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Microbial Biochemistry for Stable 5-keto-gluconate Fermentation

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Research aims

5-Keto-D-gluconic acid (5KGA) has been reported as a precursor for synthesis of many kinds of valuable substances[1] including Vitamin C, tartaric acid, xylaric acid, and 4-hydroxy-5-methyl-dehydrofuranone-3, a valuable flavor compound. It has been reported that *Gluconobacter* sp. can produce 5KGA and 2-keto-D-gluconic acid (2KGA) from D-gluconic acid, and some strains further oxidize 2KGA to 2,5-diketo-D-gluconic acid (25DKGA). Among several *Gluconobacter* stains, *G. suboxydans* IFO 12528 has been reported to produce 5KGA at high amount with lower amount of 2KGA. However, this strain is a mesophilic strain which has optimum temperature for 5KGA production at 20°C[2]. Therefore, thermotolerant 5KGA-producing strains are in demand for industrial production.

The aim of this study is to isolate the thermotolerant strains which are able to produce 5KGA at higher temperatures and improve their production of 5KGA. On the other hand, for easy quantifications of 5KGA and 2KGA, 5-ketogluconate reductase (5KGR) and 2-ketogluconate reductase (2KGR) are useful, but preparation of these enzymes from *Gluconobacter* is quite laborious. Therefore, overexpression of both enzymes in *Escherichia coli* was also carried out in this study.

Methods

(1) Screening 5KGA-producing thermotolerant Gluconobacter strains

All thermotolerant *Gluconobacter* strains used in screening experiment were isolated from various sources in Thailand[3]. For rough screening, a single colony of all isolates were inoculated to the medium containing 2% D-glucose, 2% sodium D-gluconate, 0.3% Polypeptone and 0.3% yeast extract (G-GA medium), and cultured for 48 hours at 30°C and 60 hours at 37°C. The strains which produced brown compound in the culture medium were screened out as 25DKGA-producing strains. Then, 200 μl of the culture medium of the remaining isolates were reacted with 1 ml of Resorcinol reagent (0.5% (w/v) Resorcinol 49 ml, conc. HCl 168 ml and distilled water 273 ml) at 80°C for 20 minutes. The reaction product of D-glucose and 5KGA with Resorcinol reagent appeared as red and dark brownish green precipitates, respectively, whereas D-gluconate and 2KGA did not generate any color products. The strains producing 5KGA were selected, pre-cultured in Potato medium for 1 day, and then 10 μl of the pre-cultures was seeded to 1 ml of the G-GA medium and incubated at 30°C for 36 hours and 37°C for 48 hours. The culture mediums were then collected and reacted with Resorcinol reagent, and finally the four strains which showed high productivity at 37°C were selected. 5KGA and 2KGA production was also examined by thin layer chromatography and enzyme assay by using 5KGR[4] and 2KGR[5], respectively.

(2) Overexpression of 5KGR

The *gno* (GOX2187) was obtained by PCR with genomic DNA of *G. suboxydans* IFO12528 (identical to *G. oxydans* ATCC621) as a template. A pair of PCR primers was designed to cover the locus *GOX2187* including the probable Shine-Dalgarno sequence (SD) of this gene. The PCR product was then cloned into pGEM-T Easy vector and sequenced. The PCR product was then cloned into pUC119 in *Sph*I and *Pst*I sites. The resulting plasmid, pUC-*gno*, has the *gno* gene inserted under *lac* promoter. 5KGR activity was not observed in *E. coli* DH5α carrying pUC-*gno* whereas a very low activity was observed at 37°C and no activity at lower temperatures (30 and 25°C) in the strains JM109 and TG1.

Next, the *gno* gene inserted in pUC-*gno* was then subcloned into pET-28a(+) at the *Nco*I and *Bam*HI sites. The resulting plasmid was designated as pET-*gno*. 5KGR activity was detected in cell-free extracts of *E. coli* BL21(DE3) carrying pET-*gno*, however, the activity observed was still low.

Finally, a pair of PCR primers was designed to cover *gno* gene from start to stop codon with additional *Nco*I site to the 5′ end and *Bam*HI site to the 3′ end of the gene. To create a new *Nco*I site at the start codon of *gno*, a base change is required, which resulted in a change of the second amino acid residue from Ser to Ala. The PCR product was cloned into pGEM-T Easy vector and sequenced, designated as pGEM-*gno*-NB. The PCR product was then subcloned into pET-28a(+) at *Nco*I and *Bam*HI site and the resulted plasmid was named as pET-*gno*-NB. This plasmid was introduced into *E. coli* BL21(DE3) and used for 5KGR production.

(3) Overexpression of 2KGR

2KGR was partially purified from the soluble fraction of *G. oxydans* IFO12528. N-terminal amino acid sequences were determined by protein sequencer PPSQ21 (Shimadzu), after blotted onto a sheet of PVDF membrane.

A pair of PCR primers was designed to cover the locus

GOX0417 from start to stop codon including the 5′ upstream region of 103 base pairs of this locus which contains SD sequence of this gene. The PCR product was cloned into pGEM-T Easy vector. The resulting plasmid, pGEM-*GOX0417*, was isolated and sequenced. 2KGR activity was observed from *E. coli* DH5α carrying pGEM-*GOX0417* at about 20 times greater than in soluble fraction of *Gluconobacter* strains, but the expression level was still very low.

Next, the inserted fragment on pGEM-*GOX0417* was subcloned into pET-28a(+) at *Xba*I and *Sal*I site, resulted in the pET-*GOX0417* plasmid. This plasmid was transformed to *E. coli* BL21 (DE3) and checked for expression. It was found that 2KGR activity was slightly increased comparing with *E. coli* DH5α carrying pGEM-*GOX0417*. And finally, the 5′ upstream sequence of GOX0417 with a space of 7 bases from start codon was changed from AATGGA to GAAGGA to improve translation efficiency by *Dpn*I-mediated site-directed mutagenesis, resulting the plasmid pET-*GOX0417* M6. This plasmid was introduced into *E. coli* BL21(DE3) and used for 2KGR production.

Results

(1) Screening 5KGA-producing thermotolerant Gluconobacter strains

Total 84 thermotolerant *Gluconobacter* strains isolated from Thailand were tested for 5KGA production in the medium containing both D-glucose and D-gluconate. Among them, 29 isolates showed the ability to produce 5KGA without producing brown pigment derived from 25DKGA at 37°C, but only four strains showed high ability to accumulate 5KGA at 37°C. Among four strains, the three isolates produced 2KGA as the major product with lower amount of 5KGA at 30°C and 37°C (Figure 1). Interestingly, the other strain was found to be defective in 2KGA production at 37°C whereas 5KGA was produced in nearly the same amount as that at 30°C. All four strains showed biphasic growth at 30°C. Production of both ketogluconates occurred simultaneously at the first growing phase and continued after the growth slowed down. The second growth phase was observed when consumption of both 2KGA and 5KGA occurred. On the other hand, such assimilation of both 2KGA and 5KGA was not observed when those strains were cultured at 37°C (Figure 1).

(2) Overexpression of 5KGR

The *gno* gene encoding 5KGR has been reported from *Gluconobacter oxydans* DSM 3503[6], and this is corresponding to GOX2178 in the genome of *G. oxydans* ATCC621H[7]. Several plasmids were constructed and the final construct, pET-*gno*-NB, showed very good expression in *E. coli* BL21 (DE3), 50% or more of proteins in the soluble fraction. It was found that the overexpressed strains

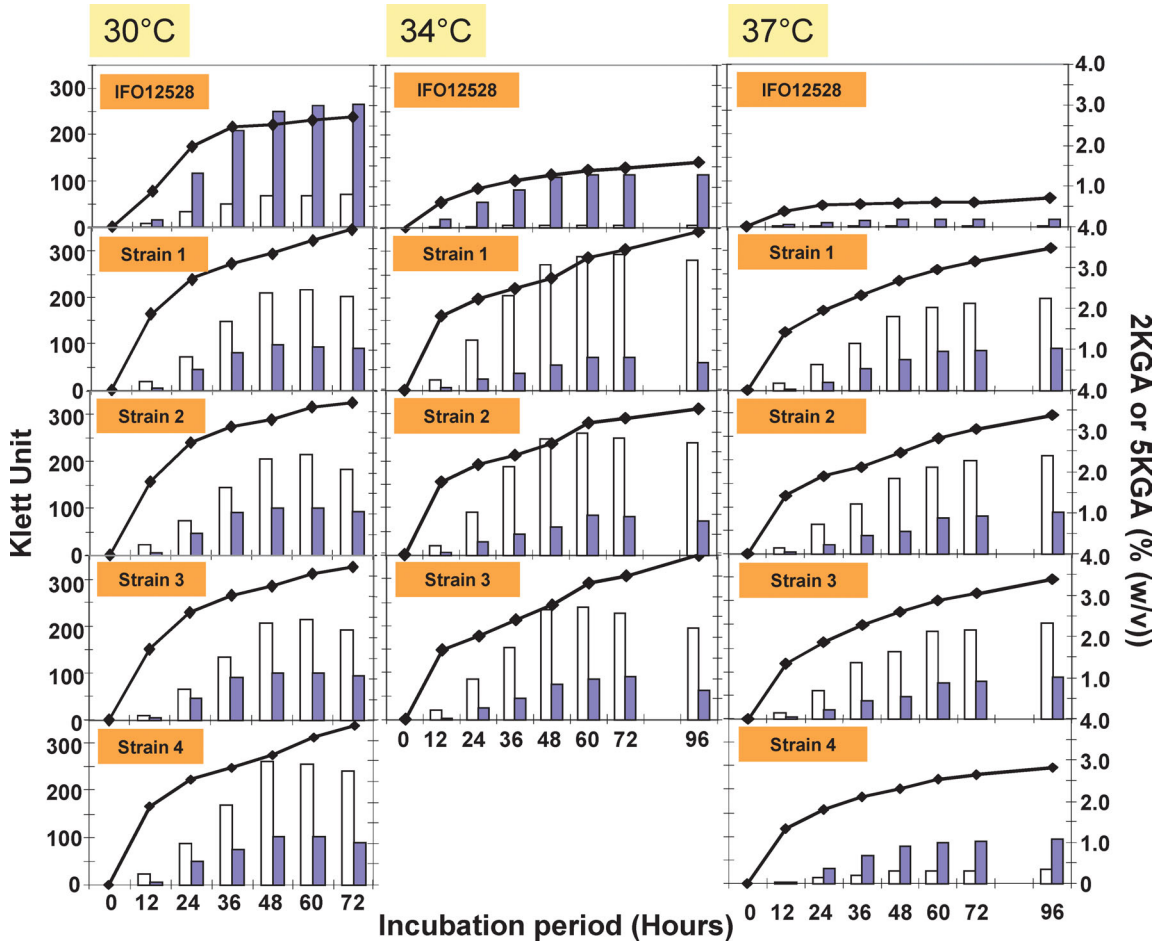

Fig. 1. Growth of thermotolerant *Gluconobacter* strains and production of 5KGA and 2KGA at different temperatures.
The strains were grown on G-GA medium. Growth (diamonds) was monitored using Klett-Summerson photometer with red filter. Small aliquots were taken from the culture and the amounts of 2KGA (white bar) and 5KGA (gray bar) in the culture supernatant were determined enzymatically using 2KGR and 5KGR, respectively.

were unstable as the activity decreased after it was subcultured for several times or kept on plate more than 1 week. Moreover, it was found that fresh cells transformed from freshly prepared competent cells give higher expression level than the old one.

The soluble fraction of 5KGR-overexpressed *E. coli* grown at 30°C was prepared in 10 mM KPB pH 6.0. This enzyme solution was applied onto DEAE-cellulose column chromatography and eluted with linear gradient of 0–0.3 M KCl. The active fractions were eluted at the concentration of KCl about 0.2 M. The partially purified 5KGR was obtained and it is ready to use for determination of 5KGA.

(3) Overexpression of 2KGR

The gene of 2KGR has not been identified yet. Therefore, N-terminal sequence of 2KGR was determined from the partially purified enzyme. The major 52 kDa protein has N-terminal amino acid sequence as AYATTNPYTGETXXTFXEAT, matched to the sequence MAYATINPYTGETLKTFPEAT encoded by the locus GOX1122, a putative NAD-dependent aldehyde dehydrogenase gene, in the genome of *G. oxydans* ATCC621H. In fact, the *E. coli* transformant which has GOX1122 showed no 2KGR activity but NADP-dependent acetaldehyde dehydrogenase activity. The 33 kDa protein has N-terminal amino acid sequence of SSXPDILAID which almost matched with the sequence MSSKPDILTID of the locus GOX0417, which is annotated as a putative 2-hydroxyacid dehydrogenase.

Several plasmids were constructed to express GOX0417 and the final construct, pET-*GOX0417* M6, showed quite good expression after the cells were grown to early-log phase and induced by IPTG for 5 hours or more. It seemed that 10% of proteins in the soluble fraction was 2KGR, although inclusion body of 2KGR observed in the precipitate fractions after collecting the cell-free extracts. The expression level at 30°C was lower than that at 37°C. The instability of 2KGR expression was also observed as in the case of 5KGR overexpression.

Partial purification of 2KGR was carried out from the soluble fraction of 2KGR-overexpressed *E. coli* grown at 37°C. After fractionated with $(NH_4)_2SO_4$, the fraction was applied onto DEAE-cellulose column chromatography which was equilibrated with 10 mM KPB pH 7.0. The active fractions obtained in passed fractions. The partially purified 2KGR thus obtained was ready to use for determination of 2KGA.

Conclusion

(1) Four strains able to produce 5KGA at 37°C were obtained from thermotolerant *Gluconobacter* strains.

(2) 5KGR was successfully overproduced in *E. coli*.

(3) The gene of 2KGR was identified. 2KGR was also succeeded to overexpress in *E. coli*.

References

1) Salusjarvi, T., Povelainen, M., Hvorsley, N., Eneyskaya, E. V., Kulminskaya, A. A., Shabalin, K. A., Neustroev, K. N., Kalkkinen, N., Miasnikov, A. N. Cloning of a gluconate/polyol dehydrogenase gene from *Gluconobacter suboxydans* IFO 12528, characterization of the enzyme and its use for the production of 5-ketogluconate in a recombinant *Escherichia coli* strain. *Appl. Microbiol. Biotechnol*. **65**: 306–314, 2004.
2) Shinagawa, E., Matsushita, K., Toyama, H., Adachi O. Production of 5-keto-D-gluconate by acetic acid bacteria is catalyzed by pyrroloquinoline quinone (PQQ)-dependent membrane-bound D-gluconate dehydrogenase. *J. Mol. Catal. B* **6**: 341–350, 1999
3) Moonmangmee, D., Adachi, O., Ano, Y., Shinagawa, E., Toyama, H., Theeragool, G., Lotong, N., Matsushita, K. Isolation and characterization of thermotolerant *Gluconobacter* strains catalyzing oxidative fermentation at higher temperatures. *Biosci. Biotechnol. Biochem*. **64**: 2306–2315, 2000
4) Ameyama, M., Adachi, O. 5-Keto-D-gluconate reductase from *Gluconobacter suboxydans*, *Methods Enzymol*. **89**: 198–202, 1982
5) Ameyama, M., Adachi, O. 2-Ketogluconate reductase from acetic acid bacteria, *Methods Enzymol*. **89**: 203–209, 1982
6) Klasen R, Bringer-Meyer S, Sahm H. Biochemical characterization and sequence analysis of the gluconate : NADP 5-oxidoreductase gene from *Gluconobacter* oxydans. *J Bacteriol*. **177**: 2637–2643, 1995
7) Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke W. F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nature Biotechnol*. **23**: 195–200, 2005