid-exponential phase, the cells were transferred with 10% (v/v) inoculum into a new seed medium with 15 g/L sucrose and 5 g/L glycerol. When the bacterium grew for ten generations in the seed medium with 15 g/L sucrose and 5 g/L glycerol, 1 mL seed liquid in mid-exponential phase was taken and diluted with sterile deionized water appropriately; the diluted seed liquid was then spread on agar plates. Afterwards, big and pure mutant bacterial colony was picked up and inoculated into a new seed medium with 10 g/L sucrose and 10 g/L glycerol for genetical screening. Successive rounds of adaptive evolution were carried out, while the concentration of sucrose was reduced gradually (10, 5, 0, 0, 0, 0, 0, 0, 0 g/L); the glycerol concentration was increased stepwise (10, 15, 20, 25, 30, 35, 40, 45, 50 g/L). Considering the influence of inorganic salts used in the fermentation medium, the glycerol concentration of the adaptive medium was 50 g/L, whereas the glycerol concentration used in the fermentation medium was 40 g/L.

2.4. Analytical methods

During the fermentation process, 5 mL fermentation broth was taken every 3 h for analysis. Samples were diluted with 15 mL deionized water and were centrifuged at 20,000×*g* for 1 h to collect cells. The supernatant was used for the determination of xanthan gum, glycerol, glucose, and sucrose.

2.4.1. Cell concentration

The collected pellets were washed with 20 mL 0.9% (w/w) sodium chloride solution and were centrifuged at 20,000×*g* for 1 h for cell collection. Cell washing was repeated three times. Finally, the collected cells were added with 5 mL deionized water for biomass determination using spectrophotometric method. The optical density was measured using a UV spectrophotometer (UV-2000; UNICO, Dayton, NJ) at 600 nm. The linearity of the dry cell weight and optical density was: DCW = 1.15 (OD_{600}) – 0.18, where DCW is the dry cell weight, g/L; and OD_{600} is the optical density at 600 nm.

2.4.2. Product and substrates concentration

The supernatant was added with three volumes (v/v) of cold ethanol to precipitate xanthan gum. The precipitated xanthan gum was dried in a vacuum oven at 40 °C and 0.1 MPa for 48 h, and the weight of the xanthan gum was measured.

The remaining alcoholic solution was transferred to a 25 mL distilling flask and was evaporated to dry at 60 °C and 0.1 MPa. Five mL deionized water was added into the distillation bottle and mixed thoroughly for substrates detection. The concentrations of glycerol, glucose, and sucrose were determined by high-performance liquid chromatography (HPLC) (Agilent 1200 series, Santa Clara, CA, USA) with a Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad Laboratories Inc, Hercules, CA, USA) and a refractive index detector. The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min at 35 °C. The sample solution was filtered through a 0.22 μm membrane with the injection volume of 20 μL.

2.5. Characterization of this new xanthan

The fermentation broth was diluted with three volumes deionized water and centrifuged at 20,000×*g* for 1 h to remove cells. Then, three volumes (v/v) of cold ethanol were added to the supernatant to precipitate xanthan gum. Part of the precipitated xanthan gum was dried with a vacuum at 40 °C and 0.1 MPa for 48 h, and was sifted through a 100 mesh sifter to study the rheological properties of this new xanthan. The commercial xanthan (Deoson Biochemical Ltd., food grade, China) was used as control. Two xanthan samples were dissolved in deionized water (1.0%, w/v) for rheological analysis by a Brookfield DV-II viscometer (USA) at 25 ± 1 °C (No. 3 rotor and 6 rpm); their transparency at OD_{600} was detected using the UV spectrophotometer (UV-2000; UNICO, Dayton, NJ) with deionized water as blank.

A part of the precipitated xanthan gum was redissolved in deionized water and sevage method was used to remove protein. Thereafter, three volumes (v/v) of cold ethanol were added to precipitate xanthan gum and remove residual organic solvents. The precipitated xanthan gum was dialyzed through a dialysis tube (8000 Da) at 25 ± 1 °C for 48 h and was freeze-dried for study of molecular characterizations of the new and commercial xanthan. Molecular weight and monosaccharide composition were analyzed according to the methods of Faria et al. (2011). The polysaccharide hydrolysate was also used for pyruvate and acetyl content analysis by HPLC. HPLC conditions are as follows: LC-2010 system (Shimadzu, Japan), Aminex HPX-87H column (Bio-Rad, USA); the mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min; the detection temperature was 35 °C and the injection volume was 20 μL. Xanthan samples for FT-IR analysis were ground with potassium bromide and were recorded using a Nicolet Nexus 470 FT-IR spectrometer (Thermo Nicolet, USA). The scanning wave was 400–4000 cm^{-1} and the results were analyzed with OMNIC-8.2 software. Xanthan samples for nuclear magnetic resonance (NMR) study were dissolved in D_2O solvent, which was detected at Shanghai Institute of Organic Chemistry (Shanghai, China). The 1D ^1H NMR spectra were acquired at 35 °C on an Agilent 800 MHz spectrometer equipped with an actively z-gradient shielded triple resonance cold probe.

2.6. Real-time reverse transcription PCR analysis

Total RNA of *X. campestris* CCTCC M2015714 and *X. campestris* NRRL B-1459 were extracted with Bacterial Total RNA Isolation Kit (Sangon, Shanghai, China) from mid-exponential phase grown in seed medium. AMV First Strand cDNA Synthesis Kit (Sangon, Shanghai, China) was used for first-stand cDNA synthesis. Prior to cDNA synthesis, the total RNA was treated with gDNase at 42 °C for 3 min to avoid the interference of genome DNA. The 16s rRNA gene was used as the internal control and RT-PCR was conducted in triplicate for each sample. A total of 30 cycles of 94 °C 30 s, 51 °C 30 s, and 72 °C 30 s were done for PCR amplification. After the cycles, an additional 72 °C for 10 min was performed to terminate the reaction. PCR product was analyzed using 2.0% (w/v) agarose gel electrophoresis and then stained with ethidium bromide. The band intensity was analyzed using Bio-Imaging System with the Quantity One 1-D analysis software (Bio-Rad, CA, USA). The primers and relevant sequences used in this work are listed in Table 1.

3. Results and discussion

3.1. Batch fermentation profiles of mutant and parent strains with sucrose and glycerol as carbon sources

If the excess crude glycerol can be used for xanthan production, the pressure on the biodiesel industry and the production cost of xanthan gum can be reduced. However, the parent strain, *X. campestris* NRRL B-1459, could not grow well and no xanthan gum was detected when glycerol as sole carbon source (Fig. 1b). Through adaptive evolution and screening of parent strain by increasing glycerol concentration gradually, a mutant strain that can utilize glycerol for xanthan production was obtained and was named *X. campestris* CCTCC M2015714 (Fig. 1d).

To identify the physiological and biochemical differences between the mutant and parent strain in carbon source utilization, the strains B-1459 and M2015714 were batch-cultured with sucrose and glycerol, respectively. As shown in Fig. 1a, when sucrose was used as the carbon source for parent strain, the cell growth time and fermentation time were 24 h and 60 h, and the

Table 1
Primers and relevant sequences used in this work.

Primers	Relevant sequences
RTf-*glpF*	5′ CTTCGGTATCTTCGCCATCA 3′
RTr-*glpF*	5′ TTCATTGCCCAGGCTTCC 3′
RTf-*glpK*	5′ CCAAGGTCAAGTGGATTCTCG 3′
RTr-*glpK*	5′ AGGTTCCAGATCAGCCAGCTA 3′
RTf-*glpD*	5′ CGCATCGTGTTTGCCATT 3′
RTr-*glpD*	5′ TGTAGTTCCACACCACATCCTG 3′
RTf-*fbp*	5′ CGGCATCTTCATCTACCCCT 3′
RTr-*fbp*	5′ GCAGTTGCGTCGGTTGAATA 3′
RTf-16s	5′ CCTACGGGAGGCAGCAG 3′
RTr-16s	5′ ATTACCGCGGCTGCTGG 3′

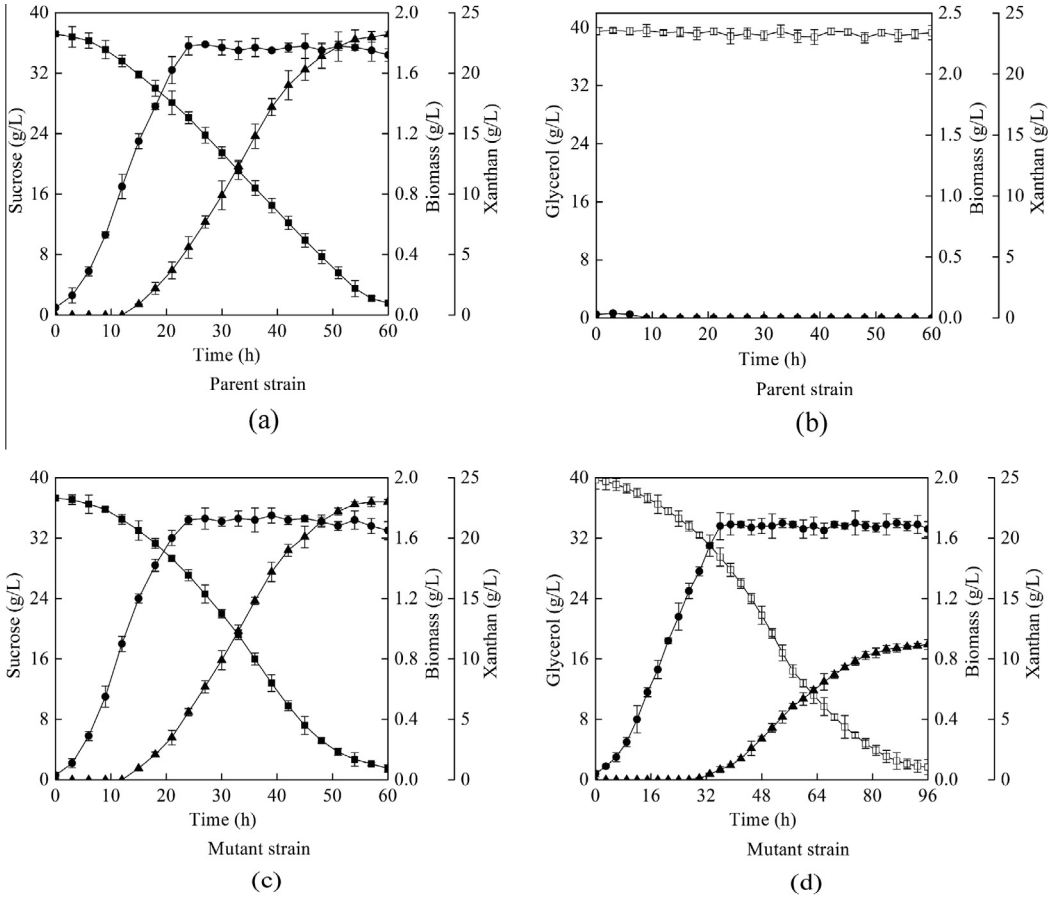

Fig. 1. Batch fermentation profiles of *X. campestris* NRRL B-1459 and *X. campestris* CCTCC M2015714 with 40 g/L sucrose (a, c) or 40 g/L glycerol (b, d) as carbon sources. The symbols represent: sucrose (■), glycerol (□), biomass (●), xanthan (▲).

dry cell weight was 1.72 g/L. In final fermentation broth, xanthan yield was 23.2 g/L with a 58% conversion of sucrose to xanthan gum. These results were in accordance with previous reports on xanthan gum batch fermentation with common carbon sources, such as glucose, sucrose, and corn starch (Garcia-Ochoa et al., 2000; Rosalam and England, 2006).

In this work, the capacity of M2015714 to produce xanthan gum using sucrose as carbon source was similar to B-1459 after the process of adaptive evolution. These results show that cell growth time (24 h), dry cell weight (1.71 g/L), fermentation time (60 h), and xanthan yield (23.1 g/L) of M2015714 were similar to B-1459 (Fig. 1c). However, the effect of artificial adaptive evolution is shown in Fig. 1d. The mutant M2015714 could use glycerol to grow and produce xanthan gum in comparison with the parent B-1459 (Fig. 1b). The cell growth time (36 h) and fermentation time (96 h) of M2015714, which used glycerol as carbon source, were longer compared with using sucrose. Xanthan yield was 11.0 g/L with a 27.5% conversion rate; these values were all lower than those that used sucrose as carbon source. The pathways of glycerol phosphorylated to glycerol-3-phosphate by glycerol kinase, glycerol-3-phosphate oxidized to dihydroxyacetone phosphate via aerobic glycerol-3-phosphate dehydrogenase, and generating fructose-6-phosphate from fructose-1,6-biphosphate by fructose-1,6-biphosphatase all consume a lot of energy. Given that Entner–Doudoroff pathway is the main metabolic pathway providing the precursors of xanthan gum synthesis when glucose or sucrose was used as carbon source, only a small portion of glucose or sucrose was routed via the pentose phosphate pathway; however, the two pathways consumed less energy compared with the above glycerol metabolism pathways (Li et al., 2015; Rosalam and England, 2006). This might be the reason for the long fermentation time and low conversion rate when M2015714 used glycerol for xanthan gum production. These results are in agreement with other reports regarding the use of glycerol as carbon source to synthesize products by fermentation (Bretz, 2015; Swinnen et al., 2013).

As compared to other carbon sources for xanthan fermentation (Table 2), glycerol could be used effectively as a carbon source for xanthan production. On the other hand, the sucrose concentration showed a loss of 7% after sterilization (Fig. 1a and c). This phenomenon may be due to caramelization and should be avoided by sterilizing the glucose separately. As for glycerol, the rate of loss due to sterilization was negligible (Fig. 1b and d).

3.2. Characterization of the new xanthan

To characterize the polymer produced by *X. campestris* CCTCC M2015714 from glycerol, the polysaccharide was purified and subjected to monosaccharide composition, molecular weight, pyruvate content, acetyl content, FT-IR, NMR, and rheology analysis. As shown in Table 3, the molar glucose, mannose: glucuronic acid ratio of the new polymer (2.0:1.65:1.0) is similar to that of commercial xanthan (2.0:1.85:1.0). The composition of mannose in sugar repeated unit is slightly low compared to the commercial xanthan. The reason may be that glycerol metabolism provides less UDP-mannose as sugar nucleotide donor for xanthan biosynthesis. Meanwhile, pyruvate and acetyl contents of the polymer showed little difference with those of commercial xanthan. Based on

Table 2
Xanthan gum yields in decreasing order with different types of carbon sources.

Substrate	Xanthan (g/L)	References
Glucose	14.744	Leela and Sharma (2000)
Sucrose	13.234	Leela and Sharma (2000)
Maltose	12.321	Leela and Sharma (2000)
Starch	12.10	Leela and Sharma (2000)
Glycerol	11.0	This work
Arabinose	10.958	Leela and Sharma (2000)
Potato starch	9.754	Leela and Sharma (2000)
Crude glycerol	7.9	This work
Galactose	7.129	Leela and Sharma (2000)
Residual glycerol	7.23	Brandao et al. (2014)
Sulphuric acid treated tapioca pulp	4.5–7.1	Gunasekar et al. (2014)
Raw starch	4.3–6.0	Kerdsup et al. (2011)
Crude glycerol	5.59	de Jesus Assis et al. (2014)
Xylose	5.531	Leela and Sharma (2000)
Fructose	5.232	Leela and Sharma (2000)
Inositol	1.502	Leela and Sharma (2000)
Sorbitol	1.401	Leela and Sharma (2000)
Lactose	1.008	Leela and Sharma (2000)

Table 3
Characteristics of this new xanthan and commercial xanthan.

	XG-commercial	XG-glycerol
Glc:Man:GlcA molar ratio	2.0:1.85:1.0	2.0:1.65:1.0
Molecular weight (Da)	$5.8 \pm 0.25 \times 10^6$	$3.0 \pm 0.14 \times 10^6$
Pyruvate content (%)	4.1 ± 0.05	4.0 ± 0.025
Acetyl content (%)	4.9 ± 0.045	5.1 ± 0.05
Apparent viscosity (mPa s)	12,000 ± 800	1150 ± 35
Transparency (%)	81.7 ± 0.3	92.5 ± 0.2
Temperature (0–100 °C)	+	+
pH (1–12)	+	+
Salts	+	+
Cellulose	+	+
Glucoamylase	+	+
α-Amylase	+	+

Data shows means ± standard deviations, $n = 3$. "+" represents the stability of xanthan under tested conditions.

FT-IR and NMR spectra results (data not shown), it is proved that the polymer was a xanthan gum but with high-transparency and low-viscosity.

3.3. RT-PCR analysis of glycerol metabolism genes

Under the same culture conditions, the parent strain with glycerol as carbon source could not grow well. On the other hand, the mutant strain M2015714 could grow well in glycerol and produce xanthan gum after adaptive evolution. This result indicated that the intrinsic difference between two strains was associated with the genetic changes in the mutant strain. The exact metabolic pathway of glycerol in *X. campestris* was not documented, but the glycerol metabolism genes could be found in the full genome of *X. campestris* pv. *campestris* ATCC 33913 (Supplementary Fig. S1). In this work, four genes related to glycerol metabolism for xanthan gum synthesis (*glpF*, *glpK*, *glpD*, and *fbp*) were chosen for RT-PCR analysis. Fig. 2 shows the relative transcription (RT) levels of *glpF*, *glpK*, *glpD*, and *fbp* in parent strain were all set at 1.0. The RT levels of these four genes in mutant strain *X. campestris* CCTCC M2015714 were all higher than 1.0, following the RT level order of: *glpD* (4.76) > *glpF* (3.36) > *glpK* (3.05) > *fbp* (2.53). This result indicated that glycerol metabolism pathway in the mutant strain was activated after adaptive evolution. Moreover, the RT level of *glpD* was the highest among the four genes because the metabolite DHAP not only entered into central metabolic pathway for cell growth, but also for xanthan gum synthesis. Other researchers

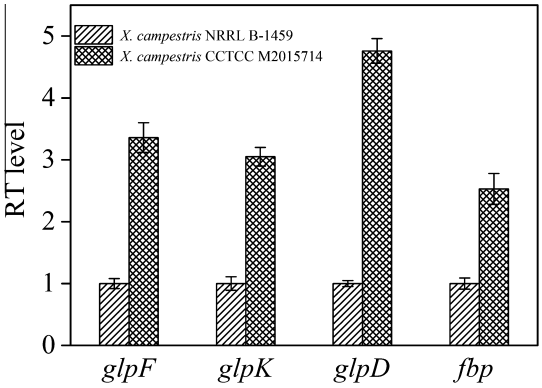

Fig. 2. Relative transcription level of glycerol metabolism related genes in two strains.

obtained similar results that spontaneous mutation of genes occurred when microorganism adapted to glycerol-based growth medium. Cheng et al. (2014) used whole-genome sequencing method to identify the causality of evolved mutant strain to new environments and found that a point mutation in *glpK* enhances glycerol metabolism. Huang et al. (2015), through metabolic flux analysis, found that the increased production of fumaric acid in *R. oryzae* G80 is associated with the activation of glycerol metabolism pathway in the *R. oryzae*.

Enzymes involved in glycerol metabolic pathway in bacteria are important for glycerol fermentation. In the production of shikimic acid by engineered *E. coli* BL21 (DE3) with glycerol, over-expression of *glpD* and *glpK* genes increased the shikimic acid yield by 5.3-fold (Yang et al., 2014). As for L-phenylalanine production by recombinant *E. coli* with glycerol, over-expression of *glpX* and *tktA* genes also increase the yield of L-phenylalanine (Gottlieb et al., 2014). These results demonstrate that efficient utilization of glycerol can increase the targeted product yield. Therefore, further researches on over-expression of the four genes (*glpF*, *glpK*, *glpD*, and *fbp*) by genetic engineering should be carried out to increase xanthan gum yield.

3.4. Influence of glycerol concentration on xanthan production by X. campestris CCTCC M2015714

Carbon sources have significant effect on cell growth and xanthan yield by *X. campestris*. Cell growth and xanthan gum production were all inhibited by 50 g/L glucose, and the glucose concentration must be controlled under a certain level (Leela and Sharma, 2000; Rosalam and England, 2006). As shown in Table 4, cell growth time were all 36 h when glycerol concentration was lower than 40 g/L, whereas cell growth time became longer when glycerol concentration was increased. Furthermore, dry cell weight decreased gradually along with the increase of glycerol

Table 4
Effect of glycerol concentration on cell growth.

Glycerol (g/L)	Cell growth time (h)	DCW (g/L)	Xanthan (g/L)
10	36	1.68 ± 0.09	0.5 ± 0.05
20	36	1.70 ± 0.11	3.7 ± 0.15
30	36	1.67 ± 0.12	7.1 ± 0.18
40	36	1.69 ± 0.10	11.5 ± 0.23
50	40	1.52 ± 0.13	10.6 ± 0.16
60	48	1.15 ± 0.08	6.9 ± 0.25
70	60	0.53 ± 0.06	2.7 ± 0.09
80	76	0.12 ± 0.03	0.3 ± 0.07

Data shows means ± standard deviations, $n = 3$.

concentration. When the mutant M2015714 were cultivated on agar plate with 20 g/L glycerol or 20 g/L sucrose as carbon source, the colony of M2015714 with glycerol (Supplementary Fig. S2b) was obviously smaller than that of with sucrose (Supplementary Fig. S2a); this suggests that the use of glycerol by M2015714 was slow even if the glycerol titer was low.

When two feeding strategies were used for fed-batch fermentation (data not shown), the yield of xanthan gum was around 9.0 g/L with M2015714 and lower than that of batch fermentation (11.0 g/L). As xanthan gum was synthesized by *X. campestris* under the conditions of nitrogen exhaustion while a high C/N ratio in the fermentation system was maintained, starvation or semi-starvation of the bacterium occurred because of the low glycerol concentration during fed-batch fermentation. Lo et al. (1997) reported the yield of xanthan gum increased from 18 g/L to 40 g/L when the mode of glucose addition was changed from periodicity to one-off. Amanullah et al. (1998) found that xanthan yield increases significantly when glucose is maintained at a high concentration after cell growth reached stationary phase.

3.5. Effect of starter substrate on glycerol batch fermentation

Many researchers have encountered the long fermentation time and low conversion rate of glycerol in previous similar studies (Bretz, 2015; Swinnen et al., 2013). Given that most microorganisms are unable to grow on glycerol as the sole carbon source, starter substrates, such as peptone, yeast extract, glucose, and sucrose, are required in glycerol fermentation for biomass formation in cultivation and to shorten fermentation time. In the preliminary work, sucrose as starter substrate improved cell growth with glycerol as carbon source. As shown in Fig. 3a, when 5 g/L sucrose was added into the fermentation medium, the cell growth time decreased from 36 h to 24 h; however, the biomass remain unchanged. This indicates that starter substrate can stimulate cells growth. Furthermore, when sucrose was used, the glycerol concentration remained stable. Generally, in dual carbon sources system with glycerol, glycerol will be consumed efficiently only at a low concentration or when the easy-to-tap carbon sources were exhausted (Li et al., 2015; Martinez-Gomez et al., 2012). This phenomenon is known as glucose effect or diauxie (Mitchell et al., 1987).

Glycerol kinase is the key enzyme in the metabolism of glycerol by bacterium. However, the activity of glycerol kinase was inhibited by high concentration of fructose-1,6-biphosphate, which is one of the metabolic intermediates of carbohydrate phosphotransferase system. Glycerol was utilized as soon as the sucrose concentration was lower than 0.5 g/L (Fig. 3a), indicating that glucose effect also existed in this experiment. At the end of fermentation, the fermentation time (90 h) and xanthan gum yield (12.5 g/L) were better than those from glycerol batch fermentation without starter substrate (Fig. 1d). As shown in Fig. 3b, with glucose as starter substrate, similar results were observed. Using the similar strategy, Pflugl et al. (2012) found that the production of 1,3-propanediol increased from 41.7 g/L to 73.7 g/L by co-feeding glucose and glycerol. Recently, Li et al. (2015) reported that with a mixture of glucose and glycerol, docosahexaenoic acid production by *Aurantiochytrium limacinum* SR21 increased by 15.24%.

3.6. Xanthan gum production by X. campestris CCTCC M2015714 with crude glycerol as carbon source

The ultimate goal of this work is to produce xanthan gum using crude glycerol and provide a solution for the excess crude glycerol from biodiesel production. However, the composition of crude glycerol from biodiesel production is complex and has many impurities, such as methanol or alcohol, salts, triglycerides, soap, and other non-glycerol organic matters (Meiswinkel et al., 2013; Yang et al., 2012), which may inhibit cell growth. For instance, Venkataramanan et al. (2012) found that the cell growth of *Clostridium pasteurianum* and targeted products (1,3-propanediol and butanol) were all negatively affected by the impurities in crude glycerol, especially unsaturated free fatty acids. Anand and Saxena (2012) compared five kinds crude glycerol and proved that *C. freundii* for 1,3-propanediol production is not tolerant to impurities in crude glycerol; thus, pretreatment of crude glycerol to remove impurities is necessary.

Crude glycerol (40 g/L) was used to study the influence of impurities on M2015714. Equivalent pure glycerol (32 g/L) was used as the control group. As shown in Fig. 4b, the cell growth time of crude glycerol was 42 h, whereas the cell growth time for pure glycerol was 36 h (Fig. 4a). The dry cell weight (1.68 g/L) was almost unchanged. The fermentation time was 90 h with xanthan yield of 7.9 g/L. The residual glycerol was about 1.0 g/L, whereas the utilization yield of crude glycerol was almost 100%. As for the control group (Fig. 4a), the yield of xanthan gum from equivalent pure glycerol was 8.7 g/L and the fermentation time was 80 h. Differences between the crude and pure glycerol may be due to the greater time needed by the mutant strain to adapt to the impurities in the culture medium. Generally, xanthan gum is biosynthesized by *X. campestris* to withstand rugged environment (Palaniraj and Jayaraman, 2011; Garcia-Ochoa et al., 2000); thus, the effect of impurities in crude glycerol on mutant strain growth was not severe. In a preliminary study by Brandao et al. (2014), the yield

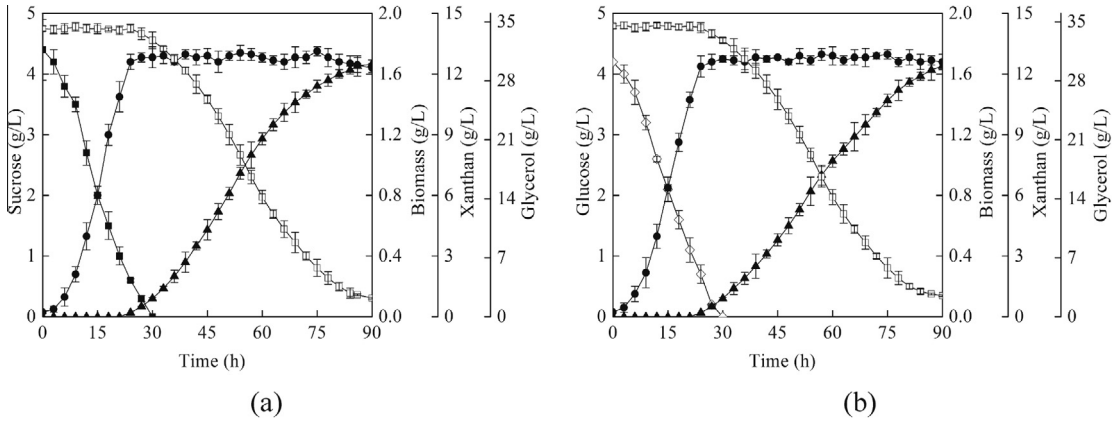

Fig. 3. Profiles of *X. campestris* CCTCC M2015714 cultured with 5 g/L sucrose + 35 g/L glycerol (a) and 5 g/L glucose + 35 g/L glycerol (b). The symbols represent: sucrose (■), glucose (◇), glycerol (□), biomass (●), xanthan (▲).

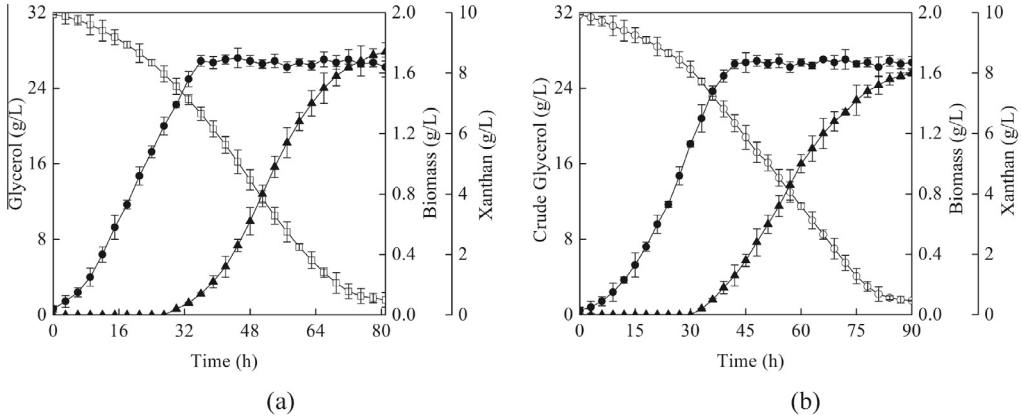

Fig. 4. Batch fermentation profiles of *X. campestris* CCTCC M2015714 with equivalent amount of glycerol (32 g/L) in crude glycerol (a) and 40 g/L crude glycerol (glycerol 80%) (b). The symbols represent: crude glycerol (○), glycerol (□), biomass (●), xanthan (▲).

of xanthan gum from residual glycerol was 7.23 g/L with a conversion rate of 36.5%. Similarly, de Jesus Assis et al. (2014) used crude glycerol as carbon source and xanthan gum yield reached 5.59 g/L at 120 h. In sum, the xanthan yield from glycerol is slightly lower compared to present commercial fermentation process.

4. Conclusions

A mutant strain *X. campestris* CCTCC M2015714, which could grow on glycerol to produce a new type xanthan gum, was obtained through adaptive evolution. RT-PCR analysis showed RT levels of glycerol metabolism related genes (*glpF*, *glpK*, *glpD*) and gene for gluconeogenesis (*fbp*) in the mutant strain were higher than those from the parent strain. Cell growth time and fermentation time were shortened by adding a small amount of sucrose or glucose, and xanthan gum yield slightly increased. The mutant strain can tolerate high titer glycerol and the impurities in crude glycerol.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.03.096.

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